Effector Cells and Mechanisms in Chronic Spontaneous Urticaria

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for the degree of Doctor of Philosophy

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To my Mother

with love and admiration

Declaration

This dissertation describes work undertaken between 2006 and 2014 at the Dermatology Department of the Norfolk & Norwich University Hospital under supervision of Dr Clive Grattan and between 2009 and 2014 at the BMRC, Norwich Medical School, University of East Anglia under joint supervision of Dr Darren Sexton, Dr Clive Grattan, Dr Cristina Fanutti and Prof. Tom Wileman and Dr Marion Dickson (an external supervisor).

I declare that this dissertation is the result of my own work, the work done in collaboration specifically indicated in the text. This thesis consists of four experimental projects: microdialysis studies in chronic spontaneous urticaria, a prospective study of the pathophysiological subsets in chronic spontaneous urticaria and their biomarkers, imaging flow cytometry studies in healthy subjects, and a retrospective study of re-evaluation of diagnostic criteria for chronic spontaneous urticaria and urticarial vasculitis.

For microdialysis studies, I declare that the protocol development was developed in collaboration with Prof. Martin Church (University of Southampton, UK) and Prof. Geraldine Clough (University of Southampton, UK) in view of their expertise in microdialysis studies, I carry out the patient recruitment and microdialysis experiments at the Norfolk & Norwich University Hospital. Sample analysis at the University of Southampton was carried out in collaboration with Dr Carolanne McGuire, Dr Andrew Walls and Dr Laurie Lau. I declare that I contributed to the sample analysis. The data analysis was carried out under supervision of Dr Allan Clark (University of East Anglia).

ImageStream studies were carried out under joint supervision by Dr Darren Sexton (University of East Anglia, UK) and Dr Roy Bongaerdts (Institute of Food Research, UK). I declare that I carried out the experimental work and the data analysis. The method development was carried out in collaboration with Dr Andy Filby (Cancer Research UK, London), Dr Richard Grenfell (Cancer Research UK, Cambridge, UK), Dr Susanne Heck and Mr PJ Chana (King's College, London, UK).

For the prospective study in chronic spontaneous urticaria, I declare that I made a major contribution to the protocol design, I carried out patient recruitment, collection of the clinical data, screening of the patients with autologous serum skin testing, sample preparation for flow cytometry studies, flow cytometry studies at the UEA, project management and data analysis. This project was carried out in collaboration with several scientists. A serum-induced basophil histamine release assay was carried out at the RefLab, National University Hospital, Copenhagen, Denmark. Basophil releasability assays and manual basophil counts were carried out by Dr Bernhard Gibbs (University of Kent, Chatham Maritime). Flow cytometry studies at the Pathology Department at the Norfolk & Norwich University Hospital were carried out by Miss Cheryl Barker. Cell sorting experiments were carried out by Dr Roy Bongaerdts. Flow cytometry method development and troubleshooting was carried out with the consultancy support by Dr Franco Falcone and Dr Roy Bongaerdts. The project was sponsored and received expert support in molecular biology by Dr Marion Dickson (GlaxoSmithKline, Stevenage, UK).

The retrospective study in urticarial vasculitis was carried out under supervision of Dr Clive Grattan (Dermatology Department, Norfolk & Norwich University Hospital, UK) and Dr Laszlo Igali (Pathology Department, Norfolk & Norwich University Hospital). I declare that I carried out patient selection in the histological database for the period 1989-20012, patient selection based on their clinical data for the same period. For this research, skin biopsy specimens stained with haematoxylin and eosin were used from the archive of the Pathology Department at the Norfolk& Norwich University Hospital. Automated immunohistochemical staining for neutrophil myeloperoxidase was carried out by Joseph Goodwill and Debra Essex. The work on the inter-observer variation of histological assessments was carried out by myself and Mathew Lofthouse (Histopathology Department, Norfolk & Norwich University Hospital). I declare that I carried out analysis of clinical data. Analysis of histological data was performed together with Dr Laszlo Igali over the period 2007-2013. The statistical analysis was carried out under supervision of Dr Allan Clark (University of East Anglia). I declare that the materials described in this dissertation have not been submitted for a degree, diploma or other qualification at any other University. Furthermore, no part of this dissertation has already been, nor is presently being submitted for any other degree, diploma or other qualification.

Data on the pathophysiological phenotyping, biomarkers in chronic spontaneous urticaria and clinical correlations generated from the prospective study in chronic spontaneous urticaria and data on the diagnostic value of immunohistochemical staining for myeloperoxidase were used as a part of the dissertation submitted for a degree of Doctor of Medical Sciences (specialty - Clinical Immunology and Allergology) at the Institute of Immunology (Moscow, Russian Federation) under supervision by Prof. Natalia Ilyina (Institute of Immunology, Moscow, Russian Federation) and Dr Clive Grattan (Norfolk & Norwich University Hospital, Norwich, UK). These data were not included as a part of this thesis. The content of both dissertations was evaluated by a panel of supervisors (Dr Darren Sexton and Dr Clive Grattan) to ensure that there is no overlap. The contents of both dissertations were submitted to the University of East Anglia to confirm different content of both dissertations.

I declare that this thesis complies with the prescribed limit for the PhD dissertations at the University of East Anglia, being less than 100,000 words (excluding figures, tables, appendices and bibliography).

Dr Elena Borzova

1 December 2014

Abstract

Background: Chronic spontaneous urticaria (CSU) is characterised by weals, angioedema, or both, which occur for six weeks or more. Itchy, red and raised weals in CSU are thought to occur as a result of skin mast cell activation, local vasodilatation and increased vascular permeability which are the cardinal features of the disease. Serum histamine-releasing activity and abnormal basophil function were implicated in the pathophysiology of CSU. We hypothesized that severe and/or persistent CSU may be associated with serum histamine-releasing activity (HRA), abnormal basophil releasability, numbers and phenotype. Furthermore, serum HRA in CSU was hypothesized to be associated with higher local concentrations of pro-inflammatory mediators (histamine, tryptase) and IL-6 in the skin, local histamine release and, possibly, neutrophil infiltration in the dermis. To test this hypothesis, we carried out cutaneous microdialysis of autologous serum skin test (ASST) response, a prospective study of basophil-related biomarkers in CSU and a retrospective histological study in CSU and urticarial vasculitis (UV).

In **cutaneous microdialysis study,** 14 CSU patients and 13 healthy subjects were evaluated to determine the baseline levels of histamine, tryptase and cytokines and their changes in response to skin testing with PBS (pH = 7.4, 20µl), autologous serum (20µl) and codeine (0.3mM, 20µl). We demonstrated a slow low-grade local histamine release after intradermal injection of autologous serum in two HRA+ CSU patients. There was elevated dermal histamine ($p=0.0193$) but normal tryptase ($p=0.1437$) and IL-6 ($p=0.1298$) concentrations in CSU compared to healthy controls. Dermal histamine concentrations were correlated to clinical scores in CSU ($r=0.602$, $p<0.05$).

The **prospective observational study** was carried out in 22 CSU patients at three time points over 6 months to elucidate the relationship between the biomarkers to disease severity and disease persistence. Laboratory assessments included serum-induced basophil HRA on healthy donor basophils, anti-IgE-induced basophil histamine release (BHR) from CSU patients, basophil flow cytometry. Baseline UAS7 correlated with serum HRA ($r=0.58$, $p=0.0045$), and anti-IgE-induced BHR ($r=0.40$, $p=0.0666$). HRA+ CSU patients (n=8) had a more severe disease than HRA- CSU patients (n=14)

(p=0.0152). Based on the ROC analysis for UAS7 at baseline, a cut-off value of 19 predicted persistence of CSU in our patient population with 63.16% accuracy (sensitivity of 60% and specificity of 66.67%). These results should, however, be interpreted with caution in view of a small sample size and the selected patient population from the secondary care dermatological setting. There was a persistent (n=3) versus transient (n=3) increase in serum HRA in CSU patients over time. Flow cytometric enumeration in CSU varied depending on choice of gating strategy for peripheral blood basophils (CCR3+CD123+ vs CCR3+CD63+ (p=0.0001), CD63+CD203c+ vs CCR3+CD123+ (p=0.0003), CD63+CD203c+ vs CCR3+CD63+ (p=0.0001)). We then examined basophil variation using **ImageStream®** in healthy subjects. ImageStream® studies confirmed the difference in basophil percentages detected by CD63+CD203c+ and CCR3+CD63+ gating strategies (0.02% vs 0.4% of basophils) in peripheral blood from a healthy donor following Ficoll-Paque density gradient centrifugation. In the CD203c+ OR CD63+ Boolean gate, we identified a basophil subpopulation with surface alterations that comprised 17.7% cells in this gate in peripheral blood sample from a healthy donor. All basophils with surface alterations were CD63+cells, 93.75% of which were CCR3+cells and 0.78% of which were CD203c+cells in this healthy donor.

In the retrospective study, we compared clinical and histological findings in CSU (n=33) and UV (n=43) patients for the presence of skin autoreactivity or serum HRA and eosinophils/neutrophil numbers per high power field (HPF). There were higher numbers of neutrophils/HPF in UV than CSU patients by haematoxylin and eosin (H&E) staining (p=0.0002) and immunohistochemical detection of myeloperoxidase (p=0.0001). Neutrophilic urticaria (more than 25 extravasated neutrophils per five HPF) was noted in 63.6% of CSU patients including two CSU patients with serum HRA.

Conclusions: In CSU, disease severity is associated with higher dermal histamine concentrations, serum HRA and abnormal basophil releasability. In ASST+ patients, an intradermal injection of autologous serum resulted in local *in vivo* histamine release suggesting that the ASST response is a useful experimental model of spontaneous wealing in CSU. Whether *in vivo* local histamine release explains the ASST response in CSU patients needs to be established in future studies. Baseline UAS7 is associated with serum HRA and anti-IgE-induced BHR and may predict disease persistence in CSU patients. Basophil phenotypic variation was demonstrated by different gating strategies which may reflect *in vivo* basophil activation in CSU. Biological and technical factors may contribute to basophil variation in imaging flow cytometry. In the lesional skin biopsies, neutrophil counts/HPF, using H&E staining or immunohistochemistry, can be useful for the diagnosis of neutrophilic urticaria and to differentiate between CSU and UV.

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I am very grateful to my colleagues from the Dermatology Department at the Norfolk & Norwich University Hospital. I am grateful for their contribution as volunteers in our research projects. I feel lucky and privileged to have worked with them and will always remember their kindness. I am indebted to fellow-researchers and PhD student-peers at the UEA. They all inspired me with their devotion to the research, their passion for excellence in the lab, their patience with clinical peers, and for being such great people to work with. I am grateful to all the patients and volunteers who took part in our studies, and whose respect for research inspired me: this research became a personal tribute to all of them.

The ImageStream studies were carried out under joint supervision by Dr Roy Bongaerts and Dr Darren Sexton. I am grateful to Roy for his support and exceptional scientific expertise. Histology studies were performed under joint supervision by Dr Clive Grattan and Dr Laszlo Igali. I am very grateful to Dr Laszlo Igali for his kind support and for all the time and effort spent on vasculitis research over many years.

I am very grateful to my collaborators: for microdialysis studies – Prof. Martin Church, Prof. Geraldine Clough, Dr Andrew Walls, and Dr Carolann McGuire (University of Southampton, UK); for biomarker studies – Dr Bernhand Gibbs (Medway School of Pharmacy, Chatham, UK), Dr Franco Falcone (University of Nottingham, UK), Ms Cheryl Barker and Dr David Walpole (Norfolk & Norwich University Hospital, UK), Dr Andrew Goldson (Institute of Food Research, Norwich), Prof. Per Skov (University of Copenhagen, Denmark), Dr Richard Grenfell (Cancer Research UK, Cambridge, UK), Dr Andy Filby (Cancer Research UK, London, UK), Dr Susanne Heck and Mr P J Chana (King's College, London, UK). It was a pleasure to work with them all: I learned a lot from them and am grateful for all their support.

I am grateful to Prof. Ebo and his colleagues at the University of Antwerpen (Antwerpen, Belgium) and Prof. Valent and his team at the University of Vienna (Vienna, Austria) for letting me visit their laboratories, teaching me basophil research techniques and sharing their protocols. It was a pleasure and a great experience to visit these departments and their kind help is remembered and highly appreciated. I thank Ben Evans from Eclipse Design (Norwich) and Peter O'Flynn (Oxford) their great help and brilliant artwork for illustrations for this thesis. Special thanks to senior statistician Allan Clark and reference team advisor Chris Bishop at the UEA. I am grateful to Phil Hearsey at the PG Office at the UEA for his kind support during my studies.

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Table of Contents

List of Tables

List of Figures

Glossary of Terms and Abbreviations

Abbreviations

A_{2b}	low-affinity adenosine receptor (class A_2 subclass b)
AMP	adenosine monophosphate
ANOVA	analysis of variance
AP Red	Alkaline Phosphate Red
APC	allophycocyanin
APC/Cy7	allophycocyanin/cyanin 7 tandem complex
ASST	autologous serum skin test
ASU	acute spontaneous urticaria
AUC	area under the curve
BAFF	B cell activating factor
BB1	basogranulin
2B4	38kDa type I transmembrane receptor (also known as CD244)
BDNF	brain-derived neurotrophic factor
BD™	Becton Dickinson™
BF	brightfield
BHR	basophil histamine release
BLT	leukotriene B4 receptor

BlyS	B-lymphocyte stimulator
BSA	bovine serum albumin
BV	Brilliant Violet
C1q	the first subcomponent of the C1 complex of the classical pathway of complement activation
C3a	complement component 3a
CR	complement receptor
C5a	complement component 5a
CCD	charge-coupled device
CCL	chemokine (C-C motif) ligand
CCR	C-C chemokine receptor
CD	cluster of differentiation
CD40L	CD40 ligand
Cε	constant domain of IgE
CGRP	calcitonin gene-related peptide
CLEC	C-type lectin
CLEC5A	C-type lectin superfamily member 5
C_{max}	peak concentration
CONGA	Consensus group on new generation antihistamines
CRP	C-reactive protein

CRTH2 chemoattractant receptor-homologous molecule expressed on Th2 cells

CSU chronic spontaneous urticaria

CXCL C-X-C chemokine ligand

CXCR C-X-C chemokine receptor

DAB 3´3-diaminobenzidine

DAO diamine oxidase

DF dilution factor

DNA deoxyribonucleic acid

dsDNA double-stranded DNA

EAACI European Academy of Allergy and Clinical Immunology

ECP eosinophil cationic protein

EDF European Dermatology Forum

EDN eosinophil-derived neurotoxin

EDTA ethylenediamine tetraacetic acid

ELISA enzyme-linked immunosorbent assay

EMLA eutectic mixture of local anesthetics

ENNP3 ectonucleotide pyrophosphate/phosphodiesterase 3

EPO eosinophil peroxidase

EPO-H_2O_2 eosinophil peroxidase – hydrogen peroxide

EPX eosinophil protein X

ESR	erythrocyte sedimentation rate
$F(ab)_2$	pepsin-digested immunoglobulin fragment bearing two antibody binding sites
FAS receptor	tumor necrosis factor receptor superfamily member 6 (also known as apoptosis antigen 1 or CD95)
Fas ligand	ligand for FAS receptor (also known as CD95L)
FACS	fluorescence-activated cell sorter
Fc	constant fragment
FcR	receptors for the Fc portion
FCAP Array™	Flow Cytometric Analysis Program Array software (a trademark of Soft Flow Hungary Ltd.)
FcεRI	high-affinity receptor for IgE
FcεRIα	alpha chain of high-affinity IgE receptor
FcεRII	low-affinity receptor for IgE (also known as CD23)
FcγRIII	low-affinity Fc receptor for IgG (also known as CD16)
FcγRI	high-affinity Fc receptor for IgG (also known as CD64)
FcγRII	low affinity IgG receptor II (also known as CD32)
FcγRIIA	low affinity IgG receptor IIa isoform
FcγRIIB	low affinity IgG receptor IIb isoform

FcγRIII	low affinity IgG receptor III (also known as CD16)
FcγRIIIb	low affinity IgG receptor IIIb isoform
FDA	US Food and Drug Administration
FITC	fluorescein isothiocyanate
fMLP	N-formyl methionyl leucyl phenylalanine
FMO	fluorescence minus one
FRET	Förster resonance energy transfer
G-CSFR	granulocyte colony-stimulating factor receptor
G_0	Gap 0 phase (resting phase) in the cell cycle
G_1	Gap-1 phase in the cell cycle
G_{11}	GTP-binding α_{11}-subunit of GPCR
GPCR	G-protein-coupled receptor
GA^2LEN	Global Allergy and Asthma European Network
GABA	gamma-aminobutiric acid
G_{as}	G-protein α_s-subunit of GPCR
GM-CSF	granulocyte macrophage colony-stimulating factor
GM-CSFR	granulocyte macrophage colony-stimulating factor receptor
gp49B1	49kDa- glycoprotein B1
G_q	G-protein α_q-subunit of GPCR

GRADE Grading of Recommendations Assessment, Development and Evaluation

Gradient RMS Gradient root mean square of the rate of change of the image intensity profile

GRE glucocorticoid-response element

H1-H4 histamine receptors 1-4

HEPES *N*-[2-hydroxyethyl]piperazine-*N′*-[2-ethansulfonic acid]

HIV human immunodeficiency virus

HLA human leukocyte antigen

HLA-DR4 human leukocyte antigen-DR

HNMT histamine – N - methyltransferase

HPF high power field

HRA histamine-releasing activity

HRP horseradish peroxidase

HUV hypocomplementaemic urticarial vasculitis

HUVS hypocomplementaemic urticarial vasculitis syndrome

ICAM-1 intracellular adhesion molecular

ICC interclass correlation coefficient

IFN interferon

IFNγ-R interferon-gamma receptor

Ig immunoglobulin

IL	interleukin
IL-10R	IL-10 receptor
IL-12R	IL-12 receptor
IL-15R	IL-15 receptor
IL-18R	IL-18 receptor
IL-1R	interleukin 1 receptor
IL-1RA	interleukin 1 receptor antagonist
IL-1RII	interleukin 1 receptor, type II (also known as CD121b)
IL-3R	IL-3 receptor (also known as CD123)
IL-4R	IL-4 receptor
IL-5R	IL-5 receptor
IL-6R	IL-6 receptor (also known as CD126)
IL-9R	IL-9 receptor (also known as CD129)
IQR	interquartile range
IRp60	inhibitory receptor protein of 60kDa (also known as CD300a)
ITAM	immunoreceptor tyrosine-based activation motif
IVIG	intravenous immunoglobulins
JAK	Janus kinase
kDa	kilodalton
K_D	dissociation constant

ng	nanogram
μg	microgram
SCF	stem cell factor (also known as c-KIT receptor ligand)
L-HDC	L-histidine decarboxylase
LAB	linker for activation of B cells
LAMP	lysosome-associated membrane protein
LFA-1	lymphocyte function-asociated antigen 1
LILRB4	leukocyte immunoglobulin-like receptor subfamily B member 4
LIR	immunoglobulin-like receptor
LL37	human cathelicidin-derived peptide of 37 amino acids with two consecutive leucine residies at its N-terminal
LOD	level of detection
LPS	lipopolysaccharide
LTB_4	leukotriene B_4
LTB4R1	leukotriene B4 receptor 1 (also known as BLT1)
LTC_4	leukotriene C_4
LTD_4	leukotriene D_4
LTE_4	leukotriene E_4
Lyn	lck/yes-related novel
M:F ratio	male : female ratio

mAb	monoclonal antibody
Mac-1	macrophage-1 antigen
MAO	monoamine oxidase
MAPK	mitogen-activated protein kinase
MBP	major basic protein
MCP	monocyte chemotactic protein
MHC	major histocompatibility complex
MC_t	mast cells (tryptase containing)
MC_{tc}	mast cells (tryptase and chymase containing)
MGUS	monoclonal gammopathy of unknown significance
mGCR	membrane-bound glucocorticoid receptor
MHC	major histocompatibility complex
MIP-1α	macrophage inflammatory protein 1α
mM	millimolar
MMP	matrix metallopeptidase
MPO	myeloperoxidase
MW	molecular weight
mRNA	messenger ribonucleic acid
MyD88	myeloid differentiation protein 88
NADPH	nicotinamide adenine dinucleotide phosphate

NB1	neutrophil antigen B1 (also known as CD177)
NETs	neutrophil extracellular traps
NF-κB	nuclear factor κB
NGF	nerve growth factor
NIH	National Institutes of Health
NK	natural killer cell
NSAID	non-steroidal anti-inflammatory drug
NUV	normocomplementaemic urticarial vasculitis
O_2	oxygen
OCT3	organic cation transporter 3
p21 Ras	rat sarcoma viral protooncogen protein of 21kDa
p38 MAPK	mitogen-activated protein kinase of 38kDa
PAC-1	platelet activation complex 1
PAF	platelet-activating factor
PAFR	platelet-activating factor receptor
PAR	protease-activated receptor
PCR	polymerase chain reaction
PBS	phosphate buffered saline
PE	phycoerythrin
PECAM-1	platelet endothelial cell adhesion molecule

PERCP	peridinin-chlorophyll α complex
PERCP/Cy5	peridinin-chlorophyll protein/cyanine 5 tandem complex
PE/Cy7	phycoerythrin-cyanine 7 tandem complex
PET	positron emission tomography
PGD_1	prostaglandin D_1
PGD_2	prostaglandin D_2
PGE_2	prostaglandin E_2
PGEP2	prostaglandin E receptor EP2 subtype
PGEP4	prostaglandin E receptor EP4 subtype
PI3K	phoshoinositide 3 – kinase
PIPES	piperazine –N,N′- bis-2-ethanesulfonic acid′
PLC-γ	phospholipase gamma
PHM	peptide histidine-methionine
PCR	polymerase chain reaction
PSGL-1	P-selectin glycoprotein lidand 1
RABGEF1	RAB guanine nucleotide exchange factor 1
RANK	receptor activator of nuclear factor kappa-B
RANTES	regulated upon activation normal T cell expressed and secreted
RNAse	ribonuclease

ROC	receiver operating curve
ROS	reactive oxygen species
RPMI 1640	Roswell Park Memorial Institute 1640 (a cell culture medium)
sgAH	second generation antihistamines
Siglec	sialic acid-binding immunoglobulin-like lectin
SHIP	Src homology 2 domain – containing inositol 5′ phosphatase
SIRPα	signal regulatory protein - α
SLE	systemic lupus erythematosus
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SSC	side scatter
ST2	IL-33 receptor (also known as T1)
STAT	signal transducer and activator of transcription
Syk	spleen tyrosine kinase
TGF-β	transforming growth factor β
T_H	helper T cell
TLR	Toll-like receptors
TM4	transmembrane 4 superfamily
TM	transmembrane (domain)
T_{max}	time to reach peak concentration

TNF-α	tumor necrosis factor -α
TNFR	TNF receptor
TPO	thyroid peroxidase
TRAIL	TNF-related apoptosis-inducing ligand
TRAIL-R	TRAIL receptor
TSLP	thymic stromal lymphopoietin
TSLPR	thymic stromal lymphopoietin receptor
UAS3	Urticaria Activity Score over 3 days
UAS7	Urticaria Activity Score over 7 days
uPAR	urokinase plasminogen activator receptor
UV	Urticarial vasculitis
VAS	visual analogue scale
VCAM-1	vascular cell adhesion molecule 1
VE-cadherin	vascular endothelial cadherin
VEGF	vascular endothelial growth factor
VLA	very late antigen
WAO	World Allergy Organisation
WBC	white blood cell

Glossary of Terms:

Basophil releasability – "the theory wheby biochemical events in basophils influence the capacity to release chemical mediators in response to activating stimuli" (Marone et al, 1986: p.19).

Sequential gating – an analysis method of flow cytometric data using sequential visualisation of a cell population of interest using dot plots and histograms (Lugli et al., 2010: p.2).

Boolean gating – "a specific approach to data processing" in flow cytometry used to define a cell population of interest by a combination of gates using Boolean logic (Lugli et al, 2010: p.7).

Frequency distribution is "a collection of observations produced by sorting observations into classes and showing their frequency of occurrence in each class" (Witte & Witte, 2010).

Normal or **Gaussian Distribution** – is a "probability density function of the continuous variable which is characterised by a continuous bell-shaped symmetrical frequency distribution centered at the mean" (Gardiner, 1997). "The intervals of one, two and three standard deviations around the mean contain the probabilities 0.683, 0.954 and 0.997, respectively" (Johnson & Tsui, 1998).

Area-under-the-curve – is an area under the plot of the concentration of solute versus time.

Mean is a measure of central tendency and represents the sum of the observations divided by the number of observations (Weiss, 1999)

Receiver operating characteristic (ROC) curve – is a plot of sensitivity on the y-axis against (1-specificity) on the x-axis for varying values of the threshold t (Zou et al., 2007).

Receiver operating characteristic (ROC) analysis is a statistical tool for assessing accuracy quantitatively or compare accuracy between tests or predictive models. ROC analysis is used to select the optimal threshold under a variety of clinical circumstances, balancing the internal tradeoffs that exist between sensitivity and specificity (Zou et al., 2007).

ANOVA – "a statistical analysis tool that separates the total variability within the data set into random and systematic factors" (such as patient group or disease severity) (Johnson & Tsui, 1998).

Chemotaxis – "a response of motile cells or organisms in which the direction of movement is affected by the gradient of a diffusible substance". (Lackie, 2007: p. 88)

Brightfield microscopy – a microscopy technique, in which "both the diffracted rays (rays that interact with the specimen) and nondiffracted rays (rays that pass undeviated through the specimen) are collected by the objective lens and contribute to image formation". (Murphy, 2001: p.112)

Darkfield microscopy – a microscopy technique, in which only diffracted rays from the specimen "are collected by the objective lens and contribute to image formation". (Murphy, 2001: p.112)

p-value – "the probability that test statistic takes a value equal to or more extreme than the value observed by chance, if the null hypothesis H_0 is true" (Johnson & Tsui, 1998).

The power of a statistical test is the probability of rejecting a false null hypothesis (Weiss, 1991).

Type I error – an error that can results from an incorrect rejection of a true null hypothesis (Gardiner, 1997)

Type II error – an error that results from an acceptance of a false null hypothesis (Gardiner, 1997).

Null hypothesis (H_0) – a statistical hypothesis that expresses that no difference or no change in the system/to the characteristic of the underlying population from previous knowledge is the answer to the research question.

Alternative (or research) hypothesis (H_a) – a statistical hypothesis that describes the response that there is a difference or a change in the system from previous knowledge.

Median – is a measure of central tendency and represents the middle value in a set of measurements expressed in order of magnitude, from smallest to largest (Gardiner, 1997).

K_d – dissociation constant which is a measure of affinity and estimates ligand concentration at which the binding of a ligand to its receptor is half-maximal (Kleinsmith & Kish, 1995)

In vivo – "Latin: describing biological phenomena that occur or are observed occurring within the bodies of living organisms" (Martin, 2010: p.387).

In vitro – "Latin: describing biological phenomena that are made to occur outside the living body (traditionally in a test tube)" (Martin, 2010: p.387).

Side scatter (SSC) – "90º light scatter by the cell or the particle as they pass through the laser beam. SSC is proportional to the granularity of the cell" (Macey, 2001: p.1).

Forward scatter (FSC) – "light scattered by a cell or a particle in the forward direction at low angles (0.5-10º) as they pass through the laser beam" (Macey, 2001: p.1).

Immunophenotyping - "the classification of normal or abnormal white blood cells according to their multiparameter surface antigen characteristics" (Givan, 2001).

Basophilic cellular sensitivity –a measure of IgE-/FceRI-mediated basophil responses which is characterised by "the (allegen) concentration inducing a distinct response" (Kleine-Tebbe et al, 2006: p. 82)

Basophilic cellular reactivity –a measure of IgE-/FceRI-mediated basophil responses which is characterised by “maximum of the basophil response” (Kleine-Tebbe et al, 2006: p.82).

Priming – “treatment that does not in itself elicit a response from a system but that induces a greater capacity to respond to a second stimulus” (Lackie, 2007: p. 341).

Degranulation – “release of secretory granule contents by fusion with the plasma membrane” (Lackie, 2007: p.117).

Receiver operating characteristic (ROC) curve – “a graph plotting the sensitivity against 1-specificity for a diagnostic test at different cut-off points” (Peacock & Peacock, 2011: p.504).

Sensitivity – “the proportion of those who have the disease who are correctly identified by the diagnostic test as positive” (Peacock & Peacock, 2011; p.504).

Specificity – “the proportion of those who do not have the disease who are correctly identified by the diagnostic test as negative” (Peacock & Peacock, 2011: p.504).

NETosis – “a form of cell death that differs from classical apoptosis and necrosis and that occurs during the formation of neutrophil extracellular traps” (Mantovani, 2011: p.522).

CHAPTER 1

General Introduction

"If I have seen a little further it is by standing on the shoulders of Giants."

— ISAAC NEWTON, 1676

1.1 Chronic spontaneous urticaria – definition and general features

In the latest EAACI/GA^{2}LEN/EDF/WAO guideline, urticaria is defined as a frequent, mast cell-driven disease, presenting with weals, angioedema or both (Zuberbier et al, 2014a) (Figure 1). The term 'urticaria' derives from Latin word for stinging nettle. In the GA^{2}LEN Task Force report, CSU is a disease with unmet clinical needs (Maurer, 2013a).

According to the EAACI/GA^{2}LEN/EDF/WAO guideline (the 2013 update), urticaria is classified as acute or chronic (Zuberbier et al, 2014a). Acute spontaneous urticaria is defined as the occurrence of spontaneous weals, angioedema, or both for less than 6 weeks. Chronic spontaneous urticaria (CSU) is defined as the occurrence of spontaneous weals, angioedema or both for 6 weeks or more due to known and unknown causes (Zuberbier et al, 2014a). Chronic urticaria subtypes, as presented in the Table 1, included chronic spontaneous urticaria and inducible urticaria. In this classification, inducible urticaria includes symptomatic dermographism, cold urticaria, delayed pressure urticaria, solar urticaria, heat urticaria, vibratory angioedema, cholinergic urticaria, contact urticaria, aquagenic urticaria.

1.2 A Historical Perspective on Research into Chronic Spontaneous Urticaria

There have been four periods in the evolution of understanding of CSU: first clinical description, then insights into disease mechanisms through research in physiology, immunology and molecular biology. Each period embraced novel scientific methods, clinical innovation and knowledge of previous experience.

Initially, CSU emerged as an entity following careful clinical observations. The ancient Chinese Manuscript "Yellow Emperors Classic of Internal Medicine" and Hippocrates' 'About Diseases' (460-377 BC) first described urticaria (Maurer, 2014). Heberden described the fleeting nature of wealing and a predilection of certain body areas to angioedema (1772) (Humphreys, 1997). The term 'urticaria' derived from *Urtica dioica* (stinging nettle in Latin) (Maurer, 2014) and was coined by Cullen (1771) who with Bateman, distinguished between acute and chronic urticarias (Humphreys, 1997).

The second period encompassed scientific discoveries of urticaria pathophysiology. Histamine's properties were studied by Dale and Laidlaw and the triple skin reaction to histamine was described by Sir Thomas Lewis in 1926 (Czarnetzki, 1989). The role of histamine was demonstrated in CSU (Kaplan, 1978). In 1946, Malmros, for the first time, described the ability of some patients with heterogeneous conditions, including CSU to induce a weal-and-flare reaction upon intradermal injection of patient's own serum (Malmros, 1946). Antihistamines were used to treat urticaria by Curtis in 1947 and corticosteroids were used in CSU in 1951 (Shelley, 1983).

The next period introduced research into the autologous serum skin test (ASST) and work on anti-FcεRIα and anti-IgE autoantibodies in CSU (Hide et al, 1993; Grattan et al, 1986). This period heralded immunomodulatory treatments, including ciclosporin, intravenous immunoglobulins and plasmapheresis in CSU (Figure 2).

Table 1. Classification of Chronic Urticaria Subtypes as recommended by the EAACI/GA^{2}LEN/EDF/WAO Guideline (the 2013 revision and update)

Chronic urticaria subtypes	
Chronic spontaneous urticaria	**Inducible urticaria**
Spontaneous appearance of wheals, angioedema, or both ≥6 weeks due to known or unknown causes	Symptomatic dermographism* Cold urticaria† Delayed pressure urticaria‡ Solar urticaria Heat urticaria§ Vibratory angioedema Cholinergic urticaria Contact urticaria Aquagenic urticaria

*also called urticaria factitia, dermographic urticaria; † also called cold contact urticaria; ‡ also called pressure urticaria; § also called heat contact urticaria.

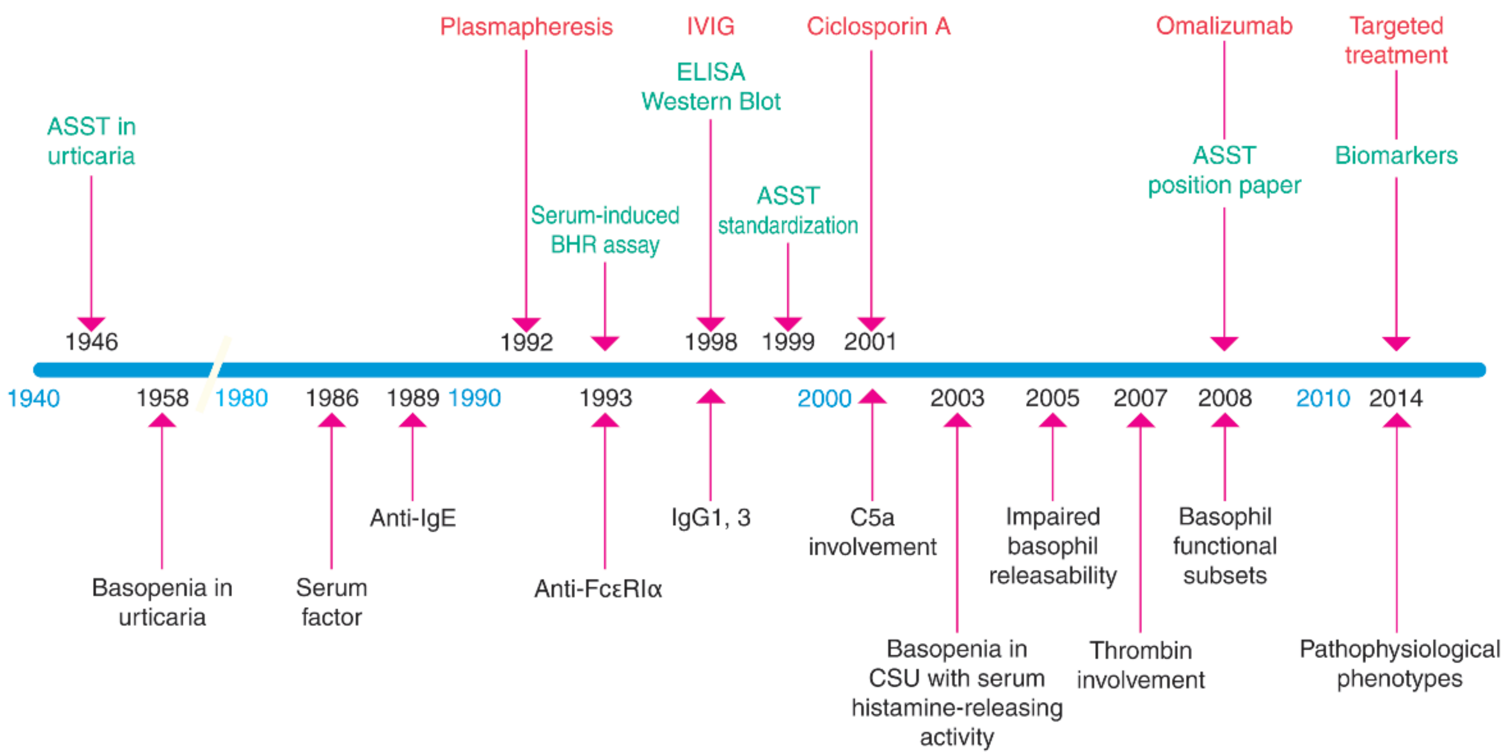
Abbrevations:
EAACI - European Academy of Allergy and Clinical Immunology
GA^{2}LEN - Global Allergy and Asthma European Network
EDF - European Dermatology Forum
WAO - World Allergy Organization

Table 1. The revised clinical classification of urticaria subtypes was suggested by the EAACI/GA^{2}LEN/EDF/WAO guideline at the structured consensus conference as a joint initiative of the EAACI Dermatology Section, GA^{2}LEN, EDF and WAO in Berlin in December 2012 (Zuberbier et al, 2014). According to this classification, acute urticaria was defined as the occurrence of spontaneous weals, angioedema, or both for less than 6 weeks. Chronic urticaria subtypes, as presented in the Table 1, included chronic spontaneous urticaria and inducible urticaria. Chronic spontaneous urticaria was characterised by spontaneous appearance of weals, angioedema, or both for 6 weeks or more due to known or unknown causes. In this classification, inducible urticaria included symptomatic dermographism, cold urticaria, delayed pressure urticaria, solar urticaria, heat urticaria, vibratory angioedema, cholinergic urticaria, contact urticaria, aquagenic urticaria. In this revised classification, previous inconsistencies were addressed so that physical urticarias were classified as a chronic urticaria subtype. Also, in this guideline, exercise-induced anaphylaxis was included in diseases related to urticaria for historical reasons and syndromes that present with hives and/or angioedema rather than in a subtype of physical urticarias as in the previous version of the guideline (Zuberbier et al, 2009).

Figure 1. Typical Urticarial Lesions

Figure 1. Extensive wealing on shoulder of a patient with acute spontaneous urticaria. Skin lesions are represented by confluent weals with surrounding flare.

Figure 2. A Historic Timeline of CSU Research



Abbrevations:
ASST - Autologous serum skin test
BHR - Basophil histamine release
CSU - Chronic spontaneous urticaria
ELISA - Enzyme-linked immunosorbent assay
IVIG - Intravenous immunoglobulins

Figure 2. Early studies in CSU focused on important observations of ASST (Malmros,1946) and basopenia (Rorsman, 1961) in CSU which became biomarkers in CSU later on. Subsequent studies reported the pathophysiological findings in CSU such as a serum histamine-releasing factor (Grattan, 1986), anti-IgE (Gruber et al, 1989) and anti-FcεRIα autoantibodies (Hide et al, 1993). Further detailed studies extended our knowledge of anti-FcεRIα antibodies as IgG1 and IgG3 isotypes (Kaplan et al, 1997) and also characterised C5a involvement in IgG-mediated basophil degranulation in CSU (Kukichi et al, 2002). Novel pathophysiological data refer to basophil functional subsets in CSU patients depending on basophil response to anti-IgE stimulation (Vonakis et al, 2007). Also, an interesting line of research in CSU is focused on the contribution of coagulation cascade to the pathophysiology of CSU (Asero et al, 2007). Current research interests involve the pathophysiological phenotyping of CSU patients (Altrichter et al, 2011; Magen et al, 2011) and a search for reliable biomarkers for disease severity and prognosis (Takahagi, 2010; Toubi 2004). Pathophysiological insights resulted in diagnostic (highlighted in green on the graph) and therapeutic (highlighted in red on the graph) advances in CSU. Studies into skin serum autoreactivity led to the standardisation of ASST and its wide use in clinical practice in CSU (Konstantinou et al, 2009). Plasmapheresis, IVIG and ciclosporin A were introduced into the clinical management of CSU patients (Grattan et al, 1992; O'Donnell et al, 1998; Grattan et al, 2000) Omalizumab was shown to be effective in CSU in several clinical trials (Saini et al, 2011; Maurer et al, 2013). The possibility of targeted treatment to certain CSU phenotypes presents an important current clinical and research interest (Kaplan et al, 2008; Maurer et al, 2011; Metz et al, 2014).

The fourth period encompasses the knowledge of basophil intracellular signalling defects delineated using molecular biology (Vonakis & Saini, 2008a) and the introduction of a biological agent (omalizumab) in the management of CSU (Spector & Tan, 2007). Each period has posed many unresolved questions to be reassessed by new generations of clinicians and researchers using careful clinical observation and novel research techniques.

1.3 Clinical Presentations of Chronic Spontaneous Urticaria

CSU is characterized by daily or almost daily itchy weals on the skin with or without angioedema for 6 weeks or more (Zuberbier et al, 2014a). The weals represent swellings of the superficial dermis. They are usually itchy with pale centres, surrounded by a red flare and resolve over hours without a mark. Swellings of the deep dermis, subcutaneous or submucosal tissues are called angioedema. Swellings are often painful rather than itchy and may persist up to 72 hours. Weals and angioedema often coexist but may occur alone.

In patients with CSU, continuous wealing and itching may lead to sleep deprivation and psychiatric comorbidities (depression), which occurs in up to 45% of patients (Weller et al, 2013a) and results in severe impairment in the quality of life to a degree comparable to that of patients with severe ischaemic heart disease waiting conorary artery bypass surgery (Poon et al, 1999).

The term urticaria is often used to describe an eruption of weals but the consensus definition now also uses it to describe a disease characterized by superficial and deep swellings. In line with this concept, urticaria is known to have diverse clinical presentations. Recognizing the clinical patterns has important implications for clinical evaluation and management without necessarily defining the aetiology.

These patterns can usually be recognized by history taking, clinical presentation and clinical investigations including physical challenge tests (for physical urticarias) and skin biopsy (if urticarial vasculitis is suspected).

The appearance, distribution and duration of weals, and any associated symptoms, are often informative. The duration of individual weals is of particular value: they usually last up to 24 hours in CSU, up to one hour in physical urticaria, two hours in contact urticaria and for longer than 24 hours in urticarial vasculitis. Patients are often unaware of the duration of their weals and it can be helpful to ask them to mark a particular weal and observe it until complete fading.

Weals may occur anywhere on the body but, in some cases, weal distribution may have a diagnostic value, especially in patients with contact or physical urticarias. For example, weals in cold-exposed areas are more likely to be observed in patients with cold urticaria. Contact urticaria starts at the site of contact with the culprit substance but can then progress to generalised urticaria in severe cases.

Weals may vary in colour from pale to red. They may be round, oval or irregular in shape, and measure from a few millimetres to many centimetres across. Although weal morphology is usually non-specific, the linear weals of dermographic urticaria and the pinpoint weals with a surrounding flare in cholinergic and aquagenic urticarias may be helpful in diagnosis. In general, the weals of patients with urticarial vasculitis may bruise and leave residual pigmentation but they may look clinically indistinguishable from spontaneous urticaria.

Different clinical patterns of urticaria can coexist in the same patient, for example, CSU and delayed pressure urticaria (Barlow et al, 1993).

1.4 Treatments of Chronic Spontaneous Urticaria

Management of CSU may be difficult. Correct recognition of the different clinical patterns helps clinical assessment and treatment. The management of urticaria involves recognition of relevant disease associations, treatment of any identifiable external causes including infection, avoidance of drug, food and physical triggers and the appropriate use of pharmacological therapies. The choice of treatment is influenced by many factors including drug licensing, safety, pattern of disease, its severity, pharmaco- economic considerations and patient preference.

Management of CSU includes nonpharmacological measures and drug therapy with a stepwise approach (Zuberbier et al, 2014a). Nonpharmacological measures include avoidance of physical triggers, infections and minimizing exposure to nonspecific aggravating factors identified by a thorough history that may include overheating, stress, alcohol, dietary pseudo-allergens and some drugs, particularly NSAIDs (grade of evidence D). Cooling lotions and 1% menthol in aqueous cream can help relieve itching. A diet low in pseudo-allergens may be effective in some CSU patients, as was demonstrated in a prospective uncontrolled and unblinded study of 140 patients (Magerl et al, 2010).

Current EAACI/GA^{2}LEN/EDF/WAO guideline recommends a step-up management algorithm for CSU patients, with the strength of recommendation being based on a modified GRADE methodology at the 4th International Consensus Meeting ("Urticaria 2012") held in Berlin, Germany in November 2012 (Maurer et al, 2013b; Zuberbier et al, 2014b). The consensus was achieved by at least 75% agreement between more than 300 participants according to an open vote (Maurer et al, 2013b). The revised treatment algorithm suggested the use of non-sedating antihistamines in standard doses as the first level treatment for all CSU patients. If there is no response within two weeks, it is recommended to up-dose non-sedating antihistamines up to fourfold daily for up to four weeks. If patients do not respond within four weeks, omalizumab, ciclosporin A or leukotriene antagonists are recommended as the third-line treatments. Short courses of systemic corticosteroids can be used for CSU flare-ups (Zuberbier et al, 2014a).

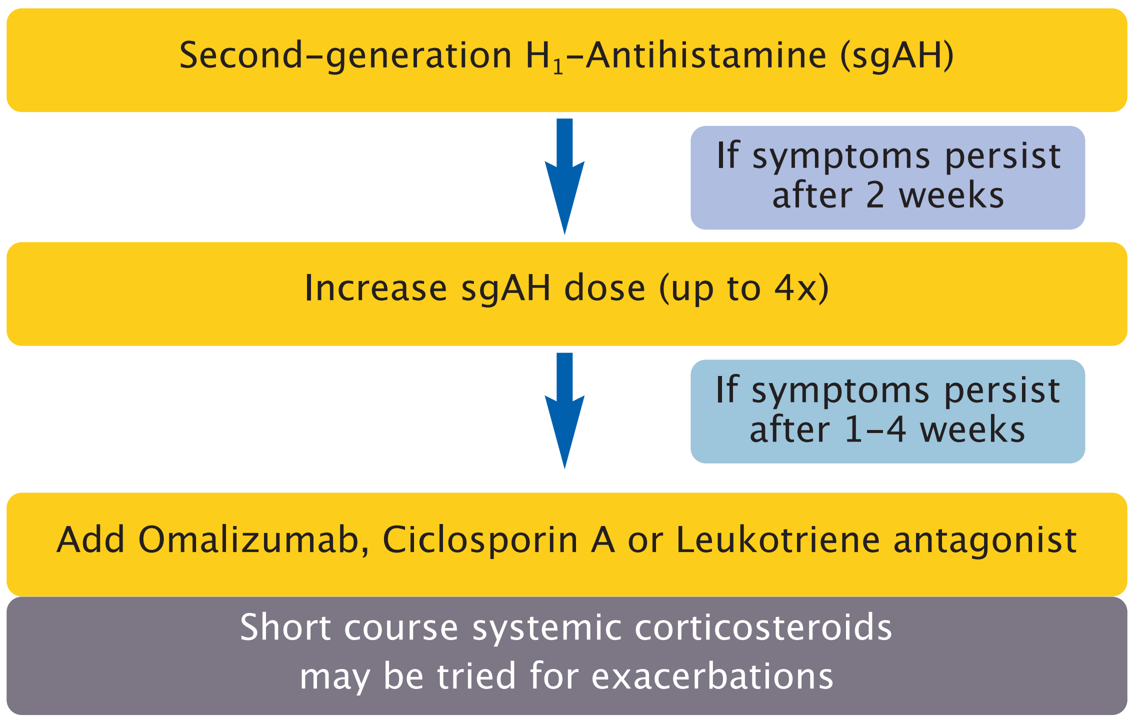
Non-sedating H1 antihistamines are the mainstay of the management of CSU (Figure 3) with their efficacy confirmed in randomized clinical trials (grade of evidence A, strong recommendation). However, some patients respond poorly to the licensed dosages of antihistamines and an increase in their daily dosages appears to be a promising strategy in a view of a wide therapeutic index and an excellent safety profile. A fourfold increase in the daily dose of non-sedating antihistamines have been endorsed by the EAACI for CSU patients with suboptimal control at the licensed doses based on wide clinical experience and general expert agreement (strength of recommendation D) (Zuberbier et al, 2009b). There is accumulating evidence for efficacy and safety of high-dose antihistamines in

CSU (Staevska et al, 2010). In real-life clinical practice, surveys of physicians in Spain (Ferrer et al, 2009) and Germany (Weller et al, 2013b) about their experience with treatment of CSU patients suggested a gradual adoption of high-dose antihistamines in the step-up treatment algorithm in CSU.

The guideline recommends the use of omalizumab, ciclosporin A or leukotriene antagonists as third-line treatments in a stepwise management algorithm for patients who responded poorly to antihistamines (Zuberbier et al, 2014a). Among these, a combination of antileukotrienes and non-sedating antihistamines appears to be the safest option but the efficacy of antileukotrienes in CSU remains to be better characterized.

Among immunomodulatory agents that have been used in CSU, the strongest evidence exists for ciclosporin A for which efficacy in CSU has been established in randomized controlled trials (Grattan et al, 2000; Vena et al, 2006). Ciclosporin A is a calcineurin inhibitor which inhibits T cell proliferation by induction of cell cycle arrest in the G0 or early G1 phase and suppresses, at a pretranslational level, the production of IL-2 (Kay, 1994; Granelli-Piperno et al, 1984). Additionally, ciclosporin A inhibits mediator release from mast cells and basophils (Marsland et al, 2005; Hultsch et al, 1990; Stellato et al, 1992; Kay, 1994). In CSU, treatment with ciclosporin was associated with reduction in serum histamine releasing activity and the ASST response to post-treatment serum (Grattan et al, 2000). Potential ciclosporin A adverse effects include hypertension, nephrotoxicity, hepatotoxicity, hyperkalaemia, hyperlipidaemia, peripheral neuropathy, gastrointestinal symptoms (diarrhoea, vomiting and dyspepsia), hirsutism and gingival hyperplasia and an increased risk of lymphomas and other malignancies (Jose, 2007) with long term use at doses used to prevent transplant rejection. Fewer side effects (mainly gastrointestinal and peripheral neuropathy) were reported at low doses (2-3 mg/kg) of ciclosporin A (Kessel & Toubi, 2009). When ciclosporin A is given at higher doses (4-5 mg/kg), side effects such as hypertension, peripheral neuropathy and increased creatinine levels were reported in 20-30% of patients (Kessel & Toubi, 2010). Therefore, careful monitoring of blood pressure and serum creatinine levels are required during treatment with ciclosporin A. Overall, ciclosporin A has been reported to be an effective treatment for severe CSU, however, there are still some unresolved issues about the optimal dosing and the duration of the treatment.

Figure 3. Management Algorithm in CSU as recommended by the EAACI/GA²LEN/EDF/WAO Guideline (the 2013 revision and update)



Abbrevations:
CSU - Chronic spontaneous urticaria
EAACI - European Academy of Allergy and Clinical Immunology
EDF - European Dermatology Forum
GA²LEN - Global Allergy and Asthma European Network
GRADE - Grading of Recommendations Assessment, Development and Evaluation
sgAH - Second-generation H_1-antihistamine
WAO - World Allergy Organization

Figure 3. Current EAACI/GA²LEN/EDF/WAO guideline recommends a step-up management algorithm for CSU patients as agreed by the expert panel members using the GRADE methodology at the 4th International Consensus Meeting ('URTICARIA 2012') held in Berlin (Germany) in November, 2012. (Maurer et al, 2013; Zuberbier et al, 2014). The consensus was achieved by at least 75% agreement between more than 300 participants according to an open vote (Maurer et al, 2013). The revised treatment algorithm suggested the use of non-sedating antihistamines in standard doses as the first level treatment for all CSU patients. If there is no response within two weeks, it is recommended to up-dose non-sedating antihistamines up to four times daily for up to four weeks. If patients do not respond within four weeks, omalizumab, ciclosporin A or leukotriene antagonists are recommended as the third-line treatments. Short trials of systemic corticosteroids can be used for CSU flare-ups.

The use of omalizumab for the treatment of CSU turned out to be the major advance in the management of CSU. Omalizumab (Xolair, Genetech/Novartis), a recombinant humanized IgG1κ monoclonal antibody, is used for biological treatment of CSU (Kaplan et al, 2008). Omalizumab is non-anaphylactogenic monoclonal antibody because it can only bind free IgE but not surface-bound IgE (Fahy, 2006). The binding of omalizumab to circulating IgE prevents IgE from interacting with the high- or low-affinity IgE receptors (Chang, 2000). The mutually exclusive binding of IgE to omalizumab and FcεRI or FcεRII is incompletely understood but is thought to be due overlapping binding sites for omalizumab and FcεRI or FcεRII within the Cε3 domain of the IgE constant region (Zheng et al, 2008; Chang, 2000).

The mechanism of action of omalizumab in CSU is incompletely understood. The putative mechanisms that may be relevant in CSU include:

1 binding to free IgE, with the reduction of surface-bound IgE (Chang et al, 2014) and, possibly, the reduction in IgE priming effects on mast cells (Kawakami & Galli, 2002);
2 down-regulation of the density of high affinity IgE receptors on the surface of mast cells and basophils (Beck et al, 2004; Chang & Shiung, 2006);
3 reduction of the release of mediators, cytokines and chemokines from mast cells and basophils (Oliver et al, 2010; Noga et al, 2008);
4 accummulation of omalizumab-IgE complexes (Hsu et al, 2010; Chang, 2000) which may have a role in sequestration of the endogenous autoantigens (Chang et al, 2014);
5 down-regulation of membrane-bound IgE-producing B cells (Chan et al, 2013) and memory B cells and, possibly, a reduction in the continuous generation of IgE-secreting plasma cells (Chan et al, 2013).

The mechanisms mediating therapeutic effects of omalizumab in CSU are difficult to explain. Treatment with omalizumab induces clinical improvement predominantly within the first week of treatment (Casale, 2014). However, it takes a longer time to down-regulate the expression of the high affinity IgE receptors on the surface of basophils (2 weeks) or mast cells (10 weeks) (Beck et al, 2004; Casale, 2014).

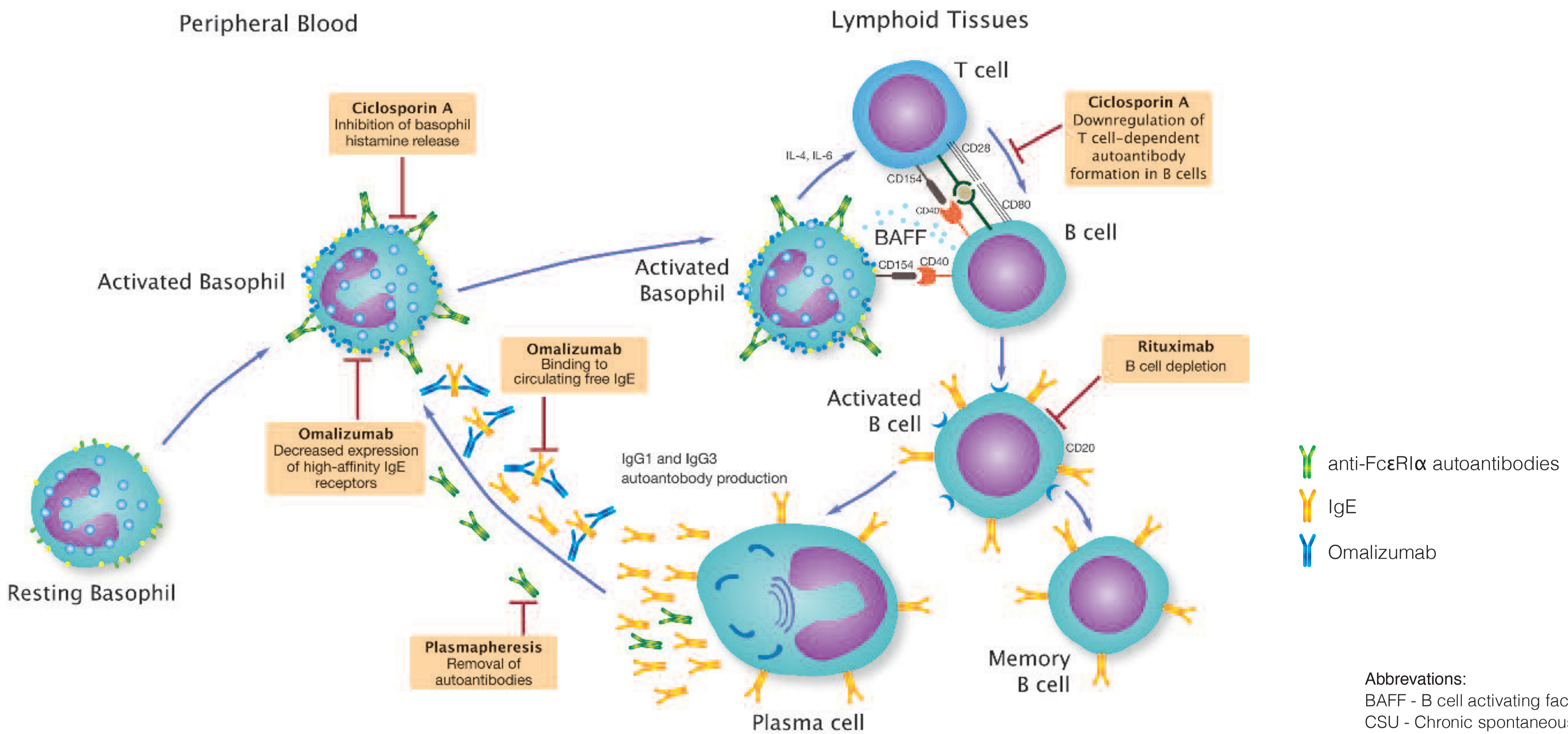
Several multicentre randomized placebo-controlled phase III clinical trials have demonstrated the efficacy of omalizumab for treatment of patients with moderate-to-severe CSU who do not respond to licensed or higher than licensed doses of H1 antihistamines (Maurer et al, 2013c, Kaplan et al, 2013). In March 2014, Xolair (omalizumab) was approved by U.S. Food and Drug Administration (FDA) for the indication of CSU in addition to asthma. Omalizumab is licensed for subcutaneous injection in adults and children aged 12 or older. The most common side effects involve local injection site reactions that occur in approximately 45% of patients (Polosa & Casale, 2012). According to the omalizumab Joint Task Force, the frequency of omalizumab-associated anaphylaxis was reported to be 0.09% of patients treated for asthma (Cox et al, 2007). The pooled analysis of the data from 67 phase I to phase IV clinical trials for asthma did not confirm an association between the use of omalizumab and risk of malignancy (Busse et al, 2012).

Short trials of systemic corticosteroids are recommended for CSU flare-ups but they are not suitable for long-term management of the disease (Zuberbier et al, 2014a). Corticosteroids exert their actions via four mechanisms: genomic mechanism; secondary non-genomic effects; membrane-bound glucocorticoid receptor (mGCR)-mediated non-genomic effects; non-specific, non-genomic effects by interaction with cellular membranes (Stahn et al, 2007). Glucocorticoids exert genomic effects by binding to the cytosolic glucocorticoid receptors that undergo nuclear translocation and bind to glucocorticoid-response elements (GRE) in the promoter region of steroid-sensitive genes. GRE affect the transcription of anti-inflammatory proteins (Barnes, 2006). Glucocorticoids act on numerous immune cells. Glucocorticoids decrease myelopoiesis and release of monocyte precursors (Stahn et al, 2007). Glucocorticosteroids reduce the synthesis of pro-inflammatory cytokines (e.g. IL-2, IL-6 and TNF-α) and prostaglandins (Stahn et al, 2007). These agents reduce the number of eosinophils and basophils whereas they increase the number of circulating neutrophils (Stahn et al, 2007). In bone marrow derived mast cells, glucocorticoid treatment suppressed the expression of FcεRIα (Benhamou & Mencia-Huerta, 1986). Additionally, glucocorticoids inhibit *c-kit*-mediated mast cell responses such as migration, p38 MAP kinase phosphorylation, TNF-α and IL-6 production (Jeong et al, 2003). Furthermore, glucocorticoids can exert rapid effects on mast cells, which are likely to occur due to their action on plasma membranes (Oppong et al, 2013). Rapid effects of glucocorticoids on mast cells include rapid decrease in histamine and calcium release (Zhou et al, 2008). Side effects of longterm glucocorticoid use are common and present a significant therapeutic limitation. Potential steroid toxicity includes osteoporosis, diabetes, abdominal obesity, glaucoma, hypertension, growth retardation in children, skin atrophy and metabolic effects (Barnes, 2006; Oakley & Cidlowski, 2013). For CSU exacerbations, prednisolone can be used as 10 mg daily or 20-25 mg every other day with tapering (Kaplan, 2009). The current guideline recommends short courses of corticosteroids for maximum 10 days (Zuberbier et al, 2014a).

Treatment-refractory CSU presents a daunting challenge for the clinicians. About 75% of patients with refractory CSU tend to respond to the treatment with ciclosporin or omalizumab (Kaplan, 2011). For CSU patients with *in vitro* serum histamine-releasing activity, intravenous immunoglobulins and plasmapheresis can be helpful (Grattan, 2004). Other treatment options such as methotrexate (Perez et al, 2010), miltefosine (Magerl et al, 2012) and rituximab (Chakravarty et al, 2011) have been tried in CSU but the place of these agents in the algorithm for the management of CSU is yet to be established. They may represent potential fourth-line treatments for CSU patients who have failed treatment with ciclosporin A or omalizumab. More evidence is needed in order to understand how these agents (methotrexate, miltefosine or rituximab) may improve stepwise management in CSU. At present, insufficient evidence base, uncertain patient selection criteria, poorly defined drug mode of action, potential toxicity and off-license toxicity limit the use of these agents for treatment of refractory CSU. Future work needs to clarify as to when to start these treatments, to define the optimal treatment duration and to elucidate the mechanisms mediating their therapeutic effects in CSU.

A summary of therapeutic targets in CSU is presented in Figure 4. Putative targets for therapeutic interventions in CSU include serum histamine-releasing activity, *in vivo* activation of peripheral blood basophils, autoantibody production and dysregulation of T and B cell response. Treatment with ciclosporin A resulted in the reduction of serum

Figure 4. Therapeutic Targets in CSU



Abbrevations:
BAFF - B cell activating factor
CSU - Chronic spontaneous urticaria

Figure 4. Potential targets for therapeutic interventions in CSU include serum histamine-releasing activity, *in vivo* activation of peripheral blood basophils, autoantibody production and dysregulation of T and B cell response. Ciclosporin A appears to induce the division arrest of T lymphocytes in the G0/early G1 phase of the cell cycle and to inhibit IL-2 production at the pre-translational level (Kay, 1994, Granelli-Piperno et al, 1984). Treatment with ciclosporin A resulted in the reduction in serum histamine-releasing activity in CSU patients (Grattan et al, 2000). *In vitro* evidence showed that ciclosporin A inhibited anti-IgE-induced histamine release from peripheral blood basophils in healthy donors (Marsland et al, 2005). Downregulation of T-cell dependent autoantibody production in B cells via suppression of T cell co-stimulatory signals was also suggested as a pathway targeted by ciclosporin A (Heidt et al, 2010). The targets of omalizumab in the context of the CSU pathophysiology are incompletely understood. The putative pathways targeted by omalizumab may include the reduction in serum levels of free IgE (Holgate et al, 2005) and a decrease in the expression of the high-affinity IgE receptor (FcεRI) on the surface of basophils and mast cells (Beck et al, 2004) and increased intrinsic sensitivity of basophils to anti-IgE-mediated stimulation (MacGlashan Jr. & Saini, 2013). Rituximab is thought to interfere with the autoantibody production in CSU by binding CD20 and depleting B cells. However, case reports of using rituximab in CSU patients showed conflicting results (Chakravarty et al, 2011; Mallipeddi & Grattan, 2007).

histamine-releasing activity (Grattan et al, 2000). *In vivo* basophil and mast cell activation may be targeted by treatment with ciclosporin A or omalizumab. Ciclosporin A was shown *in vitro* to inhibit anti-IgE-induced histamine release from peripheral blood basophils (Marsland et al, 2005). Omalizumab is known to reduce the density of FceRI receptors on the surface of mast cells and basophils (Beck et al, 2004) and may also increase intrinsic sensitivity of basophils to anti-IgE-mediated stimulation (MacGlashan Jr. & Saini, 2013). T cell-dependent autoantibody production in B cells can be down-regulated via suppression of T cell co-stimulatory signals by ciclosporin A (Heidt et al, 2010). More knowledge about the inflammation pathways in CSU would allow the identification of novel therapeutic targets in CSU.

In some patients with a poor response to antihistamines, the diagnosis of CSU should be revisited since a lack of efficacy to antihistamines may be a clinical presentation of urticarial vasculitis in 15-20% of cases (Tosoni et al, 2009). Therefore, CSU patients non-responding to antihistamine therapy should be considered for differential diagnosis with urticarial vasculitis and a skin biopsy taken.

1.5 General features of pathophysiology

CSU is considered as a disease with a complex pathophysiology which presents with recurrent wealing due to intermittent activation of skin mast cells by unknown mechanisms (Kay et al, 2014a). Several putative factors may contribute to the intermittent activation of dermal mast cells in CSU including genetic predisposition, serum histamine-releasing factors, intrinsic functional aberration of skin mast cells and the effects of the skin microenvironment. Genetic predisposition or intrinsic functional aberrations may cause a primary abnormality of skin mast cells in the pathophysiology of CSU but some argue that, in this case, the condition would be systemic rather than limited to the skin (Chang et al, 2014). Alternatively, skin mast cells are thought to be affected by factors present in the cutaneous microenvironment (Chang et al, 2014). Two possible mast cell-priming factors were suggested in CSU including factors mediating autoreactivity in CSU and monomeric IgE (Chang et al, 2014). The autoreactivity in CSU is hypothesized to be mediated by IgG autoantibodies against the high-affinity IgE receptor (FcεRI) (Figure 6), IgE or both and by autoantibodies against autoantigens, including IgE autoantibodies against thyroperoxidase and, recently reported, IgE autoantibodies against dsDNA (Hide et al, 1993; Altrichter et al, 2011; Hatada et al, 2013; Chang et al, 2014).

The role of autoantibodies in CSU. The circumstantial evidence for autoimmunity in CSU is based on the association with other autoimmune diseases within the same patient, HLA associations and clinical response to immunosuppression but direct and indirect evidence for CSU being an autoimmune disease to fulfill Witebsky's postulates is lacking (Stitt & Dreskin, 2013). CSU was noted to be linked to other autoimmune diseases including thyroid autoimmunity (Leznoff et al, 1983, O'Donnell et al, 2005). A study by Confino-Cohen et al (2013) with more than 12,778 CSU patients revealed that the odds of having one or several autoimmune diseases in CSU patients within 10 years of the diagnosis of CSU were 7.7 to 28.8 times higher than in healthy subjects. In CSU, highly significant HLA-DR4 associations were linked to positive autologous serum skin tests and *in vitro* basophil histamine releasing activity of CSU sera (O'Donnell et al, 1999). Treatment with ciclosporin, plasmapheresis and intravenous immunoglobulins were reported to be effective in CSU patients (Grattan et al, 2000; O'Donnell et al, 1998, Grattan et al, 1992).

CSU is referred to as an autoimmune disease in the current literature (Greaves, 2002; Kaplan et al, 2008; Konstantinou et al, 2013), although an animal model has not been developed and direct evidence of functional autoantibodies causing CSU in humans is not available. In the position paper by the EAACI Task Force, the proposed diagnostic criteria for 'autoimmune' CSU, based on the agreement of an expert panel, included a combination of 1) a positive bioassay (basophil histamine release assay or basophil activation marker expression) to demonstrate *in vitro* functionality and 2) positive autoreactivity demonstrated by a positive ASST and 3) a positive immunoassay for specific IgG autoantibodies against FcεRI and/or anti-IgE by Western blot or ELISA to demonstrate antibody specificity (Konstantinou et al, 2013).

Figure 5. Histamine Metabolism and Possible Alterations in CSU

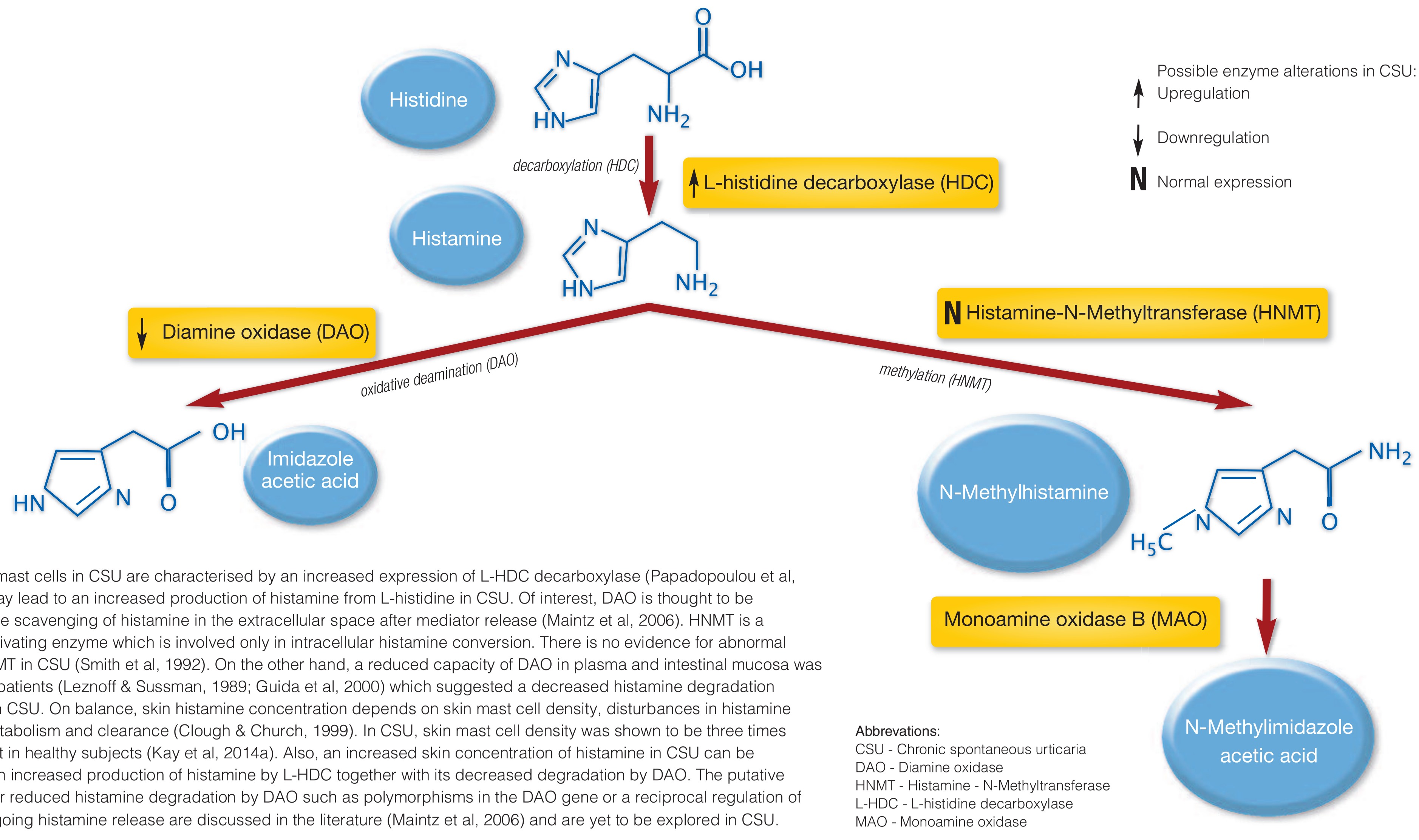


Figure 5. Skin mast cells in CSU are characterised by an increased expression of L-HDC decarboxylase (Papadopoulou et al, 2005) which may lead to an increased production of histamine from L-histidine in CSU. Of interest, DAO is thought to be implicated in the scavenging of histamine in the extracellular space after mediator release (Maintz et al, 2006). HNMT is a histamine-inactivating enzyme which is involved only in intracellular histamine conversion. There is no evidence for abnormal function of HNMT in CSU (Smith et al, 1992). On the other hand, a reduced capacity of DAO in plasma and intestinal mucosa was shown in CSU patients (Leznoff & Sussman, 1989; Guida et al, 2000) which suggested a decreased histamine degradation associated with CSU. On balance, skin histamine concentration depends on skin mast cell density, disturbances in histamine production, metabolism and clearance (Clough & Church, 1999). In CSU, skin mast cell density was shown to be three times higher than that in healthy subjects (Kay et al, 2014a). Also, an increased skin concentration of histamine in CSU can be explained by an increased production of histamine by L-HDC together with its decreased degradation by DAO. The putative mechanisms for reduced histamine degradation by DAO such as polymorphisms in the DAO gene or a reciprocal regulation of DAO by an ongoing histamine release are discussed in the literature (Maintz et al, 2006) and are yet to be explored in CSU.

Figure 6. Schematic Representation of IgE and IgE Receptors

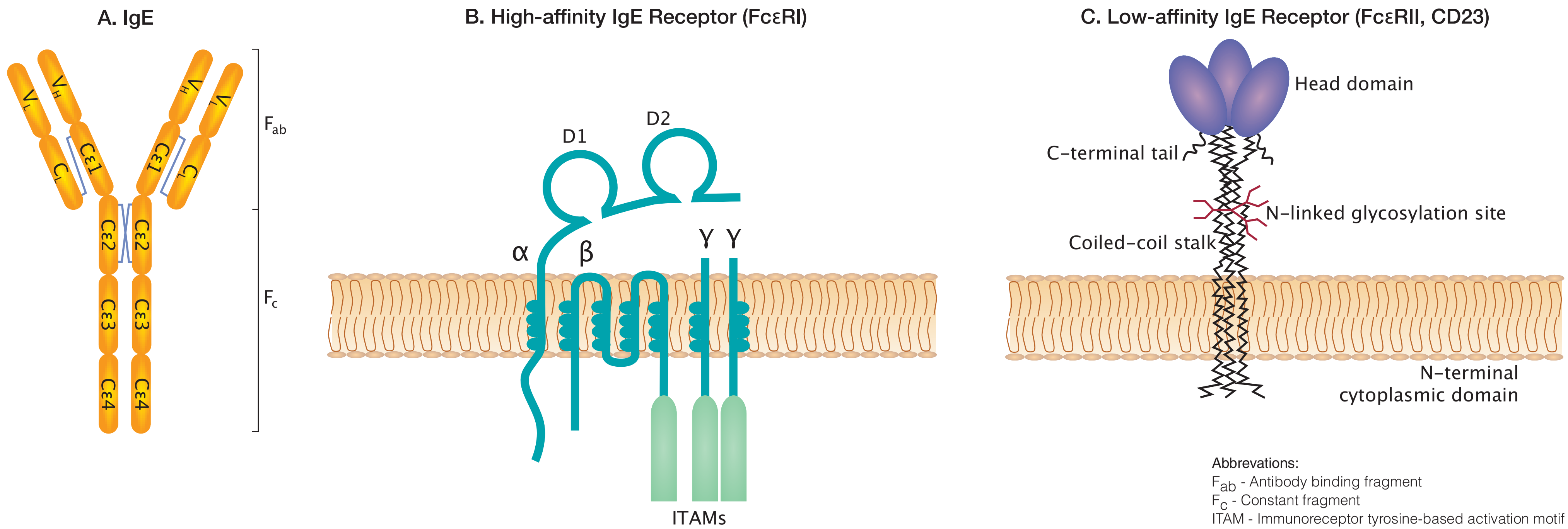


Figure 6. The structure of IgE molecule includes two light (L) chains associated with two heavy (H) chains (Figure 6A). Each heavy chain contains one variable and four constant domains (Cε1-4) (Miescher & Vogel, 2002). Heavy chain C-terminal constant domains (Cε2, Cε3 and Cε4) are known to dimerise to form the Fc fragment (Wurzburg et al, 2000). Two antigen binding sites are located within the Fab fragment of IgE.

A tetrameric FcεRI receptor contains four subunits: α, β and two γ chains (Figure 6B). The extracellular α chain contains the ligand-binding immunoglobulin-like domains D1 and D2. Transmembrane domain β domain amplifies the activation signal. Two disulphide-linked γ chains transmit the activation signal to the intracellular signalling pathways via two ITAMs. An interaction of IgE and FcεRIα occurs with high affinity ($K_d = 10^{-9}$ - 10^{-10}M) (Garman et al, 2001) via contact residues on the top of D2 domain in FcεRIα and theCε3 domain in the Fc region of IgE molecule (Turner & Kinet, 1999; Wurzburg & Jardetzky, 2001). X-ray crystallographic studies showed an asymmetrically bent conformation of IgE in its Fc fragment that occurred upon the binding to the receptor (Wat et al, 2002).

The low affinity IgE receptor (FcεRII, CD23) is a type II transmembrane glycoprotein (Acharya et al, 2010). The structure of CD23 receptor includes three lectin heads and a three-stranded, a-helical coiled-coil "stalk" and a short cytoplasmic tail (Gould et al, 2003). CD23 lectin heads display a structural homology to C-type (calcium-dependent) lectins (Gould & Sutton, 2008). IgE binding to FcεRII occurs via Cε3 domain (Acharya et al, 2010) Single lectin domain binds IgE with low affinity ($K_d = 10^{-6}$-10^{-7} M^{-1}), whereas the multipoint interaction of three lectin domains with IgE is characterised by high-affinity binding (K_d=10^{-8}-$10^{-9}$$M^{-1}$) (Gould & Sutton, 2008).

The role of anti-FcεRIα autoantibodies in the pathophysiology of CSU is less clear. Their involvement in the pathophysiology of CSU was implicated by the original study by Hide et al (1993), which was later extended by Kikuchi and Kaplan (2001), showing neutralization of serum-induced basophil histamine release by soluble recombinant α-chains of the high-affinity IgE receptor in some patients. Additional evidence comes from the studies showing that serum histamine-releasing activity in CSU is limited to IgG fraction and appears to reside in complement fixing IgG1 and IgG3 isotypes (Grattan, 1991; Kikuchi & Kaplan, 2001; Soundararajan et al, 2005). In the experimental settings, anti-FcεRIα autoantibodies were shown to activate peripheral blood basophils and skin mast cells *in vitro* (Niimi et al, 1996). Furthermore, an increased frequency of anti-FcεRIα autoantibodies was demonstrated in CSU in comparison with other diseases (Fiebiger et al, 1995). The presence of anti-FcεRIα antibodies was linked to a more severe CSU (Sabroe et al, 1999). The decrease in functional activity of CSU sera following the treatment with ciclosporin (Grattan et al, 2000), intravenous immunoglobulins (O'Donnell et al, 1998) and plasmapheresis (Grattan et al, 1992) may suggest anti-FcεRIα antibodies as a biomarker of therapeutic efficacy rather than implying causality.

On the other hand, anti-FcεRIα antibodies were also detected in healthy subjects by immunoassay (Miescher et al, 2001). Anti-FcεRIa autoantibodies, cloned from healthy subjects and patients with CSU, demonstrated the same amino acid sequence and were able to release histamine from basophils (Pachlopnik et al, 2004) although the differences in affinity and titre were not studied. The detection of anti-FcεRIa autoantibodies in healthy subjects implies that the presence of anti-FcεRIα autoantibodies may not be a sufficient component for the development of CSU. The detection of anti-FcεRIα autoantibodies in physical urticarias and urticarial vasculitis (Zuberbier et al, 2000) raises the questions about the specificity of these autoantibodies to the CSU pathophysiology. Anti-FcεRIα autoantibodies were detected in pemphigus vulgaris, dermatomyositis, SLE and bullous pemphigoid but differed in isotype specificity from those in CSU (Fiebiger et al, 1995). Anti-FcεRIα autoantibodies in CSU belong to IgG1, IgG3 and, to lesser extent, to IgG4 whereas anti-FcεRIα autoantibodies in pemphigus vulgaris, dermatomyositis, SLE and bullous pemphigoid are predominantly of the non-complement fixing IgG2 and IgG4 subtypes (Soundararajan et al, 2005, Fiebiger et al, 1998).

Furthermore, failure to demonstrate functional anti-FcεRIα antibodies in a proportion of CSU patients suggests that these antibodies may be unnecessary for the development of CSU or that better systems for detection are required. In the prospective study, anti-FcεRIα autoantibodies, as detected by an enzymatic (nonfunctional) immunoassay, did not follow the course of CSU (Eckman et al, 2008), although a selection bias might have been introduced in this study by excluding CSU patients with basopenia. Although mouse models with humanized high-affinity IgE receptor exist (Fung-Leung et al, 1996), direct evidence of wealing upon intradermal injection of anti-FcεRIα antibodies in mouse models, as required by Witebsky's criteria for autoimmune diseases, is currently lacking. Since recombinant anti-FcεRIα autoantibodies are not approved for clinical use, the evidence of skin mast cell activation by these autoantibodies *in vivo* in CSU patients or healthy subjects is not available.

Overall, there is indirect evidence to suggest that anti-FcεRIα antibodies mediate serum histamine-releasing activity in CSU based on the neutralization experiments in a few patients (Hide et al, 1993; Kikuchi & Kaplan, 2001). Although the frequency of these antibodies is increased in severe CSU, there is currently insufficient evidence to infer causality of these autoantibodies in CSU.

Several functional and binding assays have been employed for the detection of serum histamine-releasing activity and/or anti-FcεRIa autoantibodies. Functional assays (serum-induced basophil histamine release assay) rely on the detection of histamine-releasing activity in patient's serum on basophils from healthy donors. On the other hand, binding assays (immunoblotting, ELISA, immunoenzymometric assay) are based on the detection of immunoreactivity of autoantibodies in patient's serum with relevant targets (Fiebiger et al, 1998; Eckman et al, 2008). Comparative studies of functional and binding assays for IgG anti-FcεRIα autoantibodies revealed discrepancies between the results of immunoblotting, ELISA, immunoenzymometric assays and serum-induced basophil histamine release assays in CSU (Altrich et al, 2009; Ferrer et al, 1998). The possible explanations may include biological and methodological reasons. Firstly, this discrepancy may be due to the limitations of the methodology. Serum histamine-releasing assay is characterized by a considerable variability between the healthy donors, insufficient sensitivity, requires pre-selection of donors with the response to tested sera within the pre-defined range to reduce variability and the variation of laboratory protocols between the research centres, therefore, it is not standardised (MacGlashan Jr., 2013). Binding of natural autoantibodies, the lack of reactivity due to conformational change of the blotted α-chain of the high affinity IgE receptor and cross reactions of α-chain with IgG autoantibodies directed against other autoantibodies or carbohydrates may interfere with the results of binding assays (Pachlopnik et al, 2004). Secondly, the biological reasons may refer to the chosen cut-offs for positive serum histamine releasing activity in CSU in relation to healthy donor basophils and it may differ from the cut-off value relevant *in vivo* in CSU patients. The observation by Kaplan and Joseph (2007) suggests sera from CSU patients without serum histamine-releasing activity, as detected by functional assays, induces 10 times higher histamine release from healthy donor basophils than sera from healthy subjects. Participation of histamine-releasing factors other than autoantibodies could be a confounder. Finally, we do not know at present whether the presence of anti-FcεRIα autoantibodies is a primary or a secondary event in the context of CSU pathophysiology. The experiments to determine whether characterised anti-FcεRIα autoantibodies could induce weal and flare responses at physiological concentrations in CSU patients have not yet done.

The possibility of co-expression of anti-FcεRIα and anti-IgE antibodies and, for example, genetic or acquired susceptibility of effector cells to the activation with these functional antibodies as a predisposing factor for CSU in some patients should be considered. Furthermore, the interpretation of the original research twenty years ago was made in the context of background knowledge at that time. Even now, with our limited understanding of the biology and the role of these antibodies in health and CSU, we cannot fully appreciate the complexity of the CSU pathophysiology. For example, naturally occurring antibodies have been recently implicated in the apoptosis of granulocytes (von Gunten &

Simon, 2012), whether, by analogy, anti-FcεRIα antibodies play a role in basophil removal from the circulation is unknown.

In the literature and expert community in the field of CSU, the opinions about the clinical and pathophysiological relevance of anti-FcεRIα antibodies in CSU are rather polarized. This only reveals our limited knowledge of the exact molecular mechanisms underlying the clinical expression of CSU. The differences in the methodology, patient selection and study designs do not allow for direct comparisons and comprehensive interpretation of existing research in this area.

The variety of autoantibodies to diverse cellular targets involved in the pathophysiology of this disease. The most common autoantibodies include antithyroid (O'Donnell et al, 2005) and antinuclear autoantibodies (Confino-Cohen et al, 2012). The spectrum of autoantibodies in CSU include anti-endothelial autoantibodies (Grattan, 1995), anti-parietal autoantibodies (Mete et al, 2004) and autoantibodies against FcεRII receptors on the surface of eosinophils (Pucetti et al, 2005). Recently, anti-dsDNA IgE antibodies have been described to have histamine-releasing activity towards basophils in CSU patients (Hatada et al, 2013).

In vivo activation and intrinsic functional aberrations of effector cells in CSU. In the peripheral blood, there is a shift in blood differential. Patients with CSU were shown to have reduced numbers of circulating basophils and eosinophils. Lymphocyte counts were also reported to be lower in patients with CSU compared to healthy controls (Grattan et al, 2003). CSU patients with positive autologous serum skin test were divided into two groups with normal or reduced (<1500 cells/ml) lymphocyte count in the peripheral blood (Garmendia et al, 2006). The pathophysiology of CSU is characterised by *in vivo* activation of the effector cells. Basophil immunophenotyping in CSU revealed increased expression of the high affinity IgE receptor FcεRI and basophil activation markers such as CD63 and CD69 (Vasagar et al, 2006). Patients with autoreactive CSU are characterized by increased expression of CD123 on peripheral blood basophils (Dyke et al, 2008). Increased expression of CRTH2 on peripheral blood eosinophils has been suggested but needs to be further characterised (Yahara et al, 2010). T lymphocytes in

CSU appeared to have an increased expression of CD40L, whether this may be important for polyclonal activation of B lymphocytes remains to be established (Loria et al, 2001).

Furthermore, CSU is characterized by abnormal functional status of effector cells and their reactivity to the activation stimuli (Saini, 2009). Two patient subsets were recognized based on the response of their peripheral blood basophils to *in vitro* anti-IgE stimulation. Anti-IgE stimulation induced normal or reduced (<10% of total histamine) histamine release from peripheral blood basophils in some patients with CSU. In CSU abnormal capacity of immune cells for secretion of pro-inflammatory cytokines and chemokines was observed. In autoreactive CSU, aberrant functional reactivity of T and B lymphocytes in response to activation stimuli was described but needs detailed characterization in further studies (Loria et al, 2001).

Several approaches for the patient classification in CSU were proposed based on different pathophysiological parameters (Saini, 2014). The research team from the St John's Institute of Dermatology (London, UK) proposed a classification based on the detection of serum histamine-releasing activity in CSU patients (Sabroe et al, 2002). This study classified CSU patients into subsets based on the presence of serum histamine-releasing activity and anti-FcεRIα and anti-IgE autoantibodies. In this study, serum histamine-releasing activity was associated with anti-FcεRIα autoantibodies. Another pathophysiological classification was proposed by a research team from John Hopkins University (Baltimore, USA) and was based on basophil releasability to anti-IgE stimulation in CSU patients. In the work by Vonakis and associates (2007), CSU patients were subdivided on the basis of basophil functional subsets (responders and non-responders to anti-IgE stimulation). An observational study in CSU patients revealed that basophil functional phenotypes were observed regardless of the presence of anti-FcεRIα autoantibodies as detected by an immunoenzymoetric assay (Eckman et al, 2008). The authors also reported the lack of relationship between basophil functional subsets and serum histamine-releasing activity in CSU patients. Therefore, it was concluded that autoantibody-mediated desensitization of the high affinity IgE receptor appeared to be an unlikely cause for abnormal basophil releasability to anti-IgE stimulation. Patients' basophils releasing histamine to anti-IgE stimulation show reduced SHIP-1 expression level while non-responding basophils have increased SHIP-2 levels (Vonakis, et al., 2007). Also, clinical implications of basophil functional phenotypes were suggested in the study by Baker and colleagues (2008) but this needs to be further elucidated in well-designed studies. The direct comparisons between these studies is not possible due to the variation in the methodology which was reflected in the correspondence between ourselves (Grattan & Borzova, 2009) and the research team from John Hopkins University (Eckman et al, 2009). The detection of serum histamine-releasing activity relies on basophil releasability assays which are characterized by a considerable variability in basophil releasability displayed by different donors (MacGlashan Jr., 2013). In the study by Eckman et al (2008), the presence of anti-FcεRIα autoantibodies was defined by an immunoenzymometric assay. As for the binding assays, non-specific binding was reported due to the conformational changes in blotted α-chains of the high-affinity IgE receptor (Kaplan & Joseph, 2007). Both methodologies have limitations and showed a lack of correlation (Eckman et al, 2009). Hence, the use of both classification approaches in the same study population of CSU patients would be of interest may reveal the relative contribution of serum histamine-releasing activity and basophil releasability to anti-IgE stimulation to disease severity and the clinical course of disease.

Therefore, the discrepancy seems to be related to differences in patient selection and a choice of methodological approaches for detection of anti-FcεRIα autoantibodies (Grattan & Borzova, 2009). CSU patients with functional autoantibodies are known to have a profound basopenia, therefore, these patients were likely to be excluded from the studies using basophil histamine release assays to anti-IgE stimulation for technical reasons. As a result, studies into basophil functional subsets or serum histamine-releasing activity in CSU may refer to different subpopulations of CSU patients. In addition, the results of binding and functional assays for anti-FcεRIα autoantibodies do not correspond, possibly, due to the presence of natural FcεRIα autoantibodies (Pachlopnik et al, 2004). Consequently, the detection of the immunoreactivity of anti-FcεRIα autoantibodies by binding assays does not predict their functionality and, thus, their pathogenic potential.

Obviously, functional anti-FcεRIα autoantibodies and basophil functional subsets are crucial pathophysiological determinants in CSU. The pathophysiology of CSU is likely to depend on both "the seeds" (the presence of autoantibodies) and "the soil" (skin mast cells and basophils) (Grattan, 2010) Therefore, a combined methodological approach of an anti-IgE basophil histamine release assay on patient's basophils and a serum-induced basophil histamine release assay using basophils from healthy donors would enhance our understanding of a complex interaction of functional autoantibodies and their target cells (basophils and mast cells) in CSU and would help pathophysiological phenotyping of CSU patients.

Skin autoreactivity in CSU. Autologous serum skin test is characterized by a weal and flare response within 30 min of an intradermal injection of autologous serum. Autologous serum skin test is a simple cost-effective procedure which is readily available in routine clinical practice and recommended by the EAACI task force (Konstantinou et al, 2009). CSU associated with a positive autologous serum skin test is considered as autoreactive CSU. The frequency of serum autoreactivity in CSU based on cumulative data of combined analysis of 50 studies was estimated at 45.6% (Krause et al, 2009).

A few pointers for autoreactive CSU have emerged over the last 20 years. Four percent of CSU patients with a positive autologous serum skin test have a family history of CSU in first-degree relatives (Asero, 2002). The frequency of thyroid autoantibodies is higher in patients with a positive test. Patients with autoreactive CSU also show a very strong association with HLA DRB1*04 (O'Donnell et al, 1999). Patients with serum autoreactivity require higher doses of antihistamines on an as needed basis. Patients with a positive autologous serum skin test were shown to have high expression of CD123 on their basophils (Dyke, et al., 2008) and higher levels of soluble CD154 (Garmendia et al, 2004) in their serum that those with negative test results.

CSU patients with a positive autologous serum skin were found to have a longer disease duration. In prospective studies, a positive autologous serum skin test appeared to be an important predictor for a longer disease duration. A positive autologous serum skin test tends to become negative during a spontaneous remission of CSU although a positive autologous serum skin test in patients with concominant autoimmune thyroiditis (Fusari et al, 2005) is likely to persist even after a year of resolution of CSU. Further research into the natural course of disease and corresponding changes in the autologous serum skin test may help a prognosis stratification and thereby a choice of treatment strategy depending on the prognosis.

Serial testing with autologous serum may be helpful for assessing the efficacy of certain treatments in CSU. Treatment with ciclosporin was shown to affect the patient's skin response to autologous serum. In a study by DiGioacchino et al (2003), there was a significant reduction of the autologous serum skin test score in all patients who underwent treatment with ciclosporin. Following treatment, 13 out of 16 patients who developed a long-term remission for 9 months and more, showed a negative autologous serum skin test during the follow-up. O'Donnell and colleagues (1998) found that patients with a conversion to a negative autologous serum skin test within six months after treatment with intravenous immunoglobulins developed a complete sustained remission of CSU. This was also described in a case report of a patient with severe autoreactive CSU having a negative autologous serum skin test six months after therapy with intravenous immunoglobulins (Klote et al, 2005) and a positive autologous serum skin test later during relapse of disease. Repeated skin testing with autologous serum in some patients with severe autoimmune CSU after plasmapheresis revealed significantly reduced skin test responses to fresh post-exchange autologous serum compared with stored pre-exchange serum (Grattan et al, 1992). These data indicate the possibility of using an autologous serum skin test as a treatment efficacy marker and a prognostic factor for a disease remission but the optimal timing for post-treatment testing is yet to be established.

Nevertheless, clinical relevance and the pathophysiological mechanisms underlying serum autoreactivity in CSU remain incompletely understood. The positivity of autologous serum skin test was related to the presence of functional autoantibodies although other circulating pro-inflammatory factors (e.g. IL-18) may play a role in serum autoreactivity (Tedeschi et al, 2007) but their contribution is unknown. Ultrastructural studies proved mast cell degranulation at the site of intradermal skin testing with autologous serum in patients with CSU (Grattan et al, 1990). Histological studies of skin biopsies taken from the site of a positive autologous serum skin test demonstrated a mixed proinflammatory cellular infiltrate (Caproni et al, 2005). Some analogies were made with a late-phase allergic skin reaction based on histological features of skin biopsy specimens (Grattan et al, 1990). Although these two types of skin reactions are similar in their clinical and histological presentations, autologous serum skin test and late-phase allergic reactions vary in terms of timing and kinetics of their development and the composition of their cellular infiltrate (Kaplan, 2010). Moreover, a positive autologous serum skin test has been reported in several inflammatory conditions such as asthma (Taskapan et al, 2008) and nasal polyposis (Zambetti et al, 2010) using the criteria for positivity validated for CSU. However, this is in keeping with the original observation of a positive autologous serum skin test by Malmros (1946) in various inflammatory diseases, including CSU.

Further research in the pathophysiology of serum autoreactivity in CSU is crucial for our understanding of the mechanisms and clinical significance of a positive autologous serum skin test in CSU that will guide its potential use as a prognostic and predictive biomarker and a marker of therapeutic efficacy in CSU.

Skin microenvironment in CSU. Most data on the skin inflammation in CSU comes from histological studies suggesting a marked inflammatory infiltrate with increased expression of inflammatory mediators and cytokines in the dermis of CSU patients. Elevated levels of histamine were noted in the skin of CSU patients (Kaplan et al, 1978) (Figure 5). Additionally, expression of CGRP and VEGF was increased in lesional but not uninvolved skin in CSU patients compared to healthy subjects (Kay et al, 2014b). Furthermore, skin inflammation in CSU is characterised by a dense inflammatory infiltrate in the dermis. Skin mast cells demonstrated a threefold increase in the skin of CSU patients compared to healthy subjects (Kay et al, 2014a). The inflammatory infiltrate in CSU is characterised by significant increases in the intradermal CD3+, CD4+, CD8+ and CD25+ T cells, eosinophils, neutrophils, basophils and macrophages compared to nonatopic control subjects (Ying et al, 2002). Furthermore, *in situ* hybridization revealed Th0 cytokine profile, with increases in IL-4, IL-5 and IFN-γ mRNA+ cells (Ying et al, 2002). The skin of CSU patients was showed higher immunoreactivity to TNF-α and IL-3 compared to healthy controls (Hermes et al, 1999).

Functional profile of the effector cells in the skin inflammation in CSU can be explored *in vivo* by sampling techniques such as suction blister technique or cutaneous microdialysis (Figure 7). Using skin chamber technique, skin mast cell releasability was shown to fluctuate with the disease severity in CSU (Brunet et al, 1988). To our knowledge, there have been no published reports using cutaneous microdialysis in CSU. Cutaneous microdialysis is well-established technique for *in vivo* sampling of the interstitial fluid in the dermis (Clough, 1999). It enables *in vivo* monitoring of mediator release in response to various stimuli under minimally invasive conditions. The use of cutaneous microdialysis may provide novel insights into *in vivo* continuous inflammatory events in the skin of CSU patients that are of great interest and would contribute to better understanding of a dynamic process of skin pathophysiology in CSU.

1.6 Discussion of Key Elements

1.6.1 Mast cells

Development. Mast cells arise from CD13+CD34+CD117+ hematopoietic progenitor cells (Metcalfe et al, 1997). Mast cell progenitors egress from the bone marrow, enter the circulation and then migrate to various tissues to differentiate into mature mast cells (Wernersson & Pejler, 2014). In the tissues, local growth factors such as stem cell factor and IL-3 are important for mast cell differentiation (Gurish & Austen, 2012). The lifespan of mast cells in tissues ranges from weeks to months (Voehringer, 2013).

Figure 7. *In Vivo* Skin Sampling Research Techniques

A. Suction Skin Blister Technique

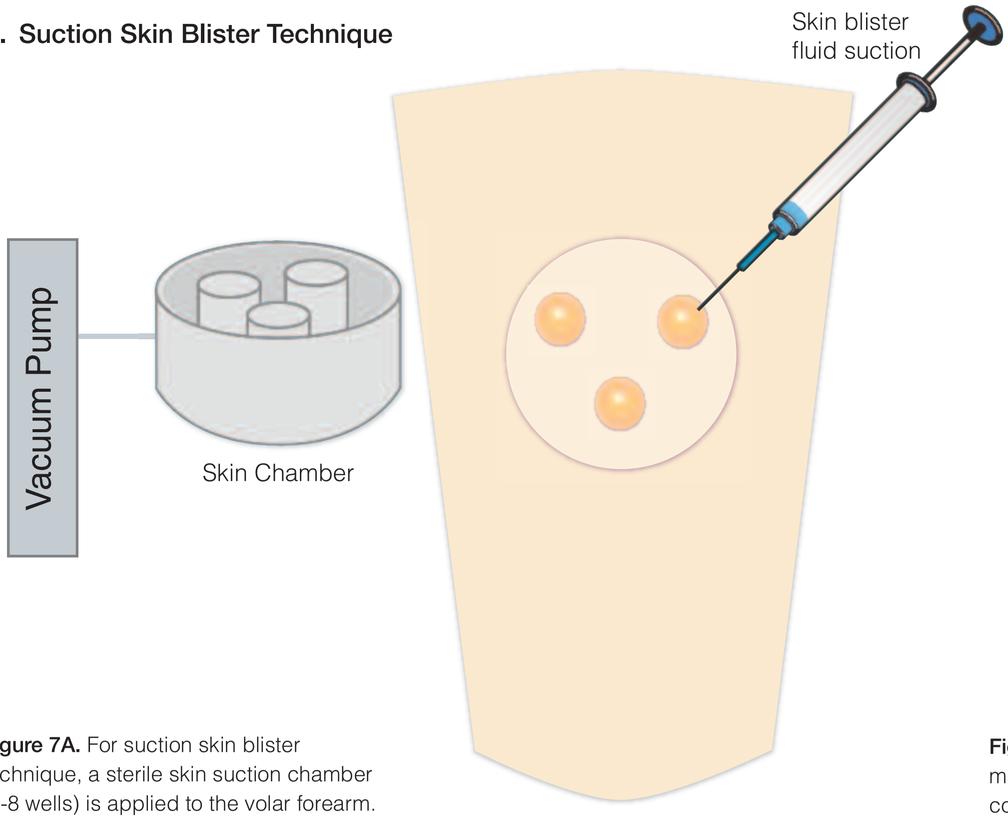


Figure 7A. For suction skin blister technique, a sterile skin suction chamber (3-8 wells) is applied to the volar forearm. Suction pressure (300-800 Pa) is delivered by a suction pump. The use of a heating pad around the suction chamber promotes blister formation. The blister fluid can be aspirated with micro syringes from fully-developed blisters for further analysis. Alternatively, the blisters can be de-roofed and a skin chamber can be applied for continuous sampling of exudation fluid or an observation of cellular migration. The advantages of the technique include site-specific and longitudinal sampling. Also, suction skin chamber technique offers an advantage of a prolonged skin challenge for studies in the skin functional state in health and skin inflammatory conditions. The limitations of the technique are associated with traumatic experimental conditions.

B. Cutaneous Microdialysis

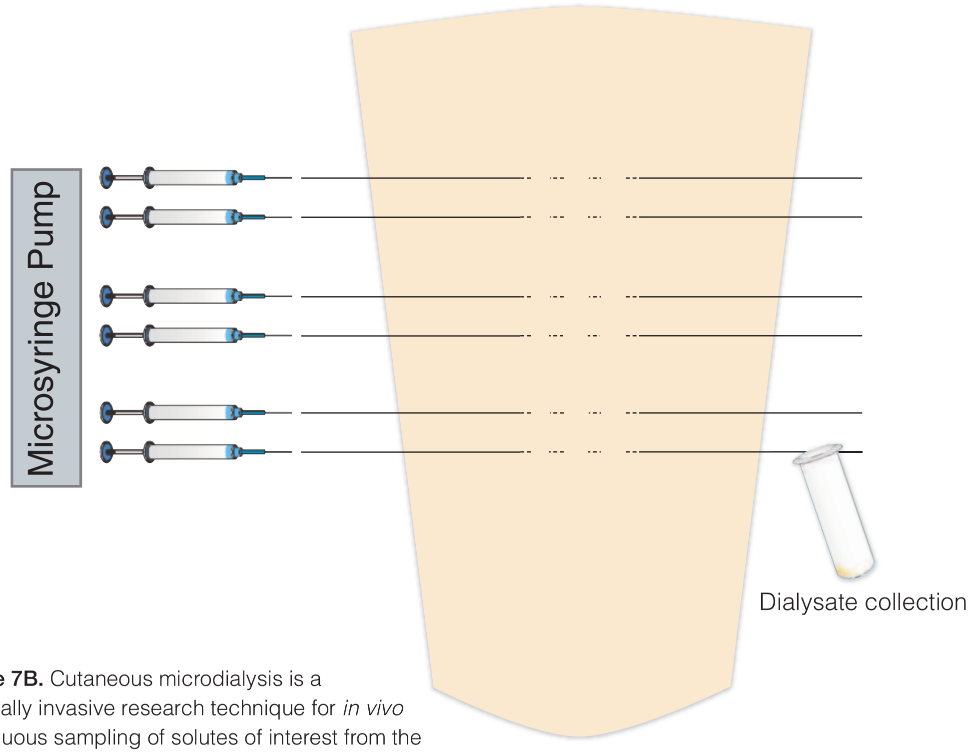


Figure 7B. Cutaneous microdialysis is a minimally invasive research technique for *in vivo* continuous sampling of solutes of interest from the extracellular space in dermis. The technique is widely applied for studies in the skin functional state, skin metabolism and pharmacokinetic studies. The technique offers an advantage of *in vivo* continuous sampling from multiple sites under minimal insertion trauma. The limitations of the technique include an exaggerated intrinsic skin response (dermal hyperreactivity) to the insertion of the microdialysis probes in some individuals and an analytical challenge of detecting low concentrations of the solute of interest in very low volumes of skin dialysates. In addition, functional microdialysis studies revealed a high intra- and inter-individual variability of mediator release in response to various stimuli. Overall, cutaneous microdialysis is a valuable sampling technique for research into skin inflammation. This sampling approach holds a promise for skin biomarker discovery and biomonitoring in various diseases.

Heterogeneity. Mast cells were classified according to their phenotype and tissue distribution. In mice, mucosal and connective tissue mast cells have been described as distinct subtypes. These mast cell subtypes differ in their granular content of proteases. Mucosal mast cells (MC_T) contains only tryptase while connective tissue mast cells (MC_{TC}) contains tryptase, chymase, cathepsin G, carboxypeptidase A3. Mucosal mast cells are located predominantly in the lung, nose and small intestine (Grant & Leonard, 2002) whereas connective tissue mast cells reside in the skin, blood vessels, gastrointestinal submucosa, heart and synovium.

Morphology. Mast cells are 6-12 µm in diameter with round or ovoid nuclear morphology (Voringher, 2013; Stone et al, 2010). By electron microscopy, mast cell morphology is characterised by electron dense secretory granules in the cytoplasm. At the ultrastructural level, cytoplasmic granules can be distinguished into scroll-, crystal- or particle-containing granules. Tryptase is known to be located in crystalline structures (Dvorak, 2005) while chymase is found predominantly in electron-dense areas (Whitaker-Menezes et al, 1995).

Surface markers. Mast cells express numerous receptors including KIT (CD117), FcεRI, FcγRI and FcγRIIa (CD32a), C3a and C5a receptors, IL-3R, IL-4R, IL-5R, IL-9R, IL-10R, GM-CSFR, IFN-γR, CCR3, CCR5, CXCR2, CXCR4, nerve growth factor receptor, and Toll-like receptors (Stone et al, 2010), β_2-adrenergic receptor, the adenosine receptor A2B, and the prostaglandin PGE_2 (Gilfillan & Beaven, 2011).

Distribution. Mast cells are distributed throughout the body in connective tissues and mucosal surfaces (Puxeddu et al, 2003) nearby blood vessels and nerve fibres. Mast cells predominantly reside in proximity to the body surfaces exposed to the environment, including skin, airway and the gastrointestinal tract (Galli et al, 2005). Mucosal mast cells are predominantly located in gastrointestinal mucosa and the respiratory tract. By contrast, connective tissue mast cells are distributed in gastrointestinal submucosa and skin.

Mediators and biological effects. Mast cells release various mediators including histamine, proteases such as tryptase, chymase and/or carboxypeptidase A3, proteoglycans (heparin and chondroitin sulfates), major basic protein, lipid-derived mediators, including PGD_2, PGE_2, LTB_4, LTC_4, LTD_4 and LTE_4, PAF and certain cytokines, chemokines and growth factors (Wernersson & Pejler, 2014; Galli & Tsai, 2012). Proteases are the most abundant constituents of the cytoplasmic granules in mast cells. The functions of serglycin proteoglycans (such as heparin) include storage of pre-formed mediators within the secretory granules as well as anticoagulation, inhibition of the complement cascade and chemoattraction of eosinophils (Grant & Leonard, 2002). Histamine effects include smooth muscle constriction, vasodilatation and increased vascular permeability, activation of nociceptive neurons and mucus secretion (Grant & Leonard, 2002; Stone et al, 2010). Tryptase is known to activate fibroblasts, degrade neuropeptides, cleave C3a, promote eosinophil and neutrophil recruitment and cause mast cell degranulation (Grant & Leonard, 2002). LTB_4 is a potent bronchoconstrictor and a chemoattractant of leukocytes (Stone et al, 2010; Grant & Leonard, 2002). PGD_2 is also a bronchoconstrictor, it increases vascular permeability and recruits eosinophils and basophils into the sites of inflammation, its active metabolite (9α, 11β-PGF_2) is known as a constrictor of coronary arteries (Stone et al, 2010; Grant & Leonard, 2002). Multiple cytokines produced by mast cells include IL-3, IL-4, IL-5, IL-6, IL-10, IL-13, IL-33, GM-CSF and TNF-α (Gilfillan & Beaven, 2011). TNF-α is a major cytokine produced by mast cells which up-regulates endothelial and epithelial adhesion molecules, attracts neutrophils, increases bronchial hyperresponsiveness and has antitumour activity (Stone et al, 2010; Grant & Leonard, 2002). Human mast cells also produce several chemokines, including CXCL8 (IL-8), CCL2, CCL3 (macrophage inflammatory protein 1α), CCL5 (Stone et al, 2010; Gilfillan & Beaven, 2011).

Mechanisms of activation. Mast cells can be activated by the cross-linking of FcεRI receptors due to the binding of a multivalent allergen to FcεRI-bound IgE on the surface of mast cells. Mast cells can be also activated via the FcγRI receptor. Other triggers for mast cell activation include complement fragments (C3a and C5a), stem cell factor, major basic protein, neuropeptides, various cytokines, bacterial peptides and certain venoms and toxins and pharmacological agents (48/80, opioids) (Metcalfe et al, 1997, Grant & Leonard, 2002). Upon activation, pre-formed mediators can be released in soluble form by compound exocytosis, proteoglycans and proteoglycan-bound compound (chymase, carboxypeptidase A3, TNF-α) can be deposited as granule remnants in the surrounding tissue (Wernersson & Pejler, 2014). In contrast, piecemeal degranulation occurs by gradual vesicular traffic through the cytoplasm (Dvorak, 2005).

Signalling. The signal transduction pathways via the FcεRI receptor involve ITAMs in the cytosolic domains of the β and γ chains that are phosphorylated by the Src family kinase Lyn. Then, the tyrosine kinases Syk and Lyn initiate the signalling that involves adaptor protein linker of activated T cells with the subsequent activation of phospholipase Cγ (PLCγ). Following this, PLCγ cleaves phosphatidylinositol 4,5 biphosphate into inositol triphosphate and diacylglycerol which leads to calcium release from the endoplasmic reticulum and, subsequently, to mast cell degranulation. Inhibitory signalling in mast cells occurs via the FcγRIIb IgG receptor, gp49B1, Siglecs, signal regulatory protein-α (SIRPα), human leukocyte immunoglobulin-like receptor, subfamily B, member 4 (LILRB4) and CD300a, TGF-β, IL-10, CD200, CD72, and intracellular signalling molecules such as LAB (linker for activation of B cells) and RABGEF1 (RAB guanine nucleotide exchange factor 1) (Gilfillan & Beaven, 2011; Kalesnikoff & Galli, 2007).

Role in health. Under physiological conditions, mast cell functions regulate epithelial secretion and permeability, peristalsis and bronchoconstriction and endothelial functions including blood flow, coagulation and vascular permeability (Kalesnikoff & Galli, 2005). Mast cells are involved in wound healing by promoting the recruitment of inflammatory cells to the site of the injury and then by stimulating re-epithelialization and angiogenesis (Moon et al, 2010; Wulff & Wilgus, 2013). Mast cells are crucial for recognition of pathogens and regulating innate immune responses (Abraham & St. John, 2010). Mast cells are responsible for pathogen recognition through pattern recognition receptors and Toll-like receptors. Also, mast cells contribute to host resistance to certain parasites

(Moon et al, 2010). Mast cells are known to play a role in host defence against toxins such as snake and honeybee venoms (Metz et al, 2006).

Role in disease. Mast cells are implicated in allergic and autoimmune diseases, cancer and fibrosis. In autoimmune diseases, for example, mast cell-derived TNF-α is likely to contribute to the amplification of local inflammation in rheumatoid arthritis (Kritas et al, 2013). Mast cells were also shown to facilitate angiogenesis and contribute to tumour survival (Kalesnikoff & Galli, 2005).

Role in allergic inflammation. The role of mast cells in the pathophysiology of allergic inflammation includes the elicitation of the early phase and contributions to the late phase by recruitment of other cells, tissue infiltration and cell activation in the tissues and participating in tissue remodelling that occurs in chronic allergic inflammation (Galli & Tsai, 2012).

Role in CSU. Mast cells are regarded as the key effector cells in eliciting and maintaining the inflammation in CSU. *In vitro* experimental evidence suggests that mast cells can be activated by functional autoantibodies against the high-affinity IgE receptor and IgE present in patient's serum (Niimi et al, 1996) although the definitive proof that serum histamine-releasing activity towards skin mast cells is attributed to anti-FcεRIα antibodies in patient's serum *in vivo* is lacking. In CSU patients, mast cell degranulation leads to the release of histamine which is a crucial mediator causing clinical manifestations of CSU. Evidence for mast cell degranulation in CSU comes from electron microscopy studies of the autologous serum skin test response (Grattan et al, 1990) and immunohistochemical detection of mast cell-derived proteases in the skin of CSU patients (Vena et al, 2002). Several lines of evidence suggested diverse effector functions of mast cells in the pathophysiology of CSU including leukocyte recruitment and control over vascular permeability. *In vitro* data demonstrated that collection of the supernatant of mast cells activated by CSU patients' sera (with or without functional autoantibodies) resulted in up-regulation of adhesion molecules on endothelial cells and vascular plasma leakage in an *in vitro* model (Bossi et al, 2011; Lee et al, 2002). Mast cells are known to induce leukocyte recruitment into the site of inflammation. In addition, immunoregulatory functions of mast cells in chronic inflammation have recently been of great interest (Metz et al, 2007). There is evidence for a crosstalk between mast cells and T-lymphocytes in CSU as demonstrated by a correlation between histamine release and IL-2 production (Hidvegi et al, 2003).

The changes in numbers of mast cells in skin of CSU patients are still debated. Some authors suggested that mast cells are increased up to 10-fold in CSU but these data were criticized for methodological reasons (Natboni et al, 1983). In general, normal or slightly elevated mast cell counts (up to 3 times) were reported in skin biopsy specimens from CSU patients (Kay et al, 2014a; Haas et al, 2001). Mast cells undergo phenotypic changes during the inflammation in CSU as was shown by their lower threshold for activation in the flare-up of the disease compared to the remission of CSU (Jacques et al, 1992). Mast cells are known to contribute to tissue remodelling in chronic inflammation (Maurer et al, 2003). Although increases in serum MMP- 9 (Kessel et al, 2005) and VEGF (Kay et al, 2014b) were reported in CSU, their effect on skin matrix composition and vessel remodelling in CSU was not studied and this aspect of mast cell biology in CSU remains unknown.

1.6.2 Basophils

Basophils are the rare granulocytes accounting for less than 1% of circulating leukocytes.

Development. Basophils develop from CD34+ pluripotent stem cells through a common basophil-eosinophil precursor (Prussin & Metcalfe, 2003). Basophils undergo differentiation and maturation in the bone marrow before their release into the circulation as fully matured cells (Prussin & Metcalfe, 2003).

Heterogeneity. There is limited evidence regarding basophil heterogeneity. In density centrifugation studies, two subpopulations of basophils were distinguished by differences in their density. Low-dense basophils differed from basophils with normal density in terms of cellular histamine content and higher sensitivity to glucocorticosteroids (Bochner, 2000). Alternatively, two basophil subsets were noted with regard to their response to anti-IgE stimulation. Releaser and nonreleaser basophils to anti-IgE stimulation were described in healthy subjects, patients with CSU and asthma (Youseff et al, 2007; Vonakis et al, 2007). Additionally, basophil functional plasticity was suggested in view of differential activation depending on cytokine stimulation (Siracusa et al, 2012). Thus, basophils produced more cytokines and chemokines following the stimulation with TSLP whereas after IL-3 stimulation basophils produced histamine (Siracusa et al, 2012).

Morphology (including granular content). Basophils are 5-7 μm in diameter. Their morphological features include a segmented nucleus with highly condensed chromatin and cytoplasmic secretory granules (Prussin and Metcalfe, 2003). In human basophils, granules contain pre-formed mediators including histamine, Charcot-Leyden crystal protein, major basic protein, cathepsin G. In basophil granules, chondroitin sulphate A is the predominant proteoglycan, which is important for the storage of preformed mediators (Grant & Leonard, 2002). In healthy subjects, there are negligible amounts of tryptase in basophil granules although basophils from allergic patients are known to have detectable levels of tryptase upon allergen challenge (Grant & Leonard, 2002).

Distribution. Basophils are found in the circulation and can be recruited into tissues in inflammation (Ito et al, 2011).

Mediators and biological effect. Basophils produce a variety of mediators and cytokines including histamine, leukotrienes (LTC_4, LTD_4, LTE_4) and PAF, IL-4, IL-13, IL-3, IL-5, IL-6, IL-8, IL-10, IL_12, TNF-α, MIP-1α and RANTES (Prussin & Metcalfe, 2003; Grant & Leonard, 2002). Histamine a potent vasoactive mediator produced by basophils. In addition, histamine has diverse effects mediated by H1-H4 receptors which are summarized in the Section 1.6.3 of the thesis. LTC_4 is a potent bronchoconstrictor, which is synthesized *de novo* in basophils from arachidonic acid and then converted to LTD_4 and LTE_4 (Harvima et al, 2014). Secretion of IL-4 contributes to Th2 differentiation (Gibbs, 2011).

Surface markers. Basophils express numerous receptors including immunoglobulin Fc receptors (FcεRI, FcγRII), cytokine receptors (IL-3R, IL-5, GM-CSF), complement receptors (CD11b, CD11c, CD35, CD88), prostaglandin receptors (CRTH2), co-stimulatory molecules (CD40L), adhesion molecules (VLA-4, VLA-5, all four β2 integrins, α4β7, L-selectin, PSGL-1 and sialyl Lewis), chemokine receptors (CCR1, CCR2, CCR3 and CXCR4) and Toll-like receptors (TLR-2 and TLR-4) (Marone et al, 2005). Basophil activation markers include CD63, CD203c, CD107a and CD107b, CD13 and CD69 (MacGlashan Jr., 2009; Yoshimura et al, 2002).

Mechanism of activation. Basophil activation can occur in response to various stimuli via activating receptors such as 2B4, LIR7, leptin receptor, FcεRI, FcγRIIA, CD200R3, C3aR, C5aR, LTB4R1, IL-3R, IL-18R, IL-33R, TSLPR (Voeringer, 2013). Allergens induce cross-linking of membrane-bound IgE on the surface of basophils whereas non-IgE-mediated basophil activation can be caused by parasitic antigens, lectins and viral superantigens (Gibbs, 2008). Basophil activation via FcεRI, C3a, C5a receptors results in histamine release, eicosanoid synthesis and cytokine IL-4 and IL-13 secretion (Prussin & Metcalfe, 2003). Signalling through the IL-3 receptor leads to basophil expansion, survival and activation in cooperation with other stimuli (Sullivan & Locksley, 2009). Additionally, IL-3, nerve growth factors, IL-33 can also induce cytokine release from human basophils (Gibbs, 2008). Basophil priming factors include IL-3, NGF, CC chemokines (eotaxin, monocyte chemoattractant protein 3, monocyte chemoattractant protein 4, RANTES), N-formyl-methionyl-leucyl-phenylalanine, IL-3, IL-5, GM-CSF (Prussin & Metcalfe, 2003; Gibbs, 2008). In basophils, granule exocytosis may occur via anaphylactic or piecemeal degranulation (Dvorak, 2005).

Signalling. The signal transduction pathways in basophils are incompletely understood. Signalling elements such as Syk and Lyn kinase are important in positive regulation of a signal transduction associated with FcεRI activation whereas lipid phosphatases SHIP-1 and SHIP-2 are negative regulators of signal transduction in basophils (Saini, 2005). SHIP expression was shown to be up-regulated via CD300a, CD200R, Siglec-8 and FcγRIIb (Gibbs, 2011). Dysregulated SHIP expression in basophils was reported in CSU (Vonakis et al, 2007). Signalling by IL-33 via a receptor complex of ST2 receptor and IL-1 receptor accessory protein is dependent on the adaptor protein MyD88 pathway

(Kakkar & Lee, 2008) and appears to be dysregulated in allergic diseases (Saluja et al, 2014).

Role in health: Basophils play a role in host defence against ticks (Voehringer, 2013) and helminths (Schwartz & Voehringer, 2011). Basophils accumulate in the dermis and epidermis at the sites of secondary infestation by ticks and confer tick resistance via yet unknown mechanisms (Voehringer, 2013). In mouse Mcpt8 models, depletion of basophils led to the loss of tick resistance (Wada et al, 2010). Basophils are accumulated in the inflamed tissues during helminth infections and may contribute to protective immunity against helminth infections by production of Th2 cytokine, proteases or by other yet unidentified mechanisms (Karasuyama et al, 2010). Basophils are also implicated in angiogenesis, presumably, via secretion of angiogenic cytokine VEGF and the expression of the high affinity urokinase plasminogen activator receptor, known to be involved in tissue remodelling and vessel sprouting (Crivellato et al, 2010).

Role in disease: Basophils are thought to be involved in allergic (eg. asthma), autoimmune (eg. lupus nephritis, rheumatoid arthritis,), inflammatory (eg. Crohn disease) diseases and haematological malignancies (acute and chronic myeloid leukemia) (Kaveri et al, 2010). In SLE, activated basophils are linked to disease severity (Charles et al, 2010). In myelodisplastic syndromes, basophilia was associated with significantly reduced survival (Wimazal et al, 2010). Basophils are also implicated in the pathophysiology of skin diseases such as CSU, atopic eczema and allergic contact eczema as well as bullous pemphigoid (Borriello et al, 2014).

Role in allergy and inflammation. Basophils are thought to be important participants in allergic inflammation (Schroeder & MacGlashan, 1997). Basophils accumulate in the inflamed tissues in allergic diseases (asthma, atopic dermatitis, allergic rhinitis) can promote ongoing allergic inflammation by secretion of Th2 cytokines (Falcone et al, 2011). In atopic eczema, basophils could contribute to the pathophysiology by producing angiogenic and tissue remodelling factors (de Paulis et al, 2006). In asthma basophils were shown to accumulate in airways of fatal asthma (Kepley et al, 2001). Basophils contribute to systemic and local allergic reactions (Voehringer, 2013). Thus, basophils were implicated in IgG-mediated anaphylaxis that occurs in repeated administration of chimeric and humanized monoclonal antibodies (Khodoun et al, 2011). Additionally, basophils are recruited to the late-phase allergic reactions in the skin and, to lesser extent, in the lungs (Macfarlane et al, 2000).

The role in CSU. Similar to mast cells, basophils are implicated in the pathogenesis of CSU (Saini, 2014). Basopenia is a hallmark feature of severe CSU, which is more profound in autoimmune CSU (Grattan et al, 1997a). Propensity of basophils to degranulate in response to physiologic stimuli was noted in the experiments with human serum (Luquin et al, 2005). Flow cytometry studies revealed *in vivo* basophil activation in the peripheral blood of CSU patients as demonstrated by their increased expression of CD69 and CD63 (Vasagar et al, 2006). Prospective evaluations of patient's basophils, using basophil histamine release assays, revealed an increase in basophil releasability to anti-IgE stimulation towards the remission of CSU (Kern & Lichtenstein, 1976). Research into basophil biology in CSU over the last decade brought out new insights into signalling aberrations of basophils associated with CSU. Two subsets of basophils were defined based on their response to anti-IgE stimulation. It was shown that the underlying defect in non-responding basophils was abnormal SHIP expression (Vonakis et al, 2007). Of interest, aberrant SHIP expression in human basophils occurred with no regard to the presence of histamine-releasing autoantibodies as defined by immunoenzyme assay (Eckman et al, 2008). The discovery of functional defects in peripheral blood basophils in CSU opened avenues for targeting basophil signalling with biological treatments. Whether newly discovered basophil functions such as contribution to immunoregulation (MacGlashan, 2008) or immunological memory (Denzel et al, 2008) are relevant in the context of CSU is unknown.

Differences between basophils and mast cells. Although basophils and mast cells phenotypic similarities such as the expression of the high-affinity IgE receptor, metachromatic staining, Th2 cytokine expression and histamine release, basophils are distinct from mast cells in terms of their origin, response to different secretagogues, mediator content, signal transduction pathways and modalities of pharmacological control (Schroeder & MacGlashan, 1997; Prussin & Metcalfe, 2003). Basophils and mast cells are believed to originate from different lineages of the haematopoietic system (Marone et al, 2002; Prussin & Metcalfe, 2003). Basophils are known to enter the bloodstream as fully differentiated cells whereas mast cells circulate as progenitors and complete their maturation in tissues under influence of local microenvironment (Li et al, 2000). Ultrastructurally, basophils are characterised by segmented nuclear morphology with condensed chromatin and fewer electron-dense granules as opposed to non-segmented nucleus and more abundant granules with characteristic patterns in mast cells (Li et al, 2000). In cytoplasmic granules, histamine is a common mediator for both cells, but they differ in their sulphated proteoglycans, proteases, cytokines. Chondroitin sulphate A is a proteoglycan in basophils whereas mast cells contain a mixture of heparin and chondroitin sulphate E (Li et al, 2000). In healthy subjects, basophils contain negligible amount of tryptase, whereas tryptase is a predominant granule constituent in mast cells. In addition, basophils are known to secrete LTC_4 but little or no PGD_2 whereas mast cells produce PGD_2 but little or no LTC_4 (Li et al, 2000).

1.6.3 Histamine

The structure and the metabolism of histamine. Histamine (2-(4-imidazolyl)ethylamine) is a biogenic amine which acts as a chemical messenger in the human body. The chemical structure of histamine comprises an imidazole ring and an ethylamine side chain (Figure 5). Main cellular sources of histamine include mast cells and basophils, gastric enterochromaffin-like cells and histaminergic neurons in the central nervous system (MacGlashan Jr., 2003; Panula & Nuutinen, 2013). Histamine is produced by decarboxylation of histidine by histidine decarboxylase (HDC). The expression of HDC can be modulated by various cytokines including IL-1, RANTES and TNF-α (Wu et al, 2004; Gutowska-Owsiak et al, 2014). Histamine is metabolized either by histamine N-methyl-transferase or by diamine oxidase (Figure 5) (Lieberman, 2011). The possible alterations of histamine metabolism in CSU are presented in the Figure 5.

Histamine receptors and their distribution. Histamine acts via four histamine receptor subtypes (H1, H2, H3 and H4) that belong to the G-protein-coupled receptor superfamily (Bongers et al, 2010). The histamine H1 receptor is mostly found on the endothelial cells in the vascular beds and on smooth muscle cells in the respiratory, gastrointestinal tracts and the vasculature (Thurmond et al, 2008). H1 receptors were also reported in various hematopoietic cells including neutrophils, eosinophils, monocytes, dendritic cells, T cells and B cells (Bongers et al, 2010; Simons & Simons, 2011). In human positron emission tomography (PET) studies, high density of H1 receptors was observed in the frontal cortex, the temporal cortex, cingulate and hippocampus whereas low density of H1 receptors was noted in cerebellum and pons (Yanai et al, 1995). The histamine H2 receptors are expressed on numerous cells including gastric parietal cells, smooth muscle cells, neurons and glial cells in the central nervous system and various tissues such as the cardiac tissue and the skin (Hil et al, 1997, Lieberman, 2011, Panula & Nuutinen, 2013; Greaves & Davies, 1982; Levi et al, 1981). The histamine H3 receptor is mainly expressed in the brain (predominantly in basal ganglia), the spinal cord and in the peripheral neurons (Hough & Rice, 2011; Panula & Nuutinen, 2013). In cardiac tissue, H3 receptors are expressed on sympathetic and sensory nerve endings (Immamura et al, 1996). The H4 receptor expression is the most prominent in various cells of hematopoietic origin including eosinophils, neutrophils, mast cells, basophils, dendritic cells and T cells (Zhang et al, 2007; Gibbs & Levi-Schaffer, 2012).

The pharmacology of histamine. Histamine binding to the histamine receptors results in the activation of specific intracellular G-proteins (Thurmond et al, 2008). Histamine binding to H1 receptor stabilizes the receptor in the active conformation and leads to signal transduction via G_q/G_{11} proteins with subsequent phospholipase C activation, inositol phosphate production and calcium mobilization (Simons & Simons, 2011; Thurmond et al, 2008). H2 receptors activate $G\alpha_s$ proteins and increase cyclic AMP levels (Simons & Simons, 2011). H3 receptors activate $G\alpha_{i/o}$ proteins, leading to the down-regulation of cyclic AMP production (Thurmond et al, 2008). H4 receptors activate signal transduction via $G\alpha_{i/o}$ proteins resulting in adenylate cyclase inhibition and a decrease in cyclic AMP levels (Simons & Simons, 2011).

Histamine exerts diverse physiological effects in human body. In the skin, an intradermal injection of histamine causes the activation of H1 and H2 receptors leading to the immediate weal and flare responses as described by Lewis (1927). The weal formation results from increased vascular permeability whereas the initial flare is caused by vasodilatation and the surrounding flare – by an axon reflex (Greaves, 2014; Williams, 1988). Histamine can induce itch by stimulating H1 receptors on free sensory nerve endings of the nonmyelinated C fibres in the skin (Metz & Ständer, 2010; Greaves, 2010). The role of H4 receptors in the itch transmission in humans needs to be characterized (Dunford et al, 2007). The activation of H1 receptors also up-regulates adhesion molecule expression (ICAM-1, VCAM-1 and P-selectin) on endothelial cells (Lo & Fan, 1987).

In the airways, histamine acts on smooth muscle cells causing bronchoconstriction (Dunford & Holgate, 2010). In clinical practice, histamine-induced bronchoconstriction is assessed in bronchial histamine challenge tests for measuring bronchial hyperresponsiveness in asthma (O'Byrne et al, 2009; de Meer et al, 2004). In nasal mucosa, histamine challenge reproduces the symptoms of the early phase of the allergen challenge including pruritus, sneezing, rhinorrhoea and nasal blockage (Taylor-Clark, 2010). The activation of H2 receptors in gastric parietal cells stimulates the proton pump H^+, K^+ ATPase resulting in gastric acid secretion (Thurmond et al, 2008). In the heart, H1 receptors mediate negative dromotropic effects of histamine whereas activation of H2 receptors has positive chronotropic and inotropic effects (Matsuda et al, 2004). In the central nervous system, H3 receptors are implicated as a neurotransmitter in the sleep-wake cycle, cognition, memory, appetite and energy regulation (Panula & Nuutinen, 2013). Presynaptic H3 receptors serve for a presynaptic autoregulation in histaminergic neurons whereas those in non-histaminergic neurons play a role in regulation of the release of other neurotransmitters such as GABA, glutamate, acetylcholine and noradrenaline (Smuda & Bryce, 2011; Panula & Nuutinen, 2013). The activation of H4 receptors mediate activation and chemotaxis of mast cells, eosinophils, dendritic cells and the cytokine secretion from T cells and dendritic cells (Thurmond et al, 2008).

Role of histamine in CSU. Histamine is considered to be the predominant mediator in CSU (Bernstein et al, 2014; Church & Maurer, 2012). Several lines of evidence support this hypothesis. Firstly, dermal histamine concentrations were shown to be increased in CSU patients (Kaplan et al, 1978). Secondly, the weals can be reproduced experimentally in healthy subjects by an intradermal injection of histamine (Lewis, 1927). Furthermore, the efficacy of H1 antihistamines in CSU was demonstrated in double-blind placebo-controlled studies (Zuberbier et al, 2010; Staevska et al, 2010, Kapp & Pichler, 2006, Kaplan et al, 2005).

Clinically, it is well known that the duration of weals in CSU is approximately 16-18 hours which is longer than that for histamine-induced weals (Kobza-Black, 1989; Greaves, 2014). Additionally, although highly effective for itching, H1 antihistamines only incompletely relieve wealing in some CSU patients (Greaves, 2014; Krause & Shuster, 1984). Furthermore, histamine-mediated effects in the dermal microvasculature are thought to be mediated by H1 and H2 receptors (Greaves et al, 1977) although the existing evidence for the efficacy of H2 antihistamines in CSU, as suggested by a Cochrane review, is weak and inconclusive (Fedorowicz et al, 2012). Taken together, this suggests that histamine is not the only mediator causing the development of weals in CSU and other mediators (leukotrienes, platelet-activating factor, calcitonin gene-related peptide, vascular endothelial growth factor, kinins, etc) are likely to contribute (Greaves, 2014; Kay et al, 2014b; Kobza Black, 1989).

Overall, the precise contribution of histamine to the pathophysiology of CSU is yet to be elucidated. Histamine is likely to initiate the weal formation and to induce itching in CSU although the persistence of weals is likely to be mediated by other mediators (Greaves, 2014). At present, several questions regarding the role of histamine in skin remodelling, immunoregulation and disease persistence in CSU remain unanswered.

Role of histamine in allergic inflammation. The importance of histamine in allergic inflammation was inferred from the detection of histamine at the sites of allergic inflammation in various allergic diseases, the reproduction of the symptoms of allergic diseases in the experimental challenge with histamine and from the control of allergic symptoms by H1 antihistamines (Bongers et al, 2010). Histamine is implicated in the pathophysiology of allergic rhinitis, conjunctivitis, anaphylaxis, urticaria and pruritus although its contribution to chronic asthma and eczema is likely to be less important in view of minimal efficacy of H1 antihistamines in these conditions (Howarth et al, 2000; Bernstein et al, 2014; Greaves, 2010; Kay, 2002).

In allergic rhinitis, histamine is thought to play a role in pruritus, sneezing, rhinorrhea and, to some extent, in nasal blockage (Holgate et al, 2003, Howarth et al, 2000; Taylor-Clark, 2010). In anaphylaxis, histamine-mediated events include vasodilatation, increased vascular permeability, decreased total vascular resistance, cutaneous and gastrointestinal symptoms, flushing, bronchoconstriction, cardiac arrhythmias and hypotension (Winbery & Lieberman, 2002). In allergic conjunctivitis, classic histamine-mediated symptoms are itching, tearing, conjunctival injection (redness) and chemosis (swelling) (Leonardi, 2002; Keane-Myers, 2001). In the airways, histamine can induce bronchoconstriction, mucosal oedema, recruitment of inflammatory cells and a local release of pro-inflammatory cytokines, these pharmacological effects of histamine may be relevant in the pathophysiology of asthma (Dunford & Holgate, 2010). However, it is pertinent to note that histamine is important but not a sole mediator of allergic inflammation (Howarth et al, 2000; Kay, 2002).

Furthermore, histamine plays a role in immunoregulation in chronic allergic inflammation by affecting immunological cells such as dendritic cells and T and B lymphocytes (Jutel et al, 2009; O'Mahony et al, 2011). For example, histamine affects maturation and antigen-presenting activity of dendritic cells. Histamine H1 and H3 receptors are known to increase antigen-presenting activity, pro-inflammatory cytokine secretion in dendritic cells which, in turn, stimulate the development of Th1 cells. By contrast, H2 receptors mediate the suppression of antigen-presenting activity and induce IL-10 secretion thereby facilitating the development of IL-10-producing T cells or Th2 cells (Mazzoni et al, 2001). Additionally, histamine may affect polarization of Th1/Th2 cells. By stimulating H2 receptors, histamine favors Th1-type responses whereas the stimulation of H2 receptors down-regulates both Th1 and Th2-type responses (Jutel et al, 2001). At the sites of local inflammation, histamine may enhance the production of pro-inflammatory cytokines and chemokines by various cells and may induce the recruitment of granulocytes (Meretey et al, 1991; Jeannin et al, 1994). Histamine was shown to induce chemotaxis of eosinophils and mast cells via H4 receptors (Hofstra et al, 2003; O'Reilly et al, 2002).

Antihistamines (1st and 2nd generation). H1 antihistamines act as inverse agonists by stabilizing the H1 receptor in the inactive conformation (Simons & Simons, 2011; Church & Maurer, 2014). According to the Consensus group on new generation antihistamines (CONGA), two generations of H1 antihistamines are currently recognized (Holgate et al, 2003). The first generation of H1 antihistamines comprises multiple compounds including promethazine, brompheniramine, chlorpheniramine, mepyramine, diphenhydramine, clemastine, cyproheptadine and hydroxyzine (Passalacqua et al, 2002). The second generation of H1 antihistamines is represented by acrivastine, epinastine, ebastine, loratadine, fexofenadine, mizolastine, desloratadine, azelastine, levocetirizine, bepotastine, alcaftadine, rupatadine and bilastine (Simons & Simons, 2011; Church & Maurer, 2014). Two second-generation H1 antihistamines (astemizole and terfenadine) were withdrawn from the market because of their cardiotoxicity. At present, the term 'third generation of anthistamines' should be reserved for future advances in the field (Holgate et al, 2003).

The first-generation H1 antihistamines have low selectivity towards H1 receptors and frequently cause anticholinergic effects such as dry mouth, blurred vision and urinary retention (Simons & Simons, 2011). Compared to the first-generation H1 antihistamines, the second-generation H1 antihistamines are characterized by a more favorable safety profile due to their high H1 receptor selectivity and little or no affinity for muscarinic cholinergic receptors (Holgate et al, 2003). The first-generation H1 antihistamines can easily cross the blood-brain barrier due to their lipophilicity, have higher H1 receptor occupancy in the brain and can often cause CNS side effects such as sedation, daytime somnolence, impaired REM phases of sleep and reduced cognitive function (Boyle et al, 2006; Yanai et al, 2011). These side effects lead to the reduced work efficiency, impaired ability to operate machinery or driving performance in adults and poor exam performance in children (Cockburn et al, 1999; Weiler et al, 2000). The second-generation H1 antihistamines have less sedative effects because of low or negligible brain penetration and their affinity for the P-glycoprotein efflux pump (Lieberman, 2009).

Anti-inflammatory effects of antihistamines. The second-generation H1 antihistamines were reported to exert various anti-inflammatory effects *in vitro* and *in vivo* (Leurs et al, 2002; Cuss, 1999). Anti-inflammatory effects of the second-generation antihistamines include the downregulation of adhesion molecules, the reduction of the leukocyte recruitment, the inhibition of the release of mediators and cytokines (Holgate et al, 2003). Mast cell stabilizing effects of non-sedating H1 antihistamines are likely to be receptor-independent. Second-generation H1 antihistamines are hypothesized to inhibit calcium ion channels and to reduce calcium ion current into the cells thereby stabilizing the membrane of mast cells (Levi-Schaffer & Eliashar, 2009; Church & Maurer, 2014). By contrast, the downregulation of leukocyte chemotaxis to the sites of inflammation and the inhibition of cytokine production are thought to be receptor-dependent effects that are likely to be mediated by the downregulation of the activation of the transcription factor NF-κB (Church & Maurer, 2014). Overall, the anti-inflammatory effects of second-generation H1 antihistamines are difficult to explain and their clinical relevance is yet to be established.

Histamine is known to be an essential mediator of skin inflammation in CSU (Greaves & Sabroe, 1996). Elevated levels of histamine were demonstrated in the skin of CSU patients (Kaplan et al, 1978). Functional studies in CSU using skin chamber technique demonstrated that histamine release in skin paralleled disease activity in CSU (Jacques et al, 1992). Effects of functional anti-FcεRIα autoantibodies found in CSU on skin mast cells activation were demonstrated *in vitro* (Niimi et al, 1996) and their contribution to activation of skin mast cells in CSU contributes to the pathophysiology of the disease. There is limited evidence suggesting abnormal skin metabolism of histamine in CSU (Figure 7). Skin mast cells in skin biopsy specimens were shown to have over-expression of histidine decarboxylase (Papandopoulou et al, 2005), which may suggest an increased generation of histamine from histidine in CSU. Reduced activity of diamine oxidase in CSU was shown in plasma (Lessoff et al, 1990) and intestinal mucosa (Guida et al, 2000) whether similar changes occur in skin is unknown. The role of genetic polymorphisms of histamine-metabolizing enzymes and histamine receptors in skin inflammation in CSU (Garcia-Martin et al, 2009) would be of interest but is not yet established. In mouse models, studies into the transporting system of histamine into mast cells revealed a role of bidirectional organic cation transporter 3 (OCT3) which was shown to be responsible for histamine uptake by mast cells (Ohtsu, 2008). In humans, basophil recovery from degranulation was studied in the work by Dvorak (2005). These studies raise fascinating questions whether the cellular machinery for histamine uptake and release in human basophils and mast cells is affected in CSU but this is an area for future studies.

1.6.4 IgE and IgE receptor

IgE structure. The structure of IgE molecule includes two light (L) chains associated with two heavy (H) chains (Figure 6A). Each heavy chain contains one variable and four constant domains (Cε1-4) (Miescher & Vogel, 2002). Heavy chain C-terminal constant domains (Cε2, Cε3 and Cε4) are known to dimerise to form the Fc fragment (Wurzburg et al, 2000). In comparison with IgG, IgE molecule has an additional Cε2 constant domain (Figure 6A). Two antigen-binding sites are located within the Fab fragment of IgE.

Biological effect of IgE. IgE antibodies recognize allergens in a membrane-bound form on B cells or basophils or mast cells and, therefore, play a role in allergic (Drinkwater et al, 2014). Monomeric IgE participates in a regulation of mast cell and basophil survival (Kawakami & Galli, 2002). IgE antibodies are important in gut immune homeostasis and play a role in transepithelial allergen transport (Li et al, 2006). Additionally, IgE antibodies have the capacity to up-regulate *in vivo* antibody response (Heyman, 2002). Furthermore, IgE antibodies are involved in protective parasite immunity (Falcone et al, 2001).

FcεRI receptor. A tetrameric FcεRI receptor contains four subunits: α, β and two γ chains (Figure 6B). The extracellular α chain contains the ligand-binding immunoglobulin-like domains D1 and D2. Transmembrane domain β domain amplifies the activation signal. Two disulphide-linked γ chains transmit the activation signal to the intracellular signalling pathways via two ITAMs.

In humans, tetrameric FcεRI receptor (abγ2) is expressed on mast cells and basophils whereas trimeric receptor (aγ2) is expressed on monocytes, dendritic cells, macrophages and Langerhan's cells, eosinophils, neutrophils, platelets, bronchial epithelial cells in patients with asthma, airway smooth muscle cells (Garman et al, 1998; Wu & Zarrin, 2014).

An interaction of IgE and FcεRIα occurs with high affinity (K_d =10^{-9} - 10^{-10}M) (Garman et al, 2001) via contact residues on the top of D2 domain in FcεRIα and the Cε3 domain in the Fc region of IgE molecule (Turner & Kinet, 1999; Wurzburg & Jardetzky, 2001). X-ray crystallographic studies showed an asymmetrically bent conformation of IgE in its Fc fragment that occurred upon the binding to the receptor (Wat et al, 2002).

Tetrameric FcεRI receptors on the surface of mast cells and basophils play a role in regulating immediate and late-phase allergic reactions, parasite immunity. Antigen-independent effects IgE alone without antigen are mediated via FcεRI and include enhanced mast cell survival and cytokine production (Kraft & Kinet, 2007, Galli & Tsai, 2012). FcεRI was demonstrated to be involved in parasitic elimination (Miescher et al, 2002). Additionally, FcεRI was implicated in the regulation of monocyte apoptosis and prevention of its differentiation into dendritic cells (Miescher et al, 2002).

The FcεRII receptor. The low affinity IgE receptor (FcεRII, CD23) is a type II transmembrane glycoprotein (Acharya et al, 2010). As demonstrated in Figure 6C, the structure of CD23 receptor includes three lectin heads and a three-stranded, a-helical coiled-coil "stalk" and a short cytoplasmic tail (Gould et al, 2003). CD23 lectin heads display a structural homology to C-type (calcium-dependent) lectins (Gould & Sutton, 2008). IgE binding to FcεRII occurs via Cε3 domain (Acharya et al, 2010). Single lectin domain binds IgE with low affinity (K_d=10^{-6}-10^{-7} M^{-1}), whereas the multipoint interaction of three lectin domains with IgE is characterised by high-affinity binding (K_d=10^{-8}-$10^{-9}$$M^{-1}$) (Gould & Sutton, 2008).

The low affinity IgE receptor is expressed on B cells, T cells, NK cells, monocytes, macrophages, follicular densritic cells, Langerhans cells, bone marrow stromal cells, neutrophils, eosinophils, platelets, airway and intestinal epithelial cells (Wu & Zarrin, 2014; Galli & Tsai, 2012).

On B cells, FcεRII receptor plays a role in positive and negative regulation of IgE production and facilitates antigen processing and presentation (Novak et al, 2001). By contrast, on macrophages and epithelial cells, the FcεRII mediates the uptake of IgE-antigen complexes across the intestinal epithelium (Wu & Zarrin, 2014). Furthermore, CD23 was shown to participate in cytotoxicity against tumour cells (Karagiannis et al, 2008).

Natural/autoimmune anti-IgE. Natural autoantibodies are defined as immunoglobulins produced by B cells without external antigen stimulation (Lutz, 2012). Naturally occurring anti-IgE autoantibodies can be found in healthy subjects, in patients with CSU, atopic dermatitis, asthma and autoimmune diseases such as rheumatoid arthritis, SLE and systemic sclerosis (Marone et al, 1999). Natural anti-IgE autoantibodies from some of the patients can induce histamine release from basophils and mast cells whereas anti-IgE autoantibodies from some patients with allergic diseases are non-anaphylactogenic (Stadler et al, 1996). In allergic diseases, IgG anti-IgE autoantibodies mostly belong to IgG_1 and IgG_4 isotypes (Shakib et al, 1994). The biological role of natural anti-IgE autoantibodies is unknown.

Therapeutic anti-IgE. Omalizumab (Xolair®, Genetech) is a monoclonal humanized IgG1 antihuman IgE antibody (rhumAb-E25), which binds to the free human IgE with higher affinity than the binding affinity between IgE and FcεRI. Fine epitope mapping studies revealed that the epitope in the Cε3 domain of IgE recognized by omalizumab overlaps with the binding site for the high-affinity IgE receptor (Zheng et al, 2008). This non-anaphylactogenic anti-IgE antibody does not bind to surface-bound IgE (D'Amato, 2006). Pharmacological properties of omalizumab are characterized by a high degree of isotype specificity for IgE without binding to other antibody classes (D'Amato, 2006). In addition to the reduction in the level of IgE, omalizumab results in rapid the reduction of FcεRI expression on basophils and in slower downregulation of FcεRI in mast cells

(Beck et al, 2004). Furthermore, omalizumab was shown to increase the intrinsic sensitivity of human basophils to anti-IgE stimulation (MacGlashan Jr. & Saini, 2013).

In CSU, there are several putative mechanisms can mediate the effects of omalizumab (Chang et al, 2014). Firstly, by binding free IgE, omalizumab downregulates the expression of FcεRI on basophils and mast cells thereby rendering them less sensitive to the subsequent stimulation. Secondly, omalizumab binds monomeric IgE and thus reduces their priming effects on mast cells, which might be relevant in the context of CSU. Additionally, the formation of omalizumab-IgE complexes may sequester endogenous autoantigens such as TPO and dsDNA that interact with IgE. Finally, omalizumab may reduce the expression of CD23 on B-cells and downregulate IgE-expressing B lymphoblasts and memory B cells which may result in a reduction of the generation of IgE-secreting plasma cells (Chan et al, 2013).

Another example of therapeutic anti-IgE antibodies is RG7449, which is a novel, humanized monoclonal antibody that recognizes the M1 prime segment of membrane IgE on B lymphocytes before they produce IgE. RG7449 is developed by Genetech and in Phase I/II clinical trials in asthma (Polosa & Casale, 2012).

Reagent anti-IgE. For research applications, there are several monoclonal anti-IgE antibodies including BSW17, Le27 and SUS-11. Monoclonal anti-human IgE antibody (BSW17) binds to an epitope located within the C3 and C4 domains of human IgE thereby inhibiting the binding of IgE to its high affinity receptor (Stadler et al, 1996). Reagent anti-IgE can be anaphylactogenic and non-anaphylactogenic (Rudolf et al, 2000). Anaphylactogenic anti-IgE antibodies were used in evaluating IgE-mediated mediator release mechanisms in human basophils and mast cells *in vitro*. In the experimental settings, antiserum specific for IgE was used to crosslink membrane-bond IgE to study the process of 'immunological activation' of mast cells and basophils (Walls & He, 2008). Basophils and mast cells *in vitro* demonstrated a variability between basophil donors in response to anti-IgE antibodies (Lichtenstein, 1970). Anti-IgE induces histamine-release showed a different time course than fMLP or substance P. Anti-IgE antibodies induce histamine release as well as the secretion of PGD_2 and LTC_4 from skin mast cells (Benyon et al, 1989). In *in vitro* models, the effect of anti-IgE antibodies on skin mast cells was characterized by elevations of intracellular cyclic AMP in the presence of extracellular calcium (Benyon et al, 1989). In skin mast cells, the kinetics of the stimulation with anti-IgE was characterized by relatively slow histamine release that reached completion within 6 min after challenge as opposed to rapid histamine release to substance P, being completed within 20s (Church et al, 1991). In earlier experiments, it was noted that anti-IgE –induced histamine release was more than 20-fold slower that that induced by fMLP (Knol et al, 1991).

1.6.5 Neutrophils

Neutrophils represent the most abundant type of granulocytes and constitute 40-60% of leukocytes in the circulation. In the bone marrow, neutrophils are generated at the rate of 10^{10}-10^{11} cells/day (Summers et al, 2010; Rankin, 2010). In peripheral blood, mature neutrophils are terminally differentiated cells with a short half-life of 6-8 hours (Summers et al, 2010). In healthy individuals, 10^9 neutrophils/kg body weight are estimated to be released daily from the bone marrow into the bloodstream (Rankin, 2010).

Neutrophils arise from CD34+ hematopoietic stem cell committed to the myeloid lineage. In neutrophil granulopoiesis, granulocyte colony-stimulating factor is an essential growth factor for myeloid progenitor differentiation and generation of neutrophils (Manz & Boettcher, 2014). Neutrophil granulopoiesis is capable of a rapid adjustment from steady-state conditions to emergency granulopoiesis in response to severe infections (Manz & Boettcher, 2014). Overall, neutrophil counts in the circulation are determined as a net result of neutrophil granulopoiesis, bone marrow egress, margination and extravasation/clearance (Bugl et al, 2012). In human body, neutrophils are distributed in the bloodstream, the bone marrow and also as the marginated neutrophil pools in the pulmonary vascular bed, liver and spleen (Nauseef & Borregaard, 2014; Pruchniak et al, 2013). The marginated neutrophil pools can be recruited into the circulation within minutes in response to epinephrine or the CXCR4 inhibitor plerixafor (Devi, 2013).

Morphologically, mature neutrophils are cells of 12-15μm in diameter with characteristic morphological features of nuclear segmentation and cytoplasmic granules, hence, neutrophils are also called polymorphonuclear granulocytes. Neutrophils have multi-lobed nucleus with three to five lobules. Neutrophils contain primary or azurophilic, secondary or specific, tertiary or gelatinase granules and secretory vesicles. Azurophilic granules contain bactericidal permeability increasing protein, neutrophil elastase, cathepsin G, protease 3, azurocidin, myeloperoxidase (Nathan, 2006) which are implicated in phagocytosis. Also, azurophilic granules contain alarmins which can activate antigen-presenting cells and induce innate and adaptive immune responses (Kaplan, 2012). These alarmins in neutrophils include α-defensins, the cathelicidin human cationic antimicrobial protein 18 and lactoferrin (Kobayashi et al, 2009). The content of specific granules includes cathelicidins (lactoferrin, lipocalin, lysozyme, LL37) which mediate antimicrobial activities. Gelatinase granules include MMP8, MMP9 and MMP25 (Nathan, 2006), which facilitate neutrophil transmigration. Secretory vesicles are characterized by a marker protein CD35 (Kjeldsen et al, 1994) and contain CD11b/CD18, complement receptor 1, fMLP receptors, LPS/lipoteichoic acid, receptor CD14, FcγRIII CD16 and leukolysin, these neutrophil products are involved in early neutrophil-mediated inflammatory response (Cascao et al, 2009).

Neutrophils are known to express several Fc-receptors such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16). Also, neutrophils express various cytokine receptors such Type I cytokine receptors (IL-4R, IL-6R, IL-12R, IL-15R, G-CSFR, GM-CSFR), Type II cytokine receptors (interferon-α (IFN-α), IFN-β, IFN-γ, IL-10R), IL-1R family (IL-1R, IL-1RII, IL-18R) and TNFR family (TNFR1, TNFR2, Fas, LTβR, RANK, TRAIL-R2, TRAIL-R3) (Pruchniak et al, 2013, Futosi et al, 2013). Also, neutrophils express adhesion molecules such selectins/selectin ligands (L-selectin, PSGL-1) and integrins (LFA-1, MAC-1, VLA-4), which plays a role in neutrophil adhesion to bone marrow stromal cells and endothelium (Futosi et al, 2013; Summers et al, 2010). Neutrophils express numerous G-protein-coupled receptors including formyl-peptide receptors, chemoattractant receptors BLT1 and BLT2 for LTB_4, receptors for platelet activating factor and complement fragment C5a, chemokine receptors (CXCR1, CXCR2), CCR1 and CCR2 (Futosi et al, 2013). Neutrophils express membrane-bound receptors (Toll-like receptors, C-type lectin receptors) that are important in pattern recognition function at the sites of inflammation (Thomas & Schroder, 2013). Human neutrophils express all Toll-like receptors except TLR3 and TLR7 (Thomas and Schroder, 2013). Syk-coupled C-type lectin receptors expressed by neutrophils recognise carbohydrate moieties and include Dectin-1, Mincle, C-type lectin domain family 2 (CLEC2) and CLEC5A (Thomas & Schroder, 2013).

Neutrophil were reported to display phenotypic differences in response to physiological and pathophysiological conditions. There are distinct neutrophil phenotypes which differ in the expression of specific molecular markers. For example, approximately 25% of circulating neutrophils express a glycoprotein olfactomedin 4 which is considered to be a tumour suppressor (Chen et al, 2011) and a negatively regulator of the activation of several granular proteases (cathepsin C, neutrophil elastase, cathepsin G and proteinase 3) (Chen et al, 2011; Clemmensen et al, 2011). A subset of neutrophils expressing surface glycoprotein CD177 (NB1) is thought to be involved in neutrophil transmigration (Nourshargh et al, 2010). In cancer, two tumour-associated neutrophil subsets included an immunosuppressive and pro-tumorigenic neutrophil phenotype (N2) and an immunostimulatory and anti-tumour neutrophil subset (N1). (Beyrau et al, 2012).

Neutrophil activation results in their effector functions such as phagocytosis, exocytosis of cytoplasmic granules, production of reactive oxygen intermediates, chemotactic migration, cytokine release and release of neutrophil extracellular traps (Futosi et al, 2013). Neutrophil activation is a two-stage process. First, neutrophils are primed by bacterial products such as lipopolysaccharide, cytokines (TNF-α, GM-CSF, IL-8 and IFN-γ), chemokines, contact with activated endothelium or foreign surfaces (Cowburn et al, 2008). Neutrophil priming can occur within minutes of stimulation due to the mobilization of intracellular granules with pre-formed mediators. As a result, the number and sometimes the affinity of surface receptor expression increase without protein biosynthesis. By contrast, some priming agents can also induce an activation of transcription factors that will result in *de novo* expression of receptors or cytokines. Neutrophil priming facilitates rapid recognition, phagocytosis and killing bacteria or activation by immune complexes via Fcγ receptors. Then, primed neutrophils migrate to the sites of infection and inflammation following a chemotactic gradient. At the site of infection or inflammation, primed neutrophils recognise pathogens for phagocytosis by pattern recognition receptors or, more efficiently, by Fc-receptors or complement receptors if the pathogens are opsonised. This results in further neutrophil activation with phagocytosis, a respiratory burst, the release of lytic enzymes and antimicrobial products.

In neutrophils, signalling via G-protein-coupled receptors results in the chemotactic migration of neutrophils (Futosi et al, 2013). Triggering via Fcγ-receptors by Ig-opsonised pathogens requires a synergistic ligation of FcγRIIA and FcγRIIIB and involves cytoplasmic ITAM motifs which, in turn, recruits the Syk tyrosine kinase for further signalling (Futosi et al, 2013). Signalling via FcγRIIa initiates chemotaxis, phagocytosis and bacterial killing whereas FcγRIIIb is involved in the secretion of reactive oxygen species in response to immune complexes, but not in phagocytosis or killing of serum-opsonized bacteria (Wright et al, 2010). For phagocytosis, cytoplasmic granules fuse with plasma membrane with help of SNARE proteins to form the phagosome and release their contents including myeloperoxidase in the phagosome (Mollinedo et al, 1999). An NADPH-dependent oxidase generates superoxide anions for bacterial killing by electron transfer from NADPH to O_2 (Cowburn et al, 2008; Mollinedo et al, 1999). Following this, superoxide is converted into hydrogen peroxide which, in turn, is used by myeloperoxidase in the phagosome to catalize the generation of potent antimicrobial products such as hypochlorous acid (Witko-Sarsat et al, 2000).

Neutrophils are known to generate and secrete cytokines, chemokines, leukotrienes and prostaglandins. Neutrophils can synthesize and secrete IL-8, IL-1, IL-1RA, IL-6, IL-12, TGF-β, TNF-α, oncostatin M and tumour-necrosis factor-related ligand B-lymphocyte stimulator (BlyS) (Mantovani et al, 2011). Neutrophils are also an important source of leukotrienes and prostaglandins, in particular, LTB_4 and PGE_2. LTB_4 is a neutrophil chemoattractant and a mediator of vascular permeability (Sadik et al, 2011; Bray, 1982) whereas PGE_2 has immunosuppressive effects on neutrophils by down-regulating their endothelial adhesion and chemotaxis (Agard et al, 2013).

Role in health. In health, neutrophils are the first line in host defence against infections including bacteria and fungi (Sadik et al, 2011). Neutrophils are the first cells to arrive at the site of infection or inflammation. Neutrophils utilize antimicrobial molecules such as myeloperoxidase, neutrophil elastase, cathepsin G and defensins to combat infections.

Role in disease. In disease, neutrophils are implicated in autoimmunity (Nemeth & Moscai, 2012). A signature of autoimmunity is the generation of autoantibodies that can be directed against nuclear material such as dsDNA, ribonucleoproteins and histones (Bardoel et al, 2014). In the last decade, a new potential source of self-molecules has come to light with the discovery of neutrophil extracellular traps (NETs). Indeed, molecules released during NETosis are found as autoantigens in many autoimmune diseases, including rheumatoid arthritis, systemic lupus erythematosus and vasculitis. Neutrophil degranulation may also result in tissue damage while NET formation may lead to the exposure to autoantigens (Bardoel et al, 2014).

Neutrophils have been implicated in cancer pathophysiology (Mantovani et al, 2011). Neutrophils are thought to be recruited into tumours and may enhance genetic instability and promote angiogenesis. In the context of tumour, neutrophils can be reprogrammed into pro-tumoral N2 phenotype under influence by TGF-β whereas downregulation of TGF-β may promote N1 phenotype which is associated with cytotoxicity and antitumour activity (Mantovani et al, 2011).

Role in allergy. Neutrophils are known to participate in immune complex –mediated hypersensitivity (Jonsson et al, 2013). Neutrophils may modulate the inflammatory response via production of pro-inflammatory cytokines (Cassatella, 2003). Neutrophilic inflammation in the airways is thought to underlie severe asthma and to mediate steroid resistance (Ito et al, 2008). Neutrophils were implicated in IgG-mediated anaphylaxis in animal models (Jonsson et al, 2011).

Role in CSU. Neutrophil accumulation is a common feature of skin inflammation in CSU which is seen on histological examination in about 18-50% of cases (Toppe et al, 1998; Llamas-Velasco et al, 2012). This two-step process is required as a means for avoiding uncontrolled neutrophil activation due to its significant destructive potential (Condliffe et al, 1998). In CSU, neutrophil accumulation was significantly associated with increased local expression of IL-3 and TNF-α (Toppe et al, 1998). Local increase in TNF-α may create microenvironment for neutrophil priming in CSU (Hermes et al, 2003). New insights into neutrophil biology may have pathophysiological relevance in CSU. Neutrophil interaction with endothelial cells during transmigration into inflamed tissues are a focus of research interest in view of neutrophil capacity to open 'flood gates' between endothelial cells and thereby contributing to increased vascular permeability (DiStasi & Ley, 2009). These data are in keeping with the previous observation of neutrophil-mediated oedema reported by Wedmore and Williams (1981). T Signalling via FcRγIIaa initiates chemotaxis, phagocytosis and bacterial killing. FcγRIIIb has been shown to play an important role in the secretion of ROS in response to immune complexes, but no role in phagocytosis or killing serum-opsonized bacteria (Wright et al, 2010).

Neutrophil accumulation is a common feature of skin inflammation in CSU which is seen on histological examination in about 18-50% of cases (Toppe et al, 1998; Llamas-Velasco et al, 2012). It is well known that neutrophil activation occurs in two phases including priming and then stimulation for neutrophil degranulation. This two-step process is required as a means for avoiding uncontrolled neutrophil activation due to its significant destructive potential (Condliffe et al, 1998). In CSU, neutrophil accumulation was significantly associated with increased local expression of IL-3 and TNF-α (Toppe et al, 1998). Local increase in TNF-α may create microenvironment for neutrophil priming in CSU (Hermes et al, 2003). New insights into neutrophil biology may have pathophysiological relevance in CSU. These data are in keeping with the previous observation of neutrophil-mediated oedema reported by Wedmore and Williams (1981). The pathophysiological contribution of neutrophils to vascular leakage and oedema formation in CSU is unknown and is worth exploring. Another area of interest in neutrophil biology stems from the novel data suggesting that neutrophils may provide a link between innate and adaptive immunity in inflammation by their crosstalk to B- and T-lymphocytes (Mantovani et al, 2011). For example, neutrophils are known as a source of cytokines that may promote B cell activation, including B-cell activating factor (BAFF) (Mantovani et al, 2011). The soluble BAFF level was reported to be increased in CSU (Kessel et al, 2012) which may raise a question about possible interrelationship between neutrophilic CSU and CSU with serum histamine-releasing activity. Overall, histological studies in CSU suggest that neutrophilic urticaria is a distinct histological phenotype of CSU. However, the clinical significance of neutrophilic urticaria is yet to be established as well as neutrophil contribution to the pathophysiology of CSU.

1.6.6 Eosinophils

In healthy subjects, eosinophils comprise less than 4% of circulating leukocytes (Robinson, Kay & Wardlow, 2002).

Morphology. Eosinophils are approximately 8-12μm in diameter. Morphologically, eosinophils are bi-lobed granulocytes with large spherical or ovoid crystalloid granules that contain four primary cationic proteins including eosinophil peroxidase, major basic protein eosinophil cationic protein, and eosinophil-derived neurotoxin (Giembycz & Lindsay, 1999).

Development: Eosinophils develop from CD34+IL-5R+ eosinophil progenitor (Fulkerson & Rothenberg, 2013). The maturation of eosinophils occurs in bone marrow and they are released in the circulation as the fully differentiated cells.

Heterogeneity: Density-gradient centrifugation studies revealed a subset of hypodense eosinophils compared to the majority of normal density eosinophils in the peripheral blood (Conesa et al, 2002). The numbers of low-density eosinophils are increased in the presence of eosinophilia (Fukuda et al, 1985). The hypodense eosinophils are smaller, vacuolated, with more lipid bodies but less MBP (Henderson et al, 1988; Peters et al, 1988). Low–density eosinophils are hypothesized to be an activated phenotype although the evidence is contradictory (Wardlaw & Kay, 2012).

Distribution. Eosinophils represent approximately 2% of circulating leukocytes in the peripheral blood and 8% of leukocytes in the bone marrow (Stone et al, 2010). In healthy subjects, eosinophils are noted in the gastrointestinal tract, thymus, uterus, spleen and lymph nodes (Kato et al, 1998). In the inflammation, eosinophils are recruited in the inflamed tissues (Kita, 2011).

Mediators and biological effects: Eosinophils secrete multiple mediators including granular proteins, lipid mediators, chemokines, cytokines and growth factors and neuropeptides (Kay, 2005; Hogan et al, 2008). In their granules, eosinophils contain major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), eosinophil-derived neurotoxin or eosinophil protein X (EDN or EPX). In eosinophils, MBP is the most abundant protein that is located in the crystalloid core of the cytoplasmic granules. MBP is cytotoxic by causing the disruption of the integrity of lipid bilayers (Abu-Ghazaleh & Gleich, 1992). MBP also causes degranulation of mast cells, basophils and neutrophils (Zheutlin et al, 1984). ECP is a zinc-containing protein that resides in the granule matrix. ECP is cytotoxic to helminthes, causes degranulation of mast cells and basophils and activates Hageman factor, kallikrein and plasminogen (Dahl & Venge, 1979). EPO is a heme-containing haloperoxidase which is cytotoxic and, as the EPO-H_2O_2-halide system, plays a role in bacterial killing (Venge, 1990; McEwen, 1992). EPO also causes mast cell degranulation (Henderson et al, 1980). EDN effects include cytotoxicity, oxidative damage, mutagenesis of DNA and RNA (Venge, 1990). Both EPO and EDN are RNAses and possess anti-viral activity (Rothenberg et al, 2013). CLC protein (also known as galectin-10) is a lysophospholipase (Rothenberg et al, 2013). Lipid mediators include prostaglandins (PGE_2 and PGF_2), leukotrienes (LTC_4 but no or minimal amount LTB_4), thromboxane B_2 and PAF (Weller, 1997). Additionally, eosinophils secrete various cytokines including TGF-α, TGF-β, IL-3, IL-5, GM-CSF, IL-2, IL-4, IL-6, IL-10, IL-16, IFN-γ, TNF-α, VEGF (Weller, 1997). Additionally, eosinophils contain multiple enzymes including acid phosphatase, collagenase, arylsulfatase B, histaminase, phospholipase D, catalase, non-specific esterases, matrix metalloproteinases and vitamin B12-binding protein (Rothenberg et al, 2013).

Surface markers: Eosinophils express a variety of surface receptors including complement receptors (C3aR, C5aR, CD3 (Mac-1)), Fc-receptors (FcαR, FcγRII, FcεRI, FcεRII (CD23)), chemoattractant receptors (IL-8R, CCR1, CCR3, CCR4, CCR5, CCR6, CCR8, CCR9, CXCR2, CXCR3, CXCR4), cytokine receptors (IL-1R, IL-2R, IL-3R, IL-4R, IL-5R, GM-CSFR α-chain, TNFR), Toll-like receptors (TLR1, TLR2, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10), receptors for lipid mediators (PAFR, CRTH2, PGD_1 receptor, PGEP4 receptor, PGEP2 receptor, LTB_4 receptor) and some other receptors (CD69, FAS (CD95), CD40) (Hogan et al, 2008; Weller, 1997). Additionally, eosinophils express several inhibitory receptors such as FcγRIIB, LIR3, Siglec-8 and IRp60 (Munitz & Levi-Schaffer, 2007; Hogan et al, 2008).

Mechanisms of activation: Eosinophil activation can be induced by various triggers and may occur via different mechanisms. For example, priming factors include IL-5, IL-3, IL-33, GM-CSF or Notch ligands (Fulkerson & Rothenberg, 2013). Several triggers can activate eosinophils including secretory IgA and cytokines (IL-5, GM-CSF, IL-3) (Gleich, 2000). Eosinophil degranulation may occur via the following mechanisms: classical exocytosis, compound exocytosis, piecemeal degranulation and cytolysis (Lee et al, 2012). Following degranulation, eosinophils can release cationic proteins, reactive oxygen intermediates, lipid mediators, cytokines, chemokines and growth factors as well as mitochondrial DNA and antimicrobial agents (Lee et al, 2012).

Signalling. In eosinophil priming, signal transduction is thought to involve Lyn, JAK2, protein tyrosin kinase and p21 ras (Bochner, 2000; Fulkerson & Rothenberg, 2013). IL-5 mediated signal transduction involves activation of signal transducer and activator of transcription 1 (STAT1), STAT3, STAT5 pathways, leading to prolonged eosinophil survival (Fulkerson & Rothenberg, 2013). Eosinophil differentiation, degranulation and cytokine production is mediated via activation of mitogen-activated protein kinase (MAPK)-related pathway (Fulkerson & Rothenberg, 2013). Eosinophil adhesion and chemotaxis are known to be dependent on MAPK, the phosphoinositide 3-kinase (PI3K) and nuclear factor – κB (NF-κB) (Fulkerson & Rothenberg, 2013).

Role in health. In health, eosinophils are implicated in tissue homeostasis, adaptive immune responses and in host defence against infections, particularly against helminths and fungi (Rothenberg, 2007; Kita, 2011).

Role in disease. Eosinophils are known to contribute to the pathophysiology of asthma and primary hypereosinophilic syndromes (Gleich, 2000). Additionally, eosinophils are thought to be involved in tumour immune surveillance (Kay, 2005; Fulkerson & Rothenberg, 2013).

Role in allergy and inflammation: Eosinophils can accumulate in the cutaneous late-phase reaction and can secrete Th2-type cytokines, which, by autocrine effects, can prolong cell survival in the tissues and amplify local allergic inflammation (Kay et al, 1997). In asthma, eosinophils are implicated in airway hyperreactivity, the damage of airway mucosa via the release of basic proteins, lipid mediators and reactive oxygen intermediates (Kay, 2005) and in airway remodeling through the secretion of fibrogenic factors such as TGF-β, IL-11 and IL-25 (Rothenberg, 2007; Kay et al, 2004).

How eosinophils contribute to weal formation in CSU? In CSU, eosinophils were shown to accumulate in cutaneous inflammatory infiltrate (Ying et al, 2002), particularly in CSU patients without serum histamine-releasing activity (Sabroe et al, 1999). In the context of CSU pathophysiology, eosinophil activation may be caused by autoantibodies against the low affinity IgE receptor (Pucetti et al, 2005) or by mast cell products (Asero et al, 2009). On the other hand, eosinophils are known to activate mast cells by MBP and other basic products (Gangwar & Levi-Schaffer, 2014). Additionally, eosinophils were a major source of VEGF in the skin inflammation in CSU as demonstrated immunohistochemically by co-localization of VEGF with ECP in CSU (Tedeschi et al, 2009). Furthermore, activated eosinophils were implicated in triggering the tissue factor pathway of coagulation cascade (Asero et al, 2007). Overall, the precise contribution of eosinophils to weal formation in CSU is unknown. The putative role of eosinophils in weal formation in CSU may involve mast cell activation by MBP and other basic products or indirectly by their contribution to the chronic skin inflammation and, possibly, skin remodeling.

The precise contribution of eosinophils to the pathogenesis of CSU is still incompletely understood (Asero et al, 2009). Decreased eosinophil counts in the peripheral blood were reported in CSU patients (Grattan et al, 2003). In CSU, eosinophils are characterized by activated phenotype as suggested by an increased expression of CRTH2 on their surface in CSU (Yahara et al, 2010). Autoantibodies to CD23 were shown in CSU and can be a possible mechanism of eosinophil activation in CSU (Pucetti et al, 2005). CSU is associated with increased levels of eotaxin in the peripheral blood (Tedeschi et al, 2012) which can induce eosinophil (and basophil) chemotaxis (Bochner & Schleimer, 2001). Eosinophil accumulation in skin was demostrated mostly in CSU patients without serum histamine-releasing activity. Release of eosinophil granule proteins was shown in the skin of patients with CSU and was associated with the duration of weals. Eosinophil degranulation was also noted in positive serum skin tests in CSU (Grattan et al, 1997). Targeting eosinophil-predominant skin inflammation in CSU with montelukast supports the role of eosinophil- derived cysteinyl leukotriene mediators in the pathophysiology of chronic urticaria (Criado et al, 2008).

Several aspects of eosinophil biology may have potential clinical relevance and therapeutic implications. For example, experimental evidence suggested that histamine induced eosinophil chemotaxis via the production of eotaxin by the endothelial cells (Menzies-Gow et al, 2004) and through H4 histamine receptors (Ling et al, 2004) that were known to be highly expressed in human eosinophils (Zhang et al, 2007). Whether this mechanism is relevant in the context of CSU is unknown and worth exploring. Some observations suggest an inhibitory role of eosinophils for mast cell degranulation (Minai-Fleminger & Levi-Schaffer, 2009). The peculiarities of a crosstalk between mast cells and eosinophils in CSU have not been studied and would be of interest. Furthermore, eosinophils are known to be a source of PAF, VEGF and MMP- 9 (Hogan et al, 2008) which were implicated as a potential participants in the inflammation in CSU (Tedeschi et al, 2009; Kessel et al, 2005). Further research in this area may provide exciting insights into the pathophysiology of CSU. An increase in eosinophil counts on the treatment with PAF inhibitors suggested that the eosinophil count serves as a potential biomarker for its therapeutic efficacy (Maiti et al, 2011).

1.6.7 Tryptase

Tryptase is a serine protease which is expressed predominantly in mast cells and, to minor extent, in basophils (Hallgren & Pejler, 2006).

Structural analysis. Crystallography studies of the tryptase structure provided insights into its unique biophysical properties. Human β-tryptase was crystallised as a rectangular assembly of four monomers with catalytic sites facing a narrow central pore (Sommerhoff et al, 1999). The geometry of the central pore allows access to the catalytic sites for low molecular weight peptides or flexible side chains of macromolecular proteins (Rice & Moore, 2000). Furthermore, the architecture of the central pore renders tryptase resistant to endogenous protease inhibitors due to inaccessibility of tryptase catalytic sites to macromolecular proteins. The stabilisation of the tetrameric structure of tryptase is achieved by ionic interaction of tryptase cationic groove with negatively charged glycosaminoglycans of serglycin proteoglycans (Walls, 2000). The cationic patches spanning across each dimer of the tetrameric structure are predominantly expressed at neutral pH, therefore, in an acidic environment tryptase-heparin complexes dissociate into inactive monomers.

Changes in tryptase molecular weight during its maturation reflect its conformational changes and post-translational modification (Schwartz, 2006). The molecular weight of tryptase monomers is approximately 30-36kDa. The tetrameric structure of tryptase is characterized by the molecular weight of approximately 140kDa. The formation of tryptase-heparin complex results in an increase in molecular weight up to 200kDa (Schmelz et al, 1999). In *in vitro* experiments, enzymatic treatment of tryptase monomers resulted in the reduction of their molecular weight suggesting a degree of glycosylation of tryptase monomers as a post-translational modification. Understanding of the changes in the molecular weight of tryptase is crucial for effective recovery of tryptase from biological fluids in mechanistic studies.

Tryptase isoenzymes. An overview of tryptase isoenzymes is important for understanding of their antigenic sites and their detection by monoclonal antibodies in diagnostic assays. There are five isoenzymes of tryptase: α, β, γ, δ and ε (Schwartz, 2006; Hernandez-

Hernandez et al, 2012). α-tryptase is processed to the form of pro-enzyme and is constitutively secreted by mast cells together with β-protryptase. Mature β tryptase is formed from β-protryptase by autoprocessing and then by proteolysis by dipeptidyl-peptidase I (cathepsin C). Total serum or plasma tryptase is comprised of α- and β-protryptases and mature β tryptase. Mature β tryptase is released during anaphylactic degranulation of mast cells while α- and β-protryptases are considered as a marker of mast cell burden. γ-transmembrane tryptase is released by mast cells during anaphylactic degranulation and is anchored to the mast cell membrane. δ-tryptase is a truncated protein resembling mouse tryptase with low level of expression. ε-tryptase is foetal tryptase, which is not present in adults.

Tryptase substrates. Tryptase preferentially hydrolyses the peptide bonds at the carboxyl side of amino acid Arginine and Lysine residues. The tetrameric 3Å crystal structure of tryptase also explained its substrate preferences and regulation. For example, tryptase is known to hydrolyse neuropeptides such as calcitonin-gene related peptide, vasoactive intestinal peptide and PMH (Walls, 2000). Small molecular weight of these peptides makes them fit to the central pore with access to catalytic sites. Tryptase can also hydrolyze flexible chains of larger proteins including pro-stromelisine, fibronectine, fibrinogen. Proteolytic cleavage of the extracellular chain of cell-surface PAR-2 receptor results in intracellular signalling on target cells. pH optimum of tryptase differs for various substrates and may account for the differences in its proteolytic profile at neutral and acidic pH microenviroment. For example, tryptase affinity for fibrinogen is higher in acidic environment while peptide proteolysis is more active at neutral pH.

Biological role. In the skin, tryptase appears to be involved in itch transmission, leukocyte recruitment, neuropeptide degradation, extracellular matrix modification and, possibly, coagulation as suggested by animal studies (Trivedi et al, 2010; Schwartz, 2006; Payne and Kam, 2004).

The role in CSU. In CSU, evidence for local tryptase release in skin stems from suction-blister fluid studies as well as immunohistochemistry and immunofluorescence (Vena et al, 2002). In systemic circulation, tryptase is elevated in active disease and in patients whose serum has the capacity to up-regulate CD63 on basophils from healthy donors (Ferrer et al, 2010). A subset of patients with CSU with elevated plasma tryptase levels were diagnosed with systemic mastocytosis (Siles et al, 2013).

Despite recent developments in the field of tryptase research, many questions about the role of tryptase in CSU remain unanswered. The expression, storage and the enzymatic profile of skin tryptase in CSU await characterisation. It is unclear whether the local tryptase release in the skin precedes the systemic rise in tryptase in CSU and whether this reflects disease progression. Additionally, the proteolytic behaviour, the kinetics of tryptase release and substrate supply in the context of ongoing mast cell degranulation and microvascular leakage in the skin of CSU patients are yet to be elucidated. Further research into the effects of tryptase haplotypes and the relative distribution of tryptase isoenzymes may help underpin the underlying changes in mast cell load or degranulation in CSU. The use of minimally invasive skin sampling techniques and sensitive assays for selective detection of tryptase isoenzymes may provide further insights in tryptase biology and skin homeostasis in CSU. Tryptase appears to be a promising therapeutic target (Rice & Moore, 2000). Tryptase inhibitors were shown to be effective in some patients with CSU. The development of selective tryptase inhibitors may advance the management of CSU.

1.6.8 Two mediator hypothesis of inflammation

It is worth noting that in inflammation several mediators are likely to interact with each other as was suggested by a two-mediator hypothesis (Williams, 1977a). According to Williams, a vasodilator mediator is likely to potentiate the plasma exudation effects of the mediator, which enhances vascular permeability (Williams, 1977a). These synergistic interactions were noted between histamine and prostaglandins (Williams, 1977b) or CGRP (Brain & Williams, 1985). The *in vivo* synergistic interactions of mediators are likely to account for a longer duration of weals in CSU but the relevant interactions of mediators *in vivo* in CSU await characterization. There is a need for *in vivo* models of wealing in CSU to explore the relative contribution of various mediators to the development of urticarial lesions in the context of persistent dermal inflammation in CSU.

1.7 Hypothesis

For this thesis, our hypothesis suggested that severe and/or persistent CSU was associated with serum histamine-releasing activity, abnormal basophil releasability, altered basophil phenotype and absolute numbers in the circulation. We also hypothesized that, at the skin level, serum histamine-releasing activity in CSU was associated with higher local concentrations of pro-inflammatory mediators (histamine, tryptase) and cytokines, local histamine release and, possibly, neutrophil infiltration in the dermis of CSU patients.

1.8 The Scope of this Thesis

This PhD dissertation is intended to study different pathophysiological aspects of CSU based on three interlinked research projects.

The *first project* evaluates skin biochemistry at baseline and in response to skin testing with phosphate buffered saline (PBS), autologous serum skin test and codeine in CSU patients and healthy controls. The aim of this study is to examine abnormal function of skin mast cells and an altered cutaneous microenvironment in CSU. This possibility is offered by cutaneous microdialysis which is a minimally invasive research technique for sampling of the dermal extracellular fluid *in vivo*. This pilot project will establish the baseline level of inflammatory mediators (histamine and tryptase) and cytokines in CSU patients and healthy controls and will explore the pattern of skin response to skin testing with PBS, autologous serum and codeine in both groups.

The *second project* is designed as a prospective observational study to investigate the pathophysiological subsets in CSU and to explore their biomarkers. This project is intended to establish and characterize CSU subsets with regards to pathophysiological features (functional autoantibodies and basophil function) and to explore their stability over time. This project will determine the predictors for disease severity in CSU. As a part of this project, the methodology development for imaging flow cytometry studies for peripheral blood basophils in healthy controls using ImageStream technology to link immunophenotyping and morphological features of human basophils. The ImageStream studies will be presented to provide the rationale for data analysis in the prospective study in CSU patients.

The third project is planned as a retrospective study and was intended to re-evaluate the numbers of neutrophils and eosinophils per high power field in lesional skin biopsies from patients with CSU and urticarial vasculitis and to explore if there is a potential link between the histological presentation of neutrophilic CSU and CSU with serum histamine-releasing activity.

CHAPTER 2

Assessment of mediators and cytokines in the skin of healthy subjects and patients with chronic spontaneous urticaria (CSU) using cutaneous microdialysis

"It is better to know some of the questions than all of the answers."

—JAMES THURBER

Abstract

Background: The pathophysiology of weal formation in CSU is poorly understood. Serum histamine-releasing activity was demonstrated *in vitro* on human basophils (Hide et al, 1993) and skin mast cells (Niimi et al, 1996) but the evidence for *in vivo* serum-induced histamine release in the skin of CSU patients is lacking. Elevated histamine levels (Kaplan et al, 1978) and cytokine up-regulation (Hermes et al, 1999; Ying et al, 2002) were reported in the skin of CSU patients but the relevance of the changes in mediators and cytokines in the skin to weal formation in CSU is unknown. We hypothesized that weal formation in CSU was mediated by local histamine release caused by circulating serum factors, aberrant skin mast cell releasability and altered skin microenvironment.

The **purpose** of the study was to evaluate an autologous serum skin test as an experimental model for weal formation in CSU and to determine the baseline levels of histamine, tryptase and cytokines and the pharmacokinetics of histamine and tryptase

release in response to skin testing with phosphate buffered saline (PBS), autologous serum and codeine in CSU patients and healthy controls.

Methods: Cutaneous microdialysis studies were carried out in 14 CSU patients and 13 healthy controls. Six (two per site) linear microdialysis probes (molecular weight cut-off of 3,000 kDa) were inserted into the forearm skin of participants and perfused with sterile Ringer's solution at 3µl/min for *in vivo* sampling of dermal extracellular fluid. Skin testing with PBS (pH = 7.4, 20µl) was carried out along the probes at the site 1, while autologous serum (20µl) and codeine (0.3mM, 20µl) were used for skin testing at the sites 2-3, respectively. Dialysate sampling was carried out at baseline (30 min), at 40min intervals for the first 2 hours and then between 4 and 6 hours after the skin testing (Protocol 1). For Protocols 2 and 3, dialysate sampling was carried out at 5 min-intervals for 40min and 80 min after skin testing, correspondingly. Dialysate samples were analyzed for histamine by ELISA (BioSource, Belgium), for tryptase by double antibody-sandwich ELISA utilizing polyclonal rabbit anti-tryptase antibodies for capture and the mouse AA5 monoclonal antibodies for the detection (University of Southampton, UK), for cytokine levels by BD™ Cytometric Bead Array (BD Biosciences, UK). VAS for itching and wealing over 24 hours were recorded during the baseline collection. The weal area was calculated by skin planimetry using digital analysis with NIH ImageJ software.

Results: Cutaneous microdialysis was well tolerated by all but two CSU patients. Median baseline histamine concentration was higher in CSU patients (2.321 (0.855; 2.784 ng/ml) compared to that in healthy controls (0.859 (0.275; 1.327 ng/ml) (Mann-Whitney test, $p<0.05$). There was a moderate correlation between VAS for wealing and baseline histamine concentration (Pearson correlation $r=0.60$, $p<0.05$) but not for dermal tryptase or IL-6 concentrations. Two CSU patients with a positive *in vitro* serum-induced basophil histamine release assay demonstrated a slow low-grade histamine release in response to an intradermal injection of autologous serum. The area-under-the-curve analysis for 40 min after skin testing suggested a tendency for higher AUC_{40min} for PBS and ASST stimulation and lower AUC_{40min} for codeine stimulation in CSU patients compared to healthy controls. When tested on the arm without microdialysis procedures, the area of the weals was larger after skin testing with autologous serum, but not PBS or codeine, in CSU patients than that in healthy controls (two-sample Wilcoxon test, $p<0.05$).

Conclusions: The data are consistent with the ASST response being a useful model of spontaneous wealing in CSU. We demonstrated for the first time *in vivo* a slow low-grade local histamine release after an intradermal injection of autologous serum in two CSU patients with serum histamine-releasing activity. Whether this histamine release explains skin autoreactivity to serum observed in some CSU patients needs to be established in future studies. In our study, the skin microenvironment in CSU was characterized by elevated dermal histamine concentrations but normal tryptase and IL-6 concentrations compared to healthy controls. Dermal histamine concentrations were correlated to clinical scores in CSU but the underlying mechanisms remained uncertain. Aberrant skin mast cell releasability was suggested by AUC_{40min} analysis and needs to be further explored in future studies.

2.1 Background

Weal formation is associated with vasodilation and vascular hyperpermeability of post-capillary venules followed by transudation of fluid from dermal microvasulature into extracellular space. Multiple mediators were implicated in the wealing in CSU as inferred from their capacity to induce weal formation on an intradermal injection in healthy subjects. Histamine is the most researched mediator which is known to induce a triple Lewis response upon intradermal injection (Greaves & Sabroe, 1996). However, histamine is unlikely to solely account for wealing in CSU taking into account a slow resolution of weals in CSU than expected for histamine-induced weals as well as partial efficacy of anti-histamine treatments in some patients (Krause & Shuster, 1985; Kobza-Black, 1989).

Other mediators which may be involved are leukotrienes, prostaglandins, proteases, kinins, platelet activating factor and neuropeptides. In human skin, prostaglandins E_2, I_2 and D_2 are known to induce erythema and oedema that persist over an hour following an intradermal injection (Flower et al, 1976). In human skin, leukotrienes C_4 and D_4 also produced a weal-and-flare reaction upon an intradermal injection (Soter et al, 1983). The erythema induced by leukotrienes C_4 and D_4 persisted for 6 hours oedema for 2 hours after an intradermal injection (Soter et al, 1983). Skin testing with leukotriene B_4 resulted in an initial weal-and-flare reaction followed by a papule lasting for 6 hours (Soter et al, 1983). When injected in human skin, PAF induced a weal and flare reaction that peaked at 5-15 min and resolved in 2-3 hours (Archer, 1984; Krause et al, 2013). Microdialysis studies in healthy controls did not reveal histamine release associated with PAF injection (Krause et al, 2013). In microdialysis studies, an intradermal delivery of CGRP to human skin resulted in prolonged wealing (Weidner et al, 2000). Substance P also induced itching, erythema and wealing when injected in human skin (Lembeck & Holzer, 1979). However, which mediators are involved *in vivo* in CSU remains unclear.

One of the Dale criteria for inflammatory mediators includes recovery of mediators from the lesions. Elevated histamine concentrations in the skin have been shown in CSU (Kaplan et al, 1978). Exocytosed immunoreactive tryptase was demonstrated in skin biopsies from non-lesional skin in CSU patients by immunochemistry studies (Vena et al, 2002). Elevated tryptase concentration was also shown in suction blister studies in CSU (Deleuran et al, 1991). Recently, CGRP and VEGF were also reported in lesional skin in CSU (Kay et al, 2014b). Elevated concentrations of MBP were noted in up to 38% of CSU patients (Peters et al, 1983). Therefore, multiple mediators were demonstrated *in vivo* in CSU, however, their relative contribution and interactions are yet to be established.

It is worth noting that spontaneous wealing in CSU is a complex phenomenon where several mediators are likely to interact with each other as was suggested by a two-mediator hypothesis (Williams, 1977a). According to Williams, a vasodilator mediator is likely to potentiate the plasma exudation effects of the mediator, which enhances vascular permeability (Williams, 1977a). These synergistic interactions were noted between histamine and prostaglandins (Williams, 1977b) or CGRP (Brain & Williams, 1985). The *in vivo* synergistic interactions of mediators are likely to account for a longer duration of weals in CSU but the relevant interactions of mediators *in vivo* in CSU awaits characterization. There is a need for *in vivo* models of wealing in CSU to explore the relative contribution of various mediators to the development of urticarial lesions in the context of persistent dermal inflammation in CSU.

Serum histamine-releasing activity is a known pathophysiological feature of CSU that is observed in 30-50% of patients (Grattan, 2004). The relevance of serum histamine releasing factors to the weal development in CSU is suggested by the phenomenon of serum autoreactivity that occurs in 45.9% of CSU patients (Krause et al, 2009). An intradermal injection of autologous serum is known to induce a cutaneous response that resembles a weal-and-flare reaction although a close observation would highlight dissimilarities such as absence of pseudopodia, the lack or minimal itching and a slower kinetic for weal formation compared to histamine-induced wealing. The relevance of serum factors to the development of wealing if inferred from the transfer of the serum-induced weal response to healthy subjects. However, there are only two observations of passive transfer of weal response to date (Grattan, 1981; Pastore et al, 2013) although the latter did not use the recipient's own serum as a control.

Cutaneous reactions to autologous serum are thought to be mediated by anti-FcεRIα antibodies by analogy with neutralization experiments with α-chains of FcεRI receptors that block histamine release from basophils from healthy donors (Hide et al, 2013; Kaplan, 2004). However, direct proof of the contribution of anti-FcεRIα to the skin response to autologous serum in CSU is currently lacking. Anti-FcεRIα antibodies are not available for *in vivo* use. Mice models with humanized FcεRI receptor exist (Hide & Greaves, 2013) but the experiments with anti-FcεRIα antibodies are still awaited. To our knowledge, an interaction of immunoreactive anti-FcεRIα antibodies and skin mast cells has not yet been demonstrated in the skin of CSU patients. Skin priming and kinin generation during blood clotting were suggested as non-specific mechanisms that may account for skin reactions to autologous serum in CSU. This is a possibility given profound skin infiltration with inflammatory cells (Ying et al, 2002; Kay et al, 2014a) and enhanced skin sensitivity to kallikrein in CSU patients (Imai, 1989) but the demonstration of *in vivo* weal formation confined to the serum fraction of greater than 100kDa (Grattan et al, 1991) and *in vitro* histamine releasing activity towards skin mast cells attributed to IgG fractions in serum from CSU patients (Niimi et al, 1996) would argue against kinin sensitivity as the only explanation for serum autoreactivity. Additionally, the demonstration of mast cell degranulation autologous serum skin test in CSU (Grattan, 1990) suggests that other mechanisms than skin priming may also mediate serum autoreactivity. Overall, this justifies the need for further research establishing whether autologous serum- induced weal could be an experimental model of wealing in CSU and to which extent it reproduces spontaneous wealing in CSU. Research into the mechanisms underlying serum reactivity in CSU would not only provide an experimental tool to delineate certain aspects of wealing but would also provide insights into the clinical significance of autologous serum skin test in CSU which is widely used in clinical practice.

Dysregulated cytokine network are a common feature of inflammatory conditions although the data on cytokine expression in the dermis of CSU is limited. CSU patients were shown to have an upregulation of TNF-α and IL-3 in the dermis (Hermes et al, 2003). Also, increased cellular mRNA expression for IL-4, IL-5 and IFN-γ was revealed in CSU by *in situ* hybridization (Ying et al, 2002). Local generation of cytokines in the skin of CSU patients is of particular interest in view of their potential effects on skin mast cell function. The effects of the cytokine milieu on inflammatory events *in situ* are plausible but has yet to be established. For example, *in vitro* data suggest that recombinant TNF-α can release histamine and tryptase from skin mast cells (van Overweld, 1991). Whether cytokines contribute to continuous histamine release or perpetuation of the local inflammation in CSU via other mechanisms has yet to be elucidated.

Further research into cytokine network in CSU is needed to enhance our understanding of disease-specific cytokine expression patterns at skin level and their effects on skin mast cell releasability in CSU.

Skin mast cell hyperreleasability is regarded as an important determinant of clinical manifestations in CSU. In suction blister studies, skin mast cell releasability was shown to fluctuate with disease activity in CSU (Jacques et al, 1992). Codeine skin testing was used experimentally to explore skin reactivity in CSU (Cohen & Rosenstreich, 1986;

Shall & Saihan, 1992) with conflicting results. Taking into account the recent studies, we may consider that codeine sensitivity reflects not only cell responsiveness but also mast cell density in CSU (Kay et al, 2014a). Nevertheless, skin sensitivity to codeine can be an important factor that suggests skin mast cell functional abnormality in CSU. Spontaneous histamine release in CSU is poorly understood. The *in vivo* evidence is lacking while experimental data suggest an increased spontaneous histamine release from cultured mast cells in CSU patients than that in healthy subjects (Saini et al, 2009). Whether spontaneous histamine release is enhanced *in vivo* in CSU remains to be elucidated.

Overall, the biochemistry and immunobiology of the dermis of uninvolved and lesional skin in CSU is only partially understood. The skin chamber studies and immunohistochemistry laid the groundwork for our understanding of biochemical events in the dermis in CSU. The advent of newer, less traumatic techniques, like microdialysis, holds the promise that the more detailed picture of *in vivo* biochemical and immunological events in the skin associated with wealing will emerge. Further understanding of the contribution of various proinflammatory mediators and cytokines to the weal formation in CSU is clinically important to allow for better therapeutic targeting in this disease.

Cutaneous microdialysis offers an opportunity of *in vivo* studying of changes in mediators and cytokines in dermal interstitial fluid in CSU (Figure 8). Cutaneous microdialysis has been used for dermatological research since the 1990s (Anderson et al, 1992; Petersen et al, 1996; Church et al, 1997, Groth, 2006). Microdialysis sampling is based on the principle of passive diffusion across the microdialysis membrane governed by the concentration gradient of the solute (Figure 7) (Chaurasia et al, 2007). While described in details in the Methods section below, in brief, microdialysis sampling is performed by insertion of the microdialysis probes (small hollow membranes made of semipermeable fibre) into the dermis (Schmidt et al, 2008). The microdialysis probes are connected to a microdialysis pump which allows their perfusion with a pH-buffered perfusate at a constant flow rate. During the perfusion of the microdialysis probes, the solute of interest from the extracellular space diffuses into the perfusate into the lumen of the microdialysis hollow fibre down the concentration gradient. The size of molecules that can diffuse across the microdialysis membrane is limited by the size of the pores in the membrane (a molecular cut-off). Once perfused through the microdialysis membrane implanted into the dermis, the perfusate is considered as dialysate which can be analysed for the content of mediators and cytokines.

The advantage of cutaneous microdialysis includes *in vivo* continuous sampling of extracellular fluid from the dermis under minimally invasive conditions. The technique is characterized by high temporal and spatial resolution (Clough et al, 2007). Site-specific sampling permits studies into skin responses to skin testing with various stimuli or topical therapeutic interventions. Methodological difficulties of cutaneous microdialysis involve the need for thorough optimisation and validation of the microdialysis experimental protocol and analytical procedures for every research application (Andersson, 1995). The limitations of the technique are related to the recovery of large or lipophilic molecules (Clough, 2005). Very low solute concentrations in low dialysate volumes present challenges for sample analysis and require highly sensitive analytical assays (Petersen, 1997a).

For the purpose of this study, we define the inflammation in CSU as the local changes in mediators and cytokines in the skin, with or without associated skin serum autoreactivity and serum histamine releasing activity in the circulation.

The hypothesis of this research proposes that weal formation in CSU is mediated by local histamine release due to circulating serum factors, aberrant skin mast cell releasability and altered skin microenvironment. To test this hypothesis, we devised an experimental model consisting of skin challenges with PBS, autologous serum and codeine followed by monitoring of the local changes in mediators and cytokines using cutaneous microdialysis technique. The assessment of baseline levels of mediators and cytokines was used to characterize dermal microenvironment in the visibly uninvolved skin in CSU patients and healthy subjects. Autologous serum was chosen as a challenge stimulus to explore the effect of serum histamine-releasing factors on local histamine release in the

Figure 8. The Principle of Cutaneous Microdialysis

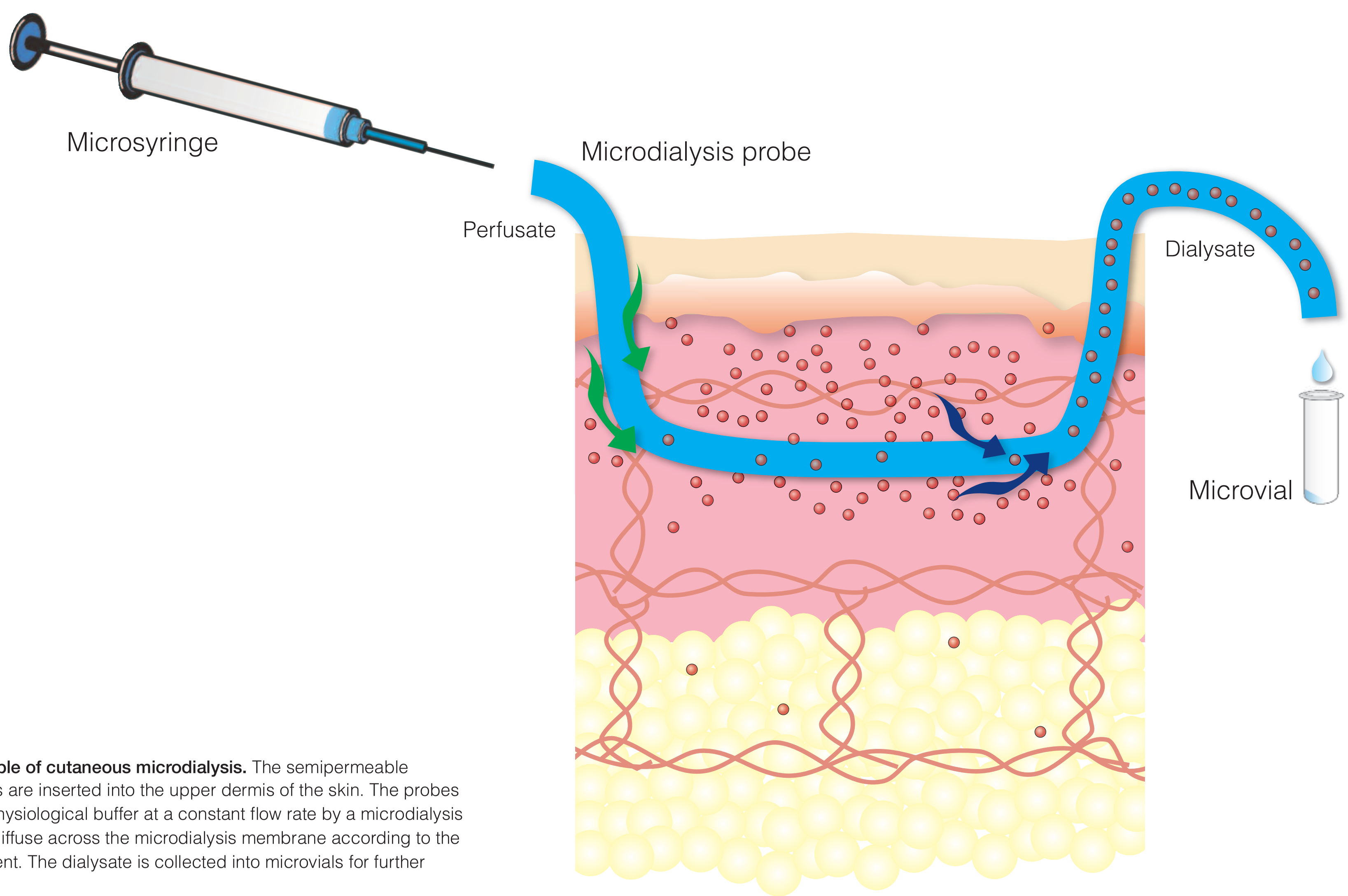


Figure 8. The principle of cutaneous microdialysis. The semipermeable microdialysis probes are inserted into the upper dermis of the skin. The probes are perfused by a physiological buffer at a constant flow rate by a microdialysis pump. The solutes diffuse across the microdialysis membrane according to the concentration gradient. The dialysate is collected into microvials for further analysis.

skin in CSU patients and healthy controls. Skin challenge with codeine was used to assess skin mast cell reactivity to non-immunological stimulation. The concentration of codeine solution was chosen following the previous experiments in healthy subjects at the University of Southampton (Cole et al, 2001). Skin testing with PBS was used as a negative control in the experimental model and also was used to assess the spontaneous histamine release in CSU and healthy controls.

Several methodological components need to be taken into account for an introduction of cutaneous microdialysis to a new research application. The pharmacokinetics of the solute of interest, the range of its dermal concentrations and an optimal temporal resolution for its sampling need to be established before using cutaneous microdialysis for full-scale clinical studies (Groth, 2006; Petersen, 1997a). The need for prior knowledge about these parameters justifies piloting cutaneous microdialysis in patients with CSU. In addition, dermal tolerance of the microdialysis procedure in patients with CSU needs to be confirmed to ensure the validity of the microdialysis data in CSU.

The purpose of this pilot study was to evaluate an autologous serum skin test as an experimental model for weal formation in CSU and to explore the pharmacokinetics of histamine, tryptase and cytokines at the baseline and in response to intradermal skin testing with PBS, autologous serum and codeine in the skin of CSU patients and healthy controls.

The objectives of the study were:

- to assess the dermal tolerance of cutaneous microdialysis in CSU;
- to develop the experimental protocol for cutaneous microdialysis studies into skin reactivity to PBS, autologous serum and codeine in CSU patients and healthy controls;
- to establish the baseline values for dermal concentrations of histamine, tryptase and cytokines in CSU patients and healthy controls;
- to explore the kinetics of histamine release in the skin of patients with CSU in response to skin testing with PBS, autologous serum and codeine;
- to choose the optimal outcome measures for future microdialysis studies in CSU.

2.2 Materials and Methods

Cutaneous microdialysis studies in CSU were carried out within the scope of the project "Microdialysis study of inflammatory mediators and cytokines in the early and late-phase of dermal response to PBS, codeine and autologous serum injections in chronic ordinary urticaria patients and healthy controls". The study was approved by the East Norfolk & Waveney Research and Governance Committee (Ref:07/Q0101/42) and the Norfolk Research Ethics Committee (Ref: 2006DERM02L(198-12-06)) (Appendix 1).

2.2.1 Participant Characteristics

A total of fourteen patients with CSU and thirteen healthy subjects completed the study. CSU patients were recruited from Cutaneous Allergy Clinics at the Dermatology Department of Norfolk & Norwich University Hospital (Norwich, UK). Healthy volunteers were recruited from the hospital staff through noticeboard advertisements. Participants were reimbursed for their traveling and meal expenses and loss of income incurred by their participation in the study. Healthy volunteers were paid for their participation in the study.

Inclusion criteria for CSU patients were age 18-70 years and continuous CSU. Exclusion criteria included pregnancy, lactation; co-morbidity of bronchial asthma treated with inhaled corticosteroids at moderate-to-high doses (e.g. beclomethasone dipropionate <400μg/day) or allergic rhinitis treated with intranasal corticosteroids; continuous treatment with potent topical and/or systemic steroids within one month or systemic steroid rescue treatment for less than 3 days within 2 weeks; treatment with ciclosporin within 3 months; current treatment with β-adrenoreceptor blockers, allergy to amide local anaesthetics, latex allergy, ongoing treatment with oral codeine, current treatment with tricyclic antidepressants (doxepin prescribed for urticaria at lower doses than for clinical depression was considered as an antihistamine rather than antidepressant) and H2 blockers prescribed for conditions other than chronic urticaria. Patients on treatment with doxepin or H2 antihistamines for their urticaria were asked to discontinue them 2 weeks or 72 hours before microdialysis experiments, respectively. Additional exclusion criteria for CSU patients included co-existing predominant physical urticarias; CSU with a suspected or confirmed infectious, allergic, drug-induced or physical cause; biopsy-proven urticarial vasculitis defined by the presence of leukocytoclasia, fibrin deposition, endothelial swelling with or without erythrocyte extravasation. Additional exclusion criteria for healthy controls were currently active atopic disease (eczema, bronchial asthma, hay fever), skin diseases or autoimmune disease, requiring treatment, including autoimmune thyroid disease. Healthy subjects were not tested for atopy in this study.

Of fourteen CSU patients who completed the study, there were hve men and nine women. The mean age of patients with CSU was 52.3 years (range 33-68 years). Of thirteen healthy controls who completed the study, there were hve men and eight women. The mean age of healthy controls was 46.2 years (range 30-60 years). All patients and healthy controls provided written informed consent before their participation in the study.

2.2.2 Experimental Set-up for Microdialysis Studies

For our microdialysis experiments, we used a high precision CMA 400 microsyringe pump (CMA Microdialysis AB, Sweden) to ensure accurate, continuous and pulseless perfusion. The pump was subjected to a safety check at the Norfolk & Norwich University Hospital. The microdialysis probes were in-house manufactured at the Immunopharmacology Unit at the University of Southampton. The probes were characterized by a 3,000 kDa cut-off. Linear microdialysis probes were manufactured using membranes from the dialysis cartridge Plasmahow ® OP Series Asahi Hollow Fiber Plasma Separator. The membrane was glued to nylon connecting tubing (Portex, France) using cyanoacrylate glue (Loctite, Ireland). Probe construction was carried out wearing sterile gloves at the dedicated area covered with laboratory benchcoat. Before the microdialysis experiments, the probes underwent ethylene oxide sterilization at the In-Health Decontamination Service (Cardiff, UK). Skin dialysates were collected into sterile DNA-free cryotubes (Greiner Bio-One, UK).

2.2.3 Cutaneous Microdialysis Procedure

H1 antihistamines were withdrawn for 72 hours, H2 blockers for 72 hours, tricyclic antidepressants (e.g. doxepin) 2 weeks before the experiment. Patients were given 1%

menthol in aqueous cream to reduce pruritus on an as needed basis. Participants were asked to attend the microdialysis procedure having applied local anaesthetic EMLA cream to three sites on their forearm and covered with occlusive Tegaderm dressing (3M Healthcare Ltd, UK) for 60 minutes before the start of microdialysis (Figure 9).

The patients and volunteers were provided with verbal and written instructions. Patients were asked to avoid strenuous exercise, eating or drinking hot or caffeine containing beverages for 8 hours before the microdialysis studies. Visual analogue scales for itching and wealing over 24 hours was assessed at baseline.

Microdialysis studies (see video: Appendix 2 on CD-R) were carried out at the day research ward at the Clinical Trials and Research Unit at the Norfolk & Norwich University Hospital (Figure 9A). Before the microdialysis experiment, patients and volunteers were asked to rest quietly lying in bed for 10 min. Six linear microdialysis probes (a molecular weight cut-off of 3,000 kDa) were inserted into non-lesional skin of the volar surface of the arm of patients and volunteers under local anaesthesia with EMLA cream (Astra AB, UK) (Figure 9B) and a short exposure to an ice cube before the probe insertion. The probe insertion was carried out using a 24 gauge guide cannula (Figure 9C) which was then removed leaving the microdialysis probe implanted in dermis (Figure 9D). The probes run for 20 mm below the surface of the skin. The probes were connected to the microdialysis pump via a plastic tubing and were perfused briefly with sterile saline solution at a flow rate of 3μl/min to test the probes for leaks, then they were disconnected from the pump and the arm was bandaged for 1 hour and 40 min for the skin to recover from local anaesthesia and initial trauma caused by probe insertion. During the sample collections the microdialysis probes were reattached to the pump and perfused with sterile Ringer's solution at the constant rate of 3μl/min.

Sterile PBS (pH=7.4) (Tayside Pharmaceuticals, UK) (20μl) was injected, using an insulin syringe, at the midpoint along each probe (1mm away from the probe) at the microdialysis site 1, autologous serum (20 μl) — at the microdialysis site 2 and codeine phosphate (20μ1 of 0.3mM codeine phosphate solution prepared in laminar flow at the Pharmacy at the Norfolk & Norwich University Hospital, UK) — at the microdialysis site

3 on the non-dominant arm after the baseline perfusion (Figure 9E). At the end of the microdialysis experiment, the probes were removed and a sterile dressing was applied.

2.2.4 Protocol Development for Microdialysis Studies

Protocol development in this study was related to the technical aspects and design issues of the microdialysis procedure. Adjustments to the experimental protocol were needed to optimize the procedure for its use in CSU. The protocol design development was focused on modifications of sampling strategy to achieve an optimal temporal resolution of microdialysis sampling for mediators of interest in skin responses to intradermal testing with PBS, autologous serum and codeine in CSU. Optimisation of sample preparation for analytical procedures was critical to ensure that the concentration range for the mediators of interest in CSU falls within the linear range of the analytical methodologies. Modifications in working research hypothesis evolved through protocol development and interim analysis of the data.

Protocol 1 (Figure 10)

Initially, the protocol was designed to establish the baseline mediator concentrations and to assess the early and late phase skin response to intradermal testing with PBS, autologous serum and codeine in CSU patients and healthy subjects. For protocol 1, microdialysis sampling in patients and healthy controls was carried out at baseline (Time period 1), within the first 2 hours (Time period 2), between 4-6 hours (Time period 3) and 24-26 hours after skin testing (Time period 4). The duration of the baseline collection was 30 min, Time periods 2-4 consisted of three 40 min dialysate collections each. Time period 1 baseline collection and Time period 2-3 collections after skin testing were performed on non-dominant arm while Time period 4 collection was carried out on the other arm. Skin testing on the opposite arm before Time period 4 collection was carried out using a template of microdialysis sites for the next day microdialysis experiment. The sites of skin testing and planned location of the probes were marked with a waterproof marker. Undiluted dermal dialysates were analysed for histamine, tryptase and cytokines.

Figure 9. Multi-step Procedure of Cutaneous Microdialysis

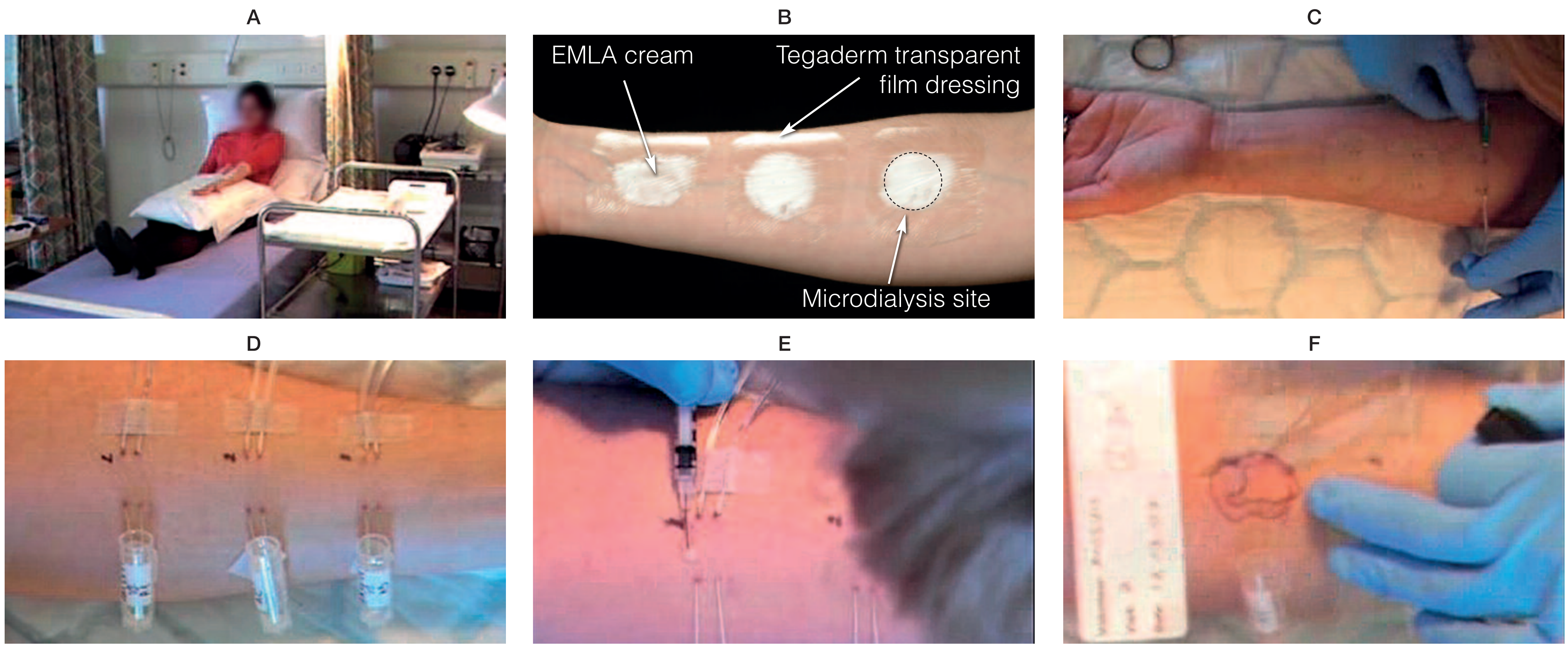
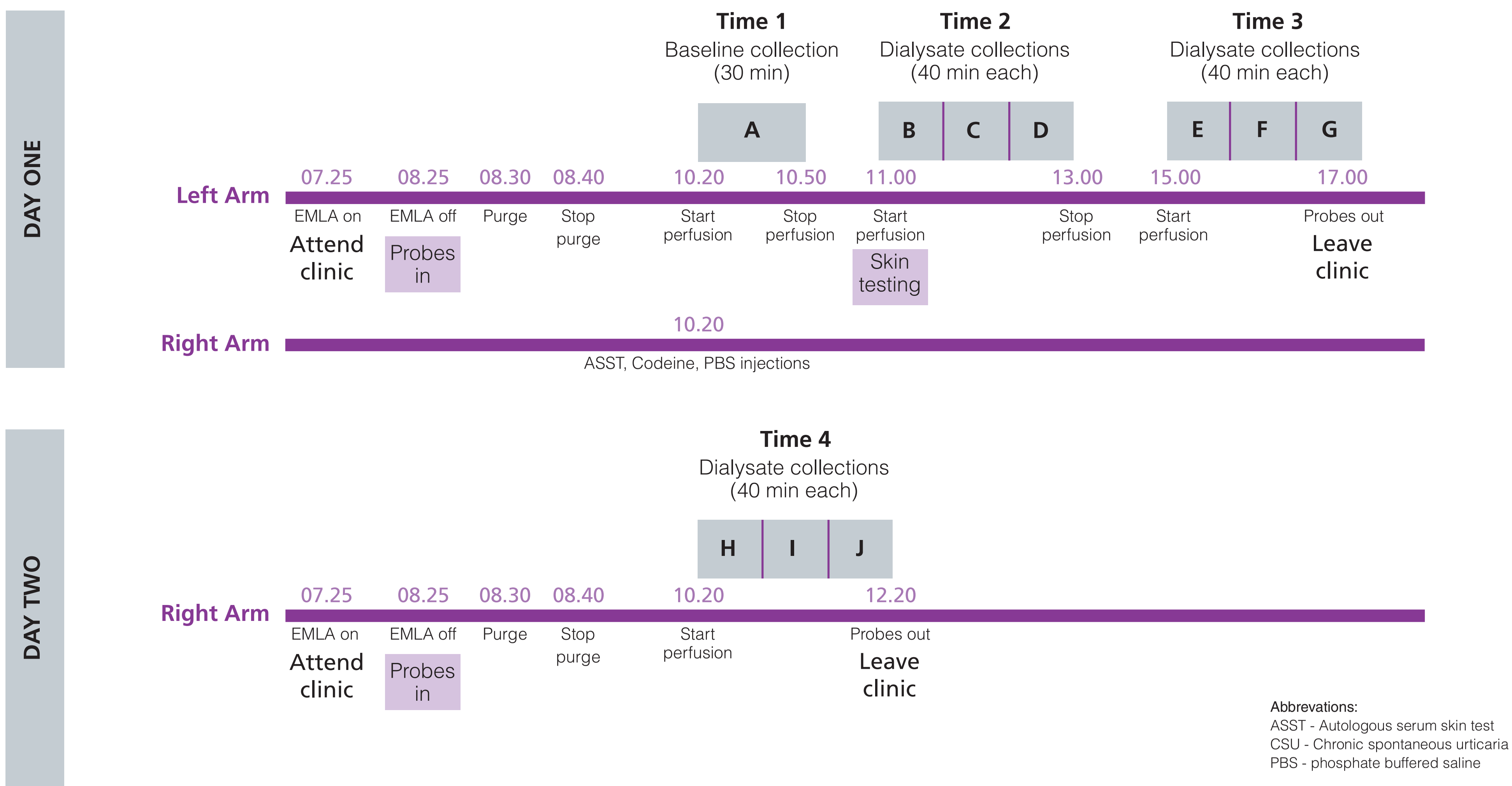


Figure 9. Multi-step procedure of cutaneous microdialysis. Microdialysis experiments were carried out at the Clinical Research and Trials Unit (Figure 9A). The EMLA cream (lidocaine 2.5%, prilocaine 2.5%) was applied to three microdialysis sites 3-5 cm apart at least an hour before the experiment (Figure 9B). The microdialysis probes were inserted using a 24-gauge needle as a guide cannula (Figure 9C). After the probes were inserted through the exit puncture of the needle, the needle was withdrawn leaving the microdialysis membrane in place in dermis (Figure 9D). After the recovery period, the probes were connected to the microdialysis pump for dialysate sampling. After 30 min baseline collection, skin testing with phosphate buffered saline, autologous serum and codeine was performed along the microdialysis probes (Figure 9E). The response to skin testing was assessed by a release of mediators in skin dialysates and by planimetry using acetate films (Figure 9F). After microdialysis sampling, the microdialysis probes were removed and the sterile bandage was applied for 12-24 hours after the microdialysis experiment.

Figure 10. Microdialysis Timeline - Protocol 1



Abbrevations:
ASST - Autologous serum skin test
CSU - Chronic spontaneous urticaria
PBS - phosphate buffered saline

Figure 10. Microdialysis timeline - Protocols 1. Protocol 1 was designed to carry out microdialysis sampling in CSU patients and healthy controls at baseline (Time period 1), within the first 2 hours (Time period 2), between 4-6 hours (Time period 3) and 24-26 hours (Time period 4) after skin testing. The baseline collection lasted for 30 mins, Time periods 2-4 consisted of three 40 min sampling intervals each.

Protocol 2 (Figure 11)

The next step of protocol development was focused on optimising the intervals of microdialysis sampling. As illustrated in the Figure 12, Protocol 2 involved measurements of skin responses in patients and healthy controls at baseline (Time period 1), within the first two hours (Time period 2), between 4-6 hours (Time period 3) after skin testing. The duration of the baseline collection was 30 min. The collection B within Time period 2 included eight 5-min dialysate collections. Collections C and D within Time period 2 and all collections within Time period 3 were each of 40 min duration. The key differences from Protocol 1 were 5-min dialysate sampling intervals for the first 40 min after skin testing and limiting the duration of the protocol by 6 hours after skin testing. Neat dermal dialysates were analysed for histamine. An introduction of 5 min dialysate collections for the first 40 min after skin testing was planned for pharmacokinetic profiling of histamine release to intradermal testing with PBS, autologous serum and codeine in CSU patients and healthy controls. This protocol modification was planned for determining optimal temporal resolution for dialysate collections after skin testing.

Protocol 3 (Figure 11)

Further protocol development was related to fine-tuning the intervals of microdialysis sampling and evaluating the dilution factor for dermal dialysates. Protocol 3 consisted of measurements of skin response in CSU patients and healthy controls at baseline (Time period 1), within the first 2 hours (Time period 2) and between 4-6 hours (Time period 3) after skin testing. The duration of the baseline collection was 30 min. The collections B and C within Time period 2 included eight 5-min dialysate collections each. Collection D within Time period 2 and all the collections within Time period 3 had duration of 40 min each. The main differences from the Protocol 2 were the extension of 5-min dialysate sampling for the first 80 min after skin testing. Also, one probe at each microdialysis site was allocated for dialysate sampling for analysis of neat dialysates to assess the baseline histamine concentration, while the other probe was used for dialysate collection for analysis of diluted samples to evaluate the peak histamine concentration after skin testing. Fine-tuning of sampling and analytical procedures in Protocol 3 was planned for

Figure 11. Microdialysis Timeline - Protocols 2 and 3

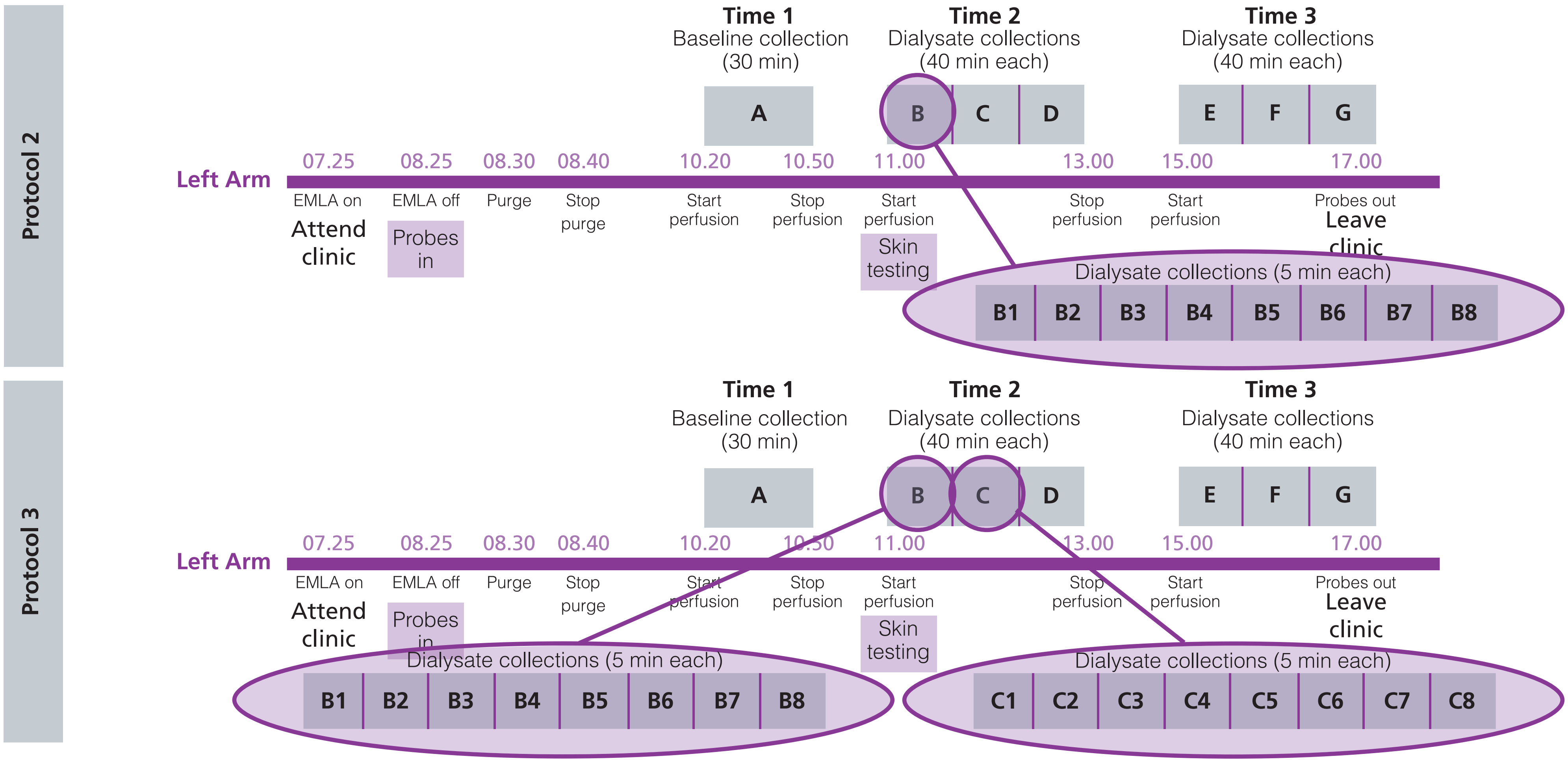
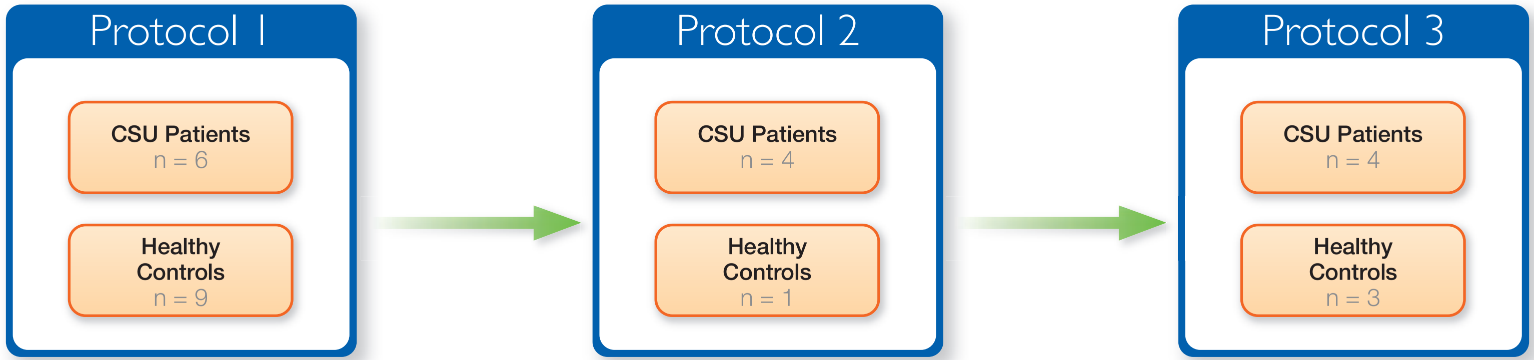


Figure 11. Microdialysis timeline - Protocols 2 and 3. There were consequent adjustments to the experimental protocol to ensure optimal dialysate sampling in CSU. In Protocol 2, key protocol modifications included 5 min dialysate sampling intervals (collections B1-B8) for the first 40 min after skin testing and limiting the duration of the protocol by 6 hours after skin testing. The introduction of 5 min dialysate collections for the first 40 min after skin testing was introduced for optimal temporal resolution and phamacokinetic profiling of histamine release to intradermal testing with PBS, autologous serum and codeine in CSU patients and healthy controls. In Protocol 3, sixteen 5-min sampling intervals were introduced within 80 min of skin testing (collections B1-B8 and C1-C8). During Protocol 3, the dilution factor for dermal dialysates was evaluated for optimal histamine detection.

Figure 12. Protocol Development for Microdialysis Studies in CSU

A. Participant flow diagram for Protocols 1-3 in a pilot study of cutaneous microdialysis in CSU



B. Adaptive study design for piloting cutaneous microdialysis in CSU

Protocol Modification	Dialysate Collections										
	Baseline	Skin Testing	Time 1			Time 2			Time 3		
Protocol 1	30 min		40 min	40 min	40 min	40 min	40 min	40 min	40 min	40 min	40 min
Protocol 2	30 min		5 min x 8	40 min	40 min	40 min	40 min	40 min			
Protocol 3	30 min		5 min x 8	5 min x 8	40 min	40 min	40 min	40 min			

Analysis of baseline extracellular histamine concentrations (Baseline cells) · Pharmacokinetic analysis (C_{max}, T_{max}, AUC) (5 min x 8 cells in the Time 1 first column)

Figure 12. Pooling the data between the experimental protocols for data analysis. The protocol development included modifications in sampling strategy and resulted in application of three experimental protocols (Protocols 1-3) throughout the study (Figure 12A). For data analysis, baseline concentrations of histamine were pooled from three protocols for microdialysis sampling (Figure 12B). Analysis of the area under the curve for 40 min after skin testing included pooled data of 5-min interval sampling from Protocols 2-3. Time 1 designates 0-2 hours after skin testing, Time 2 - 4-6 hours after skin testing and Time 3 - 24-26 hours after skin testing.

Abbrevations:
CSU - Chronic spontaneous urticaria
C_{max} - Peak concentration
T_{max} - Time to reach peak concentration

evaluation of histamine release patterns to codeine and autologous serum in CSU and for determining the optimal duration of 5 min sampling after skin testing. Determining the range of baseline and peak histamine concentrations was important for estimating the optimal dilution of dialysate samples for analytical accuracy.

The explorative pilot project (Protocol 1) required protocol modification based on the data accumulating in the study. Sequential prospective data-driven modifications to the study protocol are regarded as an adaptive study design which enhanced the efficiency and reduced the costs of the study without compromising its integrity and validity of the data (Orloff et al, 2009). An adaptive study design allowed us to improve or modify the study design during the study in a pre-planned manner guided by the interim data analysis. The adaptive design of our study also enabled us to incorporate the data of the Pilot Project (Protocol 1) into the main study (Protocols 2 and 3). However, the outcome measures throughout the study did not change, therefore, the data from different protocol modifications were pooled for analysis (Figure 12).

The microdialysis protocols were developed in collaboration with Prof. Martin Church and Prof. Geraldine Clough from the Immunopharmacology Group at the University of Southampton in view of their extensive expertise in skin mast cell biology and cutaneous microdialysis research in allergic inflammation.

2.2.5 Dialysate Handling and Analysis

Skin dialysates collected during the microdialysis experiments were snap frozen in liquid nitrogen and were stored at -70°C and then sent on dry ice for analysis to the University of Southampton by a courier delivery. Dermal dialysates were assessed for histamine by Histamine ELISA kit (BioSource, Belgium) in the Protocol 1 and for tryptase by an in-house double antibody-sandwich ELISA (University of Southampton, UK).

For histamine analysis of skin dialysates in the Protocol 2 and 3, the Histamine ELISA kit was purchased from the Cambridge Biosciences Ltd because of unavailability of the previous kit. The detection limit for Histamine ELISA was 0.12 ng/ml. For histamine analysis by ELISA, the linearity was observed in the range of concentrations between

0.74-8.48ng/ml. Histamine analysis was carried out by Dr Elena Borzova in collaboration with Dr Carolanne McGuire (University of Southampton). Histamine analysis in skin dialysates collected at 5-min intervals was carried out by Dr Laurie Lau (University of Southampton, UK).

Tryptase measurements were carried out using an in-house double antibody-sandwich ELISA utilizing polyclonal rabbit anti-tryptase antibodies for capture and the mouse AA5 monoclonal antibodies for the detection (University of Southampton, UK). Monoclonal AA5 antibodies were purified from hybridoma culture supernatant fluid by protein G chromatography. Western blot studies characterized the binding of AA5 mAbs to both human recombinant α- and β- tryptase isoforms (Buckley et al, 1997). After coating the plate with rabbit anti-tryptase serum, the plate was washed and non-specific binding was blocked with bovine serum albumin followed by another washing. The dialysate samples were added to the plate for incubation. The plates were washed and then incubated with the mouse AA5 monoclonal antibodies. After washing, biotinylated rabbit anti-mouse IgG was added and followed by washing and adding avidin-biotin-peroxidase complexes. The plate was washed and developed using p-nitrophenyl substrate. Readings of the colorimetric reaction was carried out at 450/595nm optical density. The interaction of the rabbit anti-mouse IgG and the peroxidase conjugate was enhanced by binding between avidin and biotin. The detection limit for the assay was 0.06 ng/ml. The linear region of the curve was between 0.75 ng/ml and 64 ng/ml. The tryptase assays of the dialysates were carried out by Dr Andrew Walls and Dr Zhou at the University of Southampton.

Cytokine content in the skin dialysates were analysed using BD Cytometric Bead Array Flex Sets measuring IL-5, IL-13, IL-6, IL-3, IFN-γ and TNF-α. Cytometric beads coated with specific antibodies allows for the capture of the target protein in the sample on the bead surface. In the Flex Set, several particles with discrete fluorescence intensity can be discriminated based on their size and fluorescence and, therefore, their use permits analysis of multiple target proteins in one sample, i.e. multiplexing. During the flow cytometric analysis, each bead population is designated with an alphanumeric position relative to other bead population which enables a separate flow cytometric read-out for each bead population. The fluorescence readout was carried out using FACS Calibur instrument at the Flow Cytometry facility at the University of Southampton. The data were analysed by FCAP Array™ software. The limit of detection of the assays was 20 pg/ml for selected cytokines. The cytokine assays were carried out by Dr Elena Borzova in collaboration with Dr Carolanne McGuire (University of Southampton, UK).

2.2.6 Planimetry

Reading of the weal areas was carried out by planimetry using acetate films to record the weal perimeter after skin testing with PBS, autologous serum and codeine. The planimetry imagery was then digitized and the weal area was calculated using the NIH Image J software.

2.2.7 Data Analysis

Continuous variables are presented as medians with interquartile ranges. The pharmacokinetic parameters included peak concentrations (C_{max}), time to reach the peak concentration (T_{max}) and the area under the curve (AUC) and were calculated using LabPilot 5 software (CMA Microdialysis, Sweden). Comparisons between three groups were first tested using the Kruskall-Wallis test and then Mann-Whitney U testing was used to evaluate the difference between each pair of study groups. Correlations between parameters were assessed by Pearson's correlation coefficient for variables with linear relationships and by Spearman's correlation coefficient for those with non-linear relationships. Statistical analysis was carried out using Minitab 16 (Minitab, USA). P-value <0.05 were considered to be statistically significant.

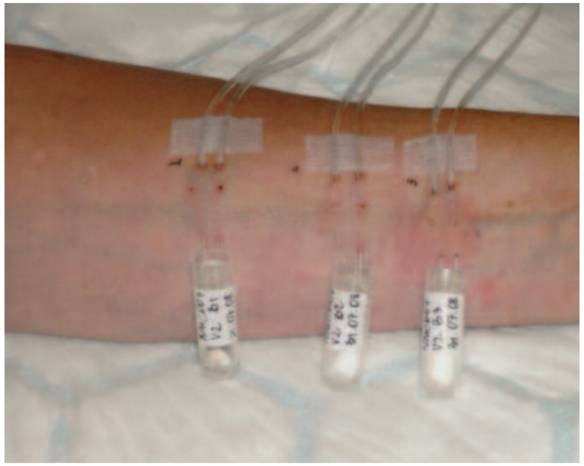
2.3 Results

2.3.1 Dermal Tolerance of Cutaneous Microdialysis Studies in CSU

Methodological validity of cutaneous microdialysis studies in CSU was supported by clinical assessment of microdialysis sites and pharmacokinetic evidence from our microdialysis experiments. Cutaneous microdialysis procedures were well tolerated in all but two patients with CSU (Figure 13). Figure 13 shows the representative microdialysis experiments in CSU patients and healthy controls suggesting dermal tolerance of cutaneous microdialysis procedures in both groups of research participants.

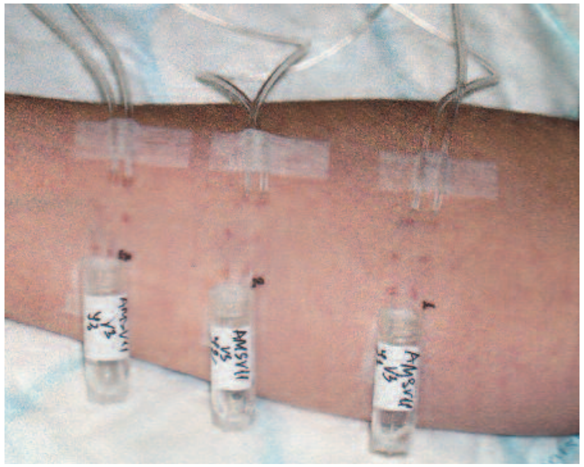
Figure 13. Representative Microdialysis Experiments in CSU Patients and Healthy Subjects

A. Representative microdialysis experiment in a CSU patient



NMCP07 - Left Arm

B. Representative microdialysis experiment in a healthy subject



AMSV11 - Right Arm

Figure 13. Photos of microdialysis experiments demonstrate dermal tolerance of microdialysis procedures in CSU patients (Figure 13A). In our pilot study, cutaneous microdialysis was well tolerated without spontaneous wealing to the insertion of the microdialysis probes or during the experiments in all (12/14) but 2 CSU patients. Clinical assessment of the microdialysis sites suggested that dermal tolerance of cutaneous microdialysis in most CSU patients was comparable to that of healthy controls (Figure 13B).

Abbrevations:
CSU - Chronic spontaneous urticaria

However, some methodological considerations regarding cutaneous microdialysis experiments arose in a few CSU patients. It was worth noting that skin reactivity to codeine may be enhanced in a few CSU patients or healthy volunteers. Acute localized urticaria around the microdialysis site was noted in one healthy control (Figure 14). Few patients with CSU developed large reactions to skin testing with codeine or autologous serum (Figure 15) and/or persistent wealing to microdialysis procedures (Figure 16). Although there was an overlap in surrounding flares, the weals did not overlap. Taking into account the previous literature (Petersen et al, 1997b), this would not affect the histamine concentrations in the adjacent sites. The insertion of the microdialysis probes resulted in local wealing in some patients (Figure 17). Spontaneous wealing at the microdialysis sites may occur before (Figure 18A) or during the microdialysis experiments (Figure 18B).

Taping of the microdialysis probes to skin resulted in spontaneous wealing in few patients with CSU. In two CSU patients and one healthy control (Figure 19), we observed increased dermal histamine or tryptase concentration in skin dialysates at baseline comparable to that after skin testing with codeine. This suggested that this dermal response was likely to occur as a result of the insertion or the presence of the microdialysis probes in the dermis. We are aware of the fact that dermal hyperreactivity to microdialysis procedures may occur in a few healthy subjects. We report this dermal response in two out of fourteen CSU patients in our study and we would recommend skin biopsy as an alternative for these patients. It may be important to include their results in the analysis because the exclusion of these patients from the studies may incur selection bias.

2.3.2 Baseline Dermal Concentrations for Histamine, and Tryptase and IL-6 wealing in a few Patients with CSU and Healthy Subjects

In our pilot study, we estimated the baseline dermal concentrations of histamine, and tryptase and IL-6 in dialysates from patients with CSU and healthy controls. The median for the baseline histamine concentration in CSU patients (n=12) was 2.32 (0.86; 2.78) ng/ml while the baseline histamine concentration for healthy controls (n=13) was 0.86 (0.28; 1.33) ng/ml. Sample analysis revealed that our study was sufficiently powered (76.5%) to detect a statistically significant difference in the baseline histamine concentration in CSU patients and healthy controls (unpaired t test, $p<0.05$). For future microdialysis studies, sample size calculation indicated that 12 participants in both groups is a sufficient sample size for demonstrating the statistically significant difference in the baseline histamine concentrations between CSU patients and healthy controls (Figure 20).

The mean baseline concentration for tryptase in skin dialysates of patients with CSU (n=9) was 2.1 (1.68; 2.77) ng/ml whereas the baseline tryptase concentration in healthy controls (n=8) was 0.70 (0.70; 1.72) ng/ml. The difference in the baseline tryptase concentrations for both groups was not statistically significant (Mann-Whitney U test, p=0.1437) (Figure 21). Sample analysis revealed that our pilot study for tryptase analysis in 9 CSU patients and 8 healthy volunteers was not sufficiently powered (41.4%) to detect the minimal significant difference in the baseline dermal tryptase concentrations between these two study groups. Baseline IL-6 concentration in CSU patients was 33.08 (22.961; 82.045) pg/ml whereas in healthy controls it was 12.81 (6.92; 31.11) pg/ml. However, the difference was not statistically significant (Figure 22).

Noteworthy, baseline histamine concentration in skin dialysates tends to be higher in CSU patients with a positive autologous serum skin test than those in with a negative autologous serum skin test and healthy controls (Table 2, Figure 23A). Also, baseline histamine concentrations in skin dialysates in CSU patients with serum histamine-releasing activity was significantly higher compared to those without serum histamine-releasing activity (Mann-Whitney U test, p=0.0262) and healthy controls (Mann-Whitney U test, p=0.008) (Figure 23B).

There was a moderate correlation between baseline histamine concentrations in skin dialysates and clinical indices for wealing in patients with CSU as assessed by visual analogue scales (Figure 24A). There was no correlation noted for the baseline tryptase or IL-6 concentration and clinical scores for wealing based on visual analogue scales in CSU patients (Figure 24B and C).

Table 2. Baseline Histamine Concentrations in Skin Dialysates in Relation to Serum Histamine-Releasing Activity and Skin Serum Autoreactivity in CSU Patients and Healthy Subjects

Patients	Serum-induced BHR (% of total cellular histamine)	ASST (positive/ negative)	Baseline Histamine (ng/ml)	Baseline Histamine (nM)	Healthy Controls	Serum-induced BHR (%) (% of total cellular histamine)	ASST (positive/ negative)	Baseline Histamine (ng/ml)	Baseline Histamine (nM)
DPP01	51	Positive	2.81	25.28	NLV04	0	Negative	1.29	11.59
MHP03	0	Negative	8.72	78.57	RAV05	0	Negative	0.17	1.55
DMP05	20	Negative	0.44	4.00	LCV06	3	Negative	2.12	19.09
DBP06	0	Positive	2.69	24.25	APV07	0	Negative	1.37	12.32
NMCP07	0	Negative	0.97	8.77	ASV09	0	Negative	0.44	3.93
EBP04	1	Positive	0.12	1.04	JEV10	9	Negative	0.24	2.13
NMP09	9	Positive	0.82	7.35	AMSV11	0	Negative	0.86	7.74
JKP10	6	Positive	2.20	19.87	ODV12	7	Negative	1.26	11.39
JRP11	2	Negative	2.79	25.15	SCV08	0	Negative	0.12	1.06
NSP12	0	Negative	9.01	81.14	CGV02	4	Negative	1.22	10.97
MLP12	46	Positive	2.44	21.97	AYV15	0	Negative	1.88	16.90
RMP20	31	Positive	2.76	24.86	IPV14	0	Negative	0.80	7.23
RPP25	37	Positive	4.57	41.20	UBV16	0	Negative	0.32	2.24
SRP24	20	Positive	1.96	17.68					

*Serum-induced BHR >16.5% is considered positive according to the Reflab criteria (Platzer et al, 2005).

Table 2. In our study, six out of fourteen CSU patients had a positive serum histamine-releasing basophil assay (Reflab, University of Copenhagen, Denmark). All but one CSU patients with serum histamine-releasing activity had a positive ASST. Of CSU patients with a negative serum-induced BHR assay, four had a positive ASST. All healthy volunteers in our study had both tests negative.

Abbrevations:
CSU - Chronic spontaneous urticaria
ASST - Autologous serum skin test
BHR - Basophil histamine release

Figure 14. Microdialysis Experiment - Technical Aspects (1)

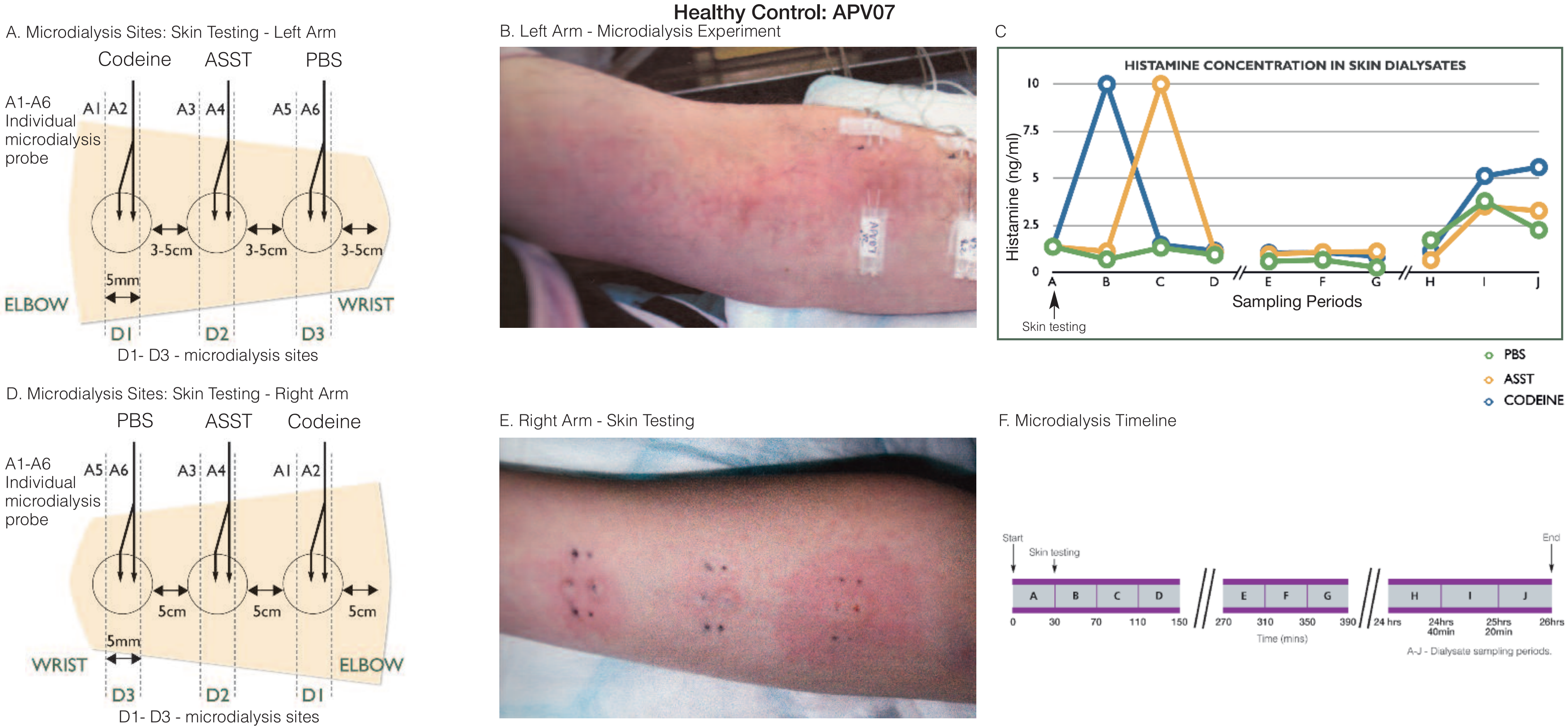
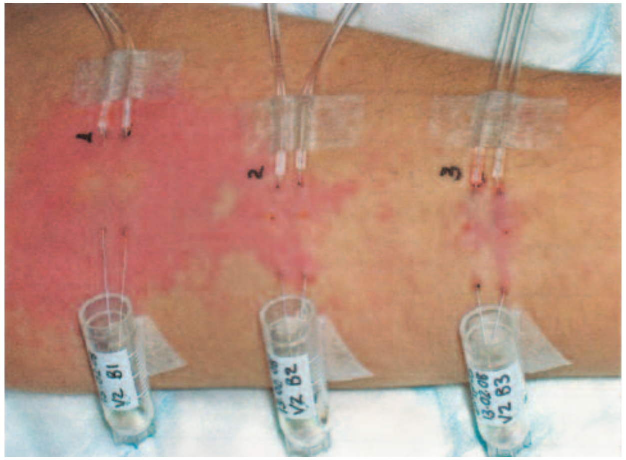


Figure 14. Acute urticaria to skin testing with codeine in a healthy subject. After skin testing with codeine (Figure 14A), a healthy volunteer developed acute localised urticaria around microdialysis site (Figure 14B). Local urticarial reaction to codeine may affect histamine concentrations from adjacent microdialysis sites (14C). Dialysate analysis revealed a pronounced histamine release at the site with ASST (Figure 14C). Skin testing with codeine on the opposite forearm (Figure 14D) as per Protocol 1 revealed pronounced reaction to codeine in this healthy volunteer (Figure 14E). The corresponding time intervals to sampling periods in the microdialysis studies are presented in Figure 14F.

Abbrevations:
ASST - Autologous serum skin test
PBS - Phosphate buffered saline

Figure 15. Microdialysis Experiment - Technical Aspects (2)

A. Patient: DMP05 - Left Arm



B. Microdialysis Sites: Skin Testing - Left Arm

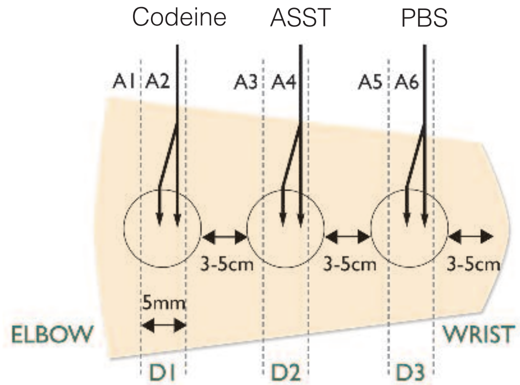


Figure 15. Large local weal-and-flare reactions to skin testing in some patients with CSU. Skin testing with codeine and autologous serum in some patients with CSU may result in large local weal-and-flare reactions (Figure 15A). Merging flare areas, but not weals, of two adjacent microdialysis sites were observed in some patients (Figure 15A). Pre-testing with codeine and autologous serum before the microdialysis experiments may reveal large local reactions in some patients and would help plan the microdialysis experiment. The distance between the microdialysis sites (Figure 15B) larger than 5cm may be recommended for the microdialysis studies measuring the mediators that spread in the flare area in CSU.

Abbrevations:
CSU - Chronic spontaneous urticaria
ASST - Autologous serum skin test
PBS - Phosphate buffered saline

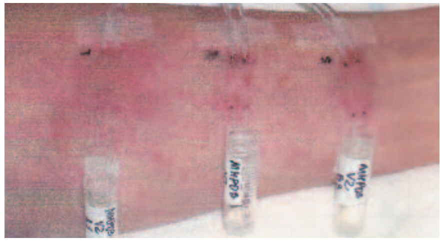
Figure 16. Microdialysis Experiment - Technical Aspects (3)

Patient: MHP03

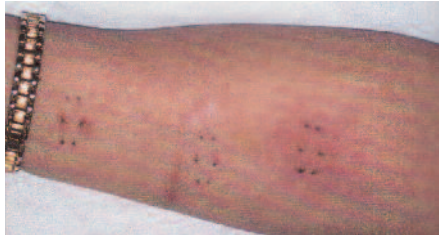
Microdialysis procedures

Skin testing

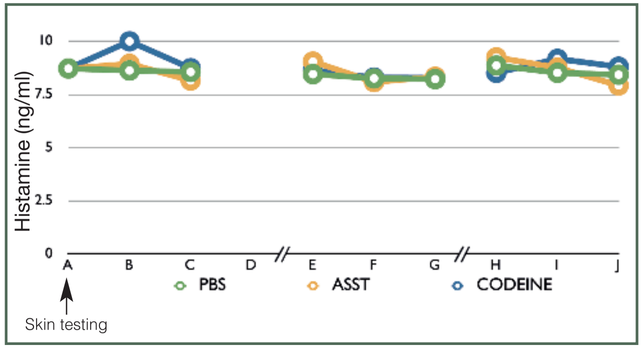
A. Left arm (Day 1). Probes in - 8.30am



C. Right arm (Day 1). Skin testing - 11.00am



E



B. Left arm (Day 1). Persistent wealing on probe removal - 5.30pm

D. Right arm (Day 1). Persistent wealing after skin testing - 5.00pm

F. Microdialysis Timeline

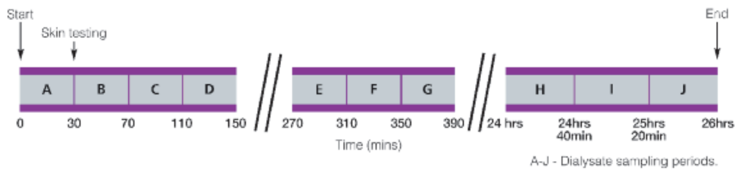


Figure 16. Persistent wealing due to codeine skin testing and a microdialysis procedure in a patient with CSU. Skin testing with codeine in this patient resulted in a large weal covering the microdialysis site which persisted from 11am (Figure 16C) till 5pm (Figure16D). This patient also developed persistent wealing during the microdialysis procedure on Day 1 (Figures 16C and 16D) and Day 2 (Figures 16E and 16F) experiments of the Protocol 1. Urticarial reaction at the microdialysis sites persisted after the removal of the microdialysis probes at the end of the experiments (Figures 16D and 16F). Dialysate analysis revealed elevated dermal histamine concentration in all dialysate samples throughout the microdialysis experiment (Figure 16E). Also, an induction of spontaneous wealing appeared to be a limitation for the use of cutaneous microdialysis in this CSU patient because the histamine concentrations in skin dialysates were affected (Figure 16E). The corresponding time intervals to sampling periods in the microdialysis studies are presented in Figure 16F. Patient pre-selection based on skin testing with codeine may be necessary for the microdialysis studies in CSU due to the possibility of persistent wealing in some patients. These patients should be included in the study and offered skin biopsy to avoid selection bias.

Abbrevations:
CSU - Chronic spontaneous urticaria
ASST - Autologous serum skin test
PBS - Phosphate buffered saline

Figure 17. Microdialysis Experiment - Technical Aspects (4)

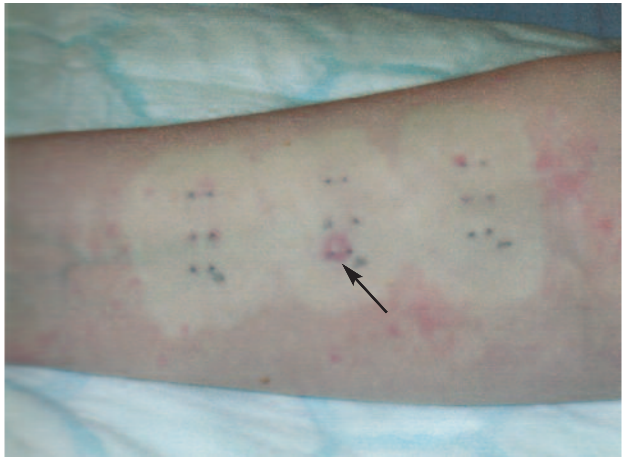
Patient JRP11 - Right Arm

Figure 17. Local wealing to the microdialysis probe insertion. The insertion of the microdialysis probes resulted in local wealing in this CSU patient. The weals tended to arise around the entry and the exit puncture sites (as indicated by an arrow on the photo) and persisted throughout the micrdialysis experiment and after the probe removal.

Abbrevations:
CSU - Chronic spontaneous urticaria

Figure 18. Microdialysis Experiment - Technical Aspects (5)

A. Patient EBP04 - Right Arm



B. Patient JKP10 - Left Arm

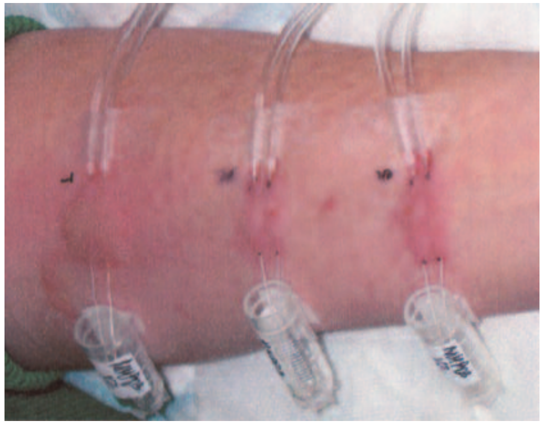
Figure 18. Spontaneous wealing at areas adjacent to microdialysis sites in some CSU patients. Some patients with severe CSU had spontaneous wealing when off antihistamines before (Figure 18A) and during (Figure 18B) the microdialysis experiment. The development of spontaneous weals at the areas selected for the microdialysis sites (as indicated by an arrow on the Figure 18A) was observed in some patients after removing the Tegaderm transparent dressing covering the EMLA cream (Figure 18A). Therefore, the application of EMLA cream to areas exceeding for 1-2cm that of the template for the microdialysis sites may help select the areas free of spontaneous wealing for the microdialysis experiment. Spontaneous wealing involving areas adjacent to the microdialysis sites (as indicated by an arrow on the Figure 18B) occurred in one CSU patient during the microdialysis experiment (Figure 18B).

Abbrevations:
CSU - Chronic spontaneous urticaria
EMLA - Eutectic Mixture of Local Anesthetics

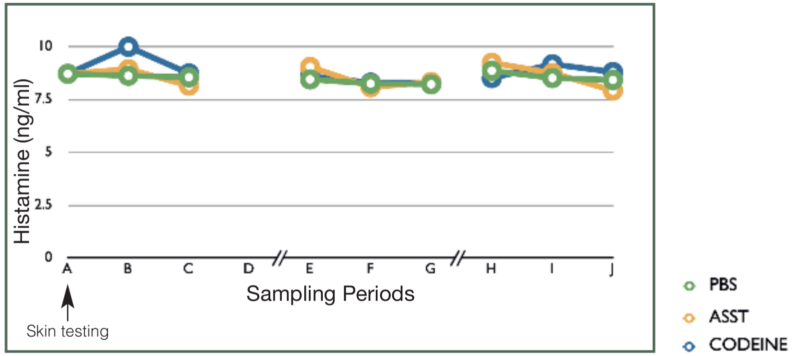
Figure 19. Microdialysis Experiment - Technical Aspects (6)

The definition of non-specific skin reactivity to microdialysis procedures

A. Patient MHP03 - Cutaneous microdialysis - Left arm



B. Histamine concentration in skin dialysates

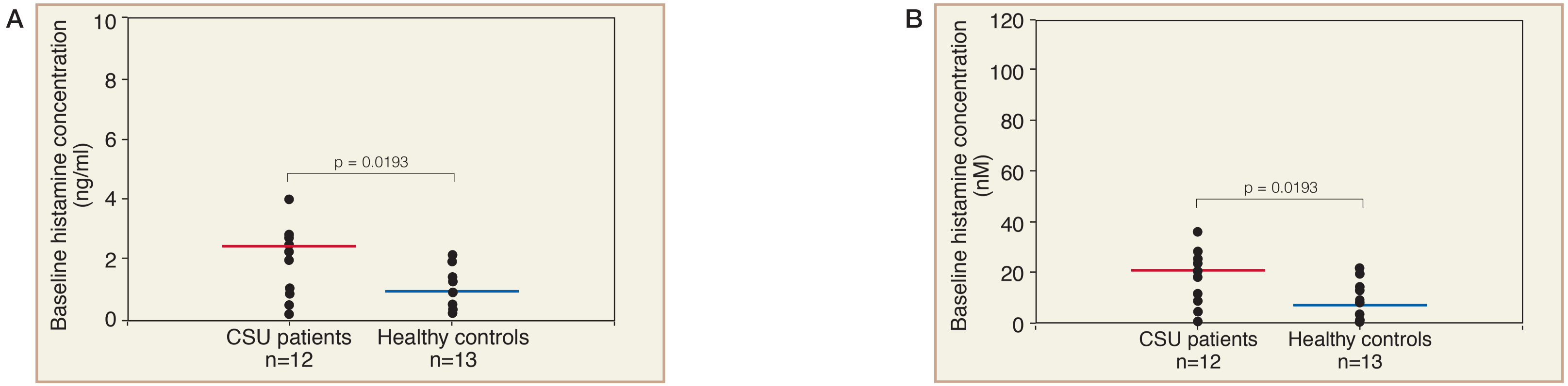


C. Microdialysis Timeline

Figure 19. Non-specific skin reactivity to microdialysis procedures. Figure 19A represents a local wealing reaction in patient MHP03 following the insertion of the microdialysis probes in the dermis. This reaction was associated with elevated dermal histamine at the baseline comparable to that after skin testing with codeine (Figure 19B). This may suggest that this dermal response may occur as a result of the insertion or the presence of the microdialysis probes in the dermis. It is important to be aware of the possibility of these reactions in few individuals. Whether this dermal response can be predicted using high resolution laser Doppler perfusion imaging needs to be established in the future studies. The corresponding time intervals to sampling periods in the microdialysis studies are presented in Figure 19C.

Abbrevations:
CSU - Chronic spontaneous urticaria
ASST - Autologous serum skin test
PBS - Phosphate buffered saline

Figure 20. Baseline Concentrations of Histamine in Skin Dialysates in CSU Patients and Healthy Subjects

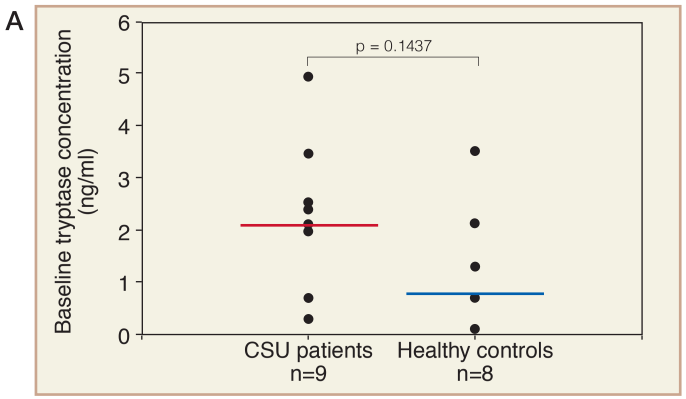


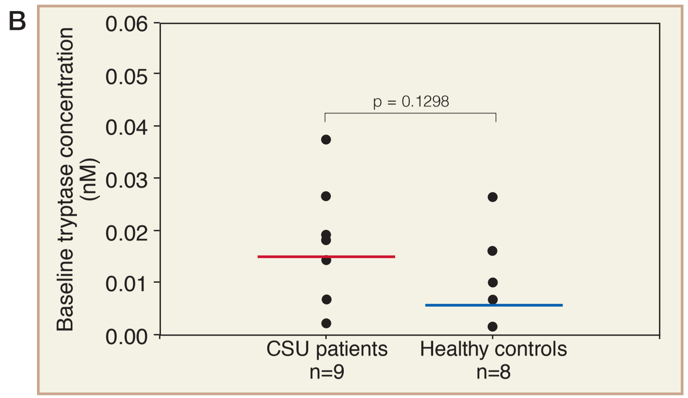
Pairwise comparisons were carried out using Mann-Whitney U test.

Abbrevations:
CSU - Chronic spontaneous urticaria
ELISA - Enzyme-linked immunosorbent assay

Figure 20. Baseline histamine concentrations in skin dialysates were shown to be significantly higher in CSU patients than in healthy controls (Mann-Whitney U test, p=0.0193). (Figure 20A). The median baseline histamine concentration in CSU patients was 2.32 (0.86; 2.78) ng/ml while median baseline histamine concentration in healthy subjects was 0.86 (0.28;1.33) ng/ml. Dermal skin dialysates were obtained using six microdialysis probes (3000kDa molecular weight cut-off) inserted into the velar surface of the forearm. Histamine concentrations in skin dialysates were determined using Histamine ELISA kit (the detection limit -0.12ng/ml). For histamine analysis, two outliers were removed. The bar represents the median. The data are expressed as medians and the interquartile ranges.

Figure 21. Baseline Concentrations of Tryptase in Skin Dialysates in CSU Patients and Healthy Subjects



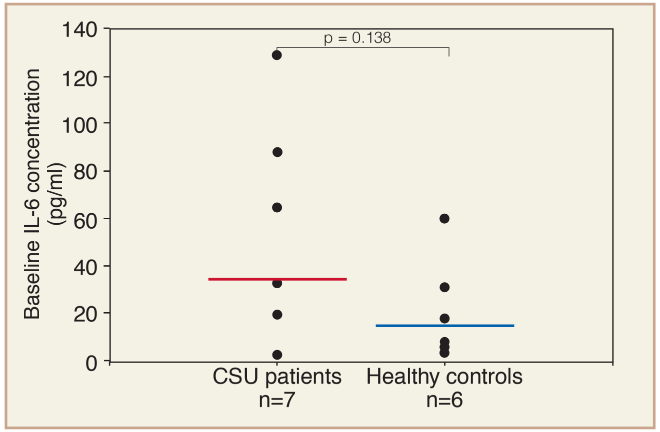


Pairwise comparisons were carried out using Mann-Whitney U test.

Abbrevations:
CSU - Chronic spontaneous urticaria
ELISA - Enzyme-linked immunosorbent assay

Figure 21. The median for baseline tryptase concentrations in CSU patients was 2.10 (1.68; 2.77)ng/ml or 0.02(0.13; 0.21) nM while the median for baseline tryptase concentrations for healthy controls was 0.70 (0.70; 1.72) ng/ml or 0.01(0.01;0.02) . The differences in baseline tryptase concentrations in CSU patients and healthy controls did not reach statistical significance. Dermal skin dialysates were obtained using six microdialysis probes (3000kDa molecular weight cut-off) inserted into the volar surface of the forearm. Tryptase concentrations were determined using an in-house double antibody sandwich ELISA utilizing polyclonal rabbit anti- tryptase antibodies for capture and mouse AA5 monoclonal antibodies for the detection (University of Southampton, UK). The level of detection for tryptase ELISA was 0.06 ng/ml. For tryptase analysis, one outlier was removed. The bar represents the median. The data are expressed as medians and the interquartile ranges.

Figure 22. Baseline Concentrations of IL-6 in Skin Dialysates in CSU Patients and Healthy Subjects



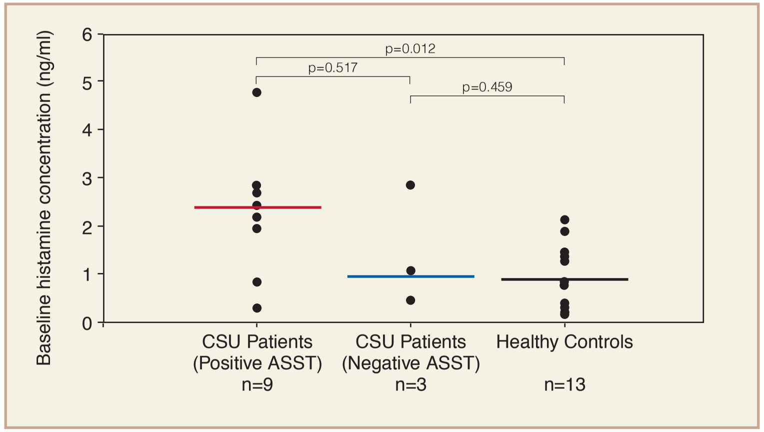
Pairwise comparisons were carried out using Mann-Whitney U test.

Abbrevations:
CSU - Chronic spontaneous urticaria
ELISA - Enzyme-linked immunosorbent assay

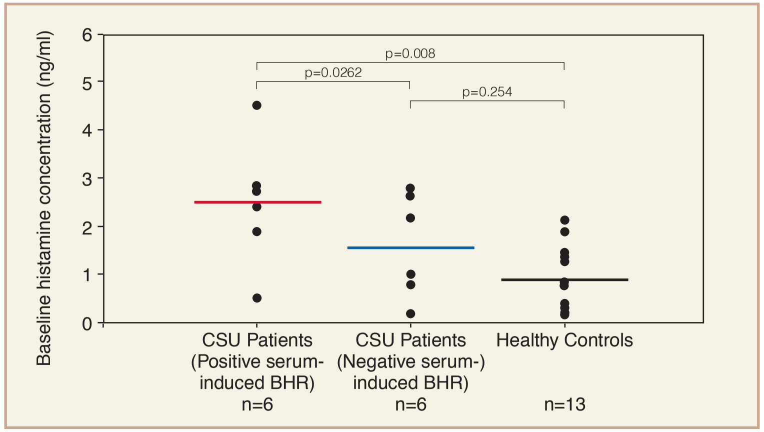
Figure 22. The median for baseline IL-6 concentrations in CSU patients was 33.08 (22.96;82.05) pg/ml whereas in healthy controls the median baseline IL-6 concentration in skin dialysates was 12.81(6.92;31.11) pg/ml. The difference in baseline IL-6 concentrations in CSU patients and healthy controls did not reach statistical significance (Mann Whitney U test, p=0.138). Dermal skin dialysates were obtained using six microdialysis probes (3000kDa molecular weight cut-off) inserted into the volar surface of the forearm. Cytokines in skin dialysates were measured using BD Cytometric Bead Array Flex Sets. Each IL-6 value represents the average of the duplicate measurements. The level of detection for BD cytometric Bead Array Flex Sets was 20 pg/ml. The bar represents the median. The data are expressed as medians and the interquartile ranges.

Figure 23. Baseline Histamine Concentrations in Skin Dialysates in Relation to Serum Histamine-Releasing Activity and Skin Serum Autoreactivity in CSU Patients and Healthy Subjects

A. Baseline histamine concentrations in skin dialysates in CSU patients with and without skin autoreactivity to autologous serum and healthy controls



B. Baseline histamine concentrations in skin dialysates in CSU patients with and without serum histamine-releasing activity



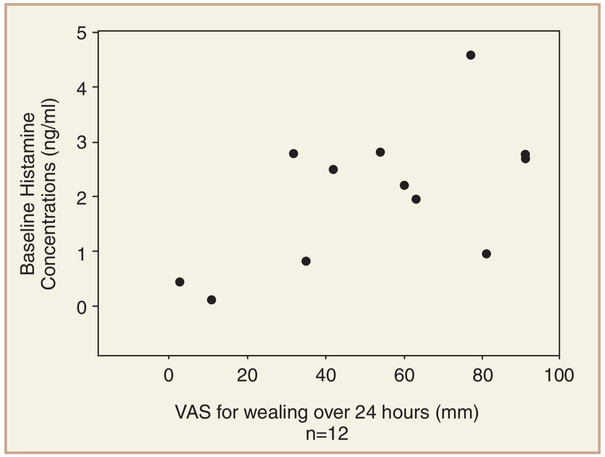
Serum-induced BHR >16.5% is considered positive according to the Reflab criteria (Platzer et al, 2005). The data were analysed using Mann-Whitney U test.

Figure 23. Baseline histamine concentrations in skin dialysates tend to be higher in CSU patients with positive ASST (median -2.44 (1.40, 2.80) ng/ml) compared to those with negative ASST (median - 0.97 (0.44, 2.79) ng/ml) and healthy controls (median - 0.86 (0.27, 1.33) ng/ml). Two outliers were removed from the group of CSU patients with a negative ASST. Baseline histamine concentrations in skin dialysates tend to be higher in CSU patients with serum histamine-releasing activity (median - 2.60 (1.58, 3.25) ng/ml) than those without serum histamine-releasing activity (median - 1.59 (0.64, 2.72) ng/ml) and healthy controls (median - 0.86 (0.28, 1.33) ng/ml. Two outliers were removed from the CSU group without serum histamine releasing activity. The bars on the graphs represent medians.

Abbrevations:
CSU - Chronic spontaneous urticaria
ASST - Autologous serum skin test
BHR - Basophil histamine release

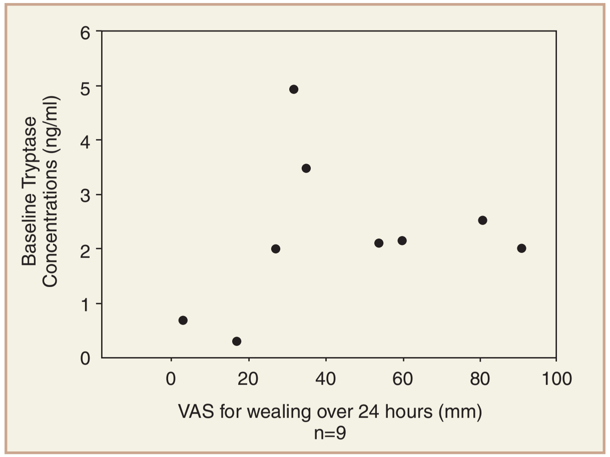
Figure 24. The Relationship between Visual Analogue Scales for Wealing over 24 hours and the Baseline Concentrations of Histamine, Tryptase and IL-6 in Skin Dialysates in CSU Patients

A. Correlation between the baseline histamine concentrations in skin dialysates and VAS for wealing in CSU patients



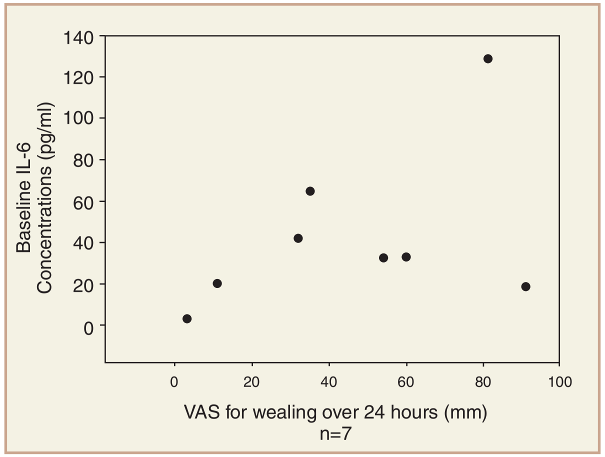
Pearson correlation r=0.602, p<0.05.

B. Correlation between the baseline tryptase concentrations in skin dialysates and VAS for wealing in CSU patients



Spearman correlation, p>0.05.

C. Correlation between the baseline IL-6 concentrations in skin dialysates and VAS for wealing in CSU patients



Spearman correlation, p>0.05.

Figure 24. Dermal histamine concentrations showed a correlation with VAS scores for wealing over 24 hours in CSU patients (Pearson correlation r=0.675, p<0.05) (Figure 24A). Dermal tryptase and IL-6 concentrations in skin dialysates showed no correlation with VAS scores for wealing (Figures 24B, C). Two outliers were removed for histamine analysis. Histamine analysis was carried out using Histamine ELISA (Biosource) (the limit of detection - 0.12 ng/ml). Dermal tryptase concentrations were measured using in-house tryptase ELISA utilising polyclonal rabbit anti-tryptase antibodies for capture and the mouse AA5 monoclonal antibodies for the detection (University of Southampton, UK) (the limit of detection -0.06 ng/ml). IL-6 concentration was measured by multiplex BD Cytometric Bead Array Flex Sets (the limit of detection -20pg/ml). VAS scores for wealing over 24 hours were measured before starting the baseline collection of the microdialysis studies. Histamine data were obtained from twelve CSU patients, tryptase data from nine patients and IL-6 data from seven patients. Two outliers were removed for histamine analysis.

Abbrevations:
CSU - Chronic spontaneous urticaria
ELISA - Enzyme-linked immunosorbent assay
IL-6 - Interleukin 6
VAS - Visual analogue scale

2.3.3 Pharmacokinetic Characteristics of Histamine Release in Response to Skin Testing with PBS, Autologous Serum and Codeine in CSU Patients and Healthy Controls

Pharmacokinetic analysis of the concentration-time profiles for histamine concentrations in response to different stimuli (PBS, autologous serum and codeine) within 40 min of skin testing was carried out on the pooled data from Protocol 2 and 3 (Figure 25).

Histamine pharmacokinetic analysis was based on eight CSU patients and three healthy controls who participated in Protocol 2 and 3. The histamine response profile to skin testing with autologous serum was noted in two CSU patients with a positive autologous serum skin test and a positive serum-induced basophil histamine release assay (MLP12 and RPP25) (Figure 26). The peak dermal extracellular histamine concentrations in these patients were 17.25 and 31.26 ng/ml, respectively (Table 3). The time to reach the peak histamine release after skin testing with autologous serum in these patients (Figure 26 and Table 3) was 15 min in both patients. Following skin testing with autologous serum, dermal extracellular histamine concentrations returned to the baseline levels within 40 min of skin testing (Figure 26).

Therefore, our data suggest a relatively slow low-grade histamine release in skin in response to skin testing with autologous serum in patients with autoreactive CSU. The pattern of histamine release induced by an intradermal injection of autologous serum differs considerably from that in response to skin testing with codeine. By contrast, skin testing with codeine resulted in the peak histamine concentrations above the linear range for Histamine Immunoassay (10 ng/ml for neat samples and 30 ng/ml for diluted dialysate samples). Following skin testing with codeine, dermal histamine release peaked within 5 min and returned to the baseline values within 20-30 min in most patients and healthy controls (Table 3). Therefore, skin testing with codeine results in a rapid, pronounced short-term histamine release in skin of CSU patients and healthy subjects.

Comparison of the box plots for AUC for histamine in response to PBS, autologous serum and codeine provided valuable information on the trends in the histamine release responses to these stimuli in CSU patients and healthy controls (Figure 25). In general,

Figure 25. Area under the Curve Analysis for Histamine Concentration in Skin Dialysates in Response to Skin Testing with PBS, Autologous Serum and Codeine in CSU Patients and Healthy Subjects

Comparison of median values of area under the curve for histamine concentration in skin dialysates in CSU patients and healthy controls

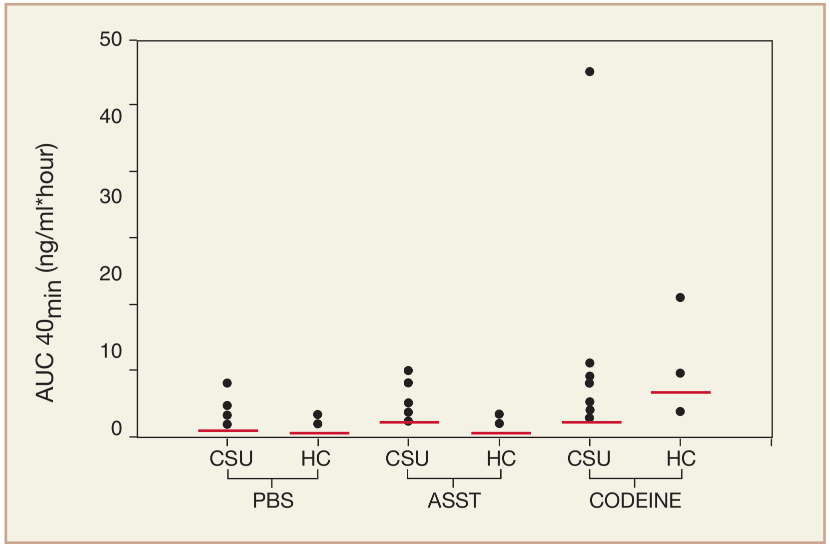


Figure 25. According to the area-under-the curve analysis, patients with CSU tend to have higher AUC_{40min} for histamine release to skin testing with PBS and autologous serum but lower AUC_{40min} for histamine release to codeine stimulation compared to healthy subjects. Only descriptive pharmacokinetic analysis was permitted because the peak concentrations for histamine release in response to codeine stimulation was above the linear range for the Histamine immunoassay. The area-under-the curve measurements were carried out using LabPilot 5 software. The bar represents the median.

Abbrevations:
CSU - Chronic spontaneous urticaria
HC - Healthy controls
PBS - Phosphate buffered saline
ASST - Autologous serum skin test
AUC - Area under the curve

Table 3. Pharmacokinetic Parameters of Histamine Concentration in Skin Dialysates in CSU Patients and Healthy Subjects

	Baseline	PBS		ASST		Codeine	
	Histamine (ng/ml)	C_{max} (ng/ml)	T_{max} (min)	C_{max} (ng/ml)	T_{max} (min)	C_{max} (ng/ml)	T_{max} (min)
Patients							
NMP09	0.82	0.97	N/A*	1.17	N/A*	7.80	5
JKP10	2.20	10.00	5	10.00	15	10.00	5
JRP11	2.79	3.00	N/A*	2.26	N/A*	4.49	5
NSP12	9.01	10.00	N/A*	10.00	N/A*	10.00	5
MLP12 (DF=3)	1.91	2.04	N/A*	17.25	15	269.00	5
RMP20 (DF=3)	3.99	6.32	5	4.86	20	66.92	5
RPP25 (DF=3)	2.46	10.00	30	31.26	15	25.12	5
SRP24 (DF=3)	2.21	2.22	N/A*	3.02	10	135.20	10
Healthy Controls							
CGV02	1.21	1.00	N/A*	1.10	N/A*	10.00	5
AYV15	1.88	1.66	N/A*	4.70	20	248.10	5
IPV14	0.80	1.43	N/A*	2.87	5	32.65	5
UBV16	0.32	0.52	N/A*	1.13	5	158.90	5

* T_{max} is not applicable as concentration-time profile is flat.

Table 3. The pharmacokinetic profiles are available only for research participants in Protocols 2 and 3. Histamine analysis was carried out on neat samples in Protocol 2 and both neat and diluted samples in Protocol 3. The peak concentration for samples obtained in Protocol 2 was limited by the linear range for the assay. Therefore, the accurate estimation of the peak concentrations was not possible but only the descriptive analysis of the pharmacokinetic data. Noteworthy, the rise in histamine concentration in response to the injection of autologous serum in three CSU patients with marked serum autoreactivity (JKP10, MLP12 and RPP25) occurred within 15 minutes after skin testing while maximum histamine release in response to codeine injection was within 5 minutes in most patients. These data suggest the different modes of histamine release underlying dermal responses to skin testing with autologous serum and codeine. It is worth noting that three CSU patients (JKP10, RPP25, RMP20) demonstrated a pronounced incease in histamine concentration in response to an injection of PBS. This indicates a high degree of effector cell releasability in the dermis in some CSU patients.

Abbrevations:
CSU - Chronic spontaneous urticaria
ASST - Autologous serum skin test
DF - Dilution factor
PBS - Phosphate buffered saline
C_{max} - maximum concentration
T_{max} - time to reach maximum concentration

Figure 26. The Pattern of Histamine Release underlying Positive ASST in Patients with CSU

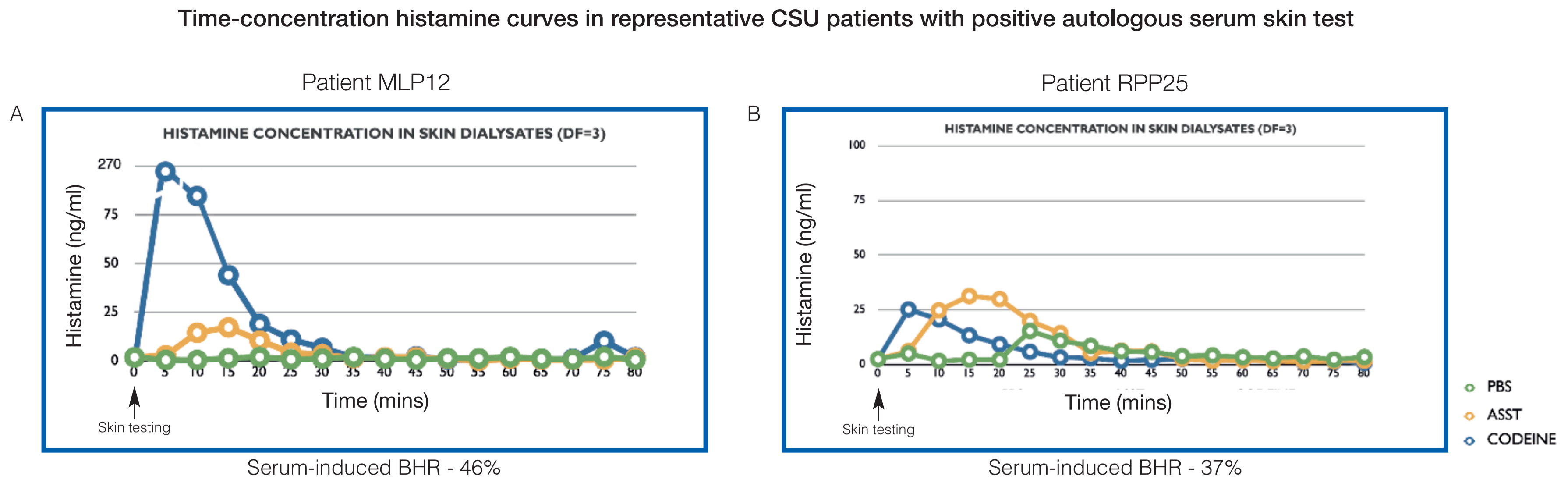


Figure 26. Time-course of dermal histamine levels measured in dialysates before and after skin testing with PBS, autologous serum and codeine. Six linear microdialysis probes (MW cut-off of 3000kDa) were inserted into non-lesional skin of the velar surface of non-dominant arm of patients with CSU and healthy volunteers. The probes run for 20mm below the surface of the skin. For skin dialysate collection, the probes were perfused with sterile saline solution at a flow rate of 3µl/min. At baseline, a 30 min collection was established before the skin testing. For the skin testing, 20 µl of each stimulus were injected along two microdialysis probes per microdialysis site. After skin testing, dialysate sampling was carried out at 5 min intervals for 40 min. Histamine concentrations were measured in skin dialysates at 5-min intervals for 40 min after skin testing using Histamine ELISA (Cambridge Biosciences Ltd). Time-concentration profiles in two CSU patients revealed a low-grade histamine release in response to skin testing with autologous serum (26A-B). Both CSU patients had a positive serum-induced basophil histamine release (see Table 1).

Abbrevations:
ASST - Autologous serum skin test
CSU - Chronic spontaneous urticaria
ELISA - Enzyme-linked immunosorbent assay
MW - Molecular weight
PBS - Phosphate buffered saline

patients with CSU tended to have higher histamine release to PBS and autologous serum but lower histamine release to codeine compared to healthy subjects. However, only descriptive pharmacokinetic analysis was possible because the peak concentration for histamine release in response to codeine stimulation was above the linear range for the histamine assay.

2.3.4 Protocol Modifications in the Cutaneous Microdialysis Study in CSU

During the study, there were two modifications of the original protocol which resulted in optimisation of cutaneous microdialysis methodology, re-appraisal of the research hypothesis and outcome measures. In this section, the interim data analysis will be reviewed as a rationale for sequential protocol modifications for our microdialysis experiments. An incremental gain and a revision of the research hypothesis will be described at each step of the protocol development. Finally, an optimal design for a cutaneous microdialysis study in CSU will be discussed based on the results of our study.

Protocol 1

An interim analysis of the data from six CSU patients and nine healthy controls accumulated during Protocol 1 revealed that the median baseline histamine concentrations (IQR) in the dermal interstitial fluid were estimated at 1.83 ng/ml (0.36, 4.28) in CSU patients and at 0.86 ng/ml (0.20, 1.33) in healthy controls. The coefficient of variation for baseline histamine concentration was 121.64% in CSU patients and 78.72% in healthy controls. There was a histamine release in B collection following intradermal testing with codeine in both study groups but no change in histamine concentration was observed after skin testing with autologous serum in patients with CSU. To ascertain if the 40-min intervals for microdialysis sampling after skin testing were suboptimal to detect the changes in mediator levels from the baseline in response to skin testing, shorter sampling intervals were proposed to overcome the limitations of the current protocol. Therefore, an alternative sampling approach was adopted and included sampling at 5-min intervals for the first 40 min after skin testing. The interim study results shifted the focus of the research on the early (0-2 hours) and the late phase (4-6 hours) biochemical events in skin after skin testing in CSU. Therefore, it was decided to proceed with the Time period collections 1-3 of the microdialysis experiments only.

Protocol 2

At this stage, the statistical power of the study was still not sufficient (52,3%) for detection of the difference in baseline histamine concentration in the dermis of CSU patients (median (IQR) — 2.24 ng/ml (0.72-4.28)) and healthy controls (median (IQR) — 1.04 ng/ml (0.22 — 1.31)). The coefficient of variation for the baseline histamine concentration in the pooled patient data from Protocols 1 and 2 was 103.43% in patients and 72.4% in healthy controls. An interim analysis at this stage revealed an increase in dermal histamine concentrations after skin testing with autologous serum in one patient with CSU. In patients and healthy controls, codeine-induced histamine release peaked at 5 min and returned to normal within 20-25 min of skin testing (Table 1).

Histamine concentrations after skin testing with autologous serum and codeine reached the maximum of the linear dynamic range for the Histamine ELISA assay (Cambridge Biosciences Limited, UK). Therefore, for *Protocol 3,* it was decided to increase the duration of 5 min-sampling up to 80 min and to use one microdialysis probe per the microdialysis site for analysis of neat samples to detect the baseline concentration of histamine and dermal dialysate collected from other probe per the microdialysis site for analysis of the diluted samples (DF=3) to look at the peak concentrations of histamine in dermis in response to skin testing. The dilution factor (DF=3) for sample dialysates was predetermined on the samples from *Protocol* 1.

Protocol 3

Final analysis included the microdialysis data accumulated in the study:

- baseline histamine concentration for 14 patients and 13 healthy controls;
- baseline tryptase data for 9 patients and 9 healthy controls;
- pharmacokinetic analysis for histamine release for 40 min for 4 patients and 3 healthy controls.

Median baseline histamine concentration was 2.32 ng/ml (0.86; 2.78) in patients with CSU and 0.86 ng/ml (0.28; 1.33). Two outliers were removed. Data analysis showed a statistically significant difference in baseline histamine concentration between patients with CSU and healthy controls (Mann-Whitney U test, $p<0.05$).

Pharmacokinetic analysis of the data from this stage showed a slow low-grade histamine release induced by autologous serum skin test in two CSU patients with serum histamine-releasing activity. Histamine release in response to autologous serum peaked at 15-20 min and lasted for up to 40 min. The pattern of histamine release to an intradermal injection of codeine was characterized by a fast and pronounced histamine release which lasted for 15-20 min.

2.3.5 Sample analysis

For protocols 2 and 3, histamine concentrations in skin dialysates collected from each microdialysis site in four CSU patients and one healthy control were analysed neat and diluted to 1:3. Pearson correlation coefficients between histamine concentrations in neat and diluted samples in CSU patients and a healthy control ranged between 83.2-97.9%.

The dilution factor (DF=3) was established by analysis of skin dialysates obtained from one patient (EBP04) and one healthy subject (SCV08) in the Protocol 1 part of the study. The Histamine ELISA assay is characterised by high sensitivity of 0.12 ng/ml in plasma samples. The log-linear range for Histamine ELISA Kit (Cambridge Biosciences Limited, UK) is between 0.74-8.48 ng/ml for neat samples and, therefore, 2.22-25.44 ng/ml for diluted samples (DF=3). It is worth noting that histamine concentrations exceed the log-linear range for this assay in diluted skin dialysates collected at 5 min intervals within 15 min of skin testing with codeine. From laboratory perspective, these samples need to be re-analysed in higher dilutions. However, this approach appears to be impractical for microdialysis studies in view of very low dialysate volumes, which do not permit the re-analysis.

2.3.6 Optimal Design of Full-scale Microdialysis Study in CSU

An optimal study design is crucial for a full-scale microdialysis study in CSU to ensure that the obtained results are valid and of biological and clinical relevance. Our pilot study confirms that cutaneous microdialysis research is suitable for research in CSU but requires protocol modifications. Our study highlighted several design factors that need to be considered for a microdialysis study in CSU. Thus, important design variables include patient population, a layout of the microdialysis sites, sampling design and the performance characteristics of analytical procedures.

Taking into account sample size calculation, the rate of dermal reactivity to the microdialysis procedures in CSU and the costs of the microdialysis experiments, the minimum number of study participants should be at least 20 patients and 20 healthy controls for sufficient statistical power of the study to detect the differences in baseline concentrations of histamine and tryptase between CSU patients and healthy controls. A balanced design for patient stratification into patients with positive and negative autologous serum skin test can be considered to test the hypothesis of a low-level histamine release underlying serum autoreactivity in CSU. Given that serum autoreactivity occurs in 45.9% of patients with CSU (Krause et al, 2009), it may be needed to balance the patient recruitment until having 10 CSU patients with a positive autologous serum skin test and 10 patients with a negative autologous serum skin test completed the study. A multi-centre study would facilitate patient accrual. Selection of the research centres with an established microdialysis set-up could reduce the costs of the study.

A certain degree of variation in the assessment of skin testing results between different centres has been addressed in several recent publications on autologous serum skin test in CSU (Konstantinou et al, 2009; Metz et al, 2009). As a suggestion, more stringent criteria for a positive autologous serum skin test, such as redness and weal diameter of more than 5 mm, may be considered for a microdialysis study in CSU in view of technical sophistication and the costs of the study to avoid borderline results of skin testing and to ensure consistency of patient recruitment in a multi-centre study. Criteria for stopping the microdialysis experiment should include intensive wealing at the areas adjacent to microdialysis sites or facial swelling during the experiment. Dermal reactivity to the microdialysis procedures should be defined as wealing at the microdialysis sites after the probe insertion or during the microdialysis experiment or a release of histamine or tryptase at the baseline comparable to that after skin challenge with codeine. A possibility of skin biopsy for study participants with dermal reactivity to cutaneous microdialysis should be discussed at the recruitment and consent for a skin biopsy, if needed, should be obtained before the microdialysis experiment. A prompt analysis of dermal dialysates after each experiment can help confirm or exclude dermal reactivity and can inform whether further arrangements for skin biopsy are necessary.

In our study, we could not demonstrate a correlation between the dermal tryptase concentrations and the clinical scores (VAS for itching and wealing) or the difference between patients with CSU and healthy subjects. First of all, small sample size could be an explanation for the lack of correlation as was demonstrated by power calculations. However, other methodological and biological reasons could provide alternative explanations. The choice of antibodies for the detection of tryptase could have an effect on concentration estimates. For example, AA5 monoclonal antibodies used in our study appears to bind to the pro and mature forms of α- and β- tryptases with equal affinity. By contrast, G5 monoclonal antibodies have greater affinity for mature β-tryptase than for the pro or mature forms of α- or β-tryptases. In CSU mast cell density in the skin was demonstrated to be about 3 times higher than that in healthy subjects (Kay et al, 2014a) as well as ongoing mast cell degranulation. Increased skin mast cell density and ongoing mast cell degranulation may contribute to the changes in dermal tryptase concentrations. It is possible that a mild degree of mast cell accumulation may not be sufficient to stimulate a detectable difference in skin tryptase levels between the groups. In addition, the mode of *in vivo* mast cell degranulation in CSU is poorly understood. A piecemeal degranulation of cutaneous mast cells or basophil degranulation in the skin could be an alternative explanation for the lack of the difference in tryptase concentrations between the groups in our study. Therefore, for future studies, the parallel use of tryptase assays using AA5 or G5 monoclonal antibodies on the same dialysate samples would provide new insights into tryptase isoform distribution in CSU. Other methodological possibilities are that tryptase may have been under-represented in the fluid recovered by microdialysis, or that availability of only small sample volumes (25µl) may have compromised the sensitivity of the assay. This justifies the allocation of larger sample volume for tryptase analysis.

In our study, there was no statistically significant difference (two-sample Wilcoxon test, $p>0.05$) in mean weal area after skin testing with PBS between CSU patients (median weal area - 0.23 (0.09-0.34 mm^2)) and healthy controls (median weal area — 0.16 (0.01-0.27 mm^2)). The weal area measurements after skin testing with codeine did not reveal any statistically significant difference (two-sample Wilcoxon test, $p>0.05$) between CSU patients (median weal area — 1.00 (0.74-1.21mm^2) and healthy controls (median weal area — 0.74 (0.47-1.36 mm^2). However, the median weal area was significantly larger (two- sample Wilcoxon test, $p<0.05$) in autologous serum-induced weals in CSU patients (median weal area — 0.40 (0.27-0.54mm^2) compared to healthy subjects (median weal area — 0.20 (0.07-0.30mm^2)). These data indicate the average weal area was within the chosen distance (3-5cm) between the microdialysis sites in our study. Although the flares did overlap between the microdialysis sites in few patients (Figure 15), this fact did not affect the results. In previous microdialysis studies, it was shown that histamine does not spread into the flare area (Petersen et al, 1997b). However, larger distance between the sites would be important in future studies measuring, for example, neuropeptides which are released within the flare area and could be affected by flare overlap.

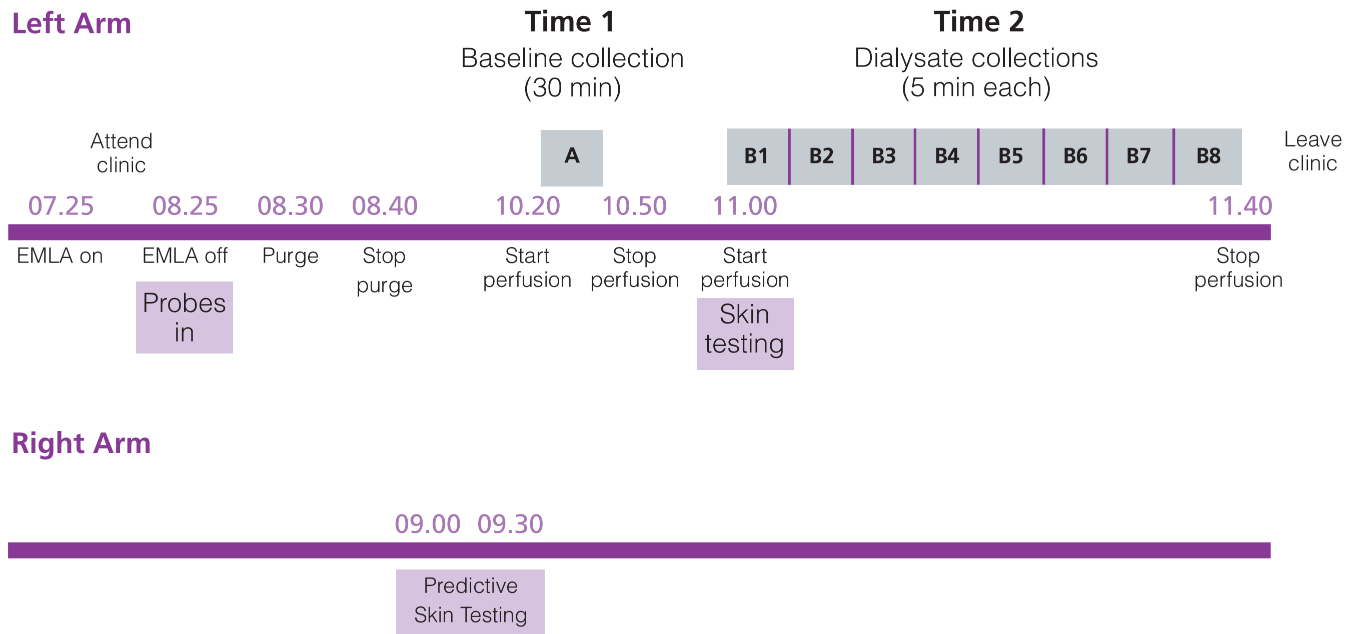
In order to assess the variability in the dialysate measurements, a two-way analysis of variance model was estimated using duplicates of baseline histamine, tryptase and IL-6 concentrations from CSU patients and healthy controls. One factor was the subject ID and another being the replicate of measurements. The analysis showed that for histamine and tryptase there was no significant variation due to replicate measurements in skin dialysates in which the tests taken but that there was significant variability between the research participants. For IL-6 measurements, there was a significant variation in the replicates and the individuals. The variability due to individuals was 3.5 times higher than the variability due to the order of measurements. These data highlight the need for larger microdialysis studies to account for inter-individual variability for all the measurements (histamine, tryptase and IL-6 concentrations). These data confirmed the reliability and precision of the measurements for histamine and tryptase in the study. This is crucial as the baseline measurements were done in dupplicates while the measurements for pharmacokinetic profiling were carried out on single samples due to the dialysate volume limitations. For cytokine concentrations, the variability in the measurements could be, possibly, reduced by using novel BD Cytometric Bead Assays with enhanced sensitivity for multiplex cytokine measurements.

Microdialysis experiment is planned on the forearm of non-dominant arm (Figure 27). Predictive skin testing with PBS, codeine and autologous serum on the opposite arm could help for adjustment of a layout of the microdialysis sites depending on the extent of reaction to codeine and autologous serum. Microdialysis sampling carried out at 5 min intervals for 40 min after skin testing is recommended for estimating the concentration over the time profile for histamine release after skin testing with PBS, autologous serum and codeine in CSU patients and healthy controls.

Optimal study outcomes may include:

- Mean baseline histamine and tryptase concentrations in the dermal interstitial fluid of CSU patients and healthy controls obtained by cutaneous microdialysis;
- Pearson's correlation coefficient for baseline histamine concentration and clinical scores (VAS for itching, VAS for wealing and UAS3);
- The difference in means of $AUC_{(40\ min)}$ for histamine release in response to PBS, autologous serum and codeine between CSU patients and healthy controls estimated by ANOVA;
- Total histamine content in skin biopsy specimens obtained from study participants with dermal reactivity to the microdialysis procedures.

Figure 27. Optimal Study Design for Full-Scale Microdialysis Study in CSU



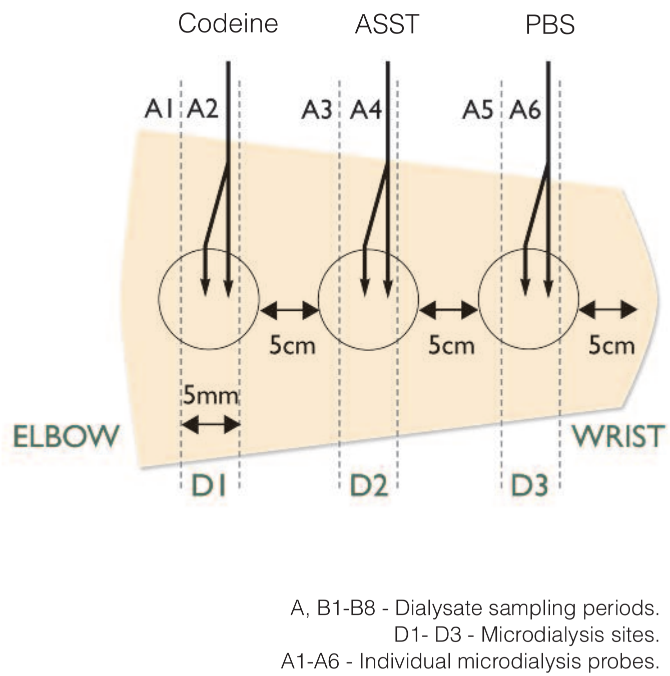


Figure 27. A full-scale microdialysis study in CSU is recommended to be designed as a multicenter comparative study in CSU patients and healthy controls. Sample size for this study would involve 20 subjects in each group with a further patient stratification for positive and negative autologous serum skin test. Microdialysis studies would include cutaneous microdialysis procedures on the forearm of non-dominant arm and a predictive skin testing on the opposite arm (Figure 27A). Microdialysis sampling at 5 min intervals for 40 min after skin testing would be optimal for estimating time-concentration profile for histamine release in response to skin testing with phosphate buffered saline, autologous serum and codeine phosphate. In Figure 27A, Time 1 represents a 30 min baseline collection (collection A), Time 2 comprises a 40 min time period following skin testing. Time 2 consists of consequent eight 5 min collections (collections B1-B8). In Figure 25B, D1-D3 designate microdialysis sites, A1-A6 designate individual microdialysis probes.

Abbrevations:
CSU - Chronic spontaneous urticaria
ASST - Autologous serum skin test
PBS - Phosphate buffered saline

2.4 Discussion

2.4.1 Experimental evaluation of Cutaneous Microdialysis in CSU

Our pilot study demonstrated that cutaneous microdialysis is a suitable research technique in CSU. Methodological, clinical and financial aspects were the main practical considerations for cutaneous microdialysis studies in CSU. Firstly, dermal tolerance of microdialysis procedures in CSU needed to be established in view of the possibility of non-specific wealing response to skin manipulations. Secondly, practical concerns were related to patient recruitment to microdialysis studies in view of the need for stopping antihistamine treatment for three days before microdialysis. Furthermore, assessment of the potential issues with experimental design and data collection in our pilot microdialysis study was of particular importance to ensure validity of future full-scale microdialysis studies in CSU. Finally, our study offered a possibility to assess the set-up and running costs for cutaneous microdialysis research in the setting of a secondary care hospital.

Excellent accrual and high patient compliance were demonstrated in our cutaneous microdialysis in CSU. Participant feedback demonstrated high motivation for research in patients with CSU due to a significant burden of the disease. In our study, withdrawal of antihistamine treatment in most patients resulted in mild-to-moderate worsening of itch and wealing but was well tolerated. In severely affected patients, stopping antihistamines was associated with severe wealing but no facial swellings were reported. Patients were informed about the possibility of withdrawing at any point if they were uncomfortable with their wealing and advised against proceeding with the experiments in case of facial swellings whilst off antihistamines. Stopping antihistamines for 72 hours before the microdialysis studies is a usual practice before autologous serum skin testing in clinical settings and 72-hour withdrawal of antihistamines for our study was approved by the Norfolk Research Ethics Committee. All patients were highly motivated and willing to proceed with the experiments due to the lack of understanding of disease mechanisms and limited efficacy of current management. In one patient, mild lip swelling was observed at the end of the microdialysis experiment which responded well to a licensed dose of cetirizine. For future microdialysis studies, withdrawal criteria from the study for patients during the wash-out period or microdialysis experiments may be considered.

Financial aspects of an introduction of cutaneous microdialysis to the research facility involves the start-up costs of microdialysis pump (the price for the CMA 400 microdialysis pump - £3,500), running costs of manufacturing and sterilization of the microdialysis probes and the costs of analytical assays. Manufacturing costs for each probe estimated at £5. For our study, manufacturing of the microdialysis probes was carried out at the University of Southampton without charge. Ethylene oxide sterilization of the microdialysis probes costed approximately £50 per half-basket. Sample analysis may incur the most substantial costs based on selected analytical assay and the number of samples according to the study protocol. For example, Protocol 1 yields 30 dialysate samples per participant, Protocol 2 -42 dialysate samples per participant and Protocol 3 — 63 samples per participant. Each Histamine immunoassay kit allows analysis of 84 samples and is marketed at the price range of £300-£500. Therefore, thorough protocol development is also important for financial feasibility of microdialysis studies in CSU.

Our study showed that cutaneous microdialysis was well tolerated in most patients with CSU but the protocol design and technical performance of the technique can be optimised for CSU. Methodological concerns are related to wealing response to the probe insertion or during the microdialysis experiments in few patients with CSU. Furthermore, enhanced response to skin testing with codeine in few patients or healthy controls which may affect the microdialysis data from the adjacent microdialysis site and may require skin pre-testing with codeine before the experiment for patient selection and optimisation of the layout of the microdialysis sites. Post-experiment eligibility criteria for patients data to be included in the final analysis may be needed based on the technical aspects of cutaneous microdialysis experiments in research participants. It may well be possible that exclusion of patients with large or persistent wealing to cutaneous microdialysis may introduce bias to the research into skin inflammation in CSU. An option of skin biopsy for these patients within the scope of future microdialysis studies may reduce the potential bias. Research into mast cell abnormality in these patients may provide some insight into skin pathophysiology of this condition. The limitations of cutaneous microdialysis studies in CSU can be improved or overcome by patient stratification, predictive skin testing before the experiments, design modifications of microdialysis experiments and possibly an option of skin biopsy for patients who are not eligible to microdialysis experiments due to non-specific wealing to the microdialysis procedures.

Cutaneous microdialysis offered a unique opportunity of continuous sampling of the extracellular fluid from the skin of patients to look at the local inflammation in the skin in CSU. Our pilot study also enabled us to explore the limitations of the technique for this novel research application — CSU. Main methodological considerations were related to an issue of dermal reactivity to the insertion of the microdialysis probes or their presence in the skin during the microdialysis experiments. In our study, dermal reactivity to the microdialysis procedures in CSU comprised a local histamine release with or without associated non-specific wealing caused by the insertion trauma or by the presence of the probes in the skin during the microdialysis experiments. Histamine release due to the probe insertion was described in the previous microdialysis studies in healthy subjects. Usually, histamine release was noted to subside during the equilibration period after the probe insertion. Further methodology research on whether the equilibration period in CSU may be of longer duration due to the pre-existing disturbances in the histamine metabolism in the skin. Dermal reactivity to the probe insertion was also reported in earlier microdialysis studies (Stenken et al, 2010; Linden et al, 2000), however, this phenomenon may well be of greater clinical relevance in CSU in a view of a lower skin threshold for wealing in this condition. Our data illustrated a good tolerance of cutaneous microdialysis in most, but two, CSU patients. However, few CSU patients and healthy controls may have skin hyper reactivity to the insertion of the microdialysis probes defined as skin wealing at the insertion sites during the experiment or histamine or tryptase release at the baseline that is similar to that after skin testing with codeine. In these individuals, cutaneous microdialysis may not be a suitable research technique to study the skin inflammation. Skin biopsy can be considered as an alternative research method to study the skin inflammation in these individuals. Skin hyperreactivity to the insertion of the microdialysis procedures in a few individuals was commonly reported in the microdialysis studies (Stenken et al, 2010; Sjogren & Anderson, 2000; Petersen, 1997a). There is an increasing interest in the recent studies whether skin hyperreactivity to the microdialysis procedures can be predicted (Linden et al, 2000).

An observation of two CSU patients with high levels of dermal extracellular histamine concentrations throughout the experiment with or without associated wealing brings to our attention an issue of skin threshold for wealing in CSU, even in the evidence of histamine release in the dermis. It is possible to assume that histamine release underlying serum autoreactivity in CSU was not detected in the previous work due to the insufficient sensitivity of histamine assay (Larsen, 2002).

Our experiments revealed higher levels of the baseline extracellular histamine concentration in dermis in patients with CSU compared to healthy controls. Our data also suggest the baseline extracellular histamine concentrations in the dermis could be used as a candidate biomarker of the intensity of local inflammation in skin in CSU in view of its relation to disease severity. This observation can be of practical importance and needs to be verified in larger scale microdialysis studies in CSU. As a suggestion, the use of urticaria activity score for three days (UAS3) when patients are off antihistamines could be a helpful parameter for clinical assessment of disease severity in addition to visual analogue scales in future microdialysis studies.

2.4.2 Histamine Concentrations in the Dermis of Patients with CSU

Therefore, the data were interpreted with caution in view of limited generalizability. Nevertheless, taking into account the usual size of cutaneous microdialysis studies, our data on the histamine pharmacokinetics in skin of CSU patients and healthy subjects were insightful and of great interest.

Study results revealed a statistically significant difference in dermal extracellular histamine concentrations in CSU patients and healthy controls. These data provided additional evidence for the minimal persistent inflammation in visibly unaffected skin in patients with CSU. Earlier studies demonstrated an increased level of histamine in the skin of CSU patients (Kaplan et al, 1978). Our study confirmed these data on a larger group of patients with CSU and showed a tendency for correlation of dermal extracellular histamine concentrations and clinical scores for itching and wealing. This is an important finding that needs to be verified in a larger study in CSU. The prospect of developing dermal histamine concentrations as a skin biomarker in CSU highlights the clinical relevance of these data.

The pilot study helped refine the research hypothesis based on the accumulating data on the pharmacokinetics of histamine release in CSU compared to that in healthy subjects. Pharmacokinetic analysis of histamine release to autologous serum and codeine in CSU patients revealed two different modes of histamine release: a fast short pronounced histamine release to codeine versus a slow low-grade histamine release to autologous serum. To our knowledge, this was the first study to report the histamine release underlying skin response to autologous serum in CSU. A slow low-grade histamine release, but not tryptase release, was described in allergic reactions following a prolonged challenge with allergen (Charlesworth et al, 1989). The persistence of functional autoantibodies to the high-affinity IgE receptor and/or anti-IgE may present a similar prolonged stimulation of the effector cells (mast cells and basophils) in CSU. These data drew attention to the activation pathways of mast cells and basophils induced by autologous serum in CSU. Hypothetically, a piecemeal degranulation of basophils and, possibly, mast cells may be an explanation for the observed pattern of histamine release.

These findings raise the question about the putative roles of elevated histamine levels in the unaffected skin in CSU. There has been recently interest in immunoregulatory effects of histamine in inflammatory conditions. H4 receptors are characterized by higher affinity towards histamine than H1 and H2 receptors, therefore histamine effects mediated via H4 receptors may occur at the local concentrations of histamine lower than required for stimulation of H1 and H2 receptors (Fung-Leung et al, 2004). Histamine effects mediated via H4 receptors include chemotaxis and cytokine and chemokine production by mast cells, eosinophils, dendritic cells and T cells (Jutel et al, 2005; Zhang et al, 2007). In mice models, triggering of H4 receptors resuted in mast cell migration towards histamine but has no effects on FcεRI-mediated degranulation (Hofstra et al, 2003). H4 receptors also mediate eosinophil chemotaxis but these effects of histamine are considerably weaker than those of CCR3-active eotaxin and eotaxin-2 (Buckland et al, 2003; Ling et al, 2004). H4 mediators also favor Th2-polarization (Schneider et al, 2002; Zhang et al, 2007) and mediate attenuation of cytokine production mediated by Toll-like receptors on dendritic cells (Zhang et al, 2007). Furthermore, histamine regulates the expression of its own receptors on endothelial cells (Shaefer et al, 1999).

2.4.3 Tryptase Concentrations in the Dermis of Patients with CSU

Previous reports of dermal tryptase concentrations estimated using cutaneous microdialysis are limited. In healthy subjects, tryptase levels in skin dialysates have been reported to be below 20 ng/ml, using a fluoroenzyme immunoassay (Pharmacia) (Nielsen et al, 2001). In dermal neurogenic inflammation, baseline tryptase concentrations in skin dialysates were estimated at 9.84±2.4ng/ml as measured by radioimmunoassay (Pharmacia) (Schmelz et al, 1999). In CSU, microdialysis data on dermal tryptase concentrations are not available to our knowledge. In suction blister studies tryptase levels in visibly unaffected skin of patients with CSU were found to be greater than those in the skin of healthy subjects as measured by Pharmacia Tryptase radioimmunoassay (Deleruan et al, 1991). In our study, the choice of the double antibody sandwich ELISA using AA5 monoclonal antibodies for the detection of tryptase was made taking into account sample volume requirements and the sensitivity of the assay.

In CSU it was observed that there was mild-to-moderate mast cell accumulation in the skin as well as ongoing mast cell degranulation. Both of these processes may contribute to the changes in dermal tryptase concentrations. α- and β-protryptases are secreted constitutively by mast cells and contribute to the total tryptase levels while β-tryptase is released upon mast cell degranulation. It is possible that a mild degree of mast cell accumulation may not be sufficient to stimulate a detectable difference in skin tryptase levels between the groups. In addition, the mode of *in vivo* mast cell degranulation in CSU is poorly understood. A low-grade continuous histamine release, without associated tryptase release, was described in the suction blister studies in the late-phase allergic reactions similar to that observed in our study. Alternatively, basophil inhltration was described in the skin of CSU patients. Basophil degranulation in the skin could result in marked histamine release but only minimal tryptase release.

Tryptase is thought to be involved in the skin inflammation in CSU, however, its precise contribution is poorly understood. Tryptase appears to be involved into microvascular leakage (He & Walls, 1998), leukocyte recruitment (Walls, 1995; Compton et al, 1999), angiogenesis (Crivellato et al, 2009) and tissue remodeling (Cairns, 1998). The role of tryptase was suggested in spreading the signal for mast cell degranulation (He et al, 1998) and inducing itch via extracellular proteolysis of PAR-2 receptors (Itoh et al, 2005) although the relevance of these processes to CSU is unknown. The presence of exocytosed immunoreactive tryptase in the skin of CSU patients was confirmed by suction-blister fluid experiments and histological studies although the expression, the storage and the relative isoenzyme distribution of skin tryptase in CSU await characterisation. In CSU, an ongoing mast cell activation may result in altered kinetics of tryptase release whereas microvascular leakage in CSU may affect the proteolytic behaviour of tryptase and the substrate supply in the skin. In CSU, serum total tryptase levels were shown to be elevated, particularly in patients with symptomatic disease and in those with serum capacity to up-regulate CD63 on basophils from healthy donors. It remains unclear whether local tryptase release in the skin precedes its systemic rise in CSU and whether this may reflect disease progression. Further research into tryptase haplotypes and activating mutations underlying hyper-releasable mast cell phenotype may explain this observation.

The potential for therapeutic targeting of β-tryptase in CSU has been indicated by a report that the non-selective protease inhibitors, nafamostat mesilate and camostat mesilate had clinical efficacy in two CSU patients (Takahagi et al, 2010). A range of tryptase inhibitors has been developed including peptidic, dibasic, zinc-mediated inhibitors and heparin antagonists (Rice & Moore, 2000). Of the inhibitors of β-tryptase developed for clinical use APC 2059, a dibasic tryptase inhibitor, was found to lack of efficacy in psoriasis but to be effective in ulcerative colitis (Tremaine et al, 2002) and asthma (Krishna et al, 2001). Novel small molecular β-tryptase inhibitors continue to be developed using combinatorial libraries. Structure-based library design can provide insights into the binding of inhibitors to catalytic sites and inducible pockets in the central cavity of the tetrameric scaffold of β-tryptase (Liang et al, 2012). The design and development of potent and highly selective tryptase inhibitors may allow better management of CSU in the future. Such innovative therapeutic approaches may provide a means for more effective anti-mediator blockade in this disease before embarking on immunomodulatory or biological therapies. Topical use of tryptase inhibitors would be an attractive approach in CSU, and should help in elucidating the contribution of β tryptase in the skin level in CSU. Protease inhibitors such as YC1015, YC1016 and YC1017 have been developed as topical formulations for atopic eczema, and their effects on dermal tryptase levels in CSU may be worth exploring. However, the potential concerns would include the risk of protein sensitization or skin irritation with topical use of β-tryptase inhibitors as well as the extent to which they may also inhibit other serine proteases.

2.4.4 Histamine Release to Codeine and Autologous Serum in CSU

Notably, both patterns of histamine release to codeine and autologous serum skin test with different pharmacokinetic profiles were characterised by the area under the curve for histamine of similar magnitude for both stimuli (autologous serum and codeine). This suggested the possibility of different modes of degranulation of mast cells and basophils to autologous serum and codeine of CSU patients. Mast cell degranulation in response to skin testing with autologous serum was shown by electron microscopy. Basophil accumulation in skin of patients with CSU was noted in histological studies, their degranulation was proved by extracellular deposition of BB1 antibodies. Piecemeal degranulation of basophils was described in various skin inflammatory conditions (Dvorak, 2005) and may serve as a possible explanation for a slow low-grade histamine release observed in response to autologous serum in some patients with autoreactive CSU.

Higher histamine release to PBS may suggest non-specific wealing due to 'twitchy' mast cells in CSU. Enhanced histamine release in response to skin testing with autologous serum demonstrated the phenomenon of serum autoreactivity which is observed in 45.9% of CSU patients (Krause et al, 2009). According to the available literature (Cohen et al, 1986; Shall & Saihan, 1992), skin responsiveness to codeine may well be a two-phase phenomenon. Initially, predisposed individuals with a tendency for intermittent wealing display high sensitivity and responsiveness to codeine skin testing while patients with clinical expression of chronic wealing (CSU) show high sensitivity but decreased skin responsiveness to codeine. This may reflect disease progression and gradual depletion of cellular pools of histamine due to continuous degranulation of mast cells and basophils.

2.4.5 Methodological issues with the detection of histamine, tryptase and cytokines

In our study, histamine release to codeine was characterised by considerable inter-individual variability beyond the dilution factor used for dialysate samples in our study. Accurate detection of both the ceiling and floor values for histamine release in response to codeine stimulation is critical for pharmacokinetic analysis of histamine release modes in CSU. Histamine ELISA (Biosource, Belgium) used in our study is highly sensitive to detect baseline extracellular histamine concentrations in skin dialysates but the dynamic range is not sufficient for the detection of the peak histamine concentrations in response to codeine. Therefore, there is a need for further development of analytical approaches to the histamine detection in dermal dialysate samples from patients with CSU and healthy subjects. For this purpose, an optimal analytical assay should be characterised by high sensitivity (low detection limit), a wide linear dynamic range for at least two log-decades (nanogram to microgram range) and low sample volume requirements.

This pilot study allowed us to refine the research hypothesis and focus our research questions. For future microdialysis studies, the working hypothesis could suggest that 1) the baseline extracellular histamine concentration in dermis reflects the intensity of local inflammation in CSU and 2) skin reactivity in CSU is mediated by aberrant modes of histamine release in response to skin testing with PBS, autologous serum and codeine. Several potential causes for raised extracellular histamine concentration at baseline can be explored including abnormal activity of histidine decarboxylase or histamine-metabolising enzymes. Local factors that can affect the enzyme activity could be explored such as cytokine effects on histidine decarboxylase or substrate inhibition of histamine-metabolising enzymes. In addition, parallel microdialysis and histological studies on the same patient in CSU could shed some light on contribution of local mast cell accumulation noted in CSU (Kay et al, 2014a) to increased dermal histamine concentration in unaffected skin. Aberrant skin mast cell releasability is a likely mechanism underlying aberrant modes of histamine release in response to PBS, autologous serum and codeine. Signaling defects or activating mutations resulting in hyperreleasable skin mast cell phenotype would be worth exploring with laser capture dissection microscopy of skin biopsies in parallel with functional responses assessed by cutaneous microdialysis.

Based on our data, the baseline extracellular histamine concentrations, T_{max}, $T_{1/2}$ and AUC can be selected as optimal outcome measures for a full-scale microdialysis study in CSU to answer these questions. The use of the peak histamine concentration (C_{max}) as an outcome measure showed a few pitfalls. The peak histamine concentrations in response to codeine were characterised by a high inter-individual variability which was in keeping with the results reported in other microdialysis studies (Krause et al, 2013). In our study, the concentration ranges for histamine release in response to skin testing with codeine was beyond the linear range of the immunoassay which did not permit an accurate detection of C_{max} in response to codeine stimulation. The use of analytical methodologies with a wide dynamic range (nanogram-miligram range) would improve the validity of C_{max} as an outcome measure for the microdialysis studies in CSU.

Therefore, other analytical approaches with high sensitivity but a wide dynamic range of at least two log-decades have to be considered for the detection of histamine in skin dialysates. Accurate detection of both baseline and peak histamine concentrations in skin dialysates is important for estimating the concentration over time profiles for histamine release to different stimuli. Clinical relevance of this pharmacokinetic analysis is highlighted by an observation of different modes of histamine release after skin testing with codeine and autologous serum in CSU. Therefore, further research into the pharmacokinetic characteristics of histamine release in response to codeine and autologous serum in CSU may provide fascinating insights into the pathophysiology of serum autoreactivity and abnormal codeine sensitivity in CSU.

Recent advances in the analytical detection of histamine in biological samples include the development of Förster resonance energy transfer (FRET)-based assay which is based on methylaloamine dehydrogenase conversion of histamine, transfer of reducing equivalence to amicyanin and measurement of resultant change in amicyanin absorption by fluorescence (Gustiananda et al, 2012). This method is characterised by high sensitivity (13nM) and a wide linear detection range (13nM-225µM). However, the method was reported for samples of 100µL, which would be a limitation of its use for skin dialysate samples in our study design. Histamine assays using Luminex technology permit histamine detection within the range of 0.4-40 ng/ml with sensitivity of 0.2 ng/ml but this linear range may not be sufficient, without sample dilutions, to detect the peak histamine concentration in response to codeine stimulation. Alternative methods for histamine detection in biological samples may include surface plasmon resonance immunosensor (Li et al, 2006) or high performance liquid chromatographic method (Siegel et al, 1990). Recently, an analyzer HistaReader 510, based on glass microfiber-based technology, was developed for histamine analysis by Reflab (University of Copenhagen, Denmark). The method is characterised by sensitivity of 5 ng/ml and a dynamic range between 0 to 150 ng/ml. A high throughput capacity of HistaReader 510 makes it an attractive method for analysis of skin dialysates in healthy subjects and patients with CSU. This method was used for microdialysis studies in healthy subjects (Krause et al, 2013). However, the baseline values in healthy subjects and CSU patients fall below the detection limit of the HistaReader analyzer. By contrast, immunoassays are characterised by high sensitivity for histamine detection. For example, the sensitivity of Histamine ELISA Kit (Cambridge Biosciences, UK) is 0.12 ng/ml, which is appropriate for the baseline levels of histamine. There may be a rationale for using both techniques for skin dialysate analysis in CSU. In the future microdialysis studies, dialysate collection by each probe per a microdialysis site can be allocated either for histamine analysis for the baseline concentration by immunoassay (high sensitivity) or for peak concentration analysis by the HistaReader technology (a wide dynamic range).

In our study, we could not demonstrate correlation between the dermal tryptase concentrations and the clinical scores (VAS for itching and wealing) or the difference between CSU patients and healthy subjects. First of all, small sample size could be an explanation for the lack of correlation as was demonstrated by power calculations.

However, other methodological and biological reasons could provide alternative explanations. The choice of antibodies for the detection of tryptase could have an effect on concentration estimates. For example, AA5 monoclonal antibodies used in our study appears to bind to the pro and mature forms of α- and β- tryptases with equal affinity. By contrast, G5 monoclonal antibodies have greater affinity for mature β-tryptase than for the pro forms of α- or β-tryptases. For future studies, the parallel use of tryptase assays using AA5 or G5 monoclonal antibodies on the same dialysate samples would provide new insights into tryptase isoform distribution in CSU. Other methodological possibilities are that tryptase may have been under-represented in the fluid recovered by microdialysis, or that availability of only small sample volumes (25μl) may have compromised the sensitivity of the assay.

We did not detect any difference in IL-6 concentration in skin dialysates between CSU patients and healthy controls. IL-6 is known to be increased in several inflammatory conditions as well as in response to the minimal trauma of microdialysis probe insertion (Krause et al, 2013; Stenken et al, 2010; Sjogren and Anderson, 2009). Our results suggest neither the increase in IL-6 in CSU patients at baseline compared to healthy controls, nor its correlation with disease severity. However, these results should be interpreted with caution as the analysis of variability indicated significant variation between individuals and sample replicates for IL-6 measurements in our study. Previous data suggest that IL-6 is consistently recovered and measured in skin dialysates (Krause et al, 2013; Sjogren et al, 2012; Clough et al, 2007) using both CBA bead-based immunoassays and microarrays although the direct comparisons between the studies are not possible due to the differences in the microdialysis set-up and the kits used for cytokine analysis. In our study, we attempted to measure IL-4, IL-5, IL-6, TNF-α and IL-13 in skin dialysates from both groups of study participants. TNF-α and IL-13 were detectable at low levels in few patients while IL-4 and IL-5 were not detectable in the samples. This is consistent with previous studies (Stenken et al, 2010, Clough et al, 2007). The possible reasons for poor recovery of these cytokines could be insufficient sensitivity of the assays, cytokine binding to the receptors and microdialysis membrane, impermeability of the membrane for certain cytokines. It is unknown whether cytokine detection could be improved by the use of novel bead-based immunoassays with enhanced sensitivity for cytokines (BD Biosciences) or by addition of the antibody-coated beads to the perfusate for microdialysis sampling (Clough et al, 2013).

2.4.6 Strengths and limitations of the study

The pilot study resulted in the detection of the differences between dermal histamine concentrations in CSU patients and healthy controls as well as the demonstration for the first time of *in vivo* histamine release in the skin of CSU patients in response to skin testing with autologous serum. The major strength of this study is its controlled study design. The use of histamine analysis assays with high sensitivity and reliability was another strength of the study which allowed us to detect dermal histamine release at the lower range of nanoscale measurements that was, probably, missed in an earlier microdialysis study in CSU (Larsen, 2002). Microdialysis studies provide stronger evidence for mediator participation in dermal responses compared to the circumstantial evidence derived from blockade of the response by antihistamines (Clough & Church, 2002). Protocol development through adaptive design was also a strength of the study which permitted the use of the same outcome measures from different stages of protocol development.

The exploratory nature of this pilot study defined the study limitations such as small participant numbers per each protocol modification and limited generalisability of the results. Also, the limitations of the experimental model have to be understood as no model can fully capture and reproduce the complexity of a biological phenomenon. Lack of histamine calibration can be considered as another limitation of this study but some authors argue that histamine calibration cannot be carried out in the inflammatory conditions due to altered solute recovery by plasma extravasation after skin testing (Petersen, 1997a). This study did not use codeine titration which limited the interpretation of observed histamine release in dermal response to the stimulation with autologous serum and its relevance to the skin autoreactivity. Analytical limitations included the lack of the detection of most cytokines except IL-6 in skin dialysates. Also, the peak concentrations of histamine in response to codeine were above the log-linear range for both neat and diluted samples (DF=3) and, therefore, could not be accurately estimated by the analytical approaches in this pilot study. Overall, the pilot study was successful and led to the development of optimal study protocol for a definitive confirmatory study.

2.4.7 Optimal Study Design for a Microdialysis Study in CSU

The development of an optimal protocol design (Figure 27) for a full-scale microdialysis study in CSU was related to refinement of post-experiment exclusion criteria, clinical criteria for stopping a microdialysis experiment, a layout of the microdialysis sites, predictive skin testing and analytical procedures for skin dialysates. A multi-centre study was suggested by sample size calculations for several candidate outcome measures such as the baseline concentrations of histamine or tryptase in skin dialysates and $AUC_{(40\ min)}$ for histamine release in response to PBS, autologous serum and codeine. The suggested study design will address the issues that arose during the pilot project. Whether or not this study design is successful for a multi-centre microdialysis study in CSU in real-life research setting will depend on sufficient attention to patient recruitment, technical training support for clinical investigators and analytical performance of the assays for sample analysis as well as close monitoring of the progress throughout the study.

2.4.8 Unresolved questions for future studies

Although our knowledge advanced over the last two decades, there are numerous unresolved questions about the pathophysiology of CSU. For example, what is the relative contribution of histamine and other mediators such as VEGF, CGRP and PAF to weal formation in CSU? Answers to these questions would optimize the use of existing anti-mediator treatments in CSU as well as enhance the development of combined therapeutic agents targeting several mediators.

It is important to know what are the molecular mechanisms mediating the proinflammatory effects of persistent increase in dermal histamine concentration in the skin of CSU patients? In particular, the relevance of histamine effects mediated via H4 histamine receptors is of practical significance in view of potential targeting of these effects with H4 histamine antagonists. In addition, whether histamine metabolism is perturbed in CSU would justify further research in enzymes involved in histamine synthesis and catabolism in the skin. This would provide novel insights into biochemical abnormalities which are likely to contribute to elevated histamine levels in the skin of CSU patients.

It would be interesting to know the functional profile of skin mast cells in CSU at a single cell level. The use of laser capture dissection microscopy on skin biopsies combined with molecular technologies would shed some light on the signaling mechanisms underlying skin mast cell releasability in CSU.

What is the relevance of the effects of anti-FcεRIα antibodies on skin mast cells to the phenomenon of skin autoreactivity? Whether anti-FcεRIα antibodies induces a weal- and-flare response in the skin, can be studied in mouse models with humanized high-affinity IgE receptor (Hide & Greaves, 2013). In such studies, *in vivo* dynamic visualization of an interaction between anti-FcεRIα antibodies and cutaneous mast cells using imaging techniques *in situ* would be of particular interest by analogy with the dynamic visualization of capture of luminal IgE by perivascular mast cells recently demonstrated in mouse models (Cheng et al, 2013).

Furthermore, studies into the effects of patients' serum on purified neutrophils would be an exciting line of research for future studies. Whether serum histamine-releasing activity, as detected by *in vitro* basophil assays, would also demonstrate neutrophil chemotactic effects is yet to be established. However, this suggestion is not unlikely taking into account a significant overlap in molecular weight of serum histamine-releasing factors in CSU and serum neutrophil chemotactic activity in cold-induced urticaria (Grattan et al, 1991; Soter, 1983). If this turns out to be right, this would provide an overlooked mechanism in CSU and may expand our understanding of underlying mechanisms in CSU associated with serum histamine-releasing activity.

Microdialysis studies in CSU open an avenue for entry to the field of skin proteomics. Shotgun proteomic approach was applied to skin dialysate samples obtained in would healing (Gill et al, 2011). The skin proteome in CSU remains to be defined. This would provide a more comprehensive picture of contributing mediators as well as other biochemical changes in the skin of CSU patients. This data would provide the groundwork for the network analysis which may reveal yet unknown biochemical interactions in CSU as demonstrated by network analysis in late-phase allergic reactions in the skin (Benson et al, 2006).

The concept of skin threshold for wealing and the reasons for its lowering in CSU needs further research. Careful codeine titration in active disease and in remission coupled with microdialysis sampling would provide some insights into the minimal histamine release required for weal formation in CSU. Another interesting area of future research would concern histamine receptors, their density, polymorphism and affinity to histamine in CSU. Whether aberrant sensitivity of histamine receptors contributes to weal formation in CSU is worth exploring.

Finally, the use of microdialysis studies could help elucidate the effects of topical medications in CSU such as Syk inhibitors and potentially protease inhibitors in the future. Further microdialysis studies into therapeutic modulation of skin priming and responsiveness in CSU would open up new opportunities for rational therapy in CSU.

CHAPTER 3

Pathophysiological Subsets in CSU and their Biomarkers: A prospective observational study

"Surprise is the greatest gift which life can grant us."

—Boris Pasternak

Abstract

Background: CSU is a common disease which is characterized by recurrent wealing for 6 weeks or more. CSU is a heterogeneous condition encompassing several pathophysiological phenotypes. CSU associated with serum histamine-releasing activity is thought to be mediated by functional autoantibodies against the high-affinity IgE receptor and IgE itself on the surface of basophils and dermal mast cells (Hide et al, 1993) although the definitive proof for functional autoantibodies as a causative factor in CSU is still lacking (Kaplan & Greaves, 2009). Conversely, patient subsets based on basophil histamine releasability to anti-IgE stimulation appeared to be unrelated to the presence or absence of serum histamine-releasing activity or anti-FcεRIα autoantibodies (Vonakis et al, 2007). Furthermore, *in vivo* basophil priming in CSU patients, suggested by flow cytometric studies, demonstrated the lack of relationship with serum histamine-releasing activity and anti-FcεRIa autoantibodies (Vasagar et al, 2006). From a clinical perspective, there is a need for greater understanding of the contribution of these pathophysiological factors to disease severity and the clinical course of CSU. Therefore,

we hypothesize that disease severity and a persistent course of the disease in CSU patients are associated with serum histamine-releasing activity, aberrant basophil releasability to anti-IgE stimulation and phenotypic changes in peripheral blood basophils.

The **aim** of this observational study was to carry out prospective longitudinal assessments of clinical and pathophysiological parameters in CSU patients and to elucidate their relation to disease severity and the persistent course of disease.

Materials and Methods: The study was designed as a prospective observational longitudinal study with data collection at three time points over the study period of 6 months. Twenty two patients (M:F ratio – 5:18) recruited in the study and were treated with their usual antihistamine with or without antileukotrienes throughout the study. Serum histamine-releasing activity was detected by a serum-induced basophil histamine release assay (RefLab, Denmark). Basophil functional subsets were defined by anti-IgE-induced basophil histamine release assays (Medway School of Pharmacy, UK). Basophil flow cytometry studies were carried out using a lyse-no-wash protocol with the microbead technology on FACS Canto™ II at the Norfolk & Norwich University Hospital. Acquired data were analyzed using Kaluza® software (version 1.1.).

Results: Based on basophil releasability assays, CSU patients were classified into basophil responders (n=8), non-responders (n=7) to anti-IgE stimulation and a subset of patients (n=7) with total cellular histamine below the level of detection by spectrofluorimetry at all points of a dose-response curve. In addition, CSU patients were divided into those with (n=8) and without (n=14) serum histamine-releasing activity. Patients with serum histamine-releasing activity were clustered in the subset with total cellular histamine below the level of detection by spectrofluorimetry (chi-squared test, p=0.004).

At baseline, CSU patients with total cellular histamine below the level of detection demonstrated a more severe disease compared to basophil responders (Mann-Whitney U Test, p=0.055) or non-responders (Mann-Whitney U test, p=0.025) to anti-IgE stimulation. The baseline UAS7 score was significantly higher in CSU patients with serum histamine-releasing activity compared to those without serum histamine-releasing activity (Mann-Whitney U test, p=0.0152). Baseline UAS7 correlated with serum histamine releasing activity (Spearman correlation r=0.58, p=0.0045), and with anti-IgE-induced BHR (Spearman correlation r=0.40, p=0.0666). In our study, 9 patients had a persistent CSU and 10 patients had a clinical improvement. Based on the ROC analysis for UAS7 at baseline, the cut-off value of 19 predicted the persistent course of CSU with 63.16% accuracy (sensitivity of 60% and specificity of 66.67%). In longitudinal analysis, we showed persistent (n=3) versus transient (n=3) increase in serum histamine-releasing activity in CSU patients over time. The use of different gating strategies for flow cytometric basophil enumeration in the same sample from each CSU patient resulted in statistically significant differences in absolute counts depending on the basophil phenotype (CCR3+CD123+ vs CCR3+CD63+ (p=0.0001), CD63+CD203c+ vs CCR3+CD123+ (p=0.0003), CD63+CD203c+ vs CCR3+CD63+ (p=0.0001)). There was no correlation between absolute basophil counts detected by different gating strategies with disease severity. There was no difference in absolute basophil counts between CSU patients with a persistent disease or a clinical improvement.

Conclusions: Pathophysiological phenotyping of CSU patients revealed the lack of relationship between serum histamine-releasing activity and basophil releasability to anti-IgE stimulation. CSU patients with serum histamine-releasing activity had a more severe disease. Disease severity in CSU predicted the persistent course of disease. Serial testing for serum histamine-releasing activity may be helpful for monitoring inflammation in CSU patients. Increased absolute counts of CCR3+CD63+ basophil subpopulation compared to CCR3+CD123+ or CD63+CD203c basophil subpopulations may reflect *in vivo* basophil priming in CSU. Flow cytometric enumeration in CSU varied depending on the choice of gating strategy for peripheral blood basophils.

3.1 Introduction

3.1.1 The Pathophysiological Classification of CSU patients

CSU is a heterogeneous condition encompassing several pathophysiological subsets (Sabroe et al, 2002; Vonakis et al, 2007). A comprehensive patient classification into distinct pathophysiological phenotypes in CSU may help a better assessment of the underlying inflammation and a selection of targeted treatment. Several approaches for the patient classification in CSU were proposed based on different pathophysiological parameters (Saini, 2014).

The research team from the St John's Institute of Dermatology (London, UK) proposed a classification based on the detection of serum histamine-releasing activity in CSU patients (Sabroe et al, 2002). This study classified CSU patients into subsets based on the presence of serum histamine-releasing activity and anti-FcεRIα and anti-IgE autoantibodies. In this study, serum histamine-releasing activity was associated with anti-FcεRIα autoantibodies. In general, serum histamine-releasing activity is detected in 30-50% of CSU patients (Kaplan & Greaves, 2009; Grattan, 2004). In CSU, serum histamine-releasing activity was shown to be confined to IgG serum fraction (Soundararajan et al, 2005; Kikuchi & Kaplan, 2001). The inhibition of serum-induced BHR by recombinant α-chains in CSU patients was first demonstrated by Hide et al (1993), and then extended in studies by Kikuchi and Kaplan (2002). This led to an interpretation that CSU with serum histamine-releasing activity may be mediated by functional autoantibodies against the α chains of the high-affinity IgE receptors or IgE itself on the surface of basophils and mast cells in 30-50% of patients (Grattan, 2004) although the direct proof of this theory is still lacking (Kaplan & Greaves, 2009). Furthermore, this theory does not explain the CSU pathophysiology in about 50-70% of patients without serum histamine-releasing activity (Saini, 2014). In addition, serum histamine-releasing activity is not specific for CSU and was also observed in cold urticaria and urticarial vasculitis (Gruber et al, 1988). Therefore, the clinical and the pathophysiological relevance of these findings is incompletely understood and merits further research.

Another pathophysiological classification was proposed by a research team from John Hopkins University (Baltimore, USA) and was based on basophil releasability to anti-IgE stimulation in CSU patients. In the work by Vonakis et al (2007), CSU patients were subdivided on the basis of basophil functional subsets (responders and non-responders to anti-IgE stimulation). An observational study in CSU patients revealed that basophil functional phenotypes were observed regardless of the presence of anti-FcεRIα autoantibodies as detected by an immunoenzymometric assay (Eckman et al, 2008). The authors also reported the lack of relationship between basophil functional subsets and serum histamine-releasing activity in CSU patients. Therefore, it was concluded that autoantibody-mediated desensitization of the high-affinity IgE receptor appeared to be an unlikely cause for abnormal basophil releasability to anti-IgE stimulation. Patients' basophils releasing histamine to anti-IgE stimulation show reduced SHIP-1 expression level while non-responding basophils have increased SHIP-2 levels (Vonakis et al, 2007). Also, clinical implications of basophil functional phenotypes were suggested in the study by Baker et al (2008) but this needs to be further elucidated in well-designed studies.

Based on the previous work, both mechanisms, serum histamine-releasing activity and basophil releasability to anti-IgE stimulation, are thought to contribute to the disease severity. The direct comparisons between these studies is not possible due to the variation in the methodology which was reflected in the correspondence between ourselves (Grattan & Borzova, 2009) and the research team from John Hopkins University (Eckman et al, 2009). The detection of serum histamine-releasing activity relies on basophil releasability assays which are characterized by a considerable variability in basophil releasability displayed by different donors (MacGlashan Jr., 2013). In the study by Eckman et al (2007), the presence of anti-FcεRIα autoantibodies was defined by an immunoenzymometric assay. As for the binding assays, non-specific binding was reported due to the conformational changes in blotted α-chains of the high-affinity IgE receptor (Kaplan & Joseph, 2007). Both methodologies have limitations and showed a lack of correlation (Eckman et al, 2009). In addition, methodological concerns were raised that CSU patients with basopenia might have been excluded from the functional studies using basophils from CSU patients. This may have resulted in the recruitment of different patient populations between the studies. Hence, the use of both classification approaches in the same study population of CSU patients may reveal the relative contribution of serum histamine-releasing activity and basophil releasability to anti-IgE stimulation to disease severity and the clinical course of disease. The interrelationship between these classification approaches in CSU patients, although currently unclear, may be potentially important for identifying underlying pathophysiology linked to severe and/or persistent CSU.

3.1.2 *In vivo* basophil priming in CSU

Another phenomenon of interest in the pathophysiology of CSU is *in vivo* priming of peripheral blood basophils in CSU as suggested by Vasagar et al (2006). In practice, activated basophils can be recognized by up-regulation of surface activation markers using flow cytometric analysis. Currently, several surface activation markers have been described for human basophils including CD63, CD203c, CD107a, CD107b, CD164 and CD13 (MacGlashan Jr., 2010a; Valent, 2010). CD63 (gp53) is a lysosomal membrane glycoprotein which belongs to a transmembrane-4 superfamily (Valent, 2010) and is expressed by basophils, mast cells, platelets and macrophages (Nieuwenhuis, et al., 1997; Metzelaar et al, 1991; Valent, 2010). In basophils, CD63 is located in the membranes of the cytoplasmic granules and is considerably up-regulated by fusion of granules with the plasma membrane upon anaphylactic degranulation of basophils (MacGlashan Jr., 2010b). CD203c (neural cell surface differentiation antigen ENPP3) is ectonucleotide pyrophosphatase 3 which is involved in the cleavage of deoxynucleotides and nucleotide sugars (Bollen et al, 2000). CD203c is a glycosylated type II transmembrane protein which is constitutively expressed on mature basophils, basophil and mast cell precursors (Buehring et al, 1999). The CD203c expression on resting basophils is relatively low which may hinder the identification of peripheral blood basophils using this marker (Sturm et al, 2010). CD203c is significantly upregulated on basophil degranulation via unknown mechanisms that differ from CD63-linked activation pathway (Sturm et al, 2010). The lysosome-associated membrane surface markers CD107a (LAMP1) and CD107b (LAMP2) are transmembrane lysosomal glycoproteins that were identified as novel basophil activation markers (Hennersdorf et al, 2005; Valent, 2010). CD13 is type

II transmembrane glycoprotein which is expressed on plasma membranes of most myeloid cells including basophils. CD13 belongs to zinc-binding metalloproteinases and is also known as aminopeptidase N. This marker was also reported as a novel basophil activation marker (Hennersdorf et al, 2005). Surface marker CD69 belongs to a transmembrane C-type lectin domain family 2 and is known as a very early activation marker for lymphocytes, especially for T cells (Hartnell et al, 1993). CD69 is expressed on lymphocytes, NK cells, basophils, eosinophils, platelets (Yoshimura et al, 2002). The kinetics of upregulation of basophil activation markers differs depending on the activating receptor and the mode of degranulation (MacGlashan Jr., 2010b; Hennersdorf et al, 2005).

Several gating strategies have been devised based on the combination of surface marker expression for identification of peripheral blood basophils. For example, a gating strategy is based on CD63 and CD203c on circulating basophils was described by Ebo et al (2012). The combination of these basophil surface markers was used in HistaFlow test (Ebo et al, 2012). Surface marker CCR3 (CD193) is a CC chemokine receptor-3 for eotaxin, eotaxin 2, MCP 3, MCP-4 and RANTES and is expressed on basophils, eosinophils, mast cells and T-lymphocytes (Pease and Williams, 2006). CCR3 (CD193) was characterized by less interindividual variability in its expression on basophils than other markers such as IgE or CD123 (Hausmann et al, 2011). Basophils can be also identified based on dual expression of CCR3 and CD63 (Eberlein et al, 2010). The combination of these markers is employed in the Flow CAST® assay (Bühlmann Laboratories AG, Switzerland). In addition, a combination of CCR3 and CD123 was also used for basophil enumeration in healthy subjects (Amundsen et al, 2012). CRTH2 (chemoattractant receptor-homologous molecule expressed on Th2 cells) represents the second receptor for prostaglandin D2 and is expressed on basophils, eosinophils and T lymphocytes (Boumiza et al, 2005). For basophil identification, CRTH2 is used in combination with CD3 to differentiate basophils from Th2 lymphocytes (Boumiza et al, 2005). CCR3-based gating has been actively developed and advocated as a sensitive and reliable approach to basophil gating compared to other gating strategies such as CD123/HLA-DR and anti-IgE-based gating (Hausmann et al, 2006). CD123 is IL-3 receptor α chain and has a high level of expression on human basophils. Another gating strategy for peripheral blood basophil relies on CD123 expression but the lack of HLA-DR expression (Ebo et al, 2006). However, HLA-DR expression on circulating basophils was described in SLE (Charles et al, 2010). IgE is commonly used as a basophil surface marker in combination with CD63 or CD203c for gating for peripheral blood basophils (de Weck et al, 2002). The IgE density varies among individuals but generally results in reliable basophil identification (MacGlashan Jr. et al, 2013). Several novel surface markers for human basophils were described such as CD164 (Chirumbolo, 2011; Wolanczyk-Medrala et al, 2011) but the information about the gating strategies using these markers was not available at the time when this study commenced. At present, comparisons of each basophil activation markers has been carried out in CSU but the use of gating expression based on two surface markers can provide further insights in basophil phenotypes and absolute counts as defined by selected gating strategy. This approach may provide novel information on the *in vivo* basophil immunephenotype and ultimately on *in vivo* basophil priming in CSU.

Activation status of peripheral blood basophils was noted in CSU (Vasagar et al, 2006), asthma (Ono et al, 2010) and venom allergy (Gober et al, 2007). In this study by Vasagar et al (2006), flow cytometric analysis demonstrated up-regulation of CD63 and CD69 but not CD203c on the surface of peripheral blood basophils in CSU patients. Recent research has shown significant up-regulation of FcεRIα on the surface of peripheral blood basophils in CSU (Lourenco et al, 2008). Basophil activation markers CD63 and CD203c on peripheral blood basophils from CSU patients were up-regulated regardless of ASST results while increased CD123 expression was associated only with skin autoreactivity in CSU patients (Lourenco et al, 2008). Of interest, Ono et al (2010) described CD203c expression on circulating basophils in asthma exacerbations. Among the patients allergic to insect venom, baseline CD63 expression was significantly higher in patients with systemic reactions during immunotherapy (Gober et al, 2007). In these patients, up-regulation of CD69 and CD203c was noted following a sting challenge (Gober et al, 2007). In patients with asthma, circulating basophils were characterized by higher levels of CD69 expression than basophils from healthy subjects (Yoshimura et al, 2002).

Furthermore, basophils recovered by bronchoalveolar lavage fluid from patients with asthma showed higher levels of CD69 expression than peripheral blood basophils from the same patients (Yoshimura et al, 2002). Overall, basophils with a vast number of receptors on their surface have a capacity of sensing the microenvironment in circulation as well as during their maturation. The resulting basophil phenotype in the circulation in patients with various diseases may reflect occurring inflammatory changes in the microenvironment. Therefore, flow cytometric immunophenotyping of peripheral basophils in CSU may be informative for disease-specific changes in peripheral blood basophil phenotype.

3.1.3 Factors associated with disease severity and the clinical course in CSU

Several pathophysiological parameters have been assessed in relation to disease severity in CSU in cross-sectional and longitudinal studies. CSU patients with serum histamine-releasing activity tended to have a more severe disease (Sabroe et al, 2002). Autoantibodies against the high affinity IgE receptors were also noted to occur at increased frequencies in patients with a more severe disease (Sabroe et al, 2002). The relationship between basophil functional subsets and disease severity in CSU patients was less clear. Some evidence exist that CSU patients with basophil responder phenotype had a more severe itching and higher frequency of patient visits to the emergency departments than basophil non-responders but both patient subsets did not differ in disease severity (Baker et al, 2008). In prospective observations, basophil numbers were also shown to fluctuate in parallel with disease severity and the effect of treatment with antihistamines or steroids (Grattan et al, 2003). Basophil releasability to anti-IgE stimulation was reported to increase towards the remission of the disease (Eckman et al, 2009) while serum histamine-releasing activity fluctuated with the treatment-induced changes in disease severity by ciclosporin (Grattan et al, 2000) and plasmapheresis (Grattan et al, 1992). In a systematic review, plasma levels of prothrombin fragment 1+2, D-dimers and C-reactive protein were evaluated as markers for disease severity (Takahagi et al, 2010). To our knowledge, longitudinal studies into the level of serum histamine-releasing activity in relation to disease severity have not been undertaken.

The natural history of CSU ranges from a spontaneous remission to a persistent course over years. Factors associated with a longer disease duration include the severe disease, the presence of angioedema, co-existence of CSU with physical urticarias and skin autoreactivity (Maurer et al, 2011). A systematic review of factors associated with disease duration confirmed a disease severity as an important predictor for a persistent course of disease (Rabelo-Filardi et al, 2013). There is a considerable variation in CSU duration between different studies into the natural course of disease which is likely to reflect the differences in patient selection and the period of observation. Nevertheless, there is generally considered that CSU in most patients would continue for longer than one year and in considerable number of patients for more than 5 years as reported in a GA^2LEN Task Force report on unmet clinical needs in CSU (Maurer et al, 2013a). Although a persistent clinical course occurs only in a subset of CSU patients, its impact on patient's wellbeing and healthcare costs makes it crucial to identify patients at risk of a persistent disease.

3.1.4 The hypothesis of the study

For this thesis, we hypothesize that disease severity and the persistent course of CSU is associated with serum histamine-releasing activity, aberrant basophil releasability to anti-IgE stimulation and *in vivo* basophil priming in the circulation. To test this hypothesis, we designed a prospective longitudinal observational study with the assessments of clinical and pathophysiological parameters at three time points throughout the observational period.

3.1.5 The rationale for the choice of biomarkers in the study

In this study, we used previously published UAS7 score (range 0-49) to assess disease severity in CSU patients. However, during this study, clinical guidelines recommended UAS7 score (range 0-42) for clinical assessments of disease severity in CSU patients (Zuberbier et al, 2009). There is no current definition for the persistent CSU. In our study, we defined a persistent disease if UAS7 score at the Visit 3 was greater or equal to that at the Visit 1 whereas a clinical improvement was defined if UAS7 score at Visit 3 in the study was less that that at Visit 1.

Serum histamine-releasing activity was chosen, with an understanding of its limitations, as an integral functional parameter reflecting the activity of histamine-releasing autoantibodies and potentially other factors such as chemokines, cytokines on peripheral blood basophils from healthy subjects. From a clinical point of view, the use of serum histamine-releasing activity was chosen because it was considered as an important determinant of disease severity in the previous research by Sabroe et al (2002). Basophil releasability assays to anti-IgE stimulation were used to apply the patient classification approach described in the previous work by Vonakis et al (2007). It was reasoned that the use of both classification approaches would reveal the details of an interaction of serum histamine-releasing factors and the effector cells, basophils, in the same patient. *In vivo* basophil priming was assessed in CSU patients to extend the findings published by Vasagar et al (2006). In this thesis, we used the detection of basophil phenotypes as defined by three previously published gating strategies (CCR3+CD123+ (Amundsen et al, 2012), CD63+CD203c+ (Ebo et al, 2002), CCR3+CD63+ (Eberlein et al, 2010) in the same whole blood sample from each CSU patient in the study. The choice and the interpretation of these pathophysiological parameters in CSU were carried out in the context of background knowledge and published evidence supporting their association with pathophysiological subsets and/or disease severity in CSU.

We did not measure the anti-FcεRIα autoantibodies in view of the limitations reported in the literature which were not, to our knowledge, resolved. These limitations include non-specific binding to IgG2 and carbohydrate moieties when blotted α-chains were used for the assay (Kaplan & Joseph, 2007). Also, the sensitivity of the autoantibody detection varies considerably between the binding assays which makes interpretation and direct comparisons difficult. Also, there are no assays at the moment that can distinguish functional from non-functional autoantibodies in CSU.

Furthermore, since there is no consensus on how to define peripheral blood basophils and the availability of several gating strategies for basophil studies, we selected well-researched basophil surface markers CD203c, CD63, CCR3, CD123 for our flow cytometric panel based on their applicability to the project aims. First, the gating strategy based on CD203c and CD63 was selected as a combination of markers representative of different modes of basophil activation as was suggested by HistaFlow assay (Ebo et al, 2012) and then demonstrated in the study by MacGlashan Jr. (2010b). CD123 was selected based on the previous data on its up-regulation in CSU with skin autoreactivity (Lourenco et al, 2008). Surface marker HLA-DR was selected as a part of the gating strategy based on CD123 and HLA-DR, however, this analysis was not carried out later in view of the possibility of HLA-DR up-regulation in CSU by analogy with SLE (Charles et al, 2010). CCR3 surface marker was included in the panel based on the initial published data on CCR3-based gating strategies for basophil identification. A dual gating strategy based on CCR3 and CD63 expression was applied later following new developments in basophil flow cytometry such as the release of Flow CAST® assay (Bühlmann Laboratories, Switzerland) employing both surface markers.

3.1.6 The aims of the study

The aims of the study included:

1 to classify CSU patients based on serum histamine-releasing activity and basophil releasability to anti-IgE stimulation and to evaluate the relationship between CSU pathophysiological subsets and disease severity;
2 to assess the natural course of disease in CSU patients and to determine the clinical and pathophysiological factors predicting the clinical course of CSU;
3 to determine absolute counts for basophil subpopulations in CSU patients identified by three flow cytometric gating strategies and to explore their association with disease severity or a clinical course of disease.

3.2 Materials and Methods

3.2.1 Study Design

The study was designed as a prospective observational longitudinal study with data collection at three time points over the study period (Figure 28). In the prospective study, there was one run-in and three follow-up visits for prospective assessment of clinical parameters and the biomarker levels in CSU patients at three time points over the study period. We allowed flexibility of 4 weeks between run-in and follow-up visits and 5 working days for the assessment visits for logistic reasons. Patients recruited to the study were treated with their usual antihistamine with the intention of staying on the same treatment throughout the study. Dose variation of antihistamine was allowed.

CSU exacerbations were treated according to standard clinical practice. The study protocol allowed short-term treatment with steroids for urticaria exacerbations if they were given within two weeks before the next scheduled study visit, the study visit should be deferred by up to 1 month provided patients went back to the original medication. Beyond this, patients would be withdrawn from the study.

As per protocol, if a clinical decision were made that patients need additional long-term second- or third- line treatment within the first three weeks of the observational periods, patients would be withdrawn from the study. If the decision was made within three weeks before the next scheduled visit, the assessment visit would be brought forward and patients would be offered a final assessment during a week of clinical work-up required before starting the second-line treatment.

3.2.2 Study Settings

The clinical study was performed at the Dermatology Department of Norfolk & Norwich University Hospital (Norwich, UK). Basophil releasability studies were carried out at Medway School of Pharmacy, University of Kent (Chatham Maritime, UK). A serum-induced basophil histamine release assay was carried our at the “RefLab” Laboratory at the Univeristy of Copenhagen (Copenhagen, Denmark). Flow cytometry studies were performed at the Pathology Department, Norfolk & Norwich University Hospital (Norwich, UK).

3.2.3 Study Population

CSU patients were recruited from the Urticaria Clinics at the Dermatology Department of Norfolk & Norwich University Hospital from December 2008 till August 2011.

Inclusion criteria for CSU patients were:

1 More than 18 years of age.
2 Continuous CSU.
3 Treatment with antihistamines with or without antileukotrienes.
4 Autologous serum skin test performed as a part of clinical routine work-up for CSU patients within one month before recruitment.
5 Willingness and capacity to give informed consent.

Exclusion criteria were:

1 Co-existing predominant physical urticarias.
2 CSU with a confirmed infectious, allergic, drug-induced or physical cause.
3 Biopsy-proven urticarial vasculitis defined by the presence of leukocytoclasia, fibrin deposition, endothelial swelling with or without red blood cell extravasation.
4 Current long-term treatment with steroids, ciclosporin or methotrexate.
5 Treatment with ciclosporin or methotrexate for urticaria or any other clinical reason within the last month before recruitment.

Criteria for patient withdrawal from the study:

1 The need for long-term steroid treatment on clinical grounds.
2 The need for a second- or third-line treatment (e.g. ciclosporin) on clinical grounds.

Figure 28. Study Design for a Prospective Study in CSU

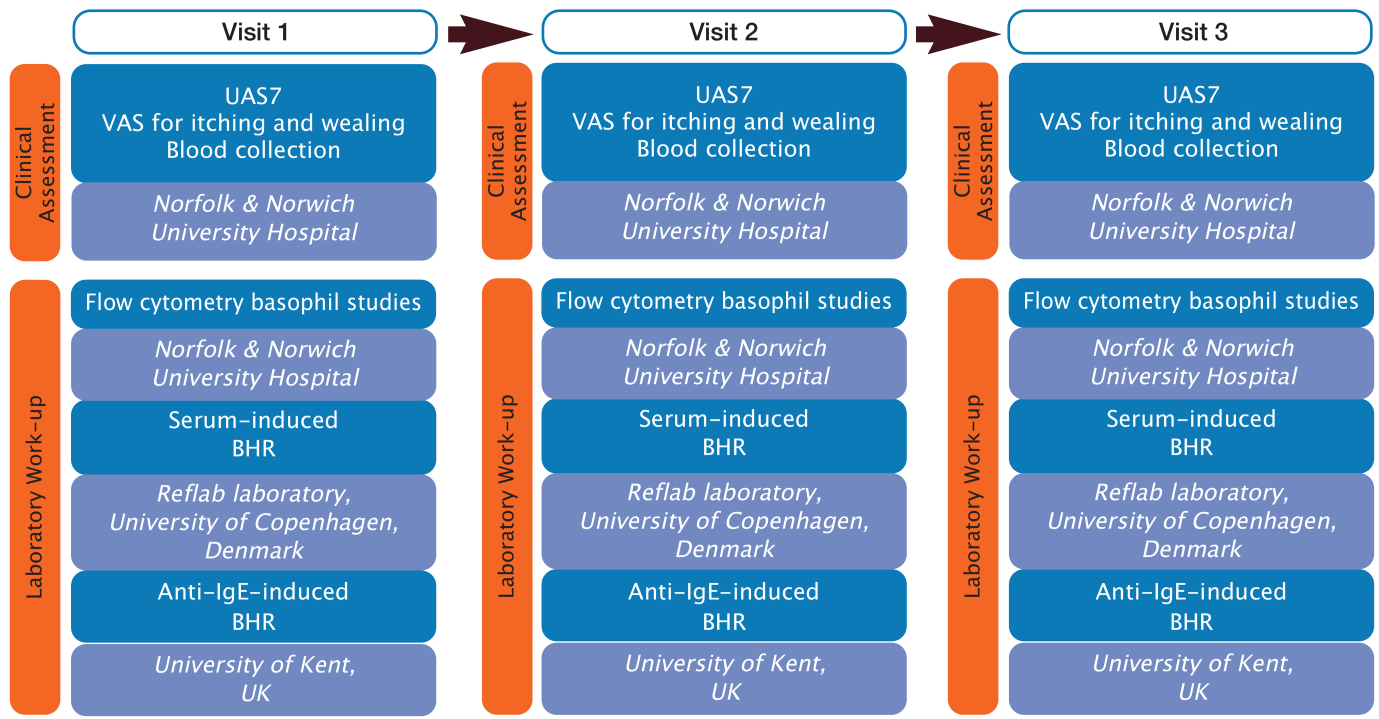


Figure 28. After run-in visit for patient recruitment, the study design included three follow-up visits for clinical examination and blood collection with approximately 10 week interval between the visits. Clinical examination on the study visit included weal count by a clinician and patient's assessment of wealing and itching based on visual analogue scales. UAS7 was also calculated based on patient's diary. Laboratory work-up comprised serum-induced BHR assay at the Reflab laboratory (Copenhagen, Denmark), flow cytometric basophil studies at the Norfolk & Norwich University Hospital (Norwich, UK), patient's basophil releasability studies and peripheral blood basophil counts at the University of Kent (Chatham Maritime, UK).

Abbrevations:
CSU - Chronic spontaneous urticaria
BHR - Basophil histamine release
UAS7 - Urticaria activity score over 7 days
VAS - Visual analogue scale

3.2.4 Study Procedures

3.2.4.1 *Clinical Activity Scores*

Patients assessed their disease activity by visual analogue scales referring to itching and wealing over the last week at each follow-up appointment. Patients recorded their symptoms daily in the self-assessments sheets. CSU activity was also assessed by UAS7 score which scores the number of weals (o=no weals, 1=1-10 small weals, 2=11-50 small weals (diameter <3cm) or 1-10 big weals (diameter > 3cm), 3=>50 small weals or 11-50 big weals, 4=almost covered) and the intensity of itching (0=none, 1=mild, 2=moderate, 3=severe) based on a patient's self-assessment sheet. The weekly score UAS7 was calculated as a sum of daily scores for itching and wealing over the last week before the blood collection. The weekly urticaria activity score ranged from 0 to 49. The number of weals on the day of the blood collection was assessed. Clinical assessment was performed by Dr Elena Borzova at the Dermatology Department of Norfolk & Norwich University Hospital.

3.2.4.2 *Serum-induced BHR assay*

For serum-induced basophil histamine release, basophils from healthy donors were incubated with 40µl patient's serum diluted 1:4 or 1:8 for 60 min at 37°C. Serum and released histamine was removed and the cells lysed using 20µl of 7% perchloric acid. After lysis of the cells 200µl PIPES were added and the samples were centrifuged at 2000g for 10 min and the histamine content in the filtrate was measured using the glass fibre method. Histamine release was expressed as a percentage of total histamine content. Serum-induced BHR assays were performed by Prof. Per Skov at the Reflab, University of Copenhagen, Denmark. Serum-induced BHR was carried out as previously described (Platzer et al, 2005).

For serum-induced BHR, the selection criteria for blood-bank buffy coats included anti-IgE induced BHR over 30% of the total cellular histamine. Then, three pools of pre-defined serum samples from CSU patients were tested on the selected buffy coat. For the serum pool 1, serum-induced BHR was required to fall within the range of 45-60% of total cellular histamine; for the serum pool 2 – within the range of 30-40%; and for the serum pool 3 – within the range of 20-30%. In addition, a pool of serum samples from healthy non-allergic patients was tested to confirm that selected buffy coat did not respond with histamine release. The test was considered negative if serum-induced BHR was below 16.5 %. In the study by Platzer et al (2005), this cut-off value of 16.5% was used to discriminate between CSU patients with positive and negative ASST and yielded negative results in non-CSU patients and healthy controls.

3.2.4.3 *Basophil purification and basophil histamine release assays*

Basophils from whole blood of CSU patients were purified by Ficoll density centrifugation. Basophil absolute counts and purity was determined by Alcian blue staining. Purified basophils were resuspended in HEPES-buffered Tyrode's solution (400 µl per tube) containing 1mmol/L of $CaCl_2$ and after a warming period (15min at 37°C) cells were stimulated with various titrations of anti-IgE or fMLP. Controls consisted of cells incubated with buffer alone. Basophils were stimulated for 15 min and reactions were terminated by adding ice-cold calcium-free HEPES buffer, followed by centrifugation and immediate transfer of supernatants into new vials. Histamine content in the supernatants, together with the cell pellets, which were diluted accordingly and lysed with perchloric acid (4%), was measured by spectrofluorimetry. BHR was considered positive if 10% or greater of the total cellular histamine was released to anti-IgE stimulation after correction for spontaneous histamine release. Total cellular histamine in the enriched basophil preparations was determined as a sum of histamine levels in supernatants and cell pellets in each experiment. Patient's basophil releasability studies were carried out by Dr Bernhard Gibbs at the Medway School of Pharmacy, University of Kent, UK.

3.2.4.4 *Flow cytometry Basophil studies*

For flow cytometric basophil studies, blood samples were drawn by venipuncture in Vacutainer tubes with sodium citrate (Beckton Dickinson, UK). The samples were incubated for 15 min at 37°C. Then, 100µl of patient's whole blood was dispensed into BD Trucount™ tubes with 100 µl of FACS Flow solution (Beckton Dickinson, UK) with 100 µl of anti-IgE solution (Sigma Aldridge, UK) or PBS. Then, the samples were mixed by inversion and incubated in waterbath for 20 min at 37°C. After this, the samples were placed on ice and the following antibodies were added to the samples: 20µl of CCR3-PE, 10µl of CD203c-APC, 20µl of CD63-FITC, 5µl of CD45-APC/Cy7, 20µl of CD123-PerCP/Cy5 (Table 4; Appendix 4, Figure 3). The samples were mixed by inversion followed by incubation on ice for 30 min in the dark. Next, 2ml of Pharmlyse solution (Beckton Dickinson, UK) was added to the sample followed by 10 min incubation at room temperature. Samples were analysed by Miss Cheryl Barker on FACS Canto II flow cytometer at the Pathology Department, Norfolk and Norwich University Hospital (Norwich, UK).

For data analysis, three gating strategies were applied on the same sample. As gating controls, fluorescence minus one (FMO) samples were used for each marker to set the gates for positive cell populations at 99th percentile (Appendix 4, Figure 4). Then, Boolean gates for each combination of two markers (CD63+CD203c+; CCR3+CD123+; CCR3+CD63+ gates) were constructed using Boolean logic by adding the gates defined for each marker by FMO samples as illustrated in Figure 5 (Appendix 4). The absolute basophil count using BD TruCount™ tubes was estimated according to the manufacturer's recommendations. For absolute count, a reverse pipetting technique was used to dispense whole blood samples. Flow cytometry data were analysed by Dr Elena Borzova (University of East Anglia) using Kaluza® Flow Analysis software (Beckman Coulter, Inc.).

As a part of the preparation for this study, a research visit to the Department of Immunology, Allergology and Rheumatology at the University of Antwerpen (Antwerpen, Belgium) (Head of the Department - Prof. Ebo) was undertaken by Dr Borzova in 2007 to study the technique and the details of the gating strategy and the data analysis. Additionally, a research visit to the Department of Hematology at the Medical University of Vienna (Vienna, Austria) (Head of the Department - Prof. Valent) was carried out by Dr. Borzova in 2008 to learn the technique for sample preparation and the technique of basophil flow cytometric studies in healthy subjects and allergic patients.

Table 4. Cellular Surface Markers used for Multiparameter Flow Cytometric Analysis of Peripheral Blood Basophils in CSU Patients

Surface Marker	Biological Family	Biological Function
CD203c	E-NNP3 (family of ectoenzymes)	Involved in hydrolysis of extracellular nucleotides
CD63	TM4 family (tetraspanin)	Expressed in late endosomes, role as an intracellular transport regulator
CCR3	Seven-transmembrane G-protein coupled receptor	C-C chemokine receptor for eotaxin, eotaxin 2, RANTES, MCP-2, -3 and -4
CD123	IL-3 receptor α-chain	Receptor for IL-3, IL-5, GM-CSF
CD45	protein tyrosine phosphatase	Leukocyte common antigen
HLA-DR	MHC class II cell surface receptor	Antigen presentation

Table 4. For flow cytometry studies, we used six-colour flow cytometric panel. This panel was designed for immunophenotyping of peripheral blood basophils using three gating strategies based on dual expression of CD63 and CD203c, CCR3 and CD63, CCR3 and CD123. Table 4 represents a selection of surface markers in the panel and their biological function.

Abbrevations:
CSU - Chronic spontaneous urticaria
CCR3 - Chemokine (C-C motif) receptor type 3
HLA-DR - D-related human leukocyte antigen (related to D-locus on the chromosome 6)
E-NNP3 - Ectonucleotide pyrophosphatase/phosphodiesterase 3
TM4 - Transmembrane 4 superfamily (tetraspanin family)
IL-3 - Interleukin 3
IL-5 - Interleukin 5
GM-CSF - Granulocyte macrophage colony-stimulating factor
MHC - Major histocompatibility complex
RANTES - Regulated upon activation normal T cell expressed and secreted
MCP - Monocyte chemotactic protein

3.2.4.5 *Statistics*

Two by three contingency table was created to determine patient distribution based on serum histamine-releasing activity and basophil releasability to anti-IgE stimulation at baseline. Distributional differences across CSU subsets based on serum histamine-releasing activity and basophil releasability to anti-IgE stimulation were tested by the chi-squared test. Continuous variables are presented as medians and the interquartile ranges. Mann-Whitney U test was used to compare continuous variables between the groups: CSU patients with or without serum histamine-releasing activity, basophil responders vs non-responders to anti-IgE stimulation, improving vs persistent disease course. Pearson's or Spearman's correlation tests were used to analyze variable correlations. Receiver-operating characteristic curves were constructed to determine the optimal threshold for differentiating between CSU patients with a persistent disease and a clinical improvement. $P<0.05$ was considered statistically significant. The analysis was performed by STATA statistical package, version 11/SE (StataCorp LP, USA).

3.3 Results

3.3.1 Study Population

Patient characteristics were presented in Figure 29. Of twenty two patients recruited to the study, nineteen patients completed the study (Table 5). The age of patients ranged from 19 and 68 years (mean – 49.2 years), the duration of CSU varied from 5 months to 42 years (mean – 8.2 years) (Table 5). The mean duration of patient's participation in the study was 174.05 ± 32.45 days (Figure 29B and C).

3.3.2 Pathophysiological Phenotypes of CSU and their relation to disease severity

The raw data for basophil histamine release assays in CSU patients are presented in the Appendix 4.

Based on basophil releasability assays, the subjects were classified into responders (n=8) and non-responders (n=7) to anti-IgE stimulation as previously described (Vonakis B.M. et al, 2007). Additionally, we identified a group of patients (n=7) with total cellular

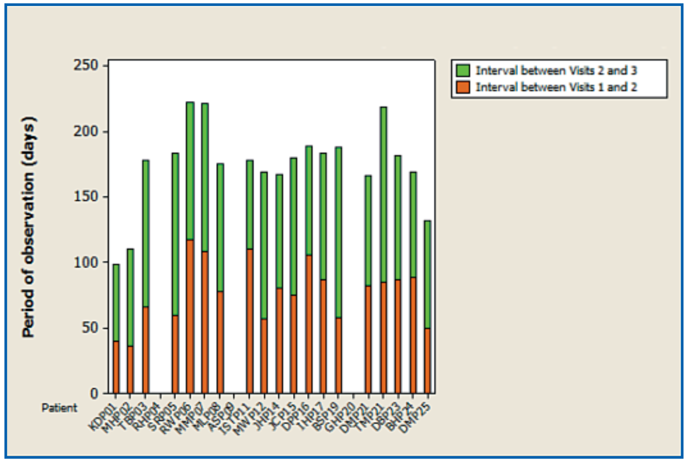
Figure 29. CSU Patient Characteristics and Period of Observation in the Prospective Study

Table 5. CSU Patient Characteristics in the prospective study

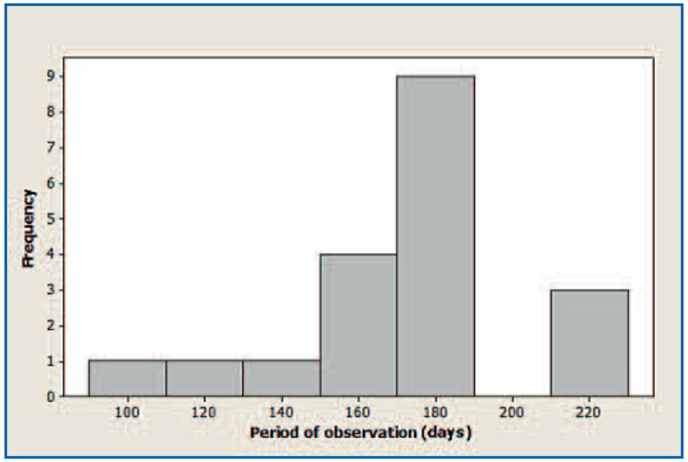
Number of patients Included in the study*	22 patients
Number of patients completed the study**	19 patients
Male:Female Ratio	5:18
Age	49.2 years (19-68 years)
Disease duration	8.2 years (5 months - 42 years)
Positive ASST	18 (78%) patients
Treatment with high dose antihistamines	16 (69.5%) patients

*CSU patients who completed the baseline visit.
**CSU patients who completed three visits in the study.

B. The period of observation of CSU patients in the prospective study



C. Histogram of the duration of the observation period for CSU patients in the study (days)



The mean period of observation in the study – 174.05 ± 32.45 days.

Figure 29. Prospective study included 22 CSU patients for pathophysiological phenotyping at baseline (Table 5). Of these, 19 CSU patients completed three visits of the study and were included in the longitudinal analysis of CSU course profiling (Table 5). The mean period of observation for CSU patients in the study was 174.05± 32.45 days (Figures 29B and C).

Abbrevations:
ASST - Autologous serum skin test
CSU - Chronic spontaneous urticaria

histamine below the level of detection by spectrofluorimetry at all points of a dose-response curve. CSU patients were divided into those with (n=8) and without (n=14) serum histamine-releasing activity. The distribution of patient across both classification approaches are presented in Figure 30A. The results for individual patients are presented in Figure 1 (Appendix 4). As expected, CSU patients with serum histamine-releasing activity were predominantly clustered in the pathophysiological subset with total cellular histamine below the level of detection by spectrofluorimetry (Figure 30B). By contrast, CSU patients without serum histamine-releasing activity were mainly distributed between CSU subsets with responding or not-responding basophils to anti-IgE stimulation. The difference in distribution of CSU patients with or without serum histamine-releasing activity between basophil releasability subsets was statistically significant (Chi-squared test, p=0.004).

Basophil responders were defined by histamine release above 10% of total cellular histamine to anti-IgE stimulation (0.1ng/ml). Basophil non-responders were defined by BHR below 10% of total cellular histamine. A subset with total cellular histamine below the level of detection by spectrofluorimetry was defined by the lack of detection of histamine at each point of dose-response curve for histamine release (the level of detection – 1ng/ml). The characteristic dose-response curve for a CSU patient with basophil response to anti-IgE stimulation was presented in Figure 22 (Appendix 4). In Figure 20 (Appendix 4), a dose response curve for histamine release to anti-IgE stimulation revealed that basophils do not respond to anti-IgE stimulation. By contrast, a flat curve for histamine release on the x-axis in Figure 11 (Appendix 4) suggested histamine concentration below the level of detection by spectrofluorimetry.

We examined the relation of CSU pathophysiological subsets to disease severity to understand if these pathophysiological subsets provide clinically meaningful information. At baseline, CSU patients with total cellular histamine below the level of detection demonstrated a more severe disease compared to basophil responders (p=0.055) or non-responders (p=0.025) to anti-IgE stimulation (Figure 31A). The baseline UAS7 score was significantly higher in CSU patients with serum histamine-releasing activity compared to those without serum histamine-releasing activity (Figure 31B). There was no difference in disease severity between basophil responders and non-responders to anti-IgE stimulation (p=0.560).

Figure 30. The Relationship between Serum Histamine-Releasing Activity and Basophil Releasability to anti-IgE Stimulation in CSU Patients

Figure 30. Based on the results of basophil releasability assays, we could differentiate three subsets of CSU patients:
- responders to anti-IgE stimulation (n=7);
- non-responders to anti-IgE stimulation (n=8);
- total cellular histamine below the LOD of spectrofluorimetry (n=7).

The distribution of CSU patients (Figure 30A) related to serum histamine-releasing activity and basophil releasability to anti-IgE stimulation is presented in Figure 30A. Serum histamine-releasing activity was significantly higher in CSU patients with total cellular histamine below the LOD for spectrofluorimetry compared to basophil responders and non-responders to anti-IgE stimulation (Figure 30B).

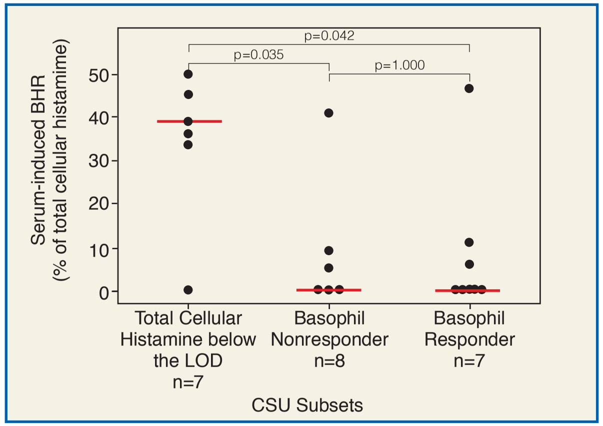
In basophil releasability assays, cells were stimulated with anti-IgE antibodies (Sigma-Aldridge, UK) at 0.1ng/ml. Serum-induced BHR was assessed on peripheral blood basophils from healthy donors at the RefLab (University of Copenhagen, Denmark). For serum-induced BHR assay, a diagnostic cut-off of 16.5% was used to detect serum histamine-releasing activity in CSU patients (Platzer et al, 2005).

A. CSU distribution related to serum histamine-releasing activity and basophil releasability to anti-IgE stimulation at baseline

	CSU with serum histamine-releasing activity	CSU without histamine-releasing activity
Basophil Responders	1	7
Basophil Non-responders	1	6
Total cellular histamine below the LOD	6	1

Chi-squared test, p=0.004

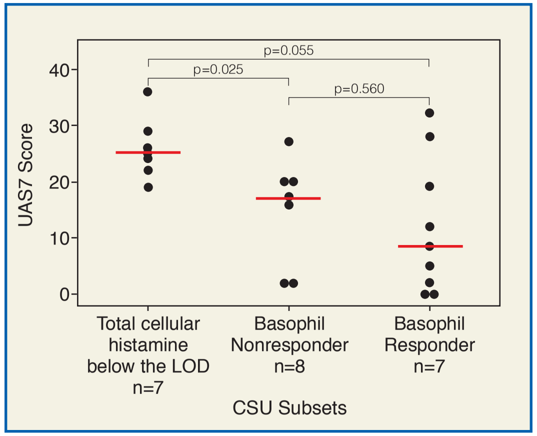
B. Serum histamine-releasing activity in CSU subsets related to basophil releasability to anti-IgE stimulation at baseline



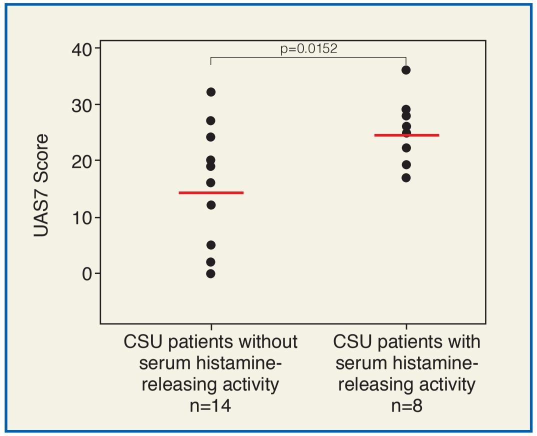
Abbrevations:
CSU - Chronic spontaneous urticaria
BHR - Basophil histamine release
LOD - Level of detection

Figure 31. The Relationship between Biomarkers and Disease Severity in CSU

Baseline UAS7 across the CSU patient subsets related to basophil releasability to anti-IgE stimulation



B. Baseline UAS7 in CSU patients with or without serum histamine-releasing activity



Red bar represents median values.

Pairwise comparisons between the pathophysiological subsets in CSU were carried out by Mann-Whitney U test. UAS7 score ranged from 0 to 49.

Abbreviations:
CSU - Chronic spontaneous urticaria
BHR - Basophil histamine release
UAS7 - Urticaria activity score over 7 days
LOD - Level of detection

Figure 31. Based on the results of basophil releasability assays, we could differentiate three subsets of CSU patients:
- responders to anti-IgE stimulation (n=7);
- non-responders to anti-IgE stimulation (n=8);
- total cellular histamine below the LOD of spectrofluorimetry (n=7).

CSU patients with total cellular histamine below the LOD for spectrofluorimetry were characterised by more severe disease than responders or non-responders to anti-IgE stimulation (Figure 31A). There was no statistically significant difference in UAS7 between responders and non-responders to anti-IgE stimulation. Anti-IgE induced BHR from peripheral blood basophils of CSU patients was carried out by Dr Bernhard Gibbs at the Medway School of Pharmacy (Chatham Maritime, UK). For basophil releasability assays, cells were stimulated with anti-IgE antibodies (Sigma-Aldridge, UK) at 0.1ng/ml. Responders to anti-IgE stimulation were defined if anti-IgE-induced BHR was above 10% of total cellular histamine while non-responders to anti-IgE stimulation were classified if anti-IgE-induced BHR was below 10% of total cellular histamine.

Based on serum-induced BHR assay, CSU patients were grouped into CSU patients with (n=8) and without (n=14) serum histamine-releasing activity. CSU patients with serum histamine-releasing activity had significantly higher UAS7 score than those without serum histamine-releasing activity (Mann-Whitney U Test, p=0.0152). Serum-induced BHR was assessed on peripheral blood basophils from healthy donors at the RefLab (University of Copenhagen, Denmark). For serum-induced BHR assay, a diagnostic cut-off of 16.5% was used to detect serum histamine-releasing activity in CSU patients (Platzer et al, 2005).

To further assess the interrelationships between these pathophysiological parameters and disease severity in CSU patients, we examined the correlations of UAS7 score with serum histamine-releasing activity and anti-IgE-induced BHR at baseline. We noted a moderate correlation between UAS7 and serum histamine releasing activity (Figure 32A), between UAS7 and basophil releasability to anti-IgE stimulation (Figure 32B). Of interest, there was a lack of correlation between serum histamine-releasing activity and anti-IgE-induced BHR in CSU patients (Figure 32C).

3.3.3 Natural course of disease in CSU patients

During follow-up, the persistent course of CSU was observed in 10 patients whereas 9 patients displayed a clinical improvement of CSU over the period of observation in our study. Based on the ROC analysis, baseline UAS7 was predictive of the persistent course of disease whereas serum histamine-releasing activity and anti-IgE-induced BHR were not informative as predictors for disease persistence in our study population. The ROC analysis for UAS7 suggested that the cut-off value of 19 allowed 63.16% accuracy in predicting the persistent course of disease, with sensitivity of 60% and specificity of 66.67% (Figure 33A). The disease profiling in CSU patients based on the ROC analysis is demonstrated in Figure 33B.

This means that, in our study, the use of a UAS7 score equal or above 19 (using the scale of 49 in total) at baseline correctly identified CSU patients with persistent disease in 60% of cases. Furthermore, baseline UAS value of 19 or higher correctly excluded patients with improving CSU in 66.67% of cases. Overall, the accuracy of prediction of the course of the disease based on baseline UAS7 score using the threshold of 19 was noted to be 63.16%.

However, ROC analysis results must be interpreted with caution in view of the limitations of ROC analysis in small samples. It is important to underscore that the threshold UAS7 value and the accuracy of prediction obtained in our study is relevant only to the studied patient population which is a small size sample from a secondary care dermatological setting. These data need to be validated in larger patient samples and also in samples from different clinical settings to explore whether a similar threshold would apply. We would expect the cut-off UAS7 threshold at baseline for predicting persistent CSU to vary depending on the sample size and the frequency of severe disease in the study population. Whether the accuracy of prediction of CSU course at baseline could be improved by using a UAS7 score in combination with inflammatory biomarkers in the skin or in the circulation, is uncertain, but its potential is worth exploring in further studies.

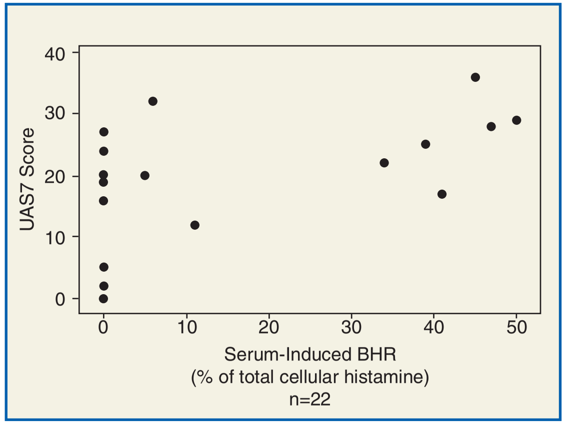
In persistent CSU, disease was more severe at baseline than in CSU patients with a clinical improvement over the observation period (Figure 34). There was no difference in serum-induced BHR or anti-IgE-induced BHR between CSU patients with persistent or improving disease (Appendix 4, Figure 5).

3.3.4 Longitudinal Changes of Serum Histamine-releasing activity and disease severity in CSU patients

Analysis of longitudinal data identified two groups of patients with different patterns of serum histamine-releasing activity over time. Three CSU patients were characterized by a persistent increase in serum histamine-releasing activity above 16.5% at all time points over the period of observation (Figures 35A-C). By contrast, three CSU patients had a transient increase of serum histamine-releasing activity above 16.5% at one or two time points only over the period of observation (Figures 35D-F).

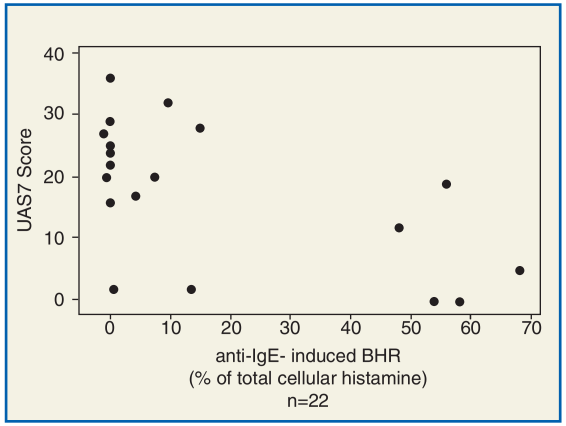
Figure 32. Correlations between Serum Histamine-Releasing Activity and Basophil Releasability to Anti-IgE Stimulation with Disease Severity In CSU

A. Correlation between UAS7 and serum histamine-releasing activity in CSU patients at baseline



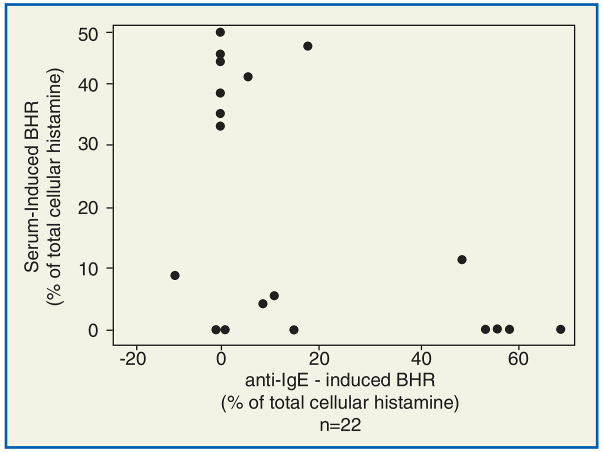
Spearman correlation r= 0.58, p=0.0045.

B. Correlation between UAS7 and anti-IgE-induced BHR from CSU patient's basophils at baseline



Spearman correlation r=-0.40, p=0.0666

C. Correlation between serum histamine-releasing activaty and anti-IgE-induced BHR from CSU patient's basophils at baseline



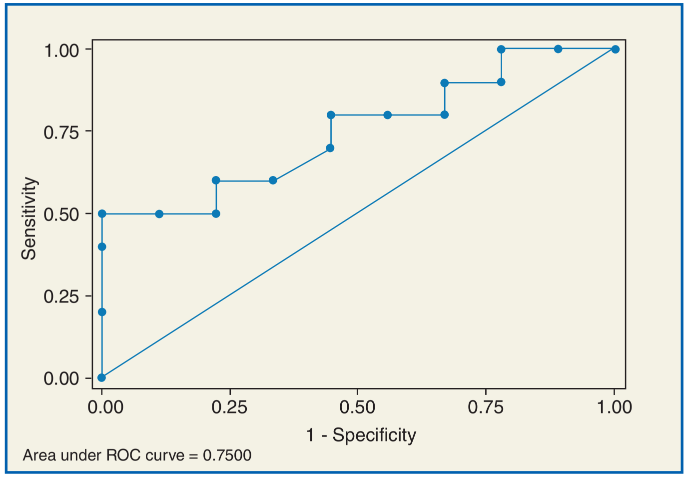
Spearman correlation r=-0.23, p=0.3092

Figure 32. At baseline, UAS7 in CSU patients showed a correlation with serum histamine-releasing activity (Spearman correlation r=0.58, p=0.0045) (Figure 32A).There was negative correlation between baseline UAS7 scores and anti-IgE-induced BHR from peripheral blood basophils in CSU patients (Spearman correlation r=0.40, p=0.0666) (Figure 30B). Noteworthy, there was no correlation between serum histamine-releasing activity and anti-IgE-induced BHR in CSU patients (Figure 32C). The analysis was carried out on 22 CSU patients who completed the baseline visit in the study. In our study, UAS7 score ranged from 0 to 49. Serum histamine-releasing activity was assessed on peripheral blood basophils from healthy donors at Reflab (University of Copenhagen, Denmark). For basophil releasability assays, cells were stimulated with anti-IgE antibodies (Sigma-Aldridge, UK) at 0.1ng/ml. Anti-IgE-induced BHR in peripheral blood basophils from CSU patients was assessed by Dr Bernhard Gibbs from Medway School of Pharmacy (Chatham Maritime, UK).

Abbrevations:
CSU - Chronic spontaneous urticaria
BHR - Basophil histamine release
UAS7 - Urticaria activity score over 7 days

Figure 33. The Clinical Course of CSU in the Prospective Study

A. ROC analysis graph for discriminating between patients with persistent CSU and those with spontaneous improvement of CSU



B. The disease course profiling in CSU patients in the prospective study

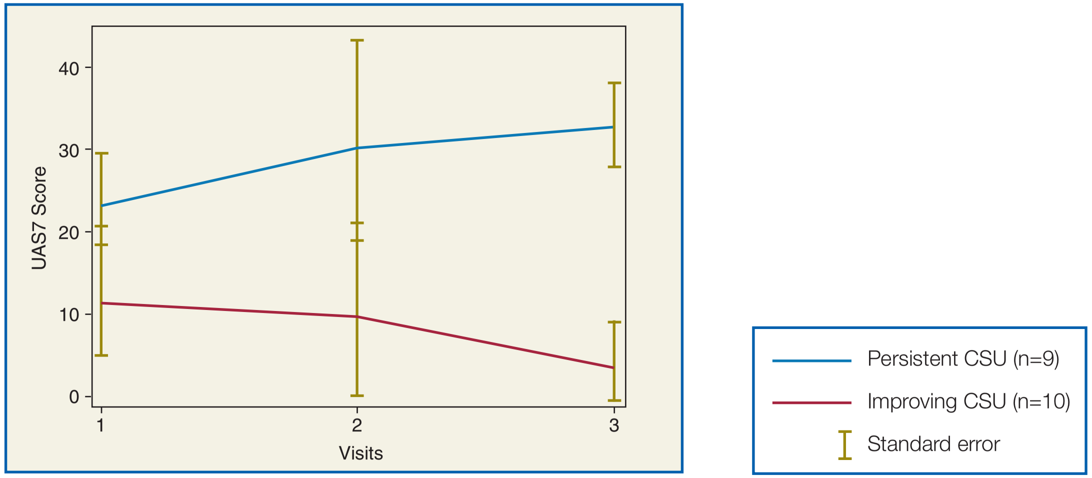
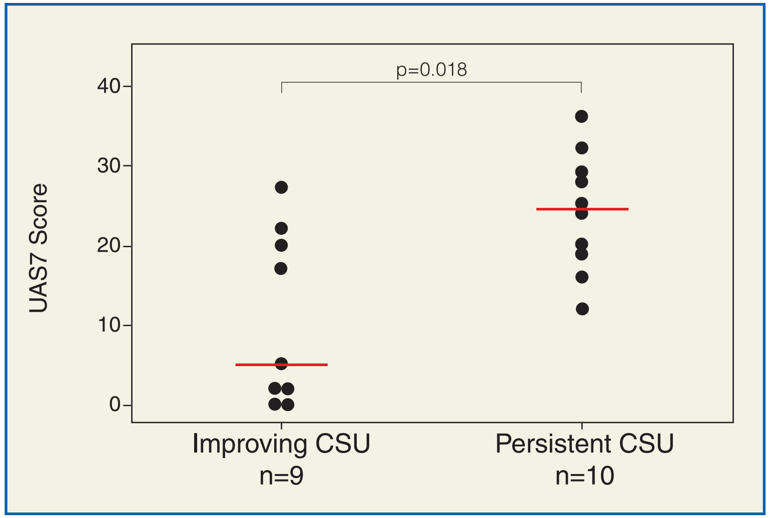


Figure 33. In our study, UAS7 appeared to be a predictor of disease course over the period of observation. The ROC graph demonstrated the reciprocal relationship between sensitivity and specificity of all possible UAS7 values (Figure 33B). Sensitivity and specificity in discriminating CSU patients with persistent course of disease from those with improving CSU was based on the disease course (Figure 33A). The ROC analysis for UAS7 suggested that the cut-off value of 19 yielded the highest accuracy for prediction of disease severity (sensitivity - 60%, specificity - 66.67%). Patients with persistent CSU (designated in blue on Figure 33B) were defined if their UAS7 score at Visit 3 was larger or equal than that at Visit 1. Patients with improving CSU were defined if their UAS7 score at Visit 3 was less than that at Visit 1 (designated in red on Figure 33B). UAS7 with score range of 0 to 49 was used in this study.

Abbrevations:
CSU - Chronic spontaneous urticaria
UAS7 - Urticaria activity score over 7 days
ROC - Receiver operating curve

Figure 34. Baseline UAS7 Score in Patients with Persistent and Improving CSU

A. Baseline UAS7 in patients with persistent or improving CSU



Red bar represents median values.
Pairwise comparisons between CSU subgroups in relation to the clinical course of disease was carried out by Mann-Whitney U test.

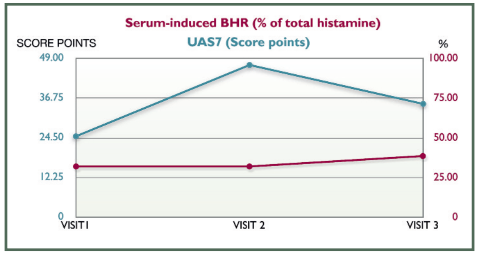
Figure 34. In our study, 19 CSU completed three visits in the observational study and were included in the longitudinal analysis of the clinical course of the disease. Of these, 9 patients had improving CSU and 10 patients had persistent CSU. At baseline, patients with persistent CSU had higher UAS7 scores than those with improving CSU (Mann-Whitney U test, p=0.018). The UAS7 score ranged from 0 to 49.

Abbrevations:
CSU - Chronic spontaneous urticaria
UAS7 - Urticaria activity score over 7 days
BD - Becton Dickinson
FACS - Fluorescence-activated cell sorting

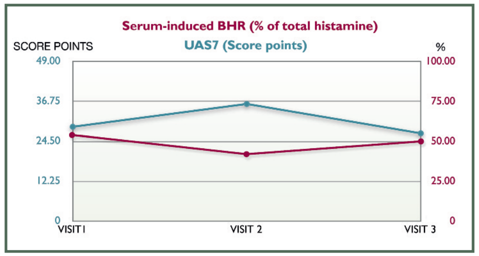
Figure 35. Longitudinal Changes in Serum Histamine-Releasing Activity and Disease Severity in CSU Patients

Persistent pattern of serum histamine-releasing activity in CSU Patients

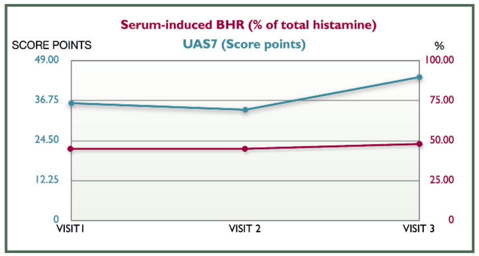
A. Patient TBP03



B. Patient DBP23

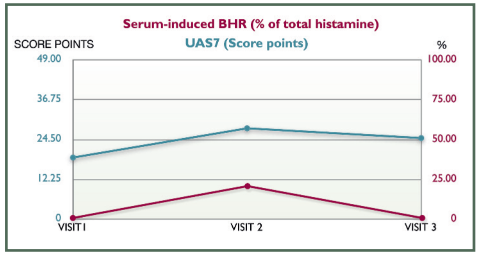


C. Patient BHP24

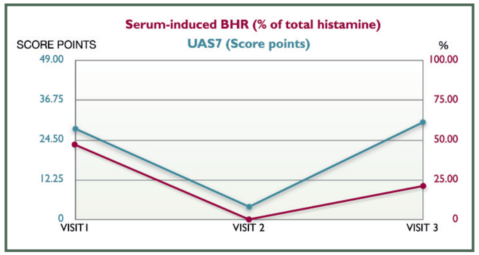


Transient pattern of serum histamine-releasing activity in CSU Patients

D. Patient DPP16



E. Patient BSP19



F. Patient DMP21

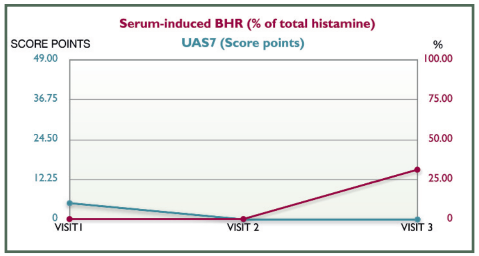


Figure 35. Longitudinal measurements revealed transient or persistent increase in serum histamine-releasing activity in CSU patients. CSU patients with a persistent increase in serum histamine-releasing activity showed serum-induced BHR assay over 16.5% at all time points of observation (Figure 35A-C). The patient group with a transient increase in serum histamine-releasing activity demonstrated serum-induced BHR above 16.5% only at one or two observation time points (Figure 35D-F). For serum-induced BHR assay, a diagnostic cut-off of 16.5% was used to detect serum histamine-releasing activity in CSU patients (Platzer et al, 2005). Serum-induced BHR assay was carried out at the RefLab (University of Copenhagen, Denmark).

Abbrevations:
BHR - Basophil histamine release
CSU - Chronic spontaneous urticaria
UAS7 - Urticaria activity score over 7 days

3.3.5 Flow cytometric enumeration of basophil subpopulations in CSU patients

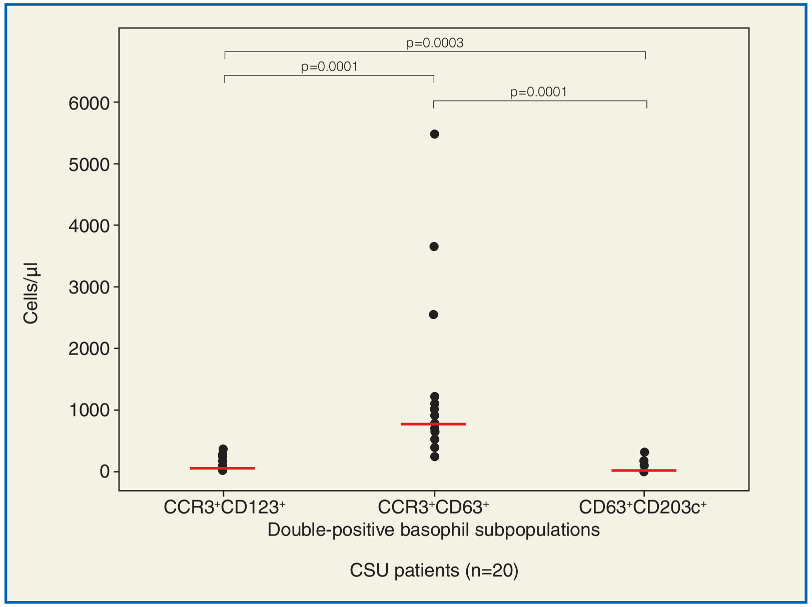
Flow cytometric quantification of basophil phenotypes in peripheral blood of CSU patients revealed statistically significant differences in absolute basophil counts detected by flow cytometric gating strategies (CCR3+CD123+ vs CCR3+CD63+ (p=0.0001), CD63+CD203c+ vs CCR3+CD123+ (p=0.0003), CD63+CD203c+ vs CCR3+CD63+ (p=0.0001)) in the same sample from each CSU patient (Figure 36). Enumeration of CCR3+CD63+ basophils by microbead technology (Figures 36A, B) yielded significantly higher absolute counts compared to CCR3+CD123+ or CD63+CD203c+ basophil subpopulations in the same peripheral blood sample from each CSU patient suggesting that absolute basophils counts depend on the gating strategy used to define peripheral blood basophil phenotype in CSU patients.

Of interest, there was no correlation between absolute basophil counts detected by CCR3+CD123+ and CCR3+CD63+ gating strategies in peripheral blood samples from CSU patients (Spearman correlation r=0.15, p=0.5269) (Figure 37A). By contrast, there was moderate correlation between absolute basophil counts determined by CD63+CD203c+ and CCR3+CD63+ gating strategies (Spearman correlation r=0.57, p=0.0085) and by CD63+CD203c+ and CCR3+CD123+ gating strategies (Spearman correlation r=0.63, p=0.0029) (Figure 37 B-C).

We examined whether basophil phenotype and absolute counts defined by different gating strategies were associated with the pathophysiological subsets, disease severity or a clinical course of CSU. We did not detect any differences in absolute basophil counts detected by three gating strategies between CSU pathophysiological subsets which was in keeping with previous data (Vonakis et al, 2007). There was a lack of correlation between baseline UAS7 and absolute basophil counts determined by three gating strategies (Appendix 4, Figure 6). Also, there was no difference in absolute basophil counts between CSU patients with persistent or improving disease (Appendix 4, Figure 7).

Figure 36. Flow Cytometric Quantification of Peripheral Blood Basophils in CSU Patients using three Gating Strategies ($CCR3^+CD123^+$, $CCR3^+CD63^+$, $CD63^+CD203^+$) on the Same Sample

A. Baseline absolute basophil counts in CSU patients using three gating strategies on the same sample



Red bar represents median values.

Pairwise comparisons were carried out using Wilcoxon signed-rank test.

B. The formula for absolute basophil count using BD Trucount Beads

$$\text{Total number of basophils/µl} = \frac{\text{Basophil Events x BD Trucount x DF}}{\text{Bead Events x Sample Volume}}$$

Where sample volume = 100 µl

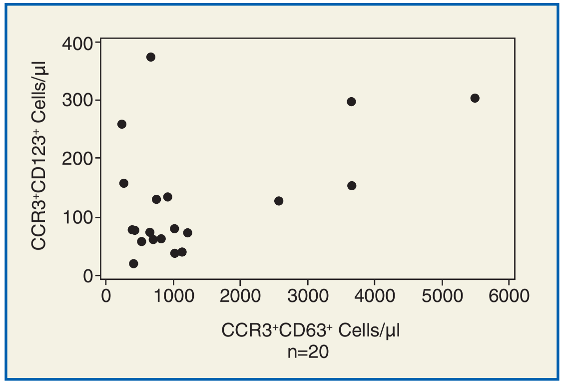
DF = 23.75

Figure 36. Flow cytometric quantification of peripheral blood basophils in CSU patients was carried out using microbead technology (BD Trucount Beads). Three gating strategies ($CCR3^+CD123^+$, $CCR3^+CD63^+$, $CD63^+CD203c^+$) for peripheral blood basophils were applied to the data analysis on the same sample from each CSU patient at baseline (Figure 36A). The absolute count of peripheral blood basophils was calculated from the dot plot values using the formula in Figure 36B. Flow cytometric data were acquired using the BD FACS Canto™ II Instrument by Miss Cheryl Barker at the Norfolk & Norwich University Hospital (Norwich, UK). Data analysis included 20 CSU patients, two CSU patients were excluded from the analysis for technical reasons with flow cytometric data acquisition.

Abbrevations:
CSU - Chronic spontaneous urticaria
DF - Dilution factor

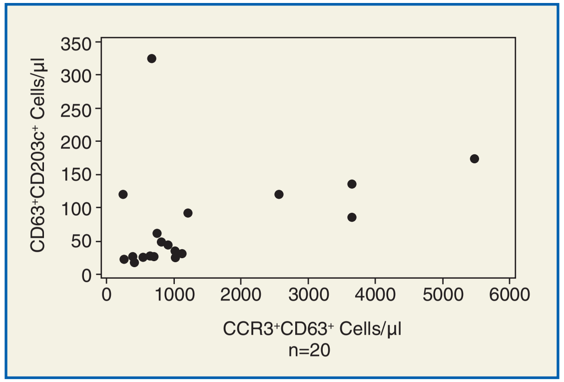
Figure 37. Correlation between Absolute Basophil Counts in Peripheral Blood of CSU Patients obtained by three Flow Cytometric Gating Strategies

A. Correlation between absolute basophil counts in CSU patients at baseline using CCR3⁺CD123⁺ and CCR3⁺CD63⁺ gating strategies



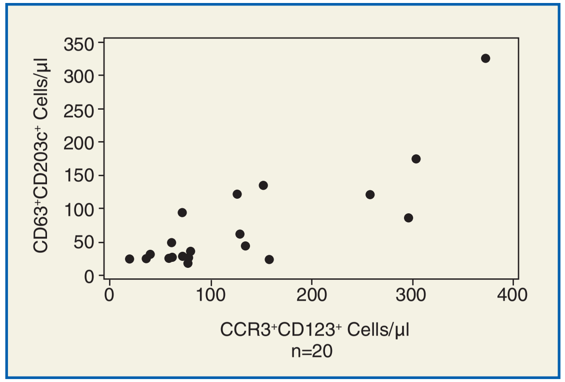
Spearman correlation r=0.15, p=0.5269

B. Correlation between absolute basophil counts in CSU patients at baseline using CD63⁺CD203c⁺ and CCR3⁺CD63⁺ gating strategies



Spearman correlation r=0.57, p=0.0085

C. Correlation between absolute basophil counts in CSU patients at baseline using CD63⁺CD203c⁺ and CCR3⁺CD123⁺ gating strategies



Spearman correlation r=0.63, p=0.0029

Figure 37. Three gating strategies for peripheral blood basophils were applied on the same sample from each CSU patient. These gating strategies included dual gating based on CCR3 and CD123, CCR3 and CD63, CD63 and CD203c. Absolute basophil counts were enumerated using BD Trucount beads. Data analysis revealed that there was no correlation between absolute basophil counts obtained by $CCR3^+CD123^+$ and $CCR3^+CD63^+$ gating strategies (Spearman correlation r=0.15, p=0.5269) (Figure 37A). Absolute basophil counts gated as $CD63^+CD203c^+$ cells correlated with those obtained by $CCR3^+CD63^+$ gating strategy (Spearman correlation r=0.57, p=0.085) (Figure 37B). There was also a correlation between absolute counts for basophils selected using $CD63^+CD203c^+$ and $CCR3^+CD123^+$ gating strategies (Spearman correlation r=0.63, p=0.0029) (Figure 37C). Flow cytometric data were acquired on BD FACS Canto™ II instrument by Miss Cheryl Barker at the Norfolk & Norwich University Hospital (Norwich, UK). Data analysis was included 20 CSU patients, two CSU patients were excluded from the analysis for technical reasons with flow cytometry data acquisition.

Abbrevations:
CSU - Chronic spontaneous urticaria
CCR3 - Chemokine (C-C motif) receptor type 3

3.4 Discussion

3.4.1 The pathophysiological phenotyping in CSU

We reported a prospective observational study in CSU patients with a focus on the pathophysiological phenotyping and the relation of the pathophysiological subsets to disease severity and a clinical course of CSU. We confirmed and extended the studies on the pathophysiological phenotyping in CSU patients by Eckman et al (2008) and Sabroe et al (2002). The observation of basophil responders and nonresponders to anti-IgE stimulation in CSU was previously reported by Eckman et al (2008) and was also confirmed by our study. We found that these basophil functional profiles were not related to serum histamine-releasing activity in CSU patients which was also consistent with the previous findings (Eckman et al, 2008). In addition, we demonstrated that serum histamine-releasing activity was predominantly clustered in the subset of CSU patients with total cellular histamine below the level of detection by spectrofluorimetry. The distribution of serum histamine-releasing activity in a CSU subset other than basophil responders and non-responders to anti-IgE stimulation suggested that serum histamine-releasing activity and aberrant basophil releasability to anti-IgE stimulation were likely to operate via independent disease pathways in CSU. This was also demonstrated by the lack of correlation between serum histamine-releasing activity and anti-IgE-induced BHR in CSU patients and may explain the previous discrepancies between the studies. However, we can interpret these results with caution as we cannot exclude the possibility that the levels of serum histamine-releasing factors, which are relevant *in vivo*, may be lower compared to the *in vitro* settings and below the levels of detection with existing diagnostic methodology. As reported by Kaplan and Joseph (2007), serum from CSU patients, classified as not having serum histamine-releasing activity, still induces BHR from healthy donor basophils 10 times higher than sera from healthy subjects. Furthermore, it remains unknown whether basophil functional defects or serum histamine-releasing activity are primary or secondary events in the pathophysiology of CSU. In general, our findings on the pathophysiological phenotyping in CSU patients highlighted the pathophysiological heterogeneity of the disease and need to be validated in larger study populations of CSU patients.

From clinical perspective, we confirmed our hypothesis that a more severe CSU was associated with serum histamine-releasing activity. The association of serum histamine-releasing activity with disease severity was in keeping with the previous work by Sabroe et al (2002) and indicated the clinical relevance of serum histamine-releasing activity as a marker for a more severe CSU. With regards to basophil functional subsets, CSU patients with total cellular histamine below the level of detection by spectrofluorimetry had a more severe disease than basophil responders or non-responders to anti-IgE stimulation. This may suggest that studies employing only basophil releasability assays from CSU patients may exclude a subset of CSU patients with basopenia and severe disease.

Longitudinal analysis of serum histamine-releasing activity in CSU patients demonstrated persistent vs transient increases of serum histamine-releasing activity in CSU patients. There are several possible explanations as to which factors may mediate serum histamine-releasing activity in CSU patients including anti-FcεRIα autoantibodies, complement components, cytokines and, possibly, yet unknown factors but the reasons for the observed persistent or transient patterns of serum histamine-releasing activity in CSU are poorly understood. At present, a large multicenter cross-sectional study in chronic autoimmune urticaria (PURIST study) is ongoing in Europe to identify and characterize novel biomarkers for chronic autoimmune urticaria in line with the proposal of diagnostic criteria by the EAACI Task Force position paper on autoimmune urticaria (Konstantinou et al, 2013). The results of this study are awaited and will likely provide some insights into this subset of CSU patients associated with serum histamine-releasing activity.

3.4.2 Natural history in CSU

Natural history of CSU is less well studied with only few observational studies reported in the literature (Toubi et al, 2004; Takahagi et al, 2010). Our study suggested a variable course of disease in CSU patients with a disease persistence in some patients and a clinical improvement in others. In our study, baseline UAS7 score was confirmed as a predictor for a persistent course of CSU which was consistent with the conclusions of the recent systematic review (Rabelo-Filardi et al, 2013). We determined, for the first time, the sensitivity and specificity of UAS7 score as predictor for persistent CSU. Our contention that serum histamine-releasing activity could be a predictor for disease persistence was not supported by this study. Neither serum histamine-releasing activity, nor anti-IgE-induced BHR predicted CSU persistence in our study. It is plausible that a persistent disease is associated with the underlying inflammation by analogy with asthma (Panattieri et al, 2008), however, we could not identify the pathophysiological predictors for a persistent course of CSU. The possible explanations could be that the association is more obvious in patient subpopulations which are likely to be treated with immunomodulatory or biological agents. These patient subpopulations were not included in our observational study. Another possibility could be that pathophysiological factors other than serum histamine-releasing activity or basophil releasability to anti-IgE stimulation may be responsible for disease persistence. For example, skin priming and remodeling associated with chronic wealing was suggested in the recent study by Kay et al (2014a) and may well be a contributing factor for a disease persistence in CSU.

Furthermore, there are only a few longitudinal observations in CSU exploring the dynamics of serum histamine-releasing activity over the clinical course of disease (Tanus et al, 1996). In longitudinal study, we demonstrated, for the first time, a persistent and a transient increase in serum histamine-releasing activity in some CSU patients over the observation period. Our data suggested that serial testing for serum histamine-releasing activity could be useful in CSU patients to differentiate between CSU patients with transient or persistent increase in serum histamine-releasing activity. The biological significance and the underlying causes for the transient or persistent production of serum histamine-releasing factors in CSU are unknown and may represent an interesting area for future research in CSU.

3.4.3 Flow cytometric analysis of circulating basophils in CSU

In flow cytometric analysis, we tested three previously published gating strategies for the identification of peripheral blood basophils in CSU patients (Ebo et al, 2012; Eberlein et al, 2009; Amundsen et al, 2012). Flow cytometric enumeration of peripheral blood basophils yielded significantly different absolute counts of basophil subpopulations depending on the gating strategy. Thus, our study revealed significantly higher absolute counts of CCR3+CD63+ basophils compared to other basophil subpopulations defined by dual expression of CCR3 and CD123 or CD63 and CD203c. There may be biological reasons for the observed variation in basophil phenotypes in CSU. The *in vivo* basophil priming was suggested by the increased expression of basophil activation markers in CSU patients as determined by flow cytometry (Vasagar et al, 2006). In the study by Vasagar et al (2006), peripheral blood basophils demonstrated up-regulation of CD63 and CD69 but not CD203c. In our study, CCR3+CD63+ basophil subpopulation appeared to be a predominant basophil phenotype in CSU patients. The biological relevance of this subpopulation is unknown although CCR3+CD63+ subpopulation may reflect basophil responses in the context of the inflammation in CSU. The biological rationale for this assumption lies in the fact that expression of CCR3 receptors may be up-regulated in response to eotaxin which is known to be an inducible chemokine in the context of the inflammation (Iikura et al, 2001). The eotaxin levels were reported to be elevated in CSU patients (Tedeschi et al, 2012) and may represent a putative mechanism for basophil recruitment into the skin. In addition, CD63 up-regulation is noted to be up-regulated in a graded fashion in response to activation stimuli (MacGlashan Jr., 1995). Hence, it is conceivable that CCR3+CD63+ basophil subpopulation may be a primed basophil phenotype undergoing chemotaxis to the inflamed skin in CSU although this assumption needs a rigorous *in vitro* testing using cutting edge chemotaxis assays (Toetsch et al, 2009). In the future, comparative studies into basophil subpopulations in healthy subjects, patients with CSU and other inflammatory conditions may provide a biological interpretation as to whether these basophil subpopulations represent a disease-specific basophil phenotype or a non-specific phenomenon in the context of inflammation.

Another important question is whether the observed basophil phenotypes in CSU represent a spectrum of peripheral blood basophils at the different stages of activation or whether they reflect basophil heterogeneity in CSU. Based on he previous work by Vasagar et al (2006), basophil phenotypes in CSU are likely to represent a primed activated state. Further studies may shed some light on molecular, functional and morphological characteristics of this subpopulation in comparison to different activation states of peripheral blood basophils. However, it is important to consider the differences between *in vivo* and *in vitro* basophil activation as was demonstrated in sting challenge in venom-allergic patients by Gober et al (2007). On the other hand, we cannot completely rule out the possibility of CCR3+CD63+ basophil subpopulation being phenotypically distinct from the remainder of the peripheral blood basophils. Functional basophil heterogeneity has been reported (Siracusa et al, 2012) but whether or not this is relevant to CSU is unknown. Classically, cellular heterogeneity relies on the distinct phenotypic signature and cellular behavior of a given cell subpopulation with a particular biological function and, possibly, clinical contribution (Altschuler & Wu, 2010; Prussin et al, 2010). In our study, we explored the association of this basophil subpopulation with disease severity or persistence but no differences were detected. Thus, we could not discern the clinically relevant contribution of the observed basophil phenotypes in CSU. In the future, this question can be addressed by single cell measurements using cutting edge technologies such as imaging flow cytometry, advanced microscopy and single cell PCR analysis. These multi-dimensional studies would allow more accurate interpretation of the observed variation in basophil phenotypes and may provide some insights into whether these different phenotypes represent the spectrum of the activation states or phenotypically distinct subpopulations.

Alternatively, several methodological reasons may also account for differences in flow cytometric assessments of circulating basophils in the peripheral blood using conventional flow cytometry. Firstly, there is a lack of consensus on flow cytometric definition of peripheral blood basophils with a wide range of surface markers being used for basophil identification. Different basophil markers are characterized by a variation of their surface expression on resting basophils which may affect the performance of these markers for basophil identification. Secondly, there is a considerable variation in the approaches to gating and data analysis. The use of different gating strategies may result in a considerable inter-laboratory variation. The lack of standardization of gating strategies hinders the comparisons between the studies as well as collaborations between the laboratories. Our data demonstrated that the choice of gating strategy affects the basophil immunophenotyping and absolute counts. Furthermore, the FMO gating is widely accepted as a technical approach to distinguish a positive population for a given marker. However, for markers with continuous expression this approach may not result in the accurate capture of biologically relevant populations. Search for basophil surface markers with discrete expression may enhance basophil identification in flow cytometry studies. Then, automated gating approaches based on the cluster recognition of cellular populations with continuous marker expression may be a more optimal approach to basophil flow cytometry research in the future. Even though flow cytometric cell enumeration by a single platform methodology is characterized by a statistical superiority compared to cell counting in microscopic slides or counting chambers, the limitations of flow cytometric absolute counts for low-frequency cell populations in whole blood are well recognized. For example, studies into flow cytometric WBC differential demonstrated insufficient accuracy of basophil detection in healthy subjects (Roussel et al, 2010). In the future, extended flow cytometric panels and standardized gating strategies may enhance basophil identification in healthy subjects and patients with various allergic or inflammatory conditions in which basophil phenotype or absolute counts can be affected.

3.4.4 The strengths and limitations of the study

This study was designed to address the specific question as to whether serum histamine-releasing activity, anti-IgE-induced basophil releasability and basophil phenotypes are related to CSU severity or persistence. The study design of prospective longitudinal assessments was the strength of the study which allowed a more accurate disease profiling at three time points over the period of observation. Novel insights were gained by the use of a combination of two classification systems based on serum histamine-releasing activity and anti-IgE-induced BHR and their relationship to disease severity and persistence although we recognize the methodological limitations of both classification approaches.

Several limitations of this study need to be reported. Firstly, as with all observational studies into the natural history of disease, it was a nonrandomized and uncontrolled study. We employed certain techniques such as extended inclusion/exclusion criteria to identify a well-defined subgroup of CSU patients in the observational study. Nevertheless, our CSU patient population may not be fully representative of a general patient population as we cannot rule out a referral bias to the secondary care settings and a selection bias inherent to the observational nonrandomized studies. In the future, the observed associations need to be confirmed in randomized controlled studies to provide a greater degree of certainty. Secondly, the rate of persistent CSU in a general patient population may differ from that in our study due to the exclusion of CSU patients on immunomodulatory or biological treatments. Another limitation arises from a small sample size of the study. Although we could detect statistically significant associations in this study, the results need to be validated in larger clinical studies. Finally, although the biological variability of serum histamine-releasing activity and anti-IgE-induced BHR in health and disease was beyond the scope of this study into a natural history of CSU, parallel assessments of the pathophysiological variables (serum histamine-releasing activity and anti-IgE-induced BHR) in healthy subjects would have strengthened a biological interpretation of the data on pathophysiological phenotyping in CSU. Overall, we believe that this study provided some insights into the natural history of disease, the markers for disease severity and the predictors of disease persistence. The observed clinical patterns and pathophysiological associations provided preliminary data for randomized controlled studies in CSU patients with a rational approach to disease phenotyping, monitoring of inflammation and, perhaps, an early therapeutic intervention in patients with a persistent disease.

3.4.5 Clinical implications of the study

The importance of pathophysiological heterogeneity in CSU is underscored by the clinical need for discerning the main pathophysiological pathways in CSU which can be targeted therapeutically. Furthermore, the identification of CSU patient subpopulations that may be responsive to different treatments underlines the importance of the pathophysiological phenotyping of the disease. Our study confirms the argument by Eckman et al (2007) that serum histamine-releasing activity and anti-IgE-induced BHR are unrelated and seem to operate in different patient subgroups. Whether this translates into differential treatment efficacy in these patient subgroups needs to be established in clinical trials.

Our data may provide the groundwork for further optimization of the diagnostic work-up in CSU patients. Testing for serum histamine-releasing activity in CSU is used in clinical practice and recommended by EAACI Task Force on autoimmune CSU (Konstantinou et al, 2013) although the clinical meaning of the test results needs to be further elucidated. Our longitudinal study, for the first time, described the transient and persistent increase in serum histamine-releasing activity in CSU patients over the period of observation. Different longitudinal patterns of serum histamine-releasing activity in CSU patients may suggest the need for serial testing for serum histamine-releasing activity in CSU patients. The results of the ongoing multicenter PURIST study into the features of CSU with serum histamine-releasing activity may provide further insights into an optimal combination of diagnostic approaches to this condition.

Our data emphasize that disease severity at presentation may predict the persistent clinical course in CSU patients. If this data are validated in larger patient populations from different clinical settings, the prediction of the persistent CSU based on UAS7 at presentation could guide a clinical decision-making for an early start of immunomodulatory or biological treatments in CSU patients with severe disease. Prognostic criteria for severe and persistent CSU will need to be explored in clinical interventional studies while the definitions for a persistent disease and a clinical remission in CSU need to be developed in clinical guidelines. From a healthcare perspective, early treatment and prevention of severe and persistent CSU in the future may reduce healthcare visits and costs that were previously estimated for the management of CSU patients (Weller et al, 2012; Delong et al, 2008, Zazzali et al, 2012).

Our flow cytometry data may provide valuable information for ongoing research into WBC differential counting by flow cytometry. Multi-parameter flow cytometry has been applied for WBC count and differential (Cherian et al, 2010; Roussel et al, 2010; van de Geijn et al, 2011) in an attempt to supersede the current reference method of manual microscopy. However, the accuracy of basophil enumeration was insufficient in WBC differential by flow cytometry (Roussel et al, 2010). For the first time, we demonstrated that absolute basophil counts in CSU varied depending on the choice of the gating strategy. These data need to be validated in healthy subjects and in patients with various allergic or inflammatory diseases before an introduction of extended flow cytometric panels for basophil identification can be recommended for flow cytometric WBC differential.

3.4.6 Unanswered questions and future studies

Although our knowledge about the natural history and the pathophysiology of CSU advances, several unanswered questions remain.

Firstly, the interrelationship and the relative contribution of serum histamine-releasing activity and anti-IgE induced basophil releasability in CSU patients are of great theoretical and clinical interest. To further understand how these factors account for the variance in disease severity in CSU, we need to design an intermediate study with the recruitment of at least 20 CSU patients per predictor in the regression equation. Furthermore, a comparative study into the variability of these two parameters in CSU patients and healthy subjects would enhance a biological interpretation of pathophysiological phenotyping in CSU patients. Larger longitudinal studies are needed to better characterize the subset of CSU patients with persistent disease and to validate the baseline predictors for disease persistence.

The causes of aberrant basophil responsiveness to anti-IgE stimulation are yet to be fully understood. For basophil responders to anti-IgE stimulation, the priming and degranulating factors in the circulation need to be further explored. The biological significance of decreased responsiveness of basophils to anti-IgE stimulation in some CSU patients is yet to be elucidated. Signaling via different activation and inhibitory receptors in peripheral blood basophils in CSU needs to be better characterized. Such integral functional characterization of peripheral blood basophils in CSU may help uncover yet unidentified signaling defects in CSU in addition to those reported by Vonakis et al (2007). What is the biological significance of different patterns of basophil releasability to anti-IgE stimulation in CSU? Do they represent the stages of the disease? Do basophils re-circulate after their migration into the inflamed skin in CSU? If they do, what are their phenotypic features and functional characteristics on re-circulation? These questions remain unanswered at present but draw an attention of scientists to these novel dimensions of inflammation in CSU.

What are the underlying mechanisms responsible for the variability of the clinical course in CSU? Although there is currently no evidence for pathophysiological determinants for persistent CSU, research into genetic predisposition, epigenetic mechanisms, skin remodelling, abnormal mast cell releasability in the context of pro-inflammatory skin microenvironment in the dermis of CSU patients with a persistent disease would be of great interest. Clinico-histopathological correlations in persistent CSU may also shed some light on the relevance of skin remodeling and the pattern of inflammatory infiltration in the dermis to the persistent disease. Research into immunomodulatory and biological treatments in CSU may help answer the question as to whether the persistent course of CSU can be therapeutically altered or prevented.

In clinical practice, absolute basophil counts in CSU patients will need to take into account basophil subpopulations as demonstrated by flow cytometric immunophenotyping in our study. Morphological and functional characteristics of basophil subpopulations using advanced microscopy, imaging flow cytometry and single-cell PCR would enhance our understanding of basophil subpopulations in health and CSU. Flow cytometric assessments of absolute basophil counts using a volumetric approach may result in higher precision of enumeration of basophil subpopulations in future studies. Monitoring changes in basophil subpopulations in CSU over the course of disease may yield novel insights into basophil biology in CSU. If basophil subpopulations can be novel therapeutic targets in CSU needs to be explored in future studies. Some lessons about basophil biology can be learned from the effects of biological agents such as omalizumab or Syk inhibitors on basophil phenotype and functional characteristics.

The mechanisms and causes for *in vivo* basophil priming are an interesting area of future research. For example, the effects of eotaxin on peripheral blood basophils in the context of CSU may represent an important line of future research. An interaction of β-chemokines with peripheral blood basophils from CSU patients and healthy subjects needs to be examined in *in vitro* using basophil activation tests and chemotaxis assays. The use of extended immunophenotyping panels and quantitative assessments of basophil surface receptors would allow better understanding of *in vivo* priming of circulating basophils in CSU. In particular, the *in vivo* expression profile of chemokine receptors in circulating basophils in CSU would also be of interest. Also, the use of novel basophil markers such as CD164 may enhance basophil identification in CSU. Furthermore, comparative studies would allow better assessments of the variability of marker expression such as HLA-DR on basophils in CSU patients and healthy subjects to understand if HLA-DR expression is affected in CSU by analogy with SLE (Charles, 2010).

Overall, translating this pathophysiological insights into clinically relevant information as markers for disease severity, predictors for a persistent course and treatment targets in CSU would certainly enhance the management of CSU patients and may allow a more targeted approach to treatment in CSU patient subgroups.

CHAPTER 4

Imaging Flow Cytometry Studies in Peripheral Blood Basophils in Healthy Subjects

"Curiosity is the ambition to go beyond."

—BARBARA M. BENEDICT

4.1 Abstract

Background: Human basophil heterogeneity has been suggested in terms of cellular density, ultrastructural morphology, immunophenotype, functional responses to anti-IgE stimulation and chemotactic stimulation with C5a. We hypothesised that the variation in basophil phenotypes in our flow cytometric studies in CSU patients may result from phenotypically and, possibly, morphologically different basophil subpopulations in the peripheral blood. In this study, we used innovative ImageStream® technology to assess basophil variation in healthy subjects by a combination of immunophenotyping and morphometric analysis at a single cell level. This study was undertaken to examine the differences in peripheral blood basophil phenotypes in healthy subjects that may explain basophil phenotypic variation in our prospective observational study in CSU using conventional flow cytometry.

The **aim** of the study was to develop methodology to characterise the immunophenotypic and morphological variation in peripheral blood basophils and differences in the basophil

yield based on different gating strategies in healthy subjects using ImageStream® technology.

Methods: For imaging flow cytometry studies, basophils were enriched by Ficoll-Paque density centrifugation (1.084 g/ml). After surface staining with a four-colour panel, samples were fixed using 0.025% glutaraldehyde solution. Data were acquired using ImageStream^{X} imaging flow cytometer (Amnis Corporation, USA). Four fluorescence images, the brightfield and the darkfield images were acquired at 40× magnification. Data analysis was carried out using IDEAS software 4.0 (Amnis Corporation, USA).

Results: The use of CD63+CD203c+ gating strategy allowed the detection of 0.02% of basophils whereas CCR3+CD63+ gating strategy identified 0.4% of basophils in the same peripheral blood sample from a healthy donor following Ficoll-Paque density gradient centrifugation. Visual inspection of cells within a Boolean gate constructed using Boolean logic CD203c+ OR CD63+ resulted in the identification of a basophil subpopulation with surface alterations that comprised 17.7% cells in this gate. In this healthy subject, single marker-based gating resulted in 0.1% of CD203c-positive cells, 1.42% CD63-positive cells and 0.89% CCR3-positive cells in the same sample. When single marker-based gates were tested for the percentages of basophil subpopulation with surface alterations in the same sample, all basophils with surface alterations were CD63-positive, 93.75% of which were CCR3-positive and 0.78% of which were CD203c-positive. We demonstrated that pre-analytical sample handling at 4°C resulted in 0.03% of basophils with surface alterations in the sample whereas sample handling at 37°C resulted in 0.16% basophils with surface alterations in the same sample from a different healthy donor. In this healthy donor, 8.41% of CD203c-positive basophils demonstrated characteristic staining with PAC-1, suggestive of platelet-basophil adhesion.

Conclusions: ImageStream® imaging flow cytometry is a useful research tool to study basophil phenotypic and morphological variation. The results of our ImageStream® basophil studies in healthy subjects suggest that the differences in the basophil yield between different gating strategies (CD63+CD203c+ versus CCR3+CD63+) may arise from biological (basophil phenotypic variation, a relative contribution of basophil subpopulation with surface alterations), technical (pre-analytical handling at different temperatures) reasons as well as effect of confounding factors (platelet-basophil adhesion). These results may indicate the putative factors contributing to basophil phenotypic variation observed in our prospective observational study in CSU using flow cytometry.

4.2 Introduction

Progress in understanding of basophil immunobiology has historically been defined by advances in research methodology. Since the discovery of basophils by Ehrlich (1879), metachromatic staining with basic dyes (toluidine blue, methylene blue, methylene violet, brilliant cresyl blue, neutral red, safranin and azure) has been the main method used for basophil identification. Further research into differential staining of human basophils with basic dyes and basophil biochemistry was accelerated in the 1950s. The direct chamber count of basophils was introduced into basophil research by Moore and James (1953) and has been widely used for studies examining variation in basophil counts in allergic and inflammatory conditions. Basophil functional studies using metachromatic staining were developed by Shelley and Juhlin (1962) and their studies resulted in a descriptive atlas of various stages of basophil granulation and degranulation. An introduction of basophil mediator release assays was an important milestone in the 1970s, and these tests remain benchmark assays for basophil functional studies to date (de Weck et al, 2008; Ebo et al, 2008). Serum-induced basophil histamine release assay was introduced for the detection of serum histamine-releasing activity in CSU in the 1990s and is used currently (Platzer et al, 2005).

Ultrastructural analysis of basophils was introduced to the field in the late 1960s and then extensively developed in 1970s-1990s (Dvorak & Ishizaka, 1995). The development of antibodies to several basophil surface markers (2D7, CD63, CD203c) as well as basophil intracellular marker BB1 in the 1990s allowed immunohistochemistry and flow cytometric studies in basophil research (Buckley et al, 2002). The advances in basophil purification in 1990s-2000s were marked by the wide use of density gradient media for basophil enrichment, basophil cell sorting and commercial kits for negative immunomagnetic basophil purification (Gibbs & Ennis, 2001). Currently, innovative technologies in the field of basophil research include live basophil allergen arrays developed as a bioassay based on the combination of protein arrays with live human basophils (Falcone et al, 2009). Future prospects in basophil research include innovative technologies (imaging flow cytometry) (Zuba-Surma et al, 2007) and single cell gene expression profiling (Livak et al, 2013) which offer novel possibilities of immunophenotyping, functional and morphometric analysis of human basophils at a single-cell level.

Basophil variations in phenotype in healthy subjects were described in terms of density, response to anti-IgE stimulation and chemotactic stimulation with C5a. Different patterns of granulation in mature and immature basophils were noted in the 1960s (Thornnard-Neumann, 1963). Comparative studies of basophil precursors and mature basophils were described in the 1970s (Parwaresch & Lennert, 1979) and then extended in 1970-1990s (Dvorak & Ishizaka, 1995). For example, basophil precursors tend to be larger in size, sensitive to metachromatic staining with basic dyes at lower pH and have less granulation compared to their mature counterparts (Parwaresch & Lennert, 1979). During activation, basophil phenotypes included fully granulated, intermediate and degranulated basophils (Dvorak, 1991). Density gradient studies revealed hypodense and hyperdense basophils (Lennart & Skeel, 1985). Additionally, chemotaxis of human basophils to the complement component C5a and other chemotactic agents was first described in two patients with myeloid leukemia and high basophil counts by Kay and Austen (1972) and it was noted in the later studies that only approximately 10% of basophils in peripheral blood respond to a chemotactic stimulation with C5a or lymphocyte-derived chemotactic factors (Lett-Brown et al, 1976; Lett-Brown & Leonard, 1977). Basophil functional heterogeneity in response to anti-IgE stimulation was described in CSU (Vonakis et al, 2007) and asthma (Youssef et al, 2007). Together, our observations and work by other researchers suggest re-visiting basophil phenotypic variation in health and disease and a re-assessment of its biological significance and, possibly, clinical contributions in allergic and inflammatory diseases including CSU.

In the context of inflammation, basophil phenotype is characterised by up-regulation of surface and intracellular markers (Bochner, 2000). For phenotypic analysis, surface activation markers include CD63, CD203c, CD107a/D107b, CD13 and CD69 (Hennersdorf et al, 2005). CD63, a member of the tetraspan family, is a highly glycosylated lysosomal-associated membrane protein (LAMP-3) which resides in cytoplasmic granules in basophils (Valent, 2010). Its up-regulation is linked, but not identical, to histamine release (MacGlashan Jr., 1995). CD63 and CD69, but not CD203c, were reported to be up-regulated on circulating basophils from CSU patients (Vasagar et al, 2006). CD69 was also up-regulated in asthma (Yoshimura et al, 2002) and venom allergy (Gober et al, 2007), however, the biological function of this activation marker is currently unknown. CD203c is an ectoenzyme which is expressed in peripheral blood basophils although the basal level on the resting basophils is low. CD203c expression increases in asthma exacerbation (Ono et al, 2010), but is unaffected in CSU patients (Vasagar et al, 2006). Basophil surface markers CD107a (also known as LAMP-1) and CD107b (also known as LAMP-2) belong to the family of lysosomal membrane proteins (Hennersdorf et al, 2005) and their up-regulation occurs via the same pathway as that for CD63 (Hennersdorf et al, 2005). The novel basophil markers CD107a/CD107b, CD13 and CD164 have not been studied in the context of CSU. The basophil intracellular granule-specific marker BB1 is the highly basic protein basogranulin (McEuen et al, 2001), however, the intracellular expression of BB1 antigen and its release has not been studied in CSU. This highlights the need for a better characterisation of basophil phenotypic variation in CSU.

Why are basophils important in CSU? Basophils have been implicated in the pathophysiology of CSU since the 1960s (Robinson & Pennington, 1966). There are several lines of evidence suggesting that basophil numbers and function are affected in CSU. The numbers of circulating basophils appear to be inversely related to the severity of CSU, and basopenia, defined by metachromatic staining, was noted to be a feature of severe disease (Grattan et al, 2003). Peripheral blood basophils in CSU patients are characterised by an abnormal sensitivity to serum from CSU patients and healthy donors in *in vitro* studies (Liquin et al, 2005). Interestingly, basopenia detected by metachromatic staining appears to be related to serum histamine-releasing activity in CSU (Grattan, 1997) while *in vivo* basophil priming and an aberrant basophil response to anti-IgE stimulation are unrelated to the presence or absence of serum histamine-releasing activity (Vasagar et al, 2006; Eckman et al, 2008). Distinct basophil functional subsets based on their histamine release to anti-IgE stimulation have been described in CSU. Basophil functional subsets were shown to be a stable feature in CSU patients but their clinical significance is poorly understood (Eckman et al, 2008; Baker et al, 2008). In longitudinal observations, basophil histamine release to anti-IgE stimulation improves towards the remission of the disease (Eckman et al, 2008). These findings suggest that basophils participate in the CSU disease process via yet unknown mechanisms. Taken together, these data led to the hypothesis that basophils, together with mast cells, are the effector cells of the inflammation in CSU, although direct proof of their involvement in weal formation in CSU is still lacking. Overall, basophils appear to be important in the pathophysiology of CSU, however, their precise contribution to weal formation, chronic skin inflammation and disease persistence remains unclear. Basophils in CSU may be targeted therapeutically (Marsland et al, 2005), particularly with advent of the biological treatments (MacGlashan Jr. et al, 2011; MacGlashan Jr. & Saini, 2013). Therefore, understanding basophil biology and function in CSU may lead to advances in their development as a biomarker or a therapeutic target.

Our prospective study also demonstrated phenotypic basophil variation in CSU patients (Chapter 3). In our study, variation in basophil phenotype depended on the choice of gating strategy used for basophil identification. We hypothesised that basophil phenotypic and, possibly, morphological variation at a single cell level may contribute to the differences in basophil phenotypes observed in our flow cytometric studies in CSU depending on the chosen gating strategy. To test this hypothesis, we used the innovative ImageStream® imaging flow cytometry to study basophil phenotypic and morphological variation in healthy subjects using the same gating strategies as in our flow cytometric study in CSU patients.

ImageStream® imaging flow cytometry (developed by Amnis Corporation and then by EMD-Millipore) is an innovative research technology (McGrath et al, 2008) that allows multiparameter immunophenotyping to be combined with morphometric analysis of basophils in the same sample at the single cell level. Analysis of cells in the suspension by imaging flow cytometry offers an advantage for CSU research because peripheral blood basophils in CSU are primed and their threshold for activation during cell sorting may be lowered. In imaging flow cytometry, analysis of cells in suspension is achieved by using a charge coupled device (CCD) camera that works in Time Delay integration mode to ensure optimal imaging of cells in flow. Furthermore, basophil research in CSU is hindered by low basophil counts in some patients. The ImageStream^X imaging flow cytometer is characterised by a maximum acquisition rate of 1,000 cells/min that makes it a high-throughput technology suitable for rare cell analysis including basophil research in CSU. Furthermore, ImageStream technology offers cell gating based on both fluorescence and morphological features. The capability of visualizing the morphology of gated cell populations identified by different gating strategies on the dot plot is missing in flow cytometry and can be used for morphometric analysis of basophil subpopulations in CSU.

The limitations of imaging flow cytometry include longer acquisition periods for rare cell research and the requirement for high-speed computers for analysis of large datasets for these experiments. Additionally, the ImageStream® technology does not allow further manipulations with cells while conventional flow cytometry offers the option of cell sorting that can be used in combination with cell culture and other techniques such as single cell PCR profiling. Therefore, the ImageStream® technology appears to be complementary to conventional flow cytometry and fluorescence microscopy.

ImageStream® may address many pressing issues in basophil research using conventional flow cytometry in CSU. The principle innovation of this technology encompasses a new level of informational content of the acquired data (Basiji et al, 2007) such as morphological and immunophenotypical data at a single cell level for cells in suspension which has not been possible before. Several important questions may be answered by the phenotypic and morphometric analysis at a single cell level, such as variability in basophil morphology, immunophenotyping of basophil subpopulations, the effect of pre-analytical sample handling on basophil morphology and immunophenotype, the selection of an appropriate gating strategy and the choice of basophil surface markers for different experimental settings. In basophil research, combined flow cytometric and morphometric studies using human basophils are rare (MacGlashan Jr., 2010b), laborious, cumbersome and require exquisite technical expertise. Low basophil counts in the peripheral blood, high water-solubility of basophil granules, a low threshold for basophil activation and degranulation during pre-analytical sample handling are the key limitations for the manipulations with human basophils using current research techniques. The use of ImageStream® technology may help circumvent these limitations and may make the combination of flow cytometric and morphological studies in human basophils more accessible.

Some limitations of imaging flow cytometry may be highlighted in comparison with conventional flow cytometry. For example, conventional flow cytometry is more widely available, provides rapid analysis and is easy to use for an experienced operator. In contrast, imaging flow cytometry is time-consuming for rare cell analysis, more difficult to operate and needs tedious optimization that requires expert consultancy at the level of highly specialized flow cytometry service. An image resolution similar to a fluorescence microscope with 40-60× lenses may be a limitation of the technique for certain aspects of cellular analysis. Additionally, data analysis for imaging flow cytometry dictates requirements for computer support comparable to that of crystallography. Therefore, this technology resides, at present, in the domain of academic settings of excellence rather than being a widely used research technique.

From clinical perspective, there are several potential applications of ImageStream® technology to basophil enumeration or basophil activation tests in the clinical settings. Firstly, current flow cytometric approaches have limited accuracy for rare cell counting (Cherian et al, 2010; Bjornsson et al, 2008) and the application of ImageStream® technology may improve absolute basophil counting through the morphological verification of gated basophil subsets combined with the use of volumetric methodology and the development of extended multi-parameter panels for basophil identification. Secondly, a better phenotypic and morphological characterisation of an activated basophil phenotype using ImageStream® technology may help the development of basophil subpopulations as biomarkers for monitoring the clinical course of allergic diseases, CSU or haematological malignancies and the effects of various treatments (Ono et al, 2010; Grattan et al, 2003; Wimazal et al, 2010; Saini & MacGlashan Jr., 2012). Thirdly, ImageStream® technology can be used as a complementary tool for standardization of the laboratory protocols for flow cytometry-based assays used for the diagnosis of food and drug allergies and for the detection of serum histamine-releasing activity in CSU (Shreffler, 2006; Hausmann et al, 2009; Platzer et al, 2005). For example, this technology may be used to evaluate the effects of fixative and lysis solutions on basophil morphology and phenotype during sample preparation or may inform and verify the bioinformatics-based approaches to automated gating (Jaye et al, 2012). Future studies may identify further areas for integrating ImageStream® technology in the basophil analysis in clinical samples.

The aims of this study were:

1. to assess and compare gating strategies for peripheral blood basophils based on dual gating for CD203c and CD63 basophil markers and a gating for CCR3 marker.
2. to assess phenotypic and morphological basophil variation in peripheral blood of healthy subjects and to determine its significance for the gating strategies used in our study.

4.3 Methods

4.3.1 Participants

Healthy volunteers were recruited from the members of staff at the Dermatology Department at the Norfolk & Norwich University Hospital and at the Biomedical Research Centre at the University of East Anglia. The study was approved by Norfolk Research Ethics Committee for method development as a part of the project "Pathophysiological subtypes in chronic ordinary urticaria and their biomarkers: a prospective observational study" (Ref. 08/H0310/53). All participants gave written informed consent before taking part in the study. In this optimisation study, healthy subjects were not tested for atopy.

4.3.2 Sample Preparation

For imaging flow cytometry studies, 35 ml of venous blood was collected from a healthy donor into four 9-ml BD Vacutainer tubes with sodium citrate. Basophils were enriched by Ficoll density centrifugation using the protocol of Valent and associates (1990) modified for our imaging flow cytometry studies. Citrated whole blood was layered over Ficoll-Paque PLUS solution (GE Healthcare, UK) with a density of 1.084 g/ml at the ratio 1:1 v/v and centrifuged at 1,800 rpm/min without brakes for 30 min at room temperature. The whole Ficoll layer was harvested for basophil studies. Samples were washed at 1,920 rpm/min without brakes for 10 min in RPMI 1640 (Invitrogen, no Phenol Red) with 3% BSA (Fraction V, Fisher Scientific, UK) at 4°C. The second wash was carried out at 980 rpm/min, no brakes, for 10 min in RPMI 1640 containing 3% BSA at 4°C.

After washing, cells were stained and counted according to the method of Kimura et al (1973). Then, the volume of cell suspension was adjusted by adding RPMI 1640 containing 3% BSA to achieve a cell density of 3-4x10^6 cells/ml per tube. Fc receptor blocking reagent for human samples (Miltenyi Biotec, UK) was added 10 µl/tube to each sample. Surface staining was performed with a 6- colour staining panel for 30 min at 4°C in the dark. Antibodies conjugated with fluorochromes used for basophil studies are presented in the Table 1 (Figure 2, Appendix 5). After staining, the cells were washed at 1450 rpm/min, no brakes, for 5 min in RPMI 1640 containing 3% BSA at 4°C. Then, the cells were fixed with 0.025% glutaraldehyde solution in PBS by adding 40µl of 0.025% glutaraldehyde to 300 µl of cell suspension. Samples were centrifuged at 200g, 4°C for 2 min and supernatant was swiftly removed. Immediately, the samples were washed in 2 ml of RPMI 1640 containing 3% BSA at 200g for 3 min at 4°C and again the supernatant was promptly removed. The second wash was carried out in 1ml of RPMI 1640 with 3% BSA at 200g for 3 min at 4°C, the supernatant was removed straight away. After this, wash was repeated once again in 0.5ml of RPMI 1640 containing 3% BSA at the same settings (200g for 3 min at 4°C). Supernatant was removed and samples left overnight at 4°C.

Next day samples were washed twice in 1 ml of RPMI 1640 containing 3% BSA for 3 min at 4°C. Cells were resuspended in 300 µl of RPMI 1640 with 3% BSA. Then Fc receptor blocking reagent for human samples (Miltenyi Biotec) was added 10 µl per tube. Samples were shipped in temperature-controlled packaging (Cool Logistics, UK) at 4°C (with temperature range between 3-8°C) for imaging flow cytometry studies.

Critical Steps

To avoid glutaraldehyde-induced autofluorescence, critical factors during sample fixation included volumes of the sample and fixative, quick supernatant removal, leaving samples overnight in RPMI 1640 with 3% BSA at 4°C and cell washing twice next day before data acquisition. Imaging flow cytometry studies required high cell density in the sample for optimal rate of data acquisition. To obtain sufficient number of cells from the defined volume of venous blood, certain precautions were undertaken for careful supernatant removal to avoid disturbing the cellular pellet and the protocol was modified to reduce cell losses during washing steps. Furthermore, at high cell density in the sample, basophils tended to be included in cellular clumps. Hence, the 3% BSA was added to RPMI 1640 at all stages of sample preparation and Fc receptor blocking reagent was added at the final steps to reduce cellular adhesion. In our studies, we found important to ship samples at 4°C but not on ice.

4.3.3 ImageStream® Data Acquisition

Imaging flow cytometry studies were carried out using ImageStream^X imaging flow cytometer (Amnis Corporation, USA). About 50,000-200,000 events per sample were acquired. To eliminate debris, only events with the minimal area of 20µm^2 in the brightfield channel were acquired. Cells were excited with a 405 nm laser (125mW), a 488 nm laser (200 mW), 561 nm laser (200 mW), a 658 nm laser (120mW) and a 795 nm laser (1.75mW) (Figure 1, Appendix 5). The brightfield imagery, the darkfield imagery and four fluorescence images per each cells were acquired at 40X magnification. Three multicolour staining panels were used for basophil studies (Figure 2A, Appendix 5). For comparative gating analysis, multicolour staining panel 1 comprised of CellMask™ Deep Red plasma membrane stain (Invitrogen, UK) and antibody conjugates anti-CD203c

Brilliant Violet 421 (Biolegend, UK), anti-CD63 FITC (BD Biosciences, UK) and anti-CCR3-PE (Biolegend) as presented in the Table 1 (Figure 2, Appendix 5). For immunophenotyping studies, Panel 2 and 3 were used as explained in Figure 2 (Appendix 5). The summary of the surface cellular markers is presented in the Table 2 (Figure 2, Appendix 5). Unfixed and fixed unstained samples were used as controls for glutaraldehyde-induced autofluorescence (Figure 4A and B, Appendix 5). Fluorochrome-conjugated antibodies against surface markers highly expressed on human basophils (anti-CD123 Brilliant Violet 421 (Biolegend, UK), anti-CD45 FITC, anti-CCR3 PE) and Cell Mask™ Deep Red plasma membrane stain (Invitrogen, UK) (Table 1, Figure 2, Appendix 5) were used for single stained controls (Figure 4, Appendix 5). Single stained controls were acquired with the brightfield illumination off to generate a compensation matrix (Figure 1B, Appendix 5). Fluorescence minus one (FMO) controls were used to set the gates for cells positive for CD203c (Figure 5A, Appendix 5), CD63 (Figure 5D, Appendix 5) and CCR3 (Figure 5G, Appendix 5) at 99.9% confidence level. Data analysis was carried out using IDEAS software 4.0 (Amnis Corporation) (Figure 3, Appendix 5).

4.4 Results

4.4.1 Comparison of two gating strategies for human basophils on the same Basophil-enriched Ficolled Peripheral Blood Samples

To compare the basophil yield by different gating strategies for human basophils, we applied two gating strategies based on 1) dual expression of CD63+CD203c+ and 2) dual expression of CCR3+CD63+ in the same Ficolled peripheral blood sample from a healthy subject. The initial gating strategy for human basophils in Ficolled sample of the peripheral blood included identification of cells in focus based on Gradient RMS (Figure 38A; for morphometric glossary – see Table 3, Appendix 5) followed by exclusion of cellular aggregates on a bivariate plot of Aspect Ratio versus Area (Figure 38B). After this, FMO-based gates for CD203c, CD63 and CCR3 surface markers were applied on the corresponding bivariate plots (Figures 38C, D, F). Boolean gates were constructed by a combination of CD203c and CD63 gates for dual gating for human basophils based on

CD203c and CD63 expression (Figure 38E) and also by a combination of CCR3 and CD63 gates for dual gating for human basophils based on CCR3 and CD63.

Thus, two gating strategies were applied to the same sample: a dual gating for CD63 and CD203c (Figure 38E) and a dual gating for CCR3 and CD63 (Figure 38G). Basophil yield between these gating strategies was different: 0.02% of basophils based on dual CD203c+CD63+ gating and 0.4% of basophil based on CCR3+CD63+ gating (Figure 38E and G). The representative imagery of basophils identified by these two gating strategies are presented in Figures 38H and 38I.

4.4.2 Identification and Phenotypic Analysis of Basophil Subpopulation with Surface Alterations in Ficolled Peripheral Blood Samples from a healthy donor

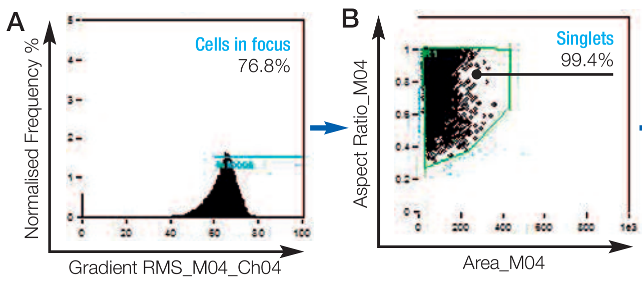
The next step of analysis included visual inspection of cells identified by a dual gating strategy constructed using a Boolean logic CD63+ OR CD203c in order to identify morphologically and phenotypically distinct basophil subpopulations by analogy with flow cytometry gating that displayed basophil variation (Ebo et al, 2012). On visual inspection, we identified a subset of basophils with surface alterations (n=128 cells per sample). In addition, we observed basophil variation in size ranging from large (mean diameter 16.7±1.2 μm) to small (mean diameter 11.4±1.7 μm) basophils. This variation was considered to be due to a normal distribution of cells, therefore, cell variation in size was not considered in further analysis.

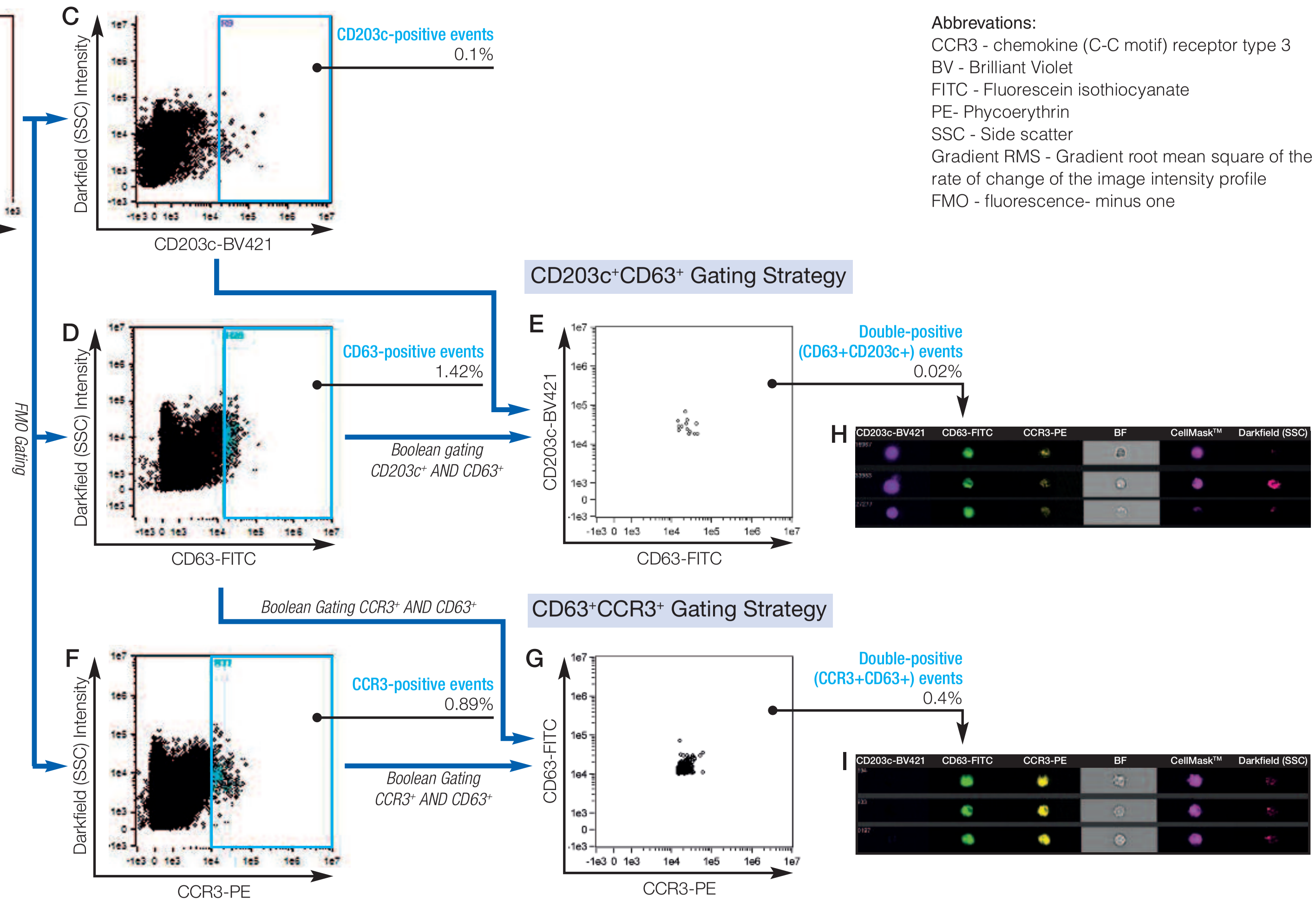
Representative brightfield image for a basophil with surface alterations is presented in Figure 39D. Basophils with surface alterations were manually tagged using IDEAS® software and then were used for comparisons with the remaining single cells in focus in the sample. Immunophenotypic analysis of the basophil subpopulation with surface alterations was performed using three samples from the same healthy donor labelled with three different staining panels (Figures 39A-C; for staining panels – see Figure 2A, Appendix 5). For immunophenotypic analysis, the histograms were plotted for basophil subpopulation with surface alterations and all single cells in the focus for each sample (Figures 39A-C). On the histograms, basophils with surface alterations were displayed as a green cell subpopulation, all single cells in focus in each sample were represented as a black cell subpopulation. The histograms for basophil subpopulation with surface alterations in three samples may suggest a bimodal distribution for the expression of CRTH2 (Figure 39A), CCR3 (Figure 39B) and CD69 (Figure 39C), with higher expression of these markers on basophils with surface alterations compared to all single cells in focus per sample although low cell counts of basophils with surface alterations should be noted.

4.4.3 Distribution of basophil subpopulation with surface alterations in gating strategies for each basophil surface marker CD203c, CD63 and CCR3 in the same Ficoll-processed Peripheral Blood Sample from a healthy donor

As the next step of the analysis, peripheral blood basophils selected by gates for each basophil surface marker CD203c, CD63 and CCR3 were compared for the yield of basophils with surface alterations (Figure 40). For this sample, a total of 128 basophils with surface alterations were identified on visual inspection of cells in the Boolean gate constructed using a Boolean logic CD203c OR CD63. For CD203c$^+$ basophil gating, only one of 128 basophils with surface alterations was identified by CD203c marker. The use of CD63-based gating resulted in all 128 basophils with surface alterations identified as CD63-positive cells. Using CCR3-based gating strategy, CCR3-positive cells comprised a total of 120 of 128 basophils with surface alterations in the sample. Therefore, CD203c-positive cells accounted for 0.78% of basophils with surface alterations (Figure 40B), basophils with surface alterations were all positive for CD63 surface marker (Figure 40D) and also CCR3-positive cells represented 93,75% of basophils with surface alterations in the sample (Figure 40F). Thus, CD63- and CCR3-based strategies in this sample yielded higher percentages of basophils with surface alterations compared to that by CD203c-based strategy. These findings suggest a variation in surface marker expression on basophils with surface alterations, with most cells being positive for CD63 and CCR3 but not for CD203c. Figure 40 demonstrates representative images of basophils with surface alterations yielded by each single basophil surface marker CD203c (Figure 40B), CD63 (Figure 40D) and CCR3 (Figure 40F).

Figure 38. Gating Strategies for Peripheral Blood Basophils in a Healthy Subject using Imaging Flow Cytometry





Abbrevations:
CCR3 - chemokine (C-C motif) receptor type 3
BV - Brilliant Violet
FITC - Fluorescein isothiocyanate
PE- Phycoerythrin
SSC - Side scatter
Gradient RMS - Gradient root mean square of the rate of change of the image intensity profile
FMO - fluorescence- minus one

Figure 38. Gating for Gradient RMS was used to select cells in focus (Figure 38A). Then, cell aggregates were excluded based on Aspect Ratio versus Area gating (Figure 38B). Two gating strategies for double-positive CD63⁺CD203c⁺ and CCR3⁺CD63⁺ basophil subpopulations were applied on the same sample. Using two gating strategies on the same sample from a healthy donor yielded different percentages of basophils (Figures 38E and G). For both gating strategies, representative images for double-positive events demonstrated in Figures 38H-I. Each cell is represented by a row of five images (from left to right): CD203c-BV421 (violet), CD63-FITC (green), CCR3-PE (yellow), BF (grey), CellMask™ Deep Red Plasma Membrane Stai (pink) and Darkfield (SSC) (red).

Basophil-enriched samples were prepared for analysis by density gradient centrifugation using Ficoll-Paque PREMIUM medium with density of 1.084g/ml (GE Healthcare, UK). All samples were acquired on an ImageStream^X imaging cytometer at X40 magnification using INSPIRE software. The gates for each surface marker were set based on FMO gating controls (Figures 38C, D and F). The gates for double-positive events were constructed using Boolean logic to combine existing populations (Figures 38E and G). Data analysis was carried out using IDEAS® software version 4.0 (Amnis Corporation).

Figure 39. Phenotypic Characterisation of Basophil Subpopulation with Surface Alterations in the Peripheral Blood from Healthy Subjects using Imaging Flow Cytometry

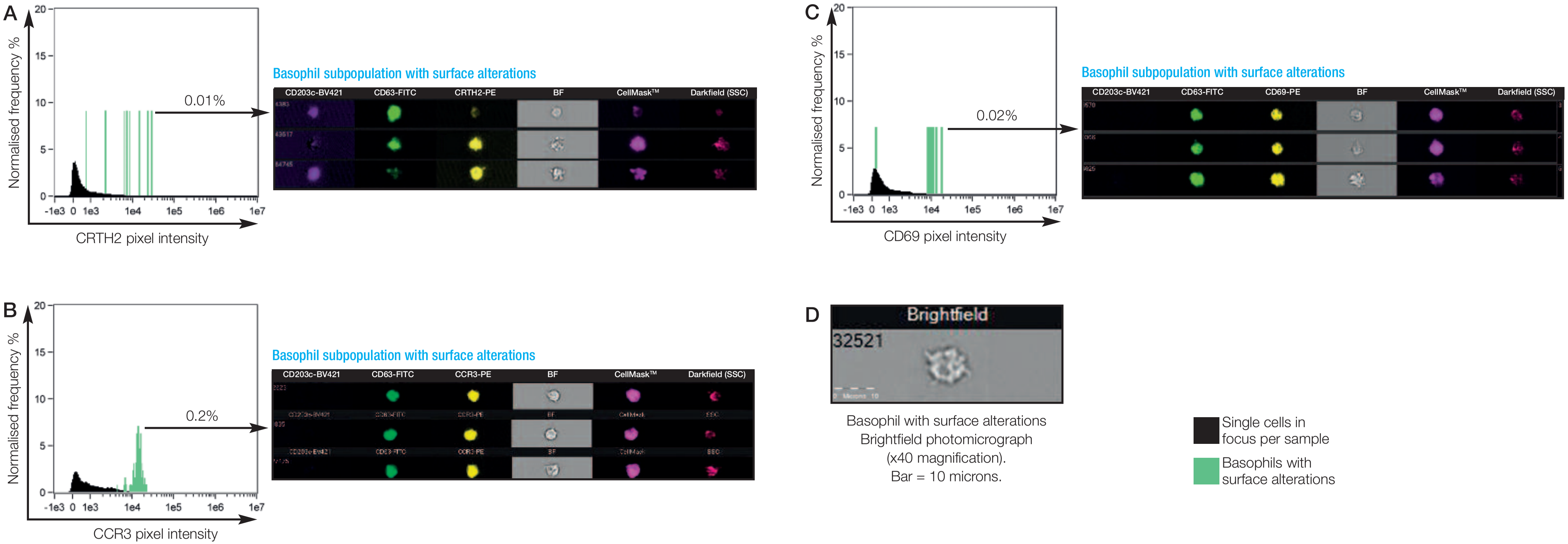


Figure 39. Frequency histograms for pixel intensity of surface markers CRTH2 (Figure 39A), CCR3 (Figure 39B) and CD69 (Figure 39C) compared basophil subpopulation with surface alterations (green histogram) and all single cells in focus (black histogram) per sample. Basophil subpopulation with surface alterations was characterised by differential expression of CRTH2, CCR3 and CD69. Brightfield photomicrograph in Figure 39D presented the morphology of basophil subpopulation with surface alterations. Basophil-enriched samples were prepared for analysis by density gradient centrifugation using Ficoll-Paque PREMIUM medium with density of 1.084g/ml (GE Healthcare, UK). . All samples were acquired on an ImageStream^{X} imaging cytometer at X40 magnification using INSPIRE software. The six-channel images of representative cells from basophil subpopulation with surface alterations are shown next to the corresponding histograms. Data analysis was carried out using IDEAS® software version 4.0 (Amnis Corporation).

Abbrevations:
CCR3 - Chemokine (C-C motif) receptor type 3
CRTH2 - Chemoattractant receptor-homologous molecule expressed on Th2 cells
BV - Brilliant Violet
FITC - Fluorescein isothiocyanate
PE - Phycoerythrin
BF - Brightfield
SSC - Side scatter

Figure 40. Distribution of Basophil Subpopulation with Surface Alterations in Different Gating Strategies on the Same Sample from a Health Donor using Imaging Flow Cytometry

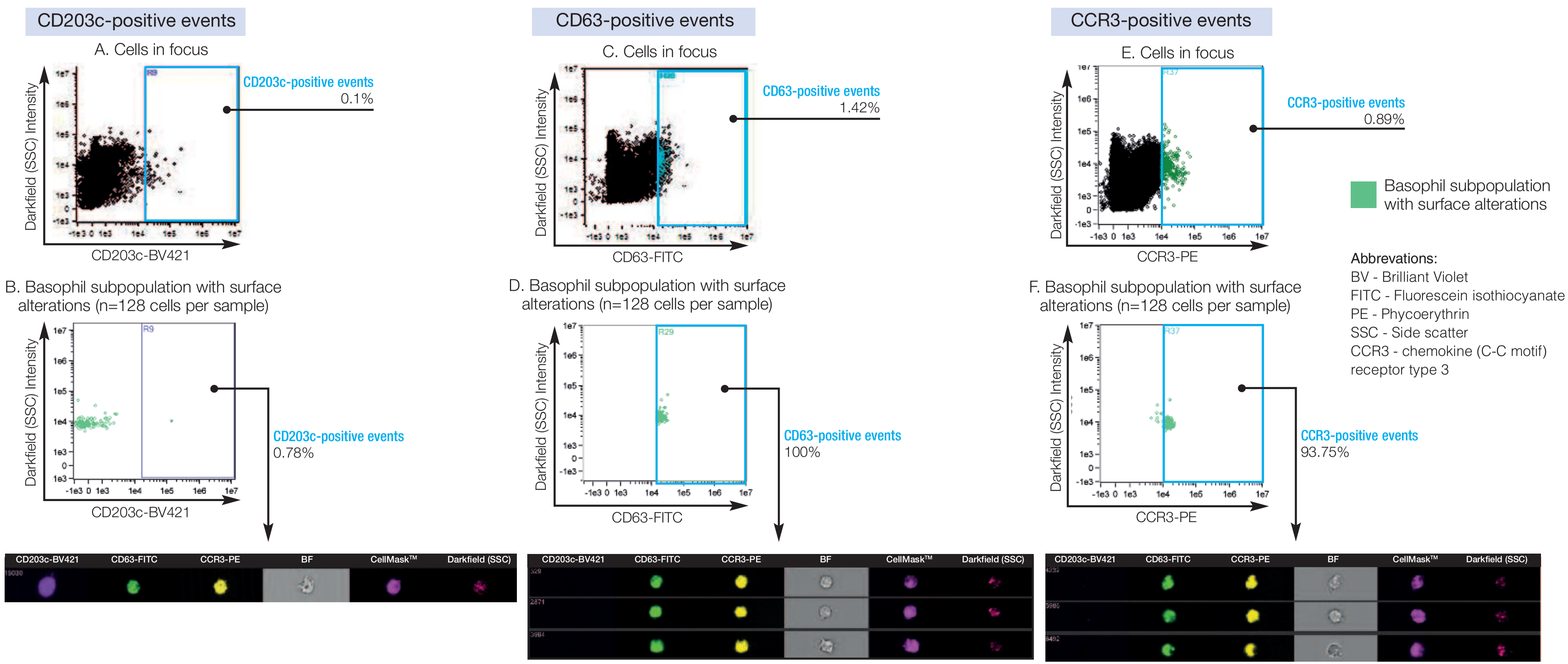


Figure 40. Three gating strategies for surface markers CD203c, CD63 and CCR3 were applied on the same sample from a healthy donor (Figures 40A, C and E). The gates for each surface marker were set based on FMO gating controls. Differential distribution of basophil subpopulation with surface alterations between gating strategies for each surface marker (CD203c, CD63 or CCR3) is displayed in Figures 40B, D and F. Representative image gallery of cells from basophil subpopulation with surface alterations in the indicated gates are shown below the corresponding plots. Each cell is represented by a row of six images (from left to right): CD203c-BV421 (violet), CD63-FITC (green), CCR3-PE (yellow), BF (grey), CellMask^{TM} Deep Red Plasma Membrane Stain (pink) and darkfield (SSC) (red). Basophil-enriched samples were prepared for analysis by density gradient centrifugation using Ficoll-Paque PREMIUM medium with density of 1.084g/ml (GE Healthcare, UK). All samples were acquired on an ImageStream^{X} imaging cytometer at X40 magnification using INSPIRE software. Data analysis was carried out using IDEAS® software version 4.0 (Amnis Corporation).

4.4.4 Platelet-basophil adhesion in basophil studies using Imaging Flow Cytometry

To explore whether CD63 expression can be confounded by platelet-basophil adhesion, we used a flow cytometric panel with platelet-specific markers. Platelet-basophil adhesion was demonstrated in imaging flow cytometry studies (Figure 41A-B) by including antibodies to platelet-specific markers (CD61 and PAC-1) into our multicolour staining panels. Co-localisation of CD63 and PAC-1 expression on platelets adherent to basophils is presented in Figure 41B. These data demonstrated that cellular interactions of platelets with human basophils can be studied using ImageStream® technology and platelet-basophil adhesion may contribute to CD63 expression in basophil studies using imaging flow cytometry.

4.5 Discussion

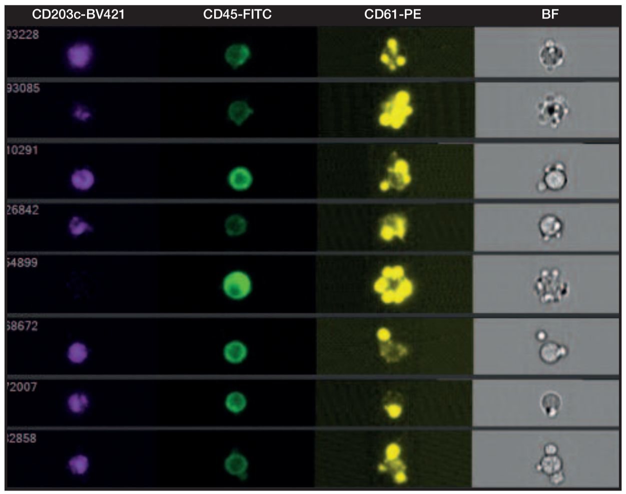
4.5.1 The relevance of ImageStream basophil studies in healthy subjects for the interpretation of flow cytometry studies in CSU patients in this thesis

For this thesis, imaging flow cytometry analysis in peripheral blood basophils was undertaken to assess basophil variation in healthy subjects and to provide a putative biological interpretation for differences in basophil phenotypes observed in flow cytometry studies in CSU patients in Chapter 3 of this thesis.

Firstly, in our ImageStream® studies we confirmed that different gating strategies yield different percentages of peripheral blood basophils, which differ phenotypically on visual inspection at a single cell level. In the ImageStream® study, gating strategy based on dual expression of CCR3 and CD63 yielded 20 times higher percentage of basophils than CD63+CD203c+ gating in the same sample of a healthy donor. These data are consistent with the results of our flow cytometric studies in CSU patients (Chapter 3) which also demonstrated significantly higher absolute counts of CD63+CCR3+ basophil phenotype compared to that of CD63+CD203c+ or CCR3+CD123+ basophils.

Figure 41. Visualisation of Platelet-Basophil Aggregates in Enriched Basophil Preparations from a Healthy Donor by Imaging Flow Cytometry

A



B

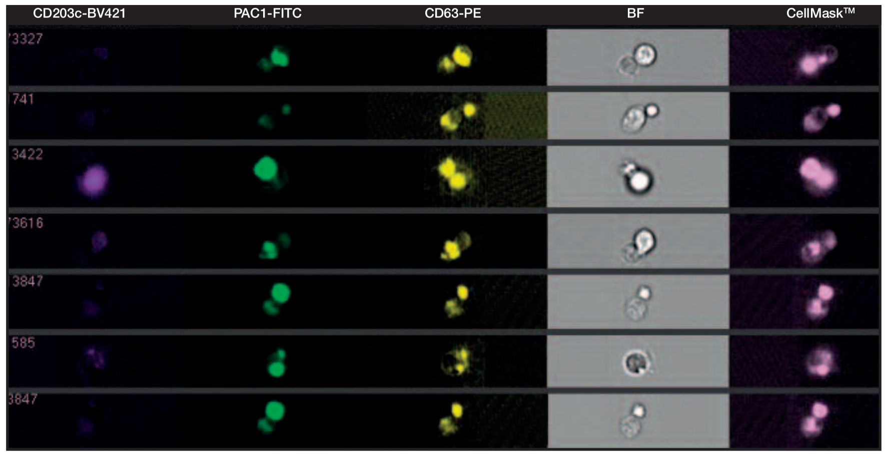


Figure 41. Platelet adhesion to peripheral blood basophils was evaluated using CD61-FITC (Figure 41A) or PAC-1-FITC (Figure 41B). The imagery of platelet-basophil aggregates in enriched basophil preparations from a healthy donor in Figures 41A and B was obtained using imaging flow cytometry. For Figure 41A, four images were generated for each cell: CD203c-BV421 (violet), CD63-FITC (green), CD61-PE (yellow) and BF (Brightfield transmitted light) (grey). For Figure 41B, five images were generated for each cell: CD203c-BV421 (violet), PAC-1-FITC (green), CD63-PE (yellow), BF (Brightfield, transmitted light) (grey) and CellMask™ Deep Red Plasma Membrane Stain (pink). Basophil-enriched samples were prepared for analysis by density gradient centrifugation using Ficoll-Paque PREMIUM medium with density of 1.084g/ml (GE Healthcare, UK). All samples were acquired on an ImageStream^{X} imaging flow cytometer at X40 magnification using INSPIRE software. Data analysis was carried out using IDEAS® software version 4.0 (Amnis Corporation).

Abbrevations:
PAC-1 Platelet Activation Complex -1
BV - Brilliant Violet
FITC- Fluorescein isocyanate
PE - Phycoerythrin
BF- Brightfield

Secondly, our ImageStream® studies offered several putative biological explanations for differences in basophil phenotype and yield depending on the choice of the gating strategies. Our data suggested that the contribution of basophils with surface alterations varies in the gates for each basophil surface marker: CD203c, CD63 or CCR3. Basophils with surface alterations were noted to be included in single marker gating for CD63 or CCR3 but not CD203c. Whether or not accumulation of basophils with surface alterations is increased in CSU and contributes to higher absolute counts of CCR3+CD63+ basophils observed in our prospective study of CSU patients needs to be explored in future studies.

Thirdly, platelet-basophil adhesion is a well-known phenomenon in conventional flow cytometry (Boumiza et al, 2005). In basophil research, platelet adhesion is believed to be a rare event which does not affect the results of basophil studies using conventional flow cytometry. Nevertheless, we could demonstrate platelet-basophil adhesion in Ficolled peripheral blood samples from healthy donors using previously described protocol for sample preparation (Valent et al, 1990). Of interest, platelet adhesion is thought to be increased in activated basophils (Shreffler et al, 2006). We do not know whether this is relevant in the context of inflammation in CSU. Taken together *in vivo* platelet activation (Palikhe et al, 2004) and basophil priming (Vasagar et al, 2006) in CSU, it is plausible that the formation of platelet-basophil aggregates may occur more frequently in CSU and, therefore, may confound the detection of CCR3+CD63+ basophil phenotype in CSU patients. By analogy, increased platelet-neutrophil aggregation was shown for activated neutrophils in patients with sickle cell disease in ImageStream® studies (Polanowska-Grabowska et al, 2010). The suggestion of increased platelet-basophil aggregation in CSU needs to be confirmed basophil studies using extended flow cytometric panels with platelet-specific markers in healthy subjects and CSU patients.

4.5.2 Novel insights into basophil phenotypic and morphological variation in healthy subjects

In our ImageStream® studies, we identified a subset of basophils with surface alterations in basophil-enriched Ficolled peripheral blood samples from healthy subjects. Could it be an activated basophil phenotype? We did not test for this in our *in vitro* studies. However, there is limited evidence that the expression of CCR3, CRTH2 and CD69 is increased in basophils with surface alterations compared with all other cells in focus in the sample. In the literature, basophils with increased CD69 expression were described in the bronchial lavage fluid obtained from patients with asthma (Yoshimura et al, 2002) and also in the peripheral blood of CSU patients (Vasagar et al, 2006). This suggests the possibility that observed basophil phenotype with surface alterations may represent activated basophils.

The formation of surface alterations by peripheral blood basophils may have biological significance. In the literature, alterations in surface morphology in leukocytes was previously reported using transmission and scanning electron microscopy. For example, neutrophils, eosinophils and monocytes demonstrated well-developed cytoplasmic projections by transmission electron microscopy and ridge-like ruffles by scanning electron microscopy (Adachi et al, 2009). *In vitro* studies demonstrated that leukocytes showed a round morphology under static conditions and respond with pseudopodia projections and cell spreading to fluid shear stress (Coughlin & Schmid-Schönbein, 2004). Previous scanning electron microscopy studies also suggested that surface morphology of circulating leukocytes was that of roughened spheres whereas leukocytes were shown to increase in diameter and to develop cytoplasmic projections after surface contact (Michaelis et al, 1971). In our studies we observed the participation of basophils with surface alterations in cellular aggregates. Whether the observed basophil subpopulation with surface alterations occurs in the context of basophil chemotaxis, adhesion and motility is unknown and worth exploring in future studies.

Imaging flow cytometry studies were performed in leukocytes and lymphocytes before, but the shape changes observed in our studies were not described. Using ImageStream® technology, neutrophils were described to increase in size with the uptake of bacteria during phagocytosis (Ploppa et al, 2011). Also, HIV-1 infected lymphocytes displayed the formation of thin filopodium-like protrusions but had the inhibition of ruffle formation (Nobile et al, 2010). Future research into basophils with surface alterations using an ImageStream® flow cytometer with the capability of higher image resolution would enhance our understanding of basophil surface morphology in health and CSU.

Certain stimuli such as anti-IgE and fMLP can induce a graded basophil activation: an induction of chemotaxis at low-grade stimulation and a degranulation at a high-grade stimulation (Suzukawa et al, 2007). Basophil activation is known to be a graded process (MacGlashan Jr., 1995) and it is conceivable that basophil chemotaxis and degranulation may be distinct steps of a graded process of basophil activation. Also, the fact that a low-grade anti-IgE stimulation may induce basophil responsiveness to a chemotactic stimulation was discussed in the literature (Yamaguchi et al, 2009).

At present, it is unclear whether basophils with surface alterations may be an activated phenotype due to chemotactic stimulation. Basophil chemotaxis occurs within multiple chemokine gradients including monocyte chemoattractant protein MCP-1, MCP-2, MCP-3, MCP-4, eotaxin, eotaxin 2, eotaxin 3, macrophage-inflammatory protein-1α, RANTES and IL-8 (Heinemann, 2000). In addition to basophil recruitment in the tissues, chemokines can cause basophil activation (Yamaguchi et al, 2009). In addition to CCR3, basophils express several chemokine receptors including CCR1, CCR2, CCR5, CXCR1 and CXCR2 and CXCR4 (Iikura et al, 2001). Among chemokines, MCP-1/CCL2 is considered as the most potent stimulus for basophil activatoin (Kuna et al, 1992; Bischoff et al, 1992) while eotaxin is the most potent signal for basophil migration (Yamada et al, 1997). Which chemotactic signals may be relevant in CSU is yet to be established. Given that the migration of basophils towards eotaxin is increased by weak FcεRI-crosslinking stimulation (Suzukawa et al, 2005), it is worth exploring whether there is a synergistic relation between eotaxin and serum histamine releasing activity in CSU in their effect on peripheral blood basophils. Extended panels with several chemotactic receptors and adhesion molecules may better characterize this basophil subpopulation in the future.

The important question remains as to whether observed basophil phenotypic variation in ImageStream studies represents a distinct subpopulation or a physiological variation according to Gaussian distribution. Statistical frequency distribution of fluorescence intensity in differential histograms is important in answering this question. It is not possible to infer from two replicates whether these data represent heterogeneity rather than temporal or population noise. However, two separate peaks in fluorescence intensity on differential histograms as opposed to continuous distribution would suggest a separate cellular subpopulation rather than a fraction of overall cell population. Therefore, basophils with surface alterations are likely to be a subpopulation while variation in cell sizes would represent population fractions according to Gaussian distribution. This suggestion is also supported by the fact that two log-decade difference in fluorescence intensity between basophils with surface alterations and the remaining cells in the sample is greater than the measurement error in flow cytometry studies. The fact that basophils with surface alterations demonstrate a separate peak of fluorescence intensity in some surface markers would suggest a link between basophil surface morphology and immunophenotype. Future studies in generalizability in healthy subjects, various diseases, by using different research techniques as well as the demonstration of biological function *in vivo* and *in vitro* would answer the question as to whether basophils with surface alterations represent a separate subpopulation (Prussin et al, 2010).

At present, the biological significance of basophils with surface alterations is unknown. They may represent a dynamic reversible state in response to microenvironmental factors as opposed to stable basophil subsets with regards to anti-IgE stimulation as described by Vonakis et al (2007). The factors inducing this cellular phenotype may include cytokines, epigenetic modifications, circulating pro-inflammatory factors and may affect precursors or terminally differentiated cells. This is likely to be a general phenomenon in the inflammation for myeloid cells or lymphocytes (Galli et al, 2012).

It is also unknown whether large basophils in the sample suggest a physiological variation during developmental changes. It is plausible in view of previous descriptions of immature basophils of larger size (Parwaresch, 1976) and our imagery of KU812 cell line with larger leukemic basophil precursors (Figure 7, Appendix 5). However, we detected only 20 large basophils in the sample which does not allow any biological interpretation.

Overall, the detection of cellular subpopulation is considered as a starting point for experimental analysis (Huang S, 2009). Re-distribution of basophils with surface alterations within the overall basophil populations may occur in inflammatory conditions (Altschuler & Wu, 2010) including CSU. Further studies into biological relevance and clinical contribution would allow this subpopulation to be developed into a biomarker or a therapeutic target.

4.5.3 Methodological Recommendations for Basophil Studies using Conventional Flow Cytometry

In our study, we confirmed that 1) basophil phenotype depends on the choice of gating strategy, 2) basophil subpopulation with surface alterations may contribute to the certain basophil phenotypes, 3) pre-analytical handling may increase the proportion of basophil subpopulation with surface alterations, 4) platelet-basophil adhesion may confound CD63 expression in basophil studies. The results of our imaging flow cytometry studies in human basophils may have some important insights into sample preparation, a panel design and gating strategies in flow cytometry studies. Technical aspects of methodology and some observations of basophil biology identified in our preliminary experiments were considered worth noting for future studies in peripheral blood basophils using imaging flow cytometry.

An optimal choice of antibody conjugates was shown to be important for protocol development for imaging flow cytometry studies in human basophils. During method development, several multicolour staining panels for basophil immunophenotyping were tried in preliminary studies. For example, signal resolution for CD63 surface marker on peripheral blood basophils in our studies was better for an antibody conjugate with FITC (BD Bioscience, UK) than with Alexa Fluor 700 (Exbio Praha, Czech Republic). Therefore, ImageStream® technology can be used for optimisation studies, in combination with conventional flow cytometry, as a visual guide for a rational construction of multiparameter flow cytometric panels based on the marker expression levels on cells as well as characteristics of both antibodies and fluorochromes.

Technical aspects of sample preparation for basophil studies such as the effect of sample pre-warming can also be addressed by imaging flow cytometry studies. As demonstrated in Figures 7A and 7B (Appendix 5), the number of basophils with surface alterations as well as their median CRTH2 fluorescence intensity was shown to increase on sample pre-warming at 37°C for 30 min (Figure 7B, Appendix 5) compared to pre-analytical sample handing at 4°C (Figure 7A, Appendix 5).

Extended immunophenotyping of basophil subpopulations may provide new insights into their function and biological significance. Noteworthy, basophils with surface alterations were noted to form cellular aggregates (Figure 8, Appendix 5) whether it suggests increased cellular adhesiveness in this basophil subpopulation is unknown and worth exploring.

Our data suggest that variation in pre-analytical sample handling may affect the composition of basophil subpopulation and their surface marker expression as demonstrated in our studies by effects of sample pre-warming on the percentage of cells with surface alterations and their CRTH2 expression. Our data consistent with method development studies in flow cytometric research for the detection of chemokine receptors on leukocytes (Berhanu et al, 2003) that found maximum expression of chemokine receptors after incubation of samples at 37°C for 30 min.

Based on our data on basophil heterogeneity, the use of extended multicolour panels may provide further insights into basophil subpopulations. At present, an addition of CCR3, CRTH2, CD69 to CD63 and CD203c may enhance basophil immunophenotyping and may help define a chemotactic basophil phenotype.

Finally, cellular interactions with human basophils can be studied using ImageStream® technology. For example, we demonstrated platelet-basophil adhesion by imaging flow cytometry studies (Figure 41A-B). In the future, the use of ImageStream® technology for studies in basophil interactions with other cells, for example, B-lymphocytes would be an important direction for future research.

4.5.4 Strengths and Limitations of the ImageStream® Basophil Study

Manual identification of basophil subpopulations, the time scale and the complexity of performed analysis do not permit several replicates of these findings and do not allow generalization of conclusions. We also cannot exclude that the proportion of basophils with surface alterations was not affected by density gradient centrifugation (Elghetany & Lacombe, 2004), pre-analytical sample handling at different temperatures or atopic predisposition in the donor and, therefore, these data need to be reproduced in larger studies.

On the other hand, the consistency of certain aspects of analysis across the samples from a different donor (an observation of cells with surface alterations in sample pre-warming studies using a different donor), the use of fixation controls, single stained controls and fluorescence minus one controls gives confidence in the observations and allows the discussion of the possible biological significance of some findings. Although the limitations of this study do not allow generalization of the findings, there are several observations made in the presented basophil studies that may be important for researchers whose work is focused on basophil immunobiology.

4.5.5 Perspectives of Imaging Flow Cytometry Studies in Basophil Research

At present, imaging flow cytometry studies in human basophils are both stimulating and challenging. These studies present us with new challenges in analytical approaches to basophil research, revealed some limitations of the technology and our incomplete understanding of basophil biology. The fact that cells with surface alterations can be seen on the brightfield imagery but can not be detected by digital masks might suggest some difficulties in a signal-noise resolution on the brightfield image for fine structures such as surface alterations. This difficulty may be resolved by further basophil studies using a higher magnification. The use of samples with a very high cellular density may be limited for basophil research due to the possibility of basophil adhesiveness under certain conditions. New imaging flow cytometers (Mark II imaging flow cytometer, Amnis Corporation) with higher throughput may allow acquisition of higher cell counts without increasing a cell density in samples for rare cell research applications. An analytical approach for discriminating basophil subpopulations based on phenotypic and morphometric features needs to be developed. More knowledge needs to be accumulated on immunophenotyping of basophil subpopulations. This seems to be easier to achieve with conventional flow cytometry and then return to imaging flow cytometry on a new level of understanding of basophil heterogeneity.

In future studies, it would be important to elucidate whether phenotypic and morphological features of basophil subpopulation with surface alterations can be reproduced *in vitro* by direct activation with various factors that can induce basophil chemotaxis without degranulation including C5a, IL-3, GM-CSF, eotaxin and eotaxin-2 (Kay & Austen, 1972; Tanimoto et al, 1992; MacGlashan Jr., 2010; Dahinden, 2000; Forssmann et al, 1997). Previous work demonstrated that suboptimal FcεRI-mediated stimulation leads to CD69 up-regulation in the presence of IL-3 (Suzukawa et al, 2007) and increased eotaxin-induced migration (Suzukawa et al, 2005). Therefore, we would expect the higher percentages of basophils with surface alterations upon activation with chemotactic factors, cytokines and suboptimal FcεRI-mediated stimulation. Additionally, it would be important to detect this subpopulation in healthy subjects, who are stratified for atopy, and in patients with allergic diseases and CSU in order to understand the biological significance and the clinical context in which this basophil subpopulation may be relevant. In patients with hay fever, basophil chemotaxis was increased during hay fever season compared to nonallergic subjects or the same allergic patients outside hay fever season (Hirsch & Kalbfleisch, 1980). Therefore, it is conceivable that basophils with surface alterations are likely to be increased during disease exacerbations in CSU or allergic diseases. It would be interesting to compare the *in vitro* findings with clinical scenarios of *in vivo* basophil activation in CSU or allergic diseases. There are limited data suggesting the greater magnitude of *in vivo* up-regulation of CD69 following intentional sting challenge in patients on venom immunotherapy compared to *in vitro* basophil activation on incubation with yellow jacket or honeybee venom (Gober et al, 2007). Additionally, in asthma patients, basophils recovered by bronchoalveolar lavage had higher CD69 expression compared to circulating basophils suggesting enhanced local basophil activation in inflamed tissues (Yoshimura et al, 2002). Therefore, future *in vitro* and *in vivo* studies may help elucidate the experimental conditions required for the *in vitro* induction of basophil subpopulation with surface alterations and to understand the inflammatory microenvironment in CSU or allergic diseases which may lead to the *in vivo* formation of this basophil subpopulation.

Overall, ImageStream® technology can provide fascinating insights into basophil heterogeneity in health and disease. A suitability of imaging flow cytometry for rare cell analysis opens up a prospect of clinical applications of this technology in conditions with low basophil counts in the peripheral blood such as CSU. From a practical perspective, a study protocol and methodology can be further adapted for basophil studies with research and clinical applications.

CHAPTER 5

Re-evaluation of Diagnostic Criteria for Chronic Spontaneous Urticaria and Urticarial Vasculitis: A retrospective study

"There is no such thing as simple. Simple is hard."

—MARTIN SCORSESE

Abstract

Background: Urticarial vasculitis (UV) is a rare disease characterized clinically by urticarial lesions with histological evidence of leukocytoclastic vasculitis. Sometimes, it can be difficult to histologically differentiate UV from acute (ASU) or chronic spontaneous urticaria (CSU), possibly, due to the variability in clinical presentations, incomplete histological presentations and limitations of routine histological assessments. Neutrophilic urticaria is a histological pattern that occurs in several urticarial conditions but its link to serum histamine-releasing activity is currently unclear.

In this thesis, we **hypothesize** that there is a difference in the density and the composition of the inflammatory infiltrate in the dermis between patients with ASU and CSU and between those with CSU and UV. We also hypothesize that CSU patients with serum histamine-releasing activity may have histological evidence of neutrophilic urticaria in lesional biopsy specimens.

To test this hypothesis, the **aims** of this retrospective study were:

1 to assess clinical characteristics of patients with ASU, CSU and UV, with the focus on skin autoreactivity or serum histamine-releasing activity in these groups;
2 to compare the numbers of eosinophils and neutrophils in the histological skin specimens between patients with ASU and CSU and between CSU and UV;
3 to characterize the subset of patients with neutrophilic urticaria and to explore whether there is an association with serum histamine-releasing activity in this group.

Materials and methods: In the retrospective analysis, patients with ASU, CSU and UV were selected based on the histopathological reports of their lesional skin biopsies. Then, clinical diagnosis was verified by a review of clinical notes. Selected patients with ASU and CSU are likely to represent a more severe phenotype because they underwent a lesional biopsy for differential diagnosis with UV on the grounds of atypical characteristics of weals, a lack of efficacy of antihistamines or laboratory findings suggestive of systemic involvement. Skin biopsy specimens stained with haematoxylin and eosin were assessed for total number of eosinophils and neutrophils per HPF using digital image morphometry. Neutrophilic urticaria was defined as 25 extravasated neutrophils per five HPF in the dermis of lesional skin according to previously published criteria (Toppe et al, 2000). HPF corresponded to 0.0326 mm^2. Skin biopsy specimens were examined by two raters in a single blinded fashion. The two raters had a good agreement for eosinophil and neutrophil counts on haematoxylin and eosin staining (ICC =0.910) and for neutrophil counts on immunohistochemistry (ICC = 0.984).

Results: We studied skin biopsy specimens from 6 patients with ASU, 33 patients with CSU and 43 patients with UV. The intermediate group of 21 patients was excluded from histological analysis in view of discrepancies between clinical and histological diagnoses and, therefore, unclear clinico-pathological correlations. In haematoxylin and eosin-stained skin biopsy specimens, there were increased numbers of neutrophils per HPF in UV patients compared to ASU (Mann-Whitney U test, p=0.062) or CSU patients (Mann-Whitney U test, p=0.0002). On immunohistochemistry, the number of myeloperoxidase-positive nucleated cells (neutrophils) per HPF was also higher in skin biopsies from UV patients than in ASU (Mann-Whitney U test, p=0.0027) or CSU patients (Mann-Whitney U test, p=0.0001). The numbers of eosinophils per HPF in the histological skin specimens did not differ between UV and ASU or CSU. In our study, neutrophilic urticaria was noted in 63.6% of CSU patients including two CSU patients with serum histamine-releasing activity.

Conclusions: The density of neutrophilic inflammatory infiltrate in the dermis was significantly higher in UV patients compared to those with ASU or CSU. In our study, neutrophilic urticaria was a common feature in histological specimens from CSU patients who underwent lesional skin biopsy for clinical reasons.

5.1 Introduction

5.1.1 Overview of UV

UV is a rare disease characterized clinically by urticarial lesions with histological evidence of leukocytoclastic vasculitis. UV occurs with peak incidence in the fourth decade of life (Aboobaker & Greaves, 1986). Characteristic urticarial lesions tend to last longer than 24 hours and leave residual bruising and hyperpigmentation on fading (Wisnieski, 2000). In some patients, weals in UV are indistinguishable from those in CSU. In addition to weals, other cutaneous presentations in UV may include livedo reticularis, Raynaud's phenomenon and very occasionally bullous lesions (Black, 1999) Angioedema frequently occurs in UV (Wisnieski, 2000) Patients with UV often present with constitutive symptoms such as fever, malaise and fatigue (Soter, 2000).

If associated with systemic involvement, UV can lead to substantial morbidity. Joint involvement is common in UV. It usually includes arthralgia and stiffness of joints and, rarely, arthritis or synovitis (Soter, 2000; Aboobaker & Greaves, 1986). Patients with UV may present with gastrointestinal features including nausea, vomiting, abdominal pain, intestinal bleeding or diarrhoea (Gammon, 1985). Some patients develop transient or persistent microscopic haematuria and proteinuria (Mehregan et al, 1992). Pulmonary symptoms may include cough, dyspnoea or haemoptysis (Berg et al, 1988). Some patients with UV may develop chronic obstructive pulmonary disease (Venzor et al, 2002). Other clinical presentations of UV may include adenopathy, splenomegaly or hepatomegaly (Soter, 2000). Rare neurological (pseudotumor cerebri, optic nerve atrophy) or ocular (episcleritis, uveitis, scleritis, conjunctivitis) manifestations may occur (Venzor et al, 2002). Of interest, a few case reports suggested a distinct association of cardiac valvulopathy, Jaccoud's arthropathy with hypocomplementemic urticarial vasculitis (Palazzo et al, 1993).

Severity of UV varies from mild to life-threatening. Patients with only cutaneous involvement are considered to have milder disease. Patients with severe UV present with hypocomplementaemia, systemic involvement or refractory disease to treatment. HUVS is at the very severe end of the spectrum (Wisnieski, 2000).

Several disease associations with UV have been described in the literature, (O'Donnell and Black, 1995) although it remains unknown whether these associations represent causality. Common associations with UV are attributed to connective tissue diseases including systemic lupus erythematosus (Asherson et al, 1991) and Sjögren's disease (Alexander & Provost, 1983). Chronic hepatitis B and C are frequent associations with UV although other infections such as infectious mononucleosis (Wands et al, 1976) and Lyme borreliosis (Olson & Esterly, 1990) have been also linked to UV.

A thorough laboratory work-up is important in patients with UV in view of potential systemic involvement and a risk of associated diseases. A spectrum of autoantibodies has been observed in UV including antinuclear antibodies, extractable nuclear antigens, (Asherson et al, 1991) antiphospholipid (Grotz et al, 2009) and anti-endothelial antibodies (D'Cruz et al, 1995). Besides, skin autoreactivity to patient's serum has been reported in UV (Athanasiadis et al, 2006). However, the pathogenic importance of these observations is unclear and further research may elucidate their clinical relevance.

Based on the presence or absence of serum hypocomplementaemia, two variants of UV can be differentiated. Normocomplementaemic UV is characterized by a better prognosis and no or minimal systemic involvement (Wisnieski, 2000). By contrast, patients with hypocomplementaemic UV tend to have a more severe disease associated with systemic involvement, including nephritis (Grotz et al, 2009). It remains unclear whether there is a transition between these clinical variants over time (Wisnieski, 2000). Therefore, serial testing of serum complement levels over time is important for distinction between normocomplementaemic and hypocomplementaemic UV.

Hypocomplementaemic UV syndrome is a distinct clinical syndrome identified in about 5% of patients with UV (Wisnieski, 2000) with the following diagnostic criteria: 1) biopsy-proven vasculitis; 2) arthralgia or arthritis; 3) uveitis or episcleritis; 4) recurrent abdominal pain; 5) glomerulonephritis; 6) decreased C1q or presence if anti-C1q autoantibodies (multidisciplinary approach) (Grotz et al, 2009).

Management of UV includes antihistamines, non-steroidal anti-inflammatory agents, oral corticosteroids, antimalarials and immunosuppressive agents. Recently, biological agents have been used in the treatment of UV. There was a case report on the use of anakinra (IL-1 receptor antagonist) in UV (Botsios et al, 2007). An open-label study demonstrated the efficacy of canakinumab (humanized monoclonal anti-IL-1β antibody) in patients with UV (Krause et al, 2013). Also, a patient with UV associated with cutaneous lupus erythematosus was treated with anti-IL-6 monoclonal antibodies (tocilizumab) with favourable outcome (Makol et al, 2012). An integration of biological agents into the management protocol for UV in the future may help overcome the issue of toxicity associated with the use of conventional treatments for UV.

5.1.2 Histopathological diagnosis of UV

Establishing a clinico-pathological correlation between urticarial lesions and biopsy-proven leucocytoclastic vasculitis is essential for the diagnosis of UV. The classical histological definition of UV include leukocytoclasis, fibrin deposition, endothelial swelling and red blood cell extravasation (O'Donnel & Black, 1995). UV tends to affect postcapillary venules of the subpapillary venular plexus in the upper dermis. Leukocytoclasis presents as neutrophil disintegration and a scatter of nuclear dust (Venzor et al, 2002). The mechanisms of leukocytoclasis are not completely understood. Fibrin deposition is thought to occur due to the endothelial damage in UV which leads to loss of endothelial anti-coagulative properties and, hence, local activation of coagulation results in subsequent fibrin deposition. Red blood cell extravasation occurs due to the damage of the vessel wall which allows the transit of red blood cells through the wall. Endothelial swelling may be caused by several factors including autoantibodies against the endothelial cells or complement membrane activation attack. Shrinkage (apoptosis) of the endothelial cells is a common feature of UV. However, a combination of these definitive features of UV does not occur in some cases (Gammon, 1985). Therefore, a concept of minimal diagnostic criteria has been introduced (Black, 1999). Usually, minimal diagnostic criteria for UV include leukocytoclasis with or without red blood cell extravasaton although there is a great variation in the combination of minimal diagnostic criteria between different studies which does not permit meaningful comparisons. Therefore, there is a need for re-evaluation and standardization of diagnostic criteria for UV.

5.1.3 Histological presentations of ASU and CSU

In clinical practice, patients with ASU or CSU do not usually undergo lesional biopsy unless there are clinical or laboratory pointers to the possibility of underlying UV. Therefore, the knowledge of the histopathological picture of ASU or CSU is limited (Stewart, 2002).

A three-fold increase in mast cells was described in CSU patients compared to healthy subjects (Kay et al, 2014). Inlfammatory infiltration in the skin of CSU patients comprises increased numbers of basophils, eosinophils, neutrophils, macrophages as well as CD3+, CD4+, CD8+, CD25+ T-lymphocytes in skin biopsy specimens from urticarial lesions in CSU patients compared to nonatopic healthy subjects (Ying et al, 2002). The in previous research by Sugita et al (2000), the extent and the composition of perivascular infiltration in CSU patients was assessed quantitatively and a correlation between eosinophils and neutrophils was noted in CSU patients.

Histological examinations of skin biopsy specimens following the injection of autologous serum revealed neutrophilic infiltration in CSU patients at the site of skin testing (Grattan et al, 1990). In histological studies in lesional skin, the analysis of lesional biopsies of CSU patients with or without serum histamine-releasing activity revealed the predominance of eosinophils in the biopsies of CSU patients without serum histamine-releasing activity (Sabroe et al, 1999). In the study by Ying et al (2002), there was no difference in the numbers of infiltrating inflammatory cells or the cytokine pattern in the lesional skin biopsies from CSU patients with or without serum histamine-releasing activity.

Neutrophilic urticaria is a histological pattern that occurs in different types of urticaria, including ASU and CSU (Toppe et al, 1998). Neutrophilic urticaria is considered to represent a more severe disease (Stewart, 2002). Neutrophilic urticaria was assessed quantitatively by Toppe et al (1998) who derived diagnostic criteria for neutrophilic urticarial of 25 extravasated neutrophils per five HPFs. Neutrophilic urticaria differs from UV by the absence of leukocytoclasis or vessel damage. The mechanisms underlying neutrophilic urticaria are poorly understood but are thought to be mediated by the expression of cytokines (TNF-α and IL-3) in the skin of CSU patients (Toppe et al, 1998). Both neutrophilic urticaria and CSU associated with serum histamine-releasing activity represent a more severe disease, however, the relation of neutrophilic urticaria to a subset of CSU patients with serum histamine-releasing activity has not been clarified. It is conceivable that these two subsets of CSU patients may overlap. The previous work by Sabroe et al (1999) demonstrated the lack of difference in neutrophil counts in skin biopsies in CSU patients regardless of serum histamine releasing activity, although the stringent criteria for neutrophilic urticaria were not applied in that study.

5.1.4 Differential diagnosis between CSU and UV

CSU is the main differential diagnosis in patients with UV. Sometimes, the differential diagnosis of urticaria and UV presents challenges in clinical practice for both the clinicians and histopathologists (Wisnieski, 2000). Why can it be difficult to differentiate between CSU and UV?

Firstly, in some patients, weals in UV are indistinguishable from those in CSU. Recent evidence suggests that UV may be an underlying process in 20% of patients with clinical presentations of CSU resistant to treatment with antihistamines (Tosoni et al, 2009).

Secondly, UV is characterized by a significant histological variability (Soter, 2000) and some of the characteristic histopathological features of UV may not be present in the skin biopsies of individual patients, thereby causing diagnostic difficulty in clinical practice. Therefore, the minimal diagnostic histological criteria for UV were discussed in the literature (Black, 1999).

Thirdly, the continuum of histological changes between urticaria and UV has been recognized (Jones et al, 1983). At the extremes of the spectrum, urticaria and UV represent distinct clinico-pathological entities with clearly defined characteristic histological and clinical features. By contrast, series of patients with intermediate histological features have been reported (Jones et al, 1983; Monroe, 1981). This suggests that there may not be a clear-cut histological distinction between these two conditions which reflect an existing gap in our knowledge of skin pathology in these two conditions and warrants further research.

Furthermore, urticaria and UV are both dynamic processes which means a series of cellular and molecular events during a histological evolution of urticarial lesions in the same patient. The knowledge about the development of UV stems from sequential histological studies which are difficult to perform due to invasiveness of the procedure and ethical considerations, on one hand side, and to practical aspects of defining the age of spontaneous weals in urticaria and UV, on the other (Kano et al, 1998; Lawlor et al, 1989). Histological patterns associated with the development of lesions in the same individual would depend on the age of the lesion and on the disease progression. Time course analysis of lesions in exercise-induced urticarial vasculitis suggested that neutrophil recruitment and eosinophil peroxidase deposition as earlier events at 10 hours and leukocytoclasis and the deposition of neutrophil elastase as later events occurring at 24 hours (Kano et al, 1996). By contrast, timed sequential biopsies in cold-induced urticaria showed significant oedema, with no changes in dermal cell populations, suggesting an exudation rather than and infiltration as a predominant mechanism in cold induced urticaria (Lawlor et al, 1999). Consistency in clinical approaches to the timing of skin biopsy in relation to the onset of the lesion formation as well as patient cooperation in timing the age of the lesion could potentially reduce this source of histological variability in urticaria and UV (Callen, 1998). Besides, the intensity of inflammation varies between the patients from minimal transient extravasation of proinflammatory cells to a dense persistent mixed perivascular infiltrate.

Finally, the detection of some histopathological features of UV may be difficult due to the limitations of the existing methodologies. For example, endothelial damage is better assessed by electron microscopy but may be challenging to detect on routine histology. The use of fluorescent dyes with high affinity to the nuclear material may improve the detection of leukocytoclasis in some cases compared to traditional haematoxylin and eosin staining. Also, the representation of affected vessels in the skin biopsy section depends on the focal plane of the section through the vessel (Carlson, 2010), thus, careful examination of several sections from the same biopsy specimen may help detect the affected vessels. Therefore, further development of diagnostic approaches may enhance the accuracy of the diagnosis of UV in difficult cases.

5.1.5 Neutrophils and their contribution to the pathophysiology of CSU and UV

Neutrophils are terminally differentiated leukocytes with a short circulating half-life of up to 12.5 hours (Kolaczkowska & Kubes, 2013). The distinctive morphological feature of neutrophils is multi-lobed nucleus. Neutrophils are known to derive from pluripotent CD34+ myeloid progenitors and to mature in bone marrow. In health, neutrophils are implicated in host defense against pathogens, although the contribution of neutrophils to adaptive immunity is also discussed (Kolaczkowska & Kubes, 2013). Neutrophils are the professional phagocytes with the capacity to generate superoxide anions via the activation of NADPH oxidase (Bardoel et al, 2014).

Neutrophils have three types of cytoplasmic granules including primary, secondary and tertiary. Primary (azurophilic) granules contain myeloperoxidase and neutrophil elastase.

Secondary (specific) granules contain lactopherin, matrix metalloproteinase-8 and pentraxin-3. Tertiary (gelatinase) granules contain gelatinase, matrix metalloproteinase-9 and MT-6 matrix metalloproteinase. Neutrophil activation occurs as a two-step process including, first, priming and then followed by activation at the sites of infection or inflammation. Upon activation, the granule contents together with ROS are discharged into the phagolysosome for bacterial killing. Neutrophils can also exocytose granule products into the extracellular environment, this mechanism of neutrophil degranulation and release of toxic products has been implicated in the pathophysiology of various diseases. Increased numbers of neutrophils in the circulation as well as neutrophil accumulation in the tissues were reported as characteristic features of various inflammatory diseases (Nemeth & Mocsai, 2012).

In the skin, neutrophil-derived enzymes may contribute to tissue damage and skin remodeling in chronic inflammation. In the skin, neutrophils are recruited in skin response to an intradermal injection of autologous serum (Grattan et al, 1990). Lesional biopsies from CSU patients showed an increase in neutrophil numbers in some patients. In the context of neutrophilic urticaria, neutrophil accumulation in the dermis occurs without the evidence of leukocytoclasis or vessel damage. The subset of patients with neutrophilic urticaria is thought to have a more severe disease. Neutrophils may contribute to chronic urticarial disease via several putative mechanisms including neutrophil-mediated oedema and the release of immune regulatory molecules.

Neutrophils can increase vascular permeability (DiStasi & Ley, 2009) and thereby may contribute to the weal formation in CSU. Neutrophil-induced oedema was described by Wedmore and Williams (1981). The contribution of neutrophils to the vasculitic process in UV may include the damage of vessels and surrounding tissue through the release of cytotoxic mediators. In the recent publication by Finsterbusch et al (2014), microvascular leakage was hypothesized to occur as a sequence of events including up-regulation and release of TNF during neutrophil adhesion and transmigration in response to chemotactic stimulation. As a result, TNF effects on endothelial cells involve the phosphorylation and endocytosis of VE-cadherin compexes in the junctions (Schulte et al, 2011) and actomyosin contraction (Yuan et al, 2002). This leads to the opening of endothelial junctions, increased endothelial cell permeability and microvascular leakage (Finsterbursch et al, 2014). Other neutrophil products may also contribute to neutrophil-mediated microvascular leakage including azurocidin (Gautam et al, 2001).

Another effector mechanism of neutrophils involves neutrophil extracellular traps (Brinkmann & Zychlinsky, 2007). Neutrophil extracellular traps were demonstrated in vascular inflammation (Phillipson & Kubes, 2011). NET formation represents a powerful method of neutrophil-mediated microbial killing. Upon activation with IL-8 or lipopolysaccharide, neutrophils undergo a discharge of nuclear chromatin together with cathelicidin antimicrobial agents. Furthermore, in the context of vasculitis, NET formation was reported in autoimmune small-vessel vasculitis (Kessenbrock et al, 2009), however, it remains unknown whether NET contributes to the development of UV.

In view of recent developments in neutrophil biology, the role of neutrophils in the pathophysiology of CSU and UV represents a promising research area. Given the growing interest in the role of neutrophils in autoimmune conditions, we re-examined the contribution of neutrophils to the inflammatory infiltrate in ASU, CSU and UV. Research into the contribution of neutrophils to the pathophysiology of CSU and UV is important in view of potential ramifications for differential diagnosis and therapeutic targeting of neutrophils in these two conditions.

5.1.6 Summary

UV is a rare disease characterized clinically by urticarial lesions with histological evidence of leukocytoclastic vasculitis. The histological presentations in ASU and CSU involve a mixed perivascular infiltration of varying intensity. The histological differential diagnosis between UV and ASU or CSU may be difficult for these reasons: incomplete presentation of histological features of UV, a histological continuum between CSU and UV, the limitations of existing methodology. Therefore, there is a need for re-assessment of histological features of ASU, CSU and UV in terms of the density and the composition of cellular infiltration in lesional skin biopsy specimens in order to evaluate the histological differences between these conditions.

The correlation between the numbers of neutrophils and eosinophils in skin biopsy specimens was demonstrated in CSU (Sugita et al, 2000). In clinical practice, a histological pattern of neutrophilic urticaria is thought to be associated with severe CSU, it differs from UV by the absence of leukocytoclasis and vascular damage. Additionally, CSU associated with serum histamine-releasing activity is linked to disease severity (Sabroe et al, 2002) although no characteristic histological features were detected (Sabroe et al, 1999; Ying et al, 2002). Furthermore, neutrophil-predominant infiltrate was associated with UV (Lee et al, 2007). Therefore, it is plausible that the histological differences between UV and ASU or CSU involve the changes in density and, possibly, the composition of the inflammatory infiltrate in the lesional skin.

Neutrophils are important effector cells of inflammation and may participate in the pathophysiology of CSU and UV through their putative contribution to the microvascular leakage, possibly, skin remodeling and vessel damage. The formation of NETs was demonstrated in vascular inflammation in autoimmune small-vessel vasculitis (Kessenbrock et al, 2009). Whether NET formation is relevant to the inflammation in UV is unknown.

5.1.7 Hypothesis and aims of the study

This thesis hypothesizes that there is a difference in the density and the composition of the inflammatory infiltrate in the dermis between patients in ASU and CSU and between CSU and UV. We also hypothesize that CSU patients with serum histamine-releasing activity may have histological evidence of neutrophilic urticaria.

To test the hypothesis stated above, the aims of this study were:

1 to assess clinical characteristics of patients with ASU, CSU and UV, with the focus on skin autoreactivity or serum histamine-releasing activity between these groups;
2 to compare the numbers of eosinophils and neutrophils in the histological skin specimens between patients with ASU and CSU and between CSU and UV;
3 to characterize the subset of patients with neutrophilic urticaria and to explore if there is any evidence for associated serum histamine-releasing activity in this group.

5.2 Materials and methods

5.2.1 Patient Selection

The patients were identified from the diagnostic database at the Pathology Department at the Norfolk & Norwich University Hospital using the keywords "urticaria" and "urticarial vasculitis" in the histological report. Patients with the histological diagnosis of urticaria or UV were selected. The clinical notes of the selected patients were reviewed. Only patients with a clinical diagnosis of ASU, CSU or UV were included (Figure 42).

Inclusion criteria for patients with urticaria:

1 Clinical diagnosis of ASU or CSU in the clinical notes.
2 Description of urticarial weals in the clinical notes.

Exclusion criteria for patients with urticaria:

1 Predominant physical urticarias.

Inclusion criteria for patients with UV:

1 Clinical diagnosis of UV in the clinical notes.
2 Description of urticarial weals consistent with clinical features of UV.

Exclusion criteria for patients with UV:

1 Other types of vasculitis

5.2.2 Specimen staining

Skin biopsy specimens stained with haematoxylin and eosin from 6 patients with ASU, 33 patients with CSU and 43 patients with UV were available for quantitative digital image analysis. Also, skin biopsy specimens from 5 patients with ASU, 31 patient with CSU and 40 patients with UV were analyzed by dual immunohistochemical staining for neutrophil myeloperoxidase and Factor VIII-related antigen.

Figure 42. Search Strategy and Patient Selection criteria for the Retrospective Histological Study

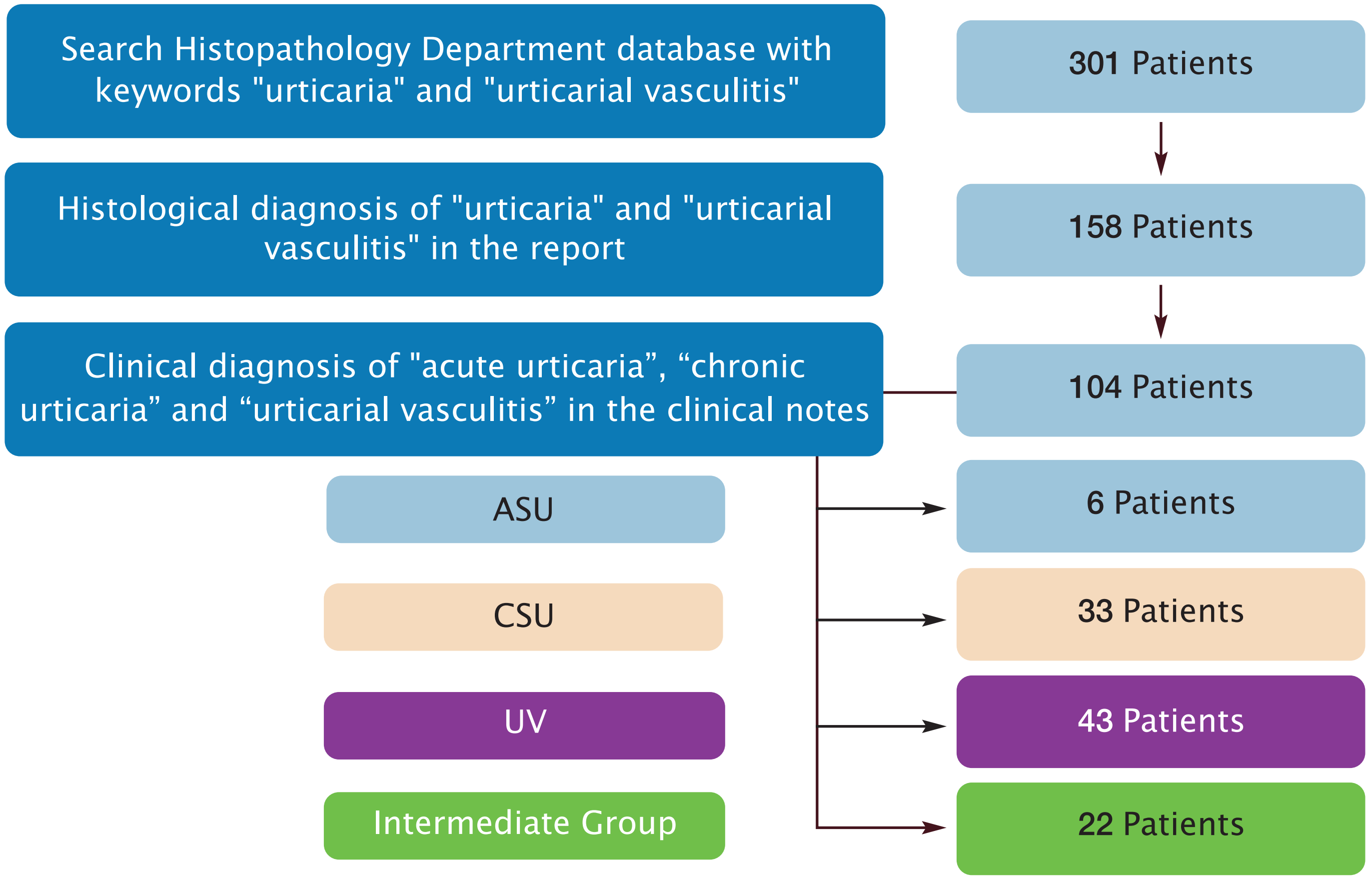


Figure 42. For patient selection into the study, 301 patients were identified from the Histopathology Department diagnostic database using keywords "urticaria" and "urticarial vasculitis" in their report. Of these, 158 patients with a histological diagnosis of urticaria or UV were selected for clinical record review. Only patients with a description or a photo of lesions in the clinical notes were included in the study. Patients with a clinical diagnosis other than urticaria or urticarial vasculitis were excluded from further study leaving 104 patients (6 patients with ASU, 33 patients with CSU, 43 patients with UV and 22 patients in the intermediate group). Patients with the intermediate group were excluded from further analysis due to unclear clinicopathological correlations.

Abbrevations:
CSU - Chronic spontaneous urticaria
UV - Urticarial vasculitis
ASU - Acute spontaneous urticaria

Dual immunohistochemical staining for neutrophil myeloperoxidase and Factor VIII-related antigen was carried out with automated immunostaining. Paraffin-embedded sections were dewaxed in xylene and hydrated using graded alcohols to tap water. Heat-induced epitope retrieval was performed using EDTA-based buffer (Bond™ Epitope Retrieval Solution 2, pH=9.0) and heat treatment at 100°C for 20 min followed by cooling to room temperature. Mouse-mouse sequential double immunoenzyme staining for Factor VIII-related antigen and neutrophil myeloperoxidase was used to study the migration of neutrophils through the vessel wall. Enzymatic visualization of alkaline phosphatase activity in red was performed using Fast Red (myeloperoxidase), visualization of horseradish peroxidase activity in brown was carried out with DAB (Factor VIII-related antigen).

Endogenous peroxidase activity in formalin-fixed, paraffin-embedded sections was blocked with 3% hydrogen peroxide for 8 min at room temperature. There was one hour incubation of primary antibody mouse anti-human monoclonal antibodies against von Factor VIII-related antigen (Leica Microsystems). Bond™ Polymer refine detection system (Leica Microsystems) was used to prepare biotin-free polymeric horseradish peroxidase (HRP)-linker antibody conjuates. After washing with Bond™ wash solution, post-primary polymer enhancer containing 10% (v/v) animal serum in Tris-buffered saline and 0.09% ProClin tm 950 was applied for 15 min at room temperature to enhance penetration of the subsequent polymer reagent. Bond™ wash solution was used as washing buffer for all further steps. After washing, polymerpoly-HRP anti-mouse/rabbit IgG containing 10% (v/v) animal serum in Tris-buffered saline and 0.09% ProClin tm 950 was applied for 15 min at room temperature to localize the primary antibody. The substrate chromogen, 3’3-diaminobenzidine (DAB) was applied for 10 min at room temperature to visualize the complex via brown precipitate. For the second staining, there was an incubation with mouse anti-human monoclonal antibody against myeloperoxidase (Leica Microsystems) for one hour. Following the same steps with post-primary polymer enhancer and polymer poly-HRP anti-mouse /rabbit IgG, Bond™ Polymer AP Red detection system was applied to prepare polymeric alkaline phosphatase – linker antibody conjugates. The substrate chromogen, Fast Red, was applied for 20 min at room temperature. Double stained specimens were counterstained using 0.02% haematoxylin for nuclear staining. Specimens were dried on a hot plate (50°C) and coverslipped with VectaMount (Vector). Immunohistochemistry staining for this project was optimized by Joseph Goodwill and was performed by Debra Essex at the Histopathology Department, Norfolk & Norwich University Hospital. Scanning and analysis of specimens was performed by Dr Elena Borzova and Dr Laszlo Igali at the Histopathology Department, Norfolk & Norwich University Hospital.

5.2.3 Digital Image Analysis

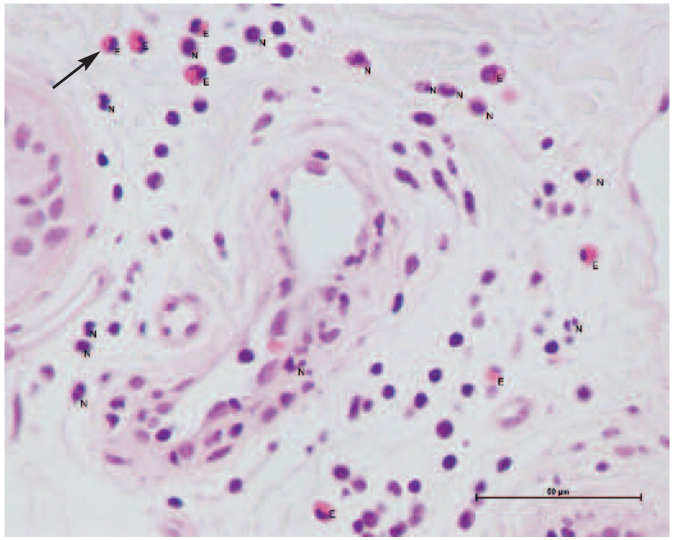
Histological specimens were analyzed using an Nikon Eclipse 80i microscope. Images of the perivascular infiltrate around a characteristic vessel in the upper dermis were digitized using Nikon DsR1 camera and processed with imaging software NIS-Elements BR 3.0. Examples of quantitative image analysis for skin biopsy specimens obtained from patients with urticaria and UV are presented in Figure 43. Inflammatory cell counting was carried out based on cell morphology using point counting morphometry (Figure 43). Cellular composition (neutrophils, eosinophils) of the perivascular inflammatory infiltrate was expressed as cell counts in 0.0326 mm^2. The microscopic sections from patients with urticaria and UV were evaluated by two observers in a blinded fashion to assess the inter-observer variability. The two raters had a good agreement for eosinophil and neutrophil counts on haematoxylin and eosin staining (ICC =0.910) and for neutrophil counts on immunohistochemistry (ICC = 0.984).

5.2.4 Statistical analysis

Statistical analysis was performed with STATA statistical package, version 11/SE (StataCorp LP, USA). The project was approved by the East Norfolk & Waveney Research and Governance Committee (Ref: 2006DERM05L (64-04-09)). The project was funded by a British Skin Foundation Small Grant Award. We used the Mann-Whitney U test to compare the neutrophil counts between two independent groups (ASU vs CSU; CSU vs UV). For inter-observer agreement, we employed inter-class correlation coefficient to detect the difference and its statistical significance for the cell counts carried out on the same histological specimens by two independent observers.

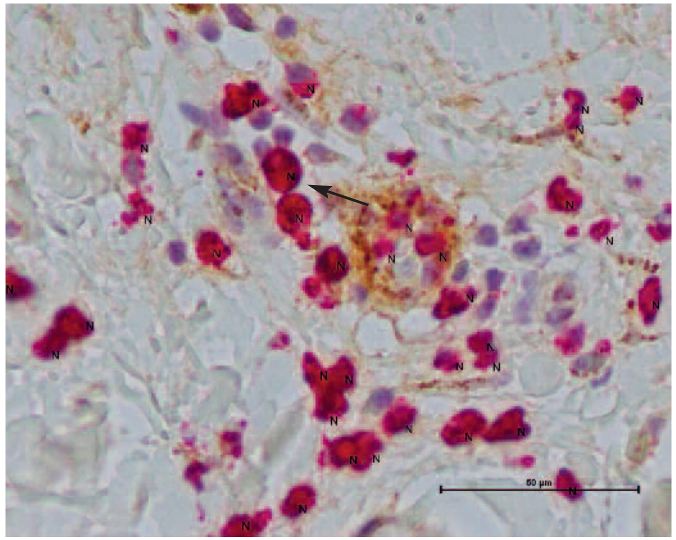
Figure 43. Quantitative Image Analysis for Skin Biopsy Specimens obtained from patients with ASU, CSU and UV

A. Point counting morphometry of neutrophils and eosinophils in skin biopsy specimens stained with hematoxylin and eosin



Haematoxylin and eosin stain.
Original magnification x400. Bar = 50μm.

B. Point counting morphometry of neutrophils in skin biopsy specimens stained immonohistochemically for myeloperoxidase and Factor VIII



Double immunohistochemistry for myeloperoxidase and Factor VIII.
Alkaline Phosphatase Red and DAB, respectively.
Original magnification x400. Bar = 50μm.

Figure 43. Point counting morphometry in skin lesional biopsy specimens was carried out using NIS-Elements BR 3.0 (Basic Research) Microscope Imaging Software (Nikon UK Ltd). Histological specimens were stained with haematoxylin and eosin (Figure 43A) and immunohistochemically for myeloperoxidase and Factor VIII (Figure 43B). In immunihistochemically stained skin biopsy specimens, myeloperoxidase-positive neutrophils were visualised with Alkaline Phosphatase Red (Figure 43B). Quantification of cells in skin lesional biopsy specimens was performed on photomicrographs by point counting morphometry as demonstrated in Figures 43A and B. For histological specimens stained with haematoxylin and eosin, eosinophils (as pointed with an arrow on the Figure 43A) and neutrophils were enumerated on photomicrographs by point morphometry as demonstrated in Figure 43A. Myeloperoxydase-positive neutrophils were counted by point morphometry on immunohistochemically stained skin biopsy specimens as demonstrated in Figure 43B. The cell counts were expressed as the number of cells in HPF corresponding to $0.0362mm^2$.

Abbrevations:
CSU - Chronic spontaneous urticaria
UV - Urticarial vasculitis
DAB - 3,3' diaminobenzidine
ASU - acute spontaneous urticaria
HPF - High power field

5.3 Results

5.3.1 Clinical Characteristics of Patients with ASU, CSU and UV

Patient selection process for this study is illustrated in Figure 42. Clinical and histological features of ASU, CSU and UV were analyzed in patients who underwent lesional skin biopsy at the Norfolk & Norwich University Hospital between 1989 and 2014. A total of 301 patient were identified from the diagnostic database at the Histopathology Department using keywords “urticaria” and “urticarial vasculitis” in the histological report. In total, 158 patients with the histological diagnosis of urticaria or UV were selected for clinical record review. Patients with the clinical diagnosis other than urticaria or UV were excluded from further study leaving 104 patients: 6 patients with ASU, 33 patients with CSU, 43 patients with UV and 22 patients indeterminate.

Clinical data of patients with ASU, CSU and UV are presented in the Table 6. The median age of ASU patients was 45 years (range 14-77 years), of CSU patients – 44 years (range 19-78 years) and of UV patients – 55 years (range 12-83 years). In UV, weals were associated with bruising (34.9%), burning or pain (23.3%) and duration over 24 hours (51.2%). Half had extracutaneous symptoms (arthralgia, abdominal pain, diarrhea, fatigue, fever or lethargy), thyroid autoimmunity (18.6%), microscopic haematuria (9.3%), abnormal liver function (23.3%), anti-nuclear antibodies (30.2%), elevated ESR/CRP (48.8%) and hypocomplementaemia (18.6%). Treatments included oral corticosteroids, dapsone, hydroxychloroquine, colchicine and methotrexate. In CSU patients, weals were associated with bruising (4.6%), burning or pain (3%) and duration over 24 hours (12.1%). All had normal C3 and C4 levels, raised ESR/CRP (39.4%), anti-nuclear antibodies (6%) and thyroid autoimmunity (24.2%). Treatments included H1-antihistamines in conventional and high doses, oral corticosteroids, ciclosporin, methotrexate and mycophenolate mofetil.

Of interest, 11 patients with CSU and five patients with UV were investigated by autologous serum skin testing or serum-induced BHR. Three of eight patients with CSU and three of five UV patients showed a positive autologous serum skin test. Serum-induced BHR was positive in two of three CSU patients and one patient with UV. As

Table 6. Clinical presentations and laboratory findings in patients with AU, CSU and UV

Parameter	ASU (n=6)	CSU (n=33)	UV (n=43)
Age	Median - 45 y.o. (range 14 - 77 y.o.)	Median - 44 y.o. (range 19 - 78 y.o.)	Median - 55 y.o. (range 12 - 83 y.o.)
Lesions:			
bruising	9 (27.3%)	2 (4.6%)	15 (34.9%)
burning or pain	1 (3%)	1 (3%)	10 (23.3%)
duration over 24 hours	9 (27.3%)	4 (12.1%)	22 (51.2%)
Angioedema	16 (48.5%)	12 (36.4%)	15 (34.8%)
NSAID intolerance	1 (3%)	1 (3%)	7 (16.3%)
Extracutaneous symptoms:			
arthralgia	2 (33.3%)	7 (21.2%)	12 (27.9%)
abdominal pain, diarrhoea	0	1 (3%)	6 (13.4%)
fatigue, fever, lethargy	7 (21.2%)	4 (12.1%)	14 (32.6%)
Laboratory findings:			
microscopic hematuria	0	0	4 (9.3%)
abnormal liver function	0	1 (3%)	10 (23.3%)
positive anti-nuclear antibodies	3 (9%)	2 (6%)	13 (30.2%)
elevated ESR and/or CRP	0	13 (39.4%)	21 (48.8%)
hypocomplementaemia	1 (3%)	0	8 (18.6%)
Underlying disease			
SLE, MGUS, lymphoma, etc	1 (16.6%)	0	11 (25.6%)
Thyroid autoimmunity	1 (16.6%)	8 (24.2%)	8 (18.6%)
Evidence for functional autoantibodies			
Positive ASST	0	3 out of 8	3 out of 5
Positive serum-induced BHR assay	0	2 out of 3	1

Table 6. Table 6 summarises clinical presentations of ASU ,CSU and UV in patients included in our retrospective histological studies. Angioedema was more frequently observed in patients with ASU compared to those with CSU and UV. Atypical characteristics of urticarial lesions and extracutaneous symptoms were more frequently noted in UV patients. Laboratory findings confirmed elevated ESR and/or CRP and thyroid autoimmunity as common features of CSU and UV. Microscopic hematuria, abnormal liver function tests and antinuclear antibodies were predominantly reported in UV patients. There were limited data on skin autoreactivity and serum histamine-releasing activity in patients with CSU and UV.

Abbrevations:
ASU - acute spontaneous urticaria
CSU - Chronic spontaneous urticaria
UV - Urticarial vasculitis
ASST - Autologous serum skin test
BHR - Basophil histamine release
SLE - systemic lupus erythematosus
MGUS - monoclonal gammopathy of unknown significance
NSAID - Non-steroidal anti-inflammatory drug

well as clinical overlap between urticaria and UV, there is limited data for skin autoreactivity and serum histamine-releasing activity in both groups, raising the possibility that they may represent different ends of a clinicopathological spectrum with similar pathogenesis.

5.3.2 Comparison of histological features between ASU and CSU patients

We found no statistically significant differences in the numbers of eosinophils or neutrophils between patients with ASU and CSU (Mann-Whitney U test, p=0.5008) (Figures 44A and B). On immunohistochemistry, the numbers of myeloperoxidase-positive neutrophils did not differ between patients with ASU and CSU (Mann-Whitney U test, p=0.8892) (Figure 45A).

5.3.3 Comparison of histological features between CSU and UV patients

To compare the histopathological features of CSU and UV, we compared the haematoxylin and eosin-stained histological specimens from 33 CSU patients and 43 UV patients. We quantified eosinophils in skin biopsies of CSU patients and those with UV and but could not detect any difference between the groups (Figure 44A). By contrast, histological examination of skin biopsies of patients with CSU and UV demonstrated significantly higher neutrophils counts per HPF in the latter group (Mann-Whitney U test, p=0.0002) (Figure 44B). These data were confirmed by immunohistochemistry showing that the numbers of myeloperoxidase-positive cells (neutrophils) per HPF in lesional biopsy are significantly higher in UV patients compared to those with CSU (Mann-Whitney U test, p=0.0001) (Figure 45A).

Analysis of histological specimen underwent immunohistochemical staining for neutrophil myeloperoxidase revealed different patterns of neutrophil involvement in urticaria and UV as illustrated in Figure 46. Histological findings in urticaria included neutrophil margination and perivascular infiltration while neutrophil involvement in UV was presented by neutrophil margination, neutrophil infiltration of the vessel wall, neutrophil perivascular infiltration and vessel destruction. Morphological changes of neutrophils in UV include loss of cellular integrity and extracellular deposition of myeloperoxidase (Figure 46B). Notably, extracellular deposition of myeloperoxidase was present mainly in patients with UV.

5.3.4 Comparison between NUV and HUV

To compare histological differences between HUV and NUV, we compared neutrophil counts in histological specimens from patients with NUV and HUV. There was a marginal statistical difference in neutrophil counts between these subsets of UV patients (Mann-Whitney U Test, p=0.053) (Figure 45B).

5.3.5 The histological pattern of neutrophilic urticaria in CSU patients

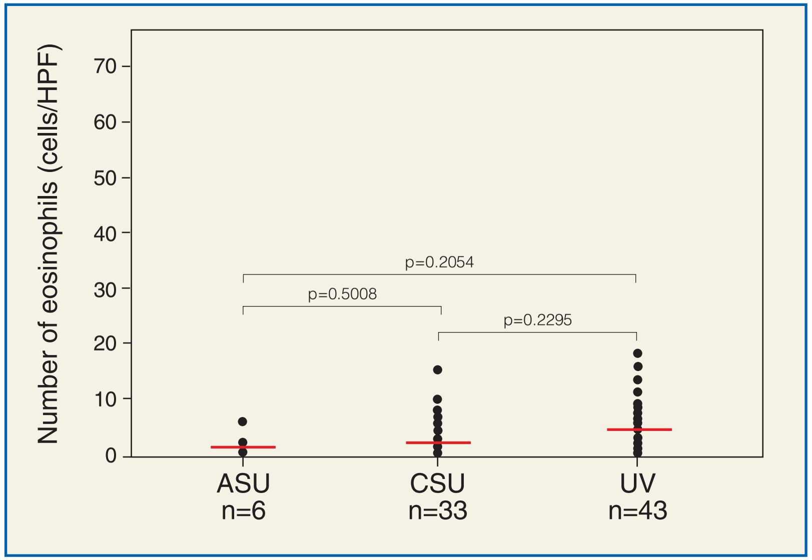
Applying the histological criteria by Toppe et al (1998), we noted neutrophilic urticaria in 21 (63.6%) of 33 CSU patients. Of patients with neutrophilic urticaria defined by 25 extravasated neutrophils in five HPF of skin biopsy specimen, a dense neutrophilic infiltrate of more than 40 neutrophils per five HPF was observed in 15 (45.5%) CSU patients.

5.3.6 The histopathology of CSU with serum histamine-releasing activity

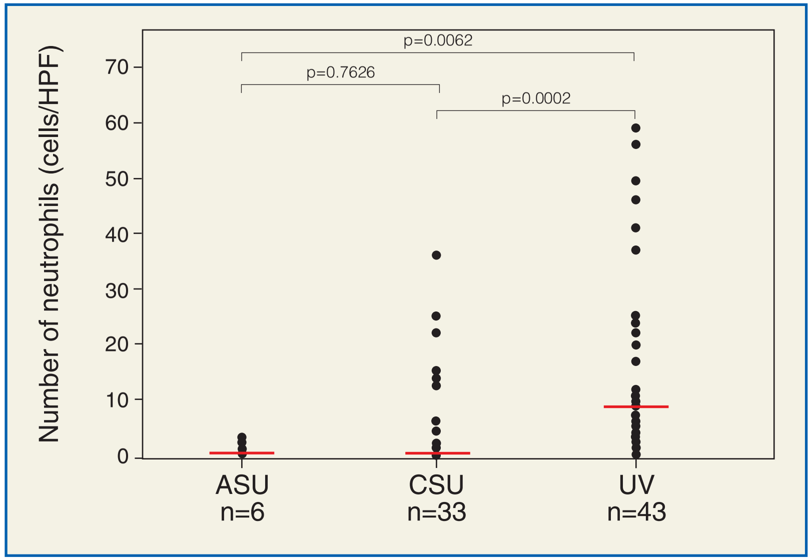
We only partially achieved our aim to assess the histological pattern of CSU with serum histamine-releasing activity because there were only two CSU patients with serum histamine-releasing activity in our study population. Both patients demonstrated the histological pattern of neutrophilic urticaria (Figure 48). Of interest, immunohistochemical staining for myeloperoxidase highlighted the extracellular deposition of myeloperoxidase in one CSU patients with serum histamine-releasing activity (Figure 48A). Whether this suggests leukocytoclasis, neutrophil degranulation or NET formation in CSU patient with serum histamine-releasing activity needs to be established in future studies.

Figure 44. The Number of Eosinophils and Neutrophils in Haematoxylin and Eosin-Stained Lesional Skin Biopsies from Patients with ASU, CSU and UV

A. The number of eosinophils per HPF in haematoxylin and eosin stained lesional skin biopsies from patients with ASU, CSU and UV



B. The number of neutrophils per HPF in haematoxylin and eosin stained lesional skin biopsies from patients with ASU, CSU and UV



Red bar represents median values.

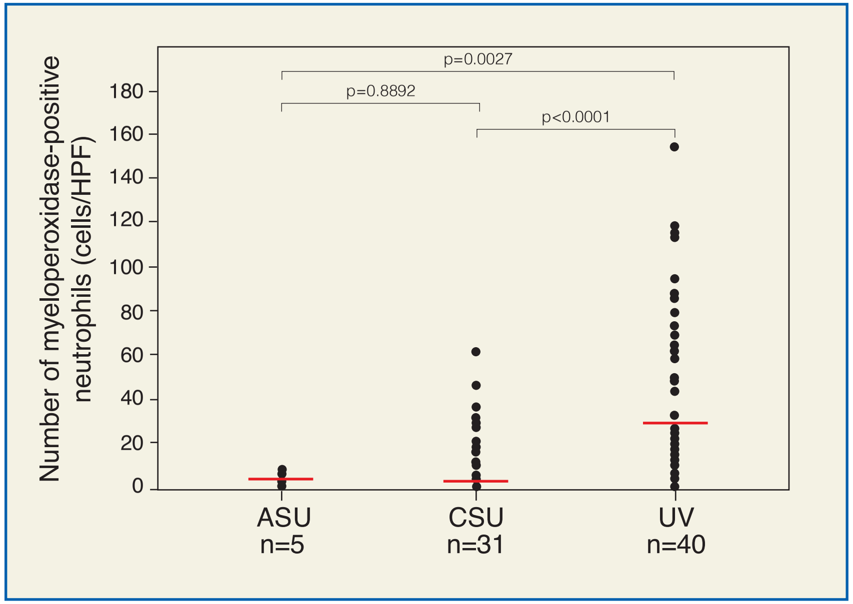
Pairwise comparisons were carried out using Mann-Whitney U test.

Figure 44. The number of extravascular eosinophils and neutrophils per HPF in histological specimens from the affected skin were compared between patients with ASU, CSU and UV. There was no statistically significant difference in eosinophil counts per HPF in lesional skin biopsy between ASU, or CSU and UV patients (Mann-Whitney U test, p>0.05) as demonstrated in Figure 44A. Patients with UV demonstrated significantly higher numbers of extravasated neutrophils than patients with ASU or CSU (Mann-Whitney U Test, p<0.001). Skin biopsies were stained with haematoxylin and eosin. The cell counts were expressed as cells per HPF (original magnification x400) corresponding to 0.0326mm^2.

Abbrevations:
ASU - Acute urticaria
CSU - Chronic spontaneous urticaria
UV - Urticarial vasculitis
HPF - High power field

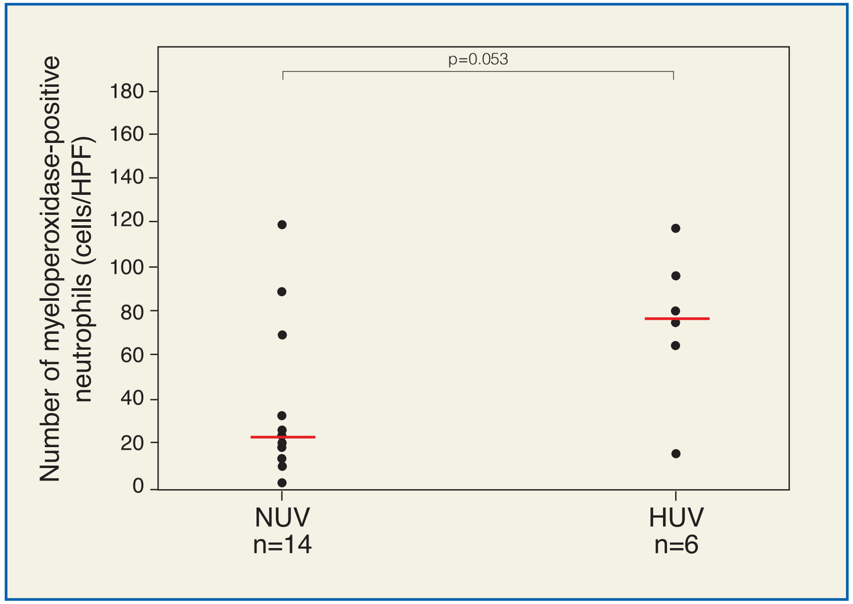
Figure 45. The Number of Myeloperoxidase-positive Neutrophils per HPF in Lesional Skin Biopsies from Patients with ASU, CSU and UV

A. The number of myeloperoxidase-positive neutrophils in skin biopsy specimens from patients with ASU , CSU and UV



Red bar represents median values.
Pairwise comparisons were carried out using Mann-Whitney U test.

B. The number of myeloperoxidase-positive neutrophils in skin biopsy specimens from patients with normocomplementaemic and hypocomplementaemic UV



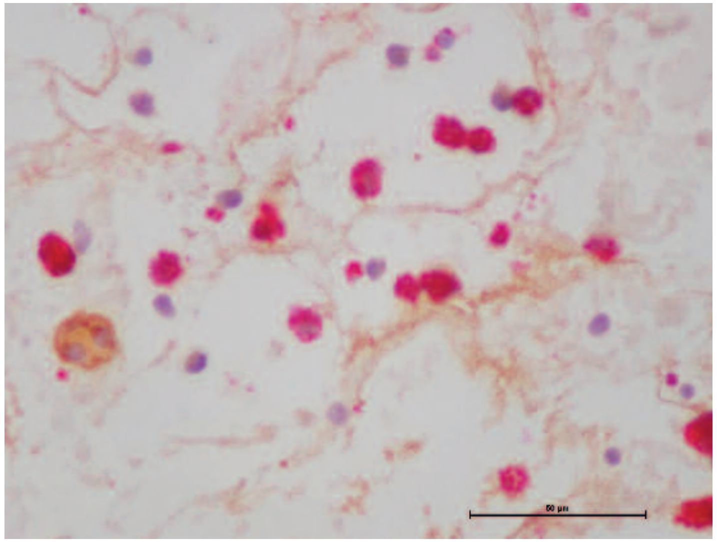
Red bar represents median values.
Pairwise comparisons were carried out using Mann-Whitney U test.

Figure 45. Lesional skin biopsies obtained from patients with ASU, CSU and UV were stained immunohistochemically for myeloperoxidase. The number of nucleated cells staining positive for myeloperoxidase was expressed per HPF (original magnification x400) corresponding to 0.0326mm^2. Only extravasated cells were counted. The median number of myeloperoxidase-positive neutrophils in skin biopsy specimens in ASU patients was 4(3;7) cells/HPF; in CSU patients - 3 (0;17) cells/HPF and UV patients - 29(14; 66) cells/HPF. There was a statistically significant difference in the numbers of extravasated cells positive for myeloperoxidase (neutrophils) per HPF between patients with ASU and UV (Mann-Whitney U test, p=0.027) and between patients with CSU and UV (Mann-Whitney U test, p<0.0001).The median neutrophil number in normocomplementaemic UV patients was 23 (3;32) cells/HPF whereas the median neutrophil number in patients with hypocomplementaemic UV was 76 (62;94) cells/HPF (Mann-Whitney U test, p=0.053). The numbers are expressed as medians and the interquartile ranges.

Abbrevations:
ASU - Acute spontaneous urticaria
CSU - Chronic spontaneous urticaria
UV - Urticarial vasculitis
NUV - Normocomplementaemic UV
HUV - Hypocomplementaemic UV
HPF - High power field

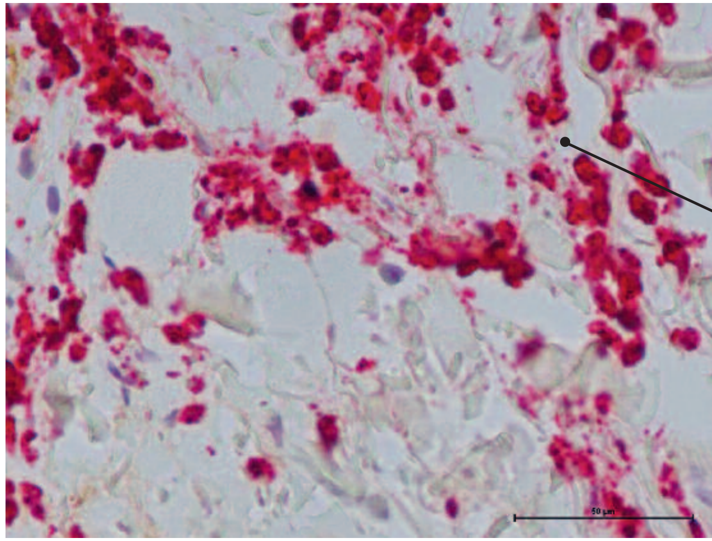
Figure 46. Neutrophil Infiltration in the Dermis in Skin Biopsy Specimens from Patients with CSU and UV

A. Representative histological specimen from a CSU patient



Double immunohistochemistry for myeloperoxidase and Factor VIII.
Alkaline Phosphatase Red and DAB, respectively.
Original magnification X400. Bar = 50µm.

B. Representative histological specimen from a UV patient



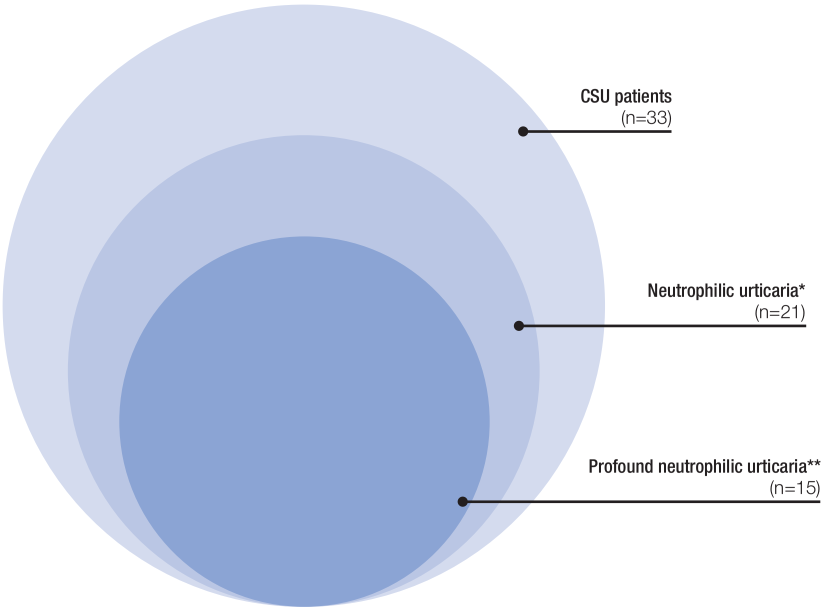
Double immunohistochemistry for myeloperoxidase and Factor VIII.
Alkaline Phosphatase Red and DAB, respectively.
Original magnification X400. Bar = 50µm.

Figure 46. Different patterns of neutrophil involvement was noted in patients with CSU and UV. Figure 46A illustrates perivascular neutrophilic infiltrate in the histological section stained immunohistochemically for myeloperoxidase from a CSU patient. Histologically, neutrophil integrity in CSU is preserved. Neutrophilic urticaria is characterised by preferential neutrophil recruitment without neutrophil disintegration. Figure 46B demonstrates neutrophilic infiltration in a UV patient with extracellular deposition of myeloperoxidase. Whether extracellular deposition of myeloperoxidase represents neutrophil degranulation, leukocytoclasis or neutrophil extracellular traps needs to be established in future studies.

Abbrevations:
CSU - Chronic spontaneous urticaria
UV - Urticarial vasculitis
DAB - 3,3' diaminobenzidine

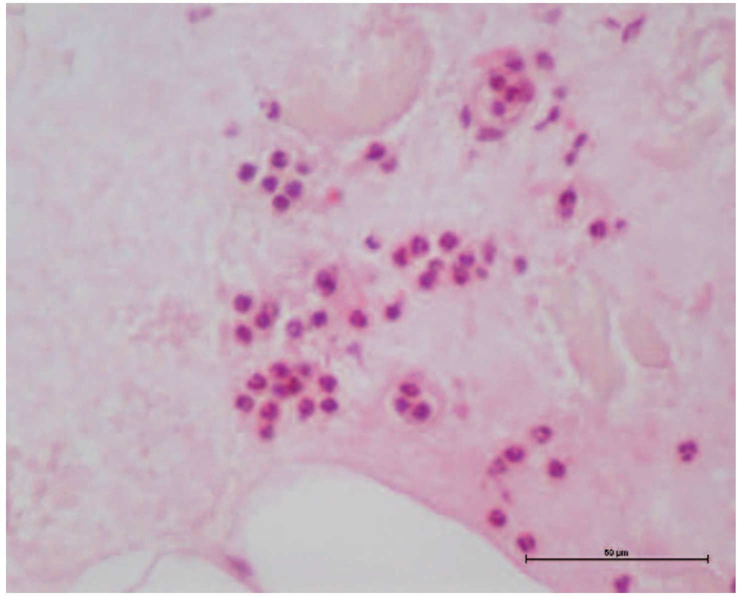
Figure 47. Histological Features and Diagnostic Criteria for Neutrophilic Urticaria

A. CSU patient distribution in relation to histological evidence for neutrophilic urticaria



* >25 extravasated neutrophils per five HPF (orginal maginification X400)
** >40 extravasated neutrophils per five HPF (orginal maginification X400)

B. Representative histological specimen from a CSU patient with histological evidence for neutrophilic urticaria



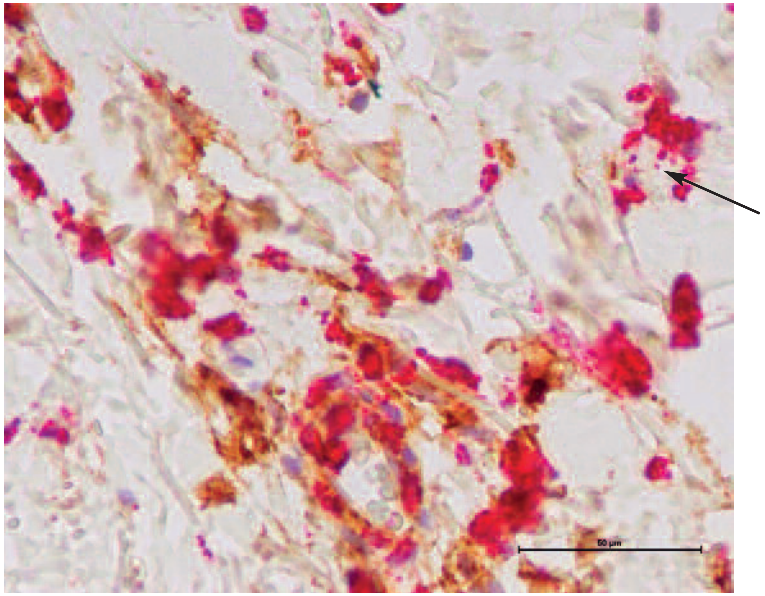
Haematoxylin and eosin stain.
Original magnification X400.
Bar =50μm.

Figure 47. In our study, 33 CSU patients were evaluated for histological evidence for neutropilic urticaria. Neutrophilic urticaria was defined by calculating 25 extravasated neutrophils per five HPF of haematoxylin and eosin stained histological specimen from a lesional biopsy at X400 original magnification (Toppe et al, 1998). On histological examination, neutrophilic urticaria was noted in 21 patient with CSU. Of these, 15 CSU patients demonstrated more than 40 extravasated neutrophils per five HPF at x400 magnification. Bar = 50μm.

Abbrevations:
CSU - Chronic spontaneous urticaria
HPF - High power field

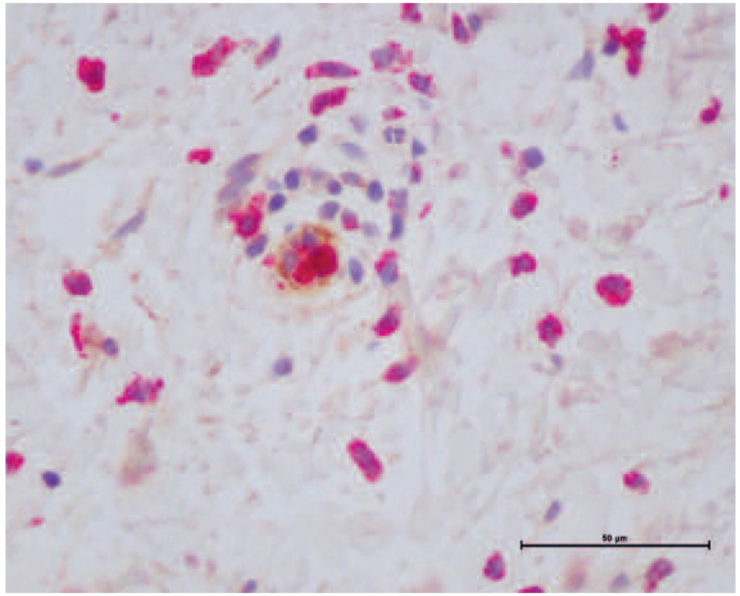
Figure 48. Histological Features of CSU with Serum Histamine-Releasing Activity

A. Patient D.M.



Double immunohistochemistry for myeloperoxidase and Factor VIII.
Alkaline Phosphatase Red and DAB, respectively.
Original magnification X400. Bar = 50µm.

B. Patient M.E.



Double immunohistochemistry for myeloperoxidase and Factor VIII.
Alkaline Phosphatase Red and DAB, respectively.
Original magnification X400. Bar = 50µm.

Figure 48. Two CSU patients with serum histamine-releasing activity demonstrated evidence for neutrophilic urticaria in lesional skin biopsy specimens. Figure 48A demonstrates dense neutrophilic infiltrate in the dermis with some extracellular deposition of myeloperoxidase (as indicated by the arrow) in the lesional skin biopsy specimen from patient D.M. Skin biopsy from urticarial lesion from patient M.E. demonstrated scattered neutrophils in the dermis. Skin biopsies were analysed using immunohistochemical detection of myeloperoxidase (visualised in red). Neutrophilic urticaria was defined as more than 25 extravasated neutrophils per five HPF (original magnification x400) (Toppe et al, 1998).

Abbrevations:
CSU - Chronic spontaneous urticaria
HPF - High power field
DAB - Diaminobenzidine

5.4 Discussion

5.4.1 The density and the cellular composition of the inflammatory infiltrate in CSU and UV

Our data suggest that the density of neutrophilic infiltrate differs between UV and ASU or CSU. Patients with UV demonstrate significantly higher numbers of extravasated neutrophils in skin biopsies compared to patients with ASU or CSU. A subset of CSU patients (63.6%) has evidence of neutrophilic urticaria in lesional biopsies from spontaneous weals. There is limited evidence for neutrophilic urticaria in two CSU patients with serum histamine-releasing activity but these data needs to be confirmed in larger studies.

Our data suggest the differences in the density and of the inflammatory infiltrate between CSU and UV and its variants which is in keeping with previously published findings. The composition of the perivascular infiltrate in CSU was studied in the previous work by Sugita et al (2000) and Toppe et al (1998). The association of neutrophil-predominant perivascular infiltrate with the histological diagnosis of UV was determined in a study by Lee et al (2007) using a multivariate analysis. More diffuse neutrophilia was also noted in hypocomplementaemic UV compared to normocomplementaemic variant (Davis et al, 1998).

Although our data and the previous work in this area suggest the increase in the number of neutrophils in UV compared to ASU or CSU, the histological variability within these groups has to be recognized. In UV patients, eosinophil-predominant inflammation can be occasionally observed in clinical practice. Also, the neutrophil counts in lesional skin biopsy in CSU patients with neutrophilic urticaria can be comparable to that in UV. These histological changes may apply only to a small patient subset and, therefore may not be detected when the whole group of CSU or UV patients is analysed. Further research in larger patient populations may provide new insights into histological heterogeneity within patient with CSU and UV.

The use of conventional haematoxylin and eosin staining and immunohistochemical detection of neutrophil myeloperoxidase enhances to the differential diagnosis of urticaria and UV which can be applicable in clinical practice. The detection of leukocytoclasis is likely to depend on the detection system. The signal to noise ratio may differ between conventional haematoxylin and eosin staining, immunohistochemistry for myeloperoxidase and, perhaps, immunofluorescence.

5.4.2 The histological pattern of neutrophilic urticaria in CSU

Neutrophilic urticaria is known as a histological pattern that occurs in patients with ASU, CSU and physical urticarias (Toppe et al, 1998). The frequency of neutrophilic urticaria in CSU was reported at 18% (Toppe et al, 1998). In our study, neutrophilic urticaria was noted in 63.6% of CSU patients. We used the same diagnostic criteria of 25 neutrophils per five HPFs as described by Toppe et al (1998). This is likely to reflect that CSU patients who are biopsied for clinical reasons have more severe disease than those in research studies. Furthermore, we illustrated that the intensity of neutrophilic infiltration may vary in CSU patients with histological evidence of neutrophilic urticaria. Therefore, the histological diagnosis of neutrophilic urticaria may depend on the threshold of neutrophil counts used as a diagnostic criterion. In our study we demonstrated that 45.5% of CSU patients had a dense neutrophilic infiltrate at more than 40 neutrophils per five HPFs. Further research into the definition and the diagnostic criteria for neutrophilic urticaria can optimize the diagnostic threshold for neutrophilic infiltrate for maximum diagnostic accuracy.

The observed difference in the numbers of extravasated neutrophil in skin biopsy specimens from CSU and UV patients raise the question as to whether perivascular neutrophilic infiltration and dermal neutrophilia may represent histological stages of the inflammatory process in CSU and UV as was suggested in previous publications (Winkelmann & Reizner, 1988; Llamas-Velasco et al, 2012; Peters & Winkelmann, 1985). Clinically, in cutaneous vasculitis, dermal neutrophilia is considered as a risk factor for systemic diseases (Carlson, 2010). Whether these associations exist in UV is yet to be established.

5.4.3 The strengths and limitations of this study

The strengths of the study include careful clinical selection of patients and a parallel use of both conventional haematoxylin and eosin staining and immunohistochemical detection of myeloperoxidase in lesional skin biopsies from the same patient. Also, a good inter-observer reliability was also a strength of the study.

However, several limitations of this study have to be acknowledged. Firstly, this study includes 43 patients with UV which may not be representative of all patients with UV. Therefore, the results have to be validated in larger patient datasets in the settings of a specialized dermatological centre, a multi-centre collaboration or using biobank research. This would increase the generalizability of the results. Also, patients with CSU and UV underwent lesional skin biopsy as a part of clinical care and therefore had only one biopsy, with unspecified timing of lesion development, which may have been a source of variation in the histological presentations in lesional skin biopsies in our study. A high rate of neutrophilic urticaria in CSU patients in our sample is likely to reflect a more severe disease in CSU patients who undergo skin biopsies for clinical reasons. Further studies in the diagnostic criteria for neutrophilic urticaria in prospective studies with the recruitment of consecutive patients is likely to result in more accurate estimate of the frequency of neutrophilic urticaria among CSU patients. Secondly, as all retrospective studies, this study has its inherent limitations of the incomplete data and a certain degree of variability in clinical approaches between the clinicians. Furthermore, we cannot exclude a referral bias and a selection bias in this study, further studies in different clinical settings would also increase the generalizability of the results.

5.4.4 Unresolved questions for future histological studies

This study poses new questions to be addressed in the pathophysiology of UV. Two main determinants of the skin inflammation in UV are the vessel damage and a participation of neutrophils mediating this damage. Immunohistochemistry staining for neutrophil myeloperoxidase employed in our study advances our understanding of the changes in neutrophil involvement in the vasculitic process in UV. The histological pattern of early leukocytoclastic changes has a definitive thread-like appearance which may suggest the neutrophil extracellular traps as a potential mechanism for these findings (Brinkmann & Zychlinsky, 2007). Neutrophil extracellular traps were implicated in the pathophysiology of autoimmune small-vessel vasculitis (Kessenbrock et al, 2009) and may be worth exploring in UV. Visualizing the neutrophil extracellular traps can be achieved by a combined immunostaining for nuclear (histone) and granular (neutrophil myeloperoxidase) components (Brinkmann et al, 2010). In our study, the detection of the nuclear component of leukocytoclasis was carried out by haematoxylin and eosin staining. In future studies, a combination of fluorescence dyes for the detection of DNA and an immunofluorescence detection of myeloperoxidase may improve the detection of leukocytoclasis in UV.

Furthermore, vessel changes during the disease progression are of great importance for defining UV (Jones & Eady, 1998) and need to be better understood in the context of dysregulated neutrophil-endothelial interactions (Hu et al, 2011) as a possible mechanism in UV. This can be achieved by combining the immunohistochemistry staining for neutrophil myeloperoxidase with endothelium-specific markers (Haller et al, 1998).

Mechanisms of the persistence of skin inflammation in severe urticaria represent another important question of the pathophysiological significance arising from our study. The plausible mechanisms for the persistence of the inflammation could be a deficiency in TGF-β (Serhan & Savill, 2005), dysregulation of regulatory T cells (Fujio et al, 2012; Nathan & Ding, 2010), defective clearance by macrophages (Nathan & Ding, 2010) and, possibly, persistent stimulation with functional autoantibodies. The persistence of skin inflammation in severe urticaria and UV may result from the persistent stimulation or a deficiency in mechanisms of tissue clearance and repair (Serhan & Savill, 2005; Nathan, 2002).

In future prospective studies, an introduction of a powerful DNA microarray approach to study systems biology in the skin of UV may enhance our understanding of the disease pathophysiology. As an example, the potential insight that can be gained by applying systems biology research to the skin inflammation has been recently demonstrated by a network-based study in late-phase allergic reaction (Bensom et al, 2006). The prospect of using the DNA microarrays to decipher the complexity of the pathophysiological pathways in UV is fascinating.

CHAPTER 6

Discussion

"We shall not cease from exploration, and the end of all our exploring will be to arrive where we started and know the place for the first time"

—T.S. ELIOT

Experimental work carried out in this thesis contributes to two main aspects of the pathophysiology of CSU: biochemical and histopathologic characteristics of the skin inflammation and the clinical course of CSU and predictors of disease severity and persistence, together with pathophysiological phenotyping based on the presence of functional autoantibodies and the aberrations of basophil biology in CSU. The experimental data in this thesis were collected as a result of three research projects: 1) cutaneous microdialysis studies in CSU; 2) a prospective observational study of the pathophysiological phenotypes in CSU and, as a method development part of this project, imaging flow cytometry studies in healthy subjects and 3) a retrospective study re-evaluating the diagnostic histological criteria for urticarial vasculitis. First, the results of the microdialysis study suggested an increased histamine concentration in the upper dermis of patients with CSU which was shown to correlate with disease activity. There was limited experimental evidence indicating a slow low-grade histamine release underlying skin autoreactivity in CSU.

Secondly, in a prospective observational study, the pathophysiological phenotyping based on the presence of histamine-releasing autoantibodies and the patterns of basophil releasability revealed three pathophysiological phenotypes in CSU including responders, non-responders to anti-IgE stimulation and a subset of patients with total cellular histamine below the level of detection by spectrofluorimetry. Patients with the latter group had a more severe CSU than responders or non-responders to anti-IgE stimulation. In addition, CSU patients were classified on the basis of the presence or absence of serum histamine-releasing activity. Patients with serum histamine-releasing activity were characterised by a more severe disease. In our study, the clinical course of CSU observed in patients included a clinical improvement and a persistent CSU. Baseline UAS7 was demonstrated to be a predictor of a persistent course of disease. The use of different gating strategies (CCR3+CD123+; CCR3+CD63+; CD63+CD203c+) for flow cytometric basophil enumeration in the same sample from each CSU patient resulted in statistically significant differences in absolute counts depending on the basophil phenotype. Absolute counts for CCR3+CD63+ basophils were significantly higher compared to CCR3+CD123+ or CD63+CD203c+ basophil subpopulations in the same peripheral blood sample from each CSU patient suggesting that absolute basophils counts depend on the gating strategy used to define peripheral blood basophil phenotype in CSU patients. Absolute basophil counts measured by microbead technology showed no association with disease severity or persistence.

Following this, imaging flow cytometry studies in healthy subjects demonstrated that the differences in the basophil detection between different gating strategies (CD63+CD203c+ versus CCR3+CD63+) may arise from biological (basophil phenotypic variation, a relative contribution of basophil subpopulation with surface alterations), technical (pre-analytical handling at different temperatures) reasons as well as effect of confounding factors (platelet-basophil adhesion). Basophil subpopulation with surface alterations was mostly detected in CCR3- or CD63-based gates. The data from one healthy subject suggests that basophils with surface alterations might have higher expression of CCR3, CRTH2 and CD69 but these data need to be verified in future studies on healthy subjects stratified for atopy.

In haematoxylin and eosin- stained skin biopsy specimens, there were increased numbers of neutrophils per HPF in UV patients compared to ASU (Mann-Whitney U test, p=0.062) or CSU patients (Mann-Whitney U test, p=0.0002). On immunohistochemistry, the number of myeloperoxidase-positive nucleated cells (neutrophils) per HPF was also higher in skin biopsies from UV patients than in ASU (Mann-Whitney U test, p=0.0027) or CSU patients (Mann-Whitney U test, p=0.0001). The numbers of eosinophils per HPF in the histological skin specimens did not differ between UV and ASU or CSU. In our study, neutrophilic urticaria was noted in 63.6% of CSU patients including two CSU patients with serum histamine-releasing activity.

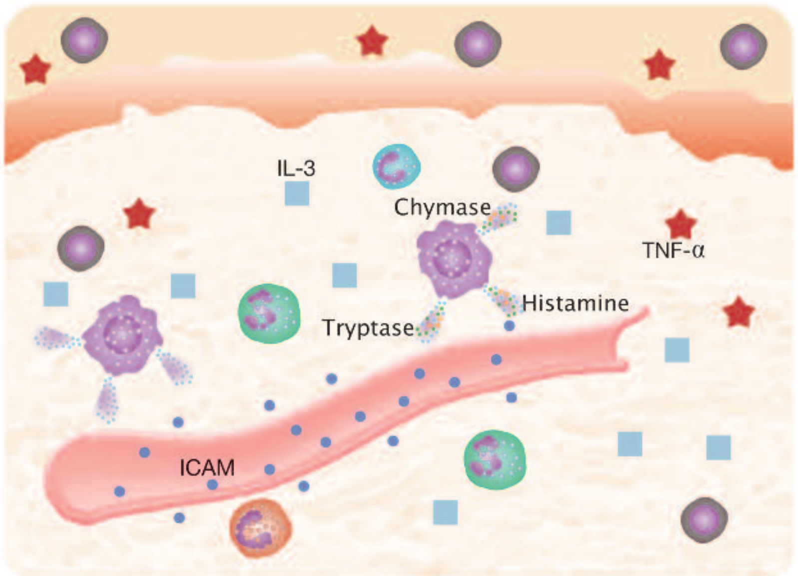
Finally, in the retrospective histological study, haematoxylin and eosin-stained staining revealed increased numbers of neutrophils per high power field in UV patients compared to ASU and CSU patients. These data were confirmed on immunohistochemistry, which demonstrated higher numbers of myeloperoxidase-positive nucleated cells (neutrophils) per high power field in UV patients compared to ASU and CSU patients. Of interest, the numbers of eosinophils per high power filed in skin biopsies from UV patients did not differ from patients with ASU and CSU. In our study, we showed a high frequency (63.6%) of neutrophilic urticaria in lesional skin biopsy in the population of CSU patients who underwent lesional skin biopsy for clinical reasons.

Several questions of pathophysiological significance arose from the cutaneous microdialysis study. Firstly, an increased histamine concentration in the extracellular space in the dermis of CSU patients was consistent with the concept of the minimal persistent inflammation (Figure 49) in CSU. Several factors may account for an increased histamine concentration in the dermis including abnormal histamine metabolism, increased local blood flow or enhanced cellular releasability of skin mast cells and basophils (Figure 50). The fact of correlation between the extracellular concentration of histamine and severity scores for itching and wealing based on visual analogue scales pointed towards the possible role of the dermal histamine concentrations as skin biomarker of underlying inflammation in CSU and therefore, to the potential clinical applications of cutaneous microdialysis in CSU. Research into H4 receptors suggested a role of histamine in inducing eosinophil chemotaxis through H4 histamine receptors (Ling et al, 2004). It was unknown whether these effects of histamine on the influx of proinflammatory cells was relevant in CSU. If proved so, then these proinflammatory effects of histamine could be potentially targeted with H_4 receptor antagonists.

Furthermore, an observation of a slow low-grade histamine release underlying serum autoreactivity in CSU invited several pathophysiological implications. By analogy, similar pattern of histamine release was noted in the previous studies in the late- phase allergic reactions by using skin chamber technique. The authors noted about the possibility of a distinct mode of histamine release in skin mast cells in the late-phase allergic reaction (Zweiman, 1998). In the work by Dvorak (1991), two modes of histamine release were described for human basophils: anaphylactic and piecemeal degranulation (Figure 51). Piecemeal degranulation was known as a feature of various inflammatory conditions and was characterized by a gradual vesicle traffic towards the cell surface. Therefore, piecemeal degranulation of skin mast cells and basophils could be a potential explanation of the observed pattern of histamine release in patients with a positive autologous serum skin test.

Figure 49. Minimal Persistent Inflammation of Skin in CSU

A. Schematic Diagram of Minimal Persistent Inflammation in CSU



B. Functional Effects of Minimal Persistent Inflammation in Skin in CSU

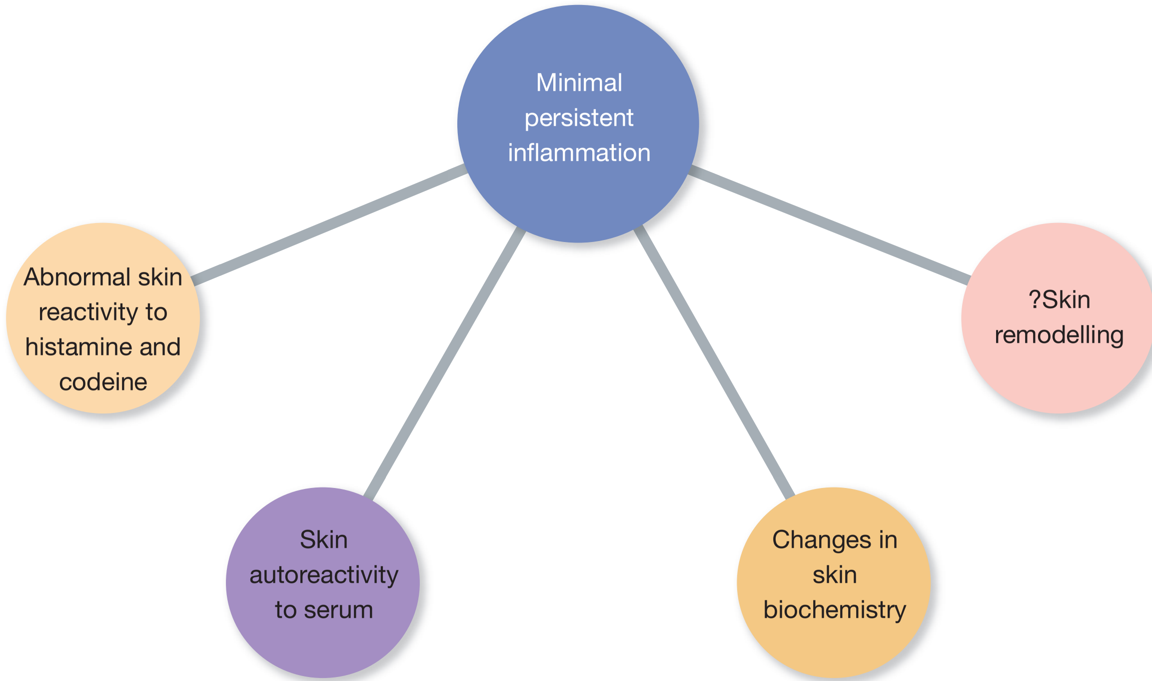


Figure 49. In CSU, minimal persistent inflammation can be defined as involvement into subclinical inflammation of visually unaffected skin. Immunohistochemical analysis of skin biopsy specimens from the areas of visibly unaffected skin in CSU patients showed over-expression of adhesion molecules (VCAM, ELAM), mast cell proteases (tryptase and chymase) and cytokines (TNF-α) (Cassano et al, 2002). This is schematically represented in Figure 49A. Minimal persistent inflammation is known to modify function of the affected organ. Theoretically, in CSU, minimal persistent inflammation could be linked to abnormal skin reactivity to autologous serum and codeine as well as to changes in skin biochemistry (histamine and tryptase levels) (Figure 49B). Some of the pro-inflammatory agents (VEGF, MMP-9, BDNF) found in the skin inflammation in CSU could be potentially related to skin remodelling but whether or not this has clinical relevance in CSU remains to be established.

Abbrevations:
MMP-9 - Matrix metallopeptidase 9
BDNF - Brain-derived neurotrophic factor
CSU - Chronic spontaneous urticaria
ICAM - Intracellular adhesion molecule
IL-3 - Interleukin 3
TNF-α - Tumor necrosis factor-alpha
VCAM - Vascular cell adhesion molecule
VEGF - Vascular endothelial growth factor

Figure 50. Key Determinants of Histamine Concentration in Skin

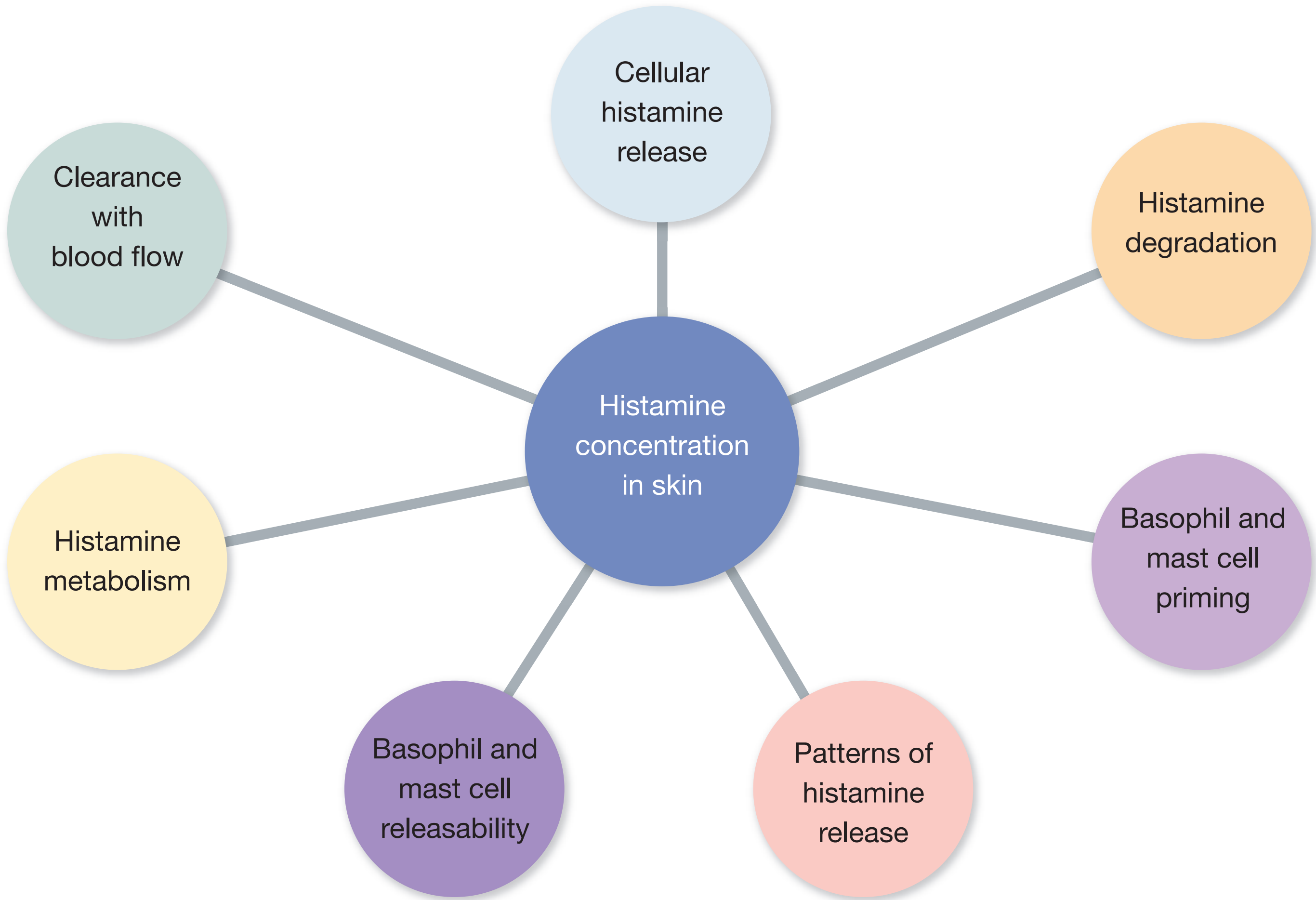


Figure 50. Skin histamine release depends on several factors including effector cell releasability (Atkins et al, 1990) and their sensitivity to the antigen and other stimuli (Atkins et al, 1990). Histamine recovery by cutaneous micrdialysis is affected by its clearance with local blood flow (Peterson, 1998). Maximum histamine release depend on basophil priming (Gentinetta et al, 2011) and may vary for different modes of histamine release in skin. Altered histamine metabolism may also account for differences in histamine concentration in skin (Greaves et al, 1981).

Figure 51. Mechanisms of Basophil Degranulation

Figure 51. The scheme of anaphylactic and piecemeal degranulation of human peripheral blood basophils (adapted from Dvorak, 1991). Following allergen stimulation, anaphylactic degranulation begins with the fusion of the granules and the formation of a degranulation sac (indicated by arrows) lled with granules in the cytoplasm followed by its fusion with the cytoplasmic membrane and extrusion of the granules into the extracellular space (MacGlashan Jr. 2010) . In contrast, piecemeal degranulation is characterised by budding of the vesicles from the granules and a gradual transport of the vesicles towards the cell surface followed by a release of their content into the extracellular space. The key feature of piecemeal degranulation of human basophils is the presence of empty granule chambers (indicated by arrows) in their cytoplasm.

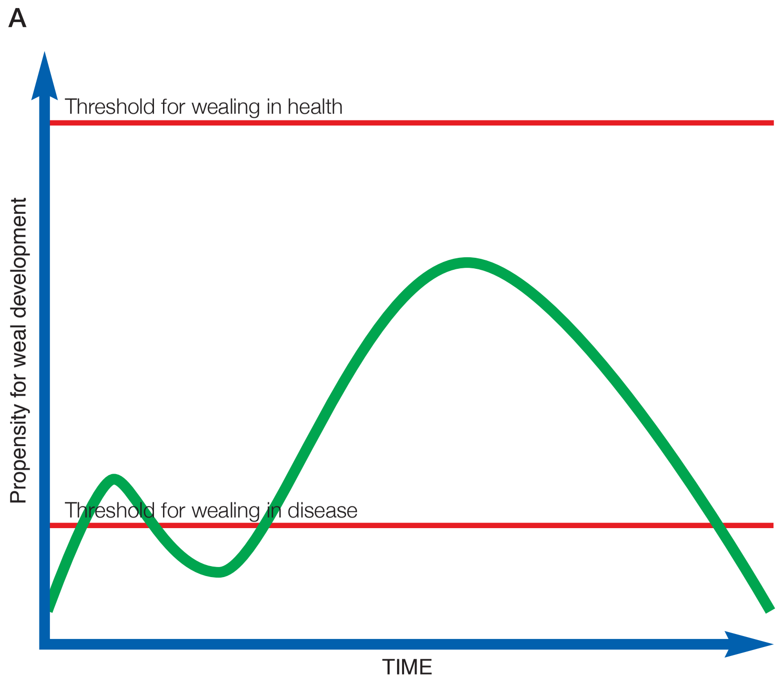
On the other hand, a slow low-grade histamine release may occur in a positive autologous serum skin test but was thought to be an unlikely solely mechanism mediating its clinical expression as indirectly confirmed by a recent observation of lack of inhibition of serum autoreactivity by antihistamines (Asero et al, 2009). Alternatively, serum autoreactivity can be mediated by other mediators and cytokines such as, for example, platelet-activating factor or vascular endothelial growth factor (Kay et al, 2014b). Other cell types may also mediate serum autoreactivity in CSU. A positive autologous serum skin test was noted to be similar to the late-phase allergic reaction. However, different kinetic of skin response to autologous serum suggests other potential explanations. For example, skin pathology of a positive autologous serum skin test is suggestive of neutrophil-predominant reaction (Grattan et al, 1991). Autologous serum is well known stimuli used to induce neutrophil chemotaxis (Paulsson et al, 2010, Follin P, 1999). In the work of Toppe et al (1998), it was mentioned that neutrophils could mediate some immediate manifestations of physical urticarias. It is tempting to suggest that an intradermal injection of autologous serum in CSU may induce a neutrophilic response with a time course that can be in keeping with the dynamics of clinical signs of a positive autologous serum skin test. Neutrophils were known to be capable of regulating vascular permeability (Wedmore & Williams, 1981) by causing vessel leakage through release of chemokines and reactive oxygen species and the products of arachidonic acid (DiStasi & Ley, 2009).

Furthermore, cutaneous microdialysis research opens up the perspectives for prospective studies in CSU looking at skin threshold for wealing (Figure 52) in susceptible individuals. Acute wealing to NSAIDs, enhanced skin response to skin testing with codeine or a positive autologous serum skin test could precede the onset of urticaria or persist in the remission of the disease. Therefore, prospective studies in skin reactivity in patients with urticaria and in predisposed individuals may help elucidate the mechanisms predisposing to CSU.

The results of a prospective observational study in CSU and preliminary imaging flow cytometry experiments suggested basophil heterogeneity in health and disease. Basophils were known to have a graded response to stimulation with anti-IgE and fMLP, the possibility of associated phenotypic changes during this graded response was discussed in the literature. A low-grade anti-IgE stimulation was noted to induce CD69 expression and eotaxin-induced migration of human basophils. Taken together with the results of our imaging flow cytometry studies, it is conceivable that the subthreshold stimulation via the high affinity IgE receptor might contribute to the phenotypic changes in peripheral blood basophils (Figure 53). This may have clinical implications in the context of inflammatory conditions like asthma or CSU. Subthreshold stimulation may render basophils chemotactic while reaching the source of the chemotactic gradient causes them to degranulate (Figure 54). Whether the morphology of basophils with surface alterations is a feature of their activation needs to be further studied. However, taking into account an increased expression of CD69 on peripheral blood basophils (Vasagar et al, 2006) in CSU and in basophils in bronchoalveolar lavage in asthma (Yoshimura et al, 2002), the contribution of basophil chemotaxis to the pathophysiology of CSU warrants further research. Moreover, patients with polymorphism of CRTH2 in CSU were shown to require higher doses of antihistamine therapy (Palikhe, 2009). This is another piece of evidence that puts the molecules involved in basophil activation, including chemotaxis, in the focus of research in further studies.

Targeted treatment is a cornerstone of personalized medicine. Patient stratification is an essential condition for successful development of personalized treatment approaches. Studies in this thesis addressed the issue of the patient stratification by the clinical course of the disease, the pathophysiological phenotyping in the prospective study in CSU as well as histopathological presentations in the retrospective evaluation of histopathological criteria for the diagnosis of urticarial vasculitis. The perspective of phenotype-guided therapy would facilitate the development of an individual treatment plans for patients with distinct abnormalities of basophil releasability, the presence of serum histamine-releasing activity or the presentations of neutrophilic urticaria in lesional skin biopsies. At present, there is limited evidence for higher efficacy of certain treatments (ciclosporin, plasmapheresis) in CSU patients with serum histamine-releasing activity (Grattan et al, 2000; Grattan et al, 1992). However, definitive comparative studies for immunomodulatory and biological treatments in the defined pathophysiological phenotypes would help incorporate the pathophysiological biomarkers into a routine work-up in patients with CSU for an informed clinical decision on the phenotype-targeted treatment.

Figure 52. Skin Wealing Thresholds in Health and CSU (modified from Grattan, 2010)



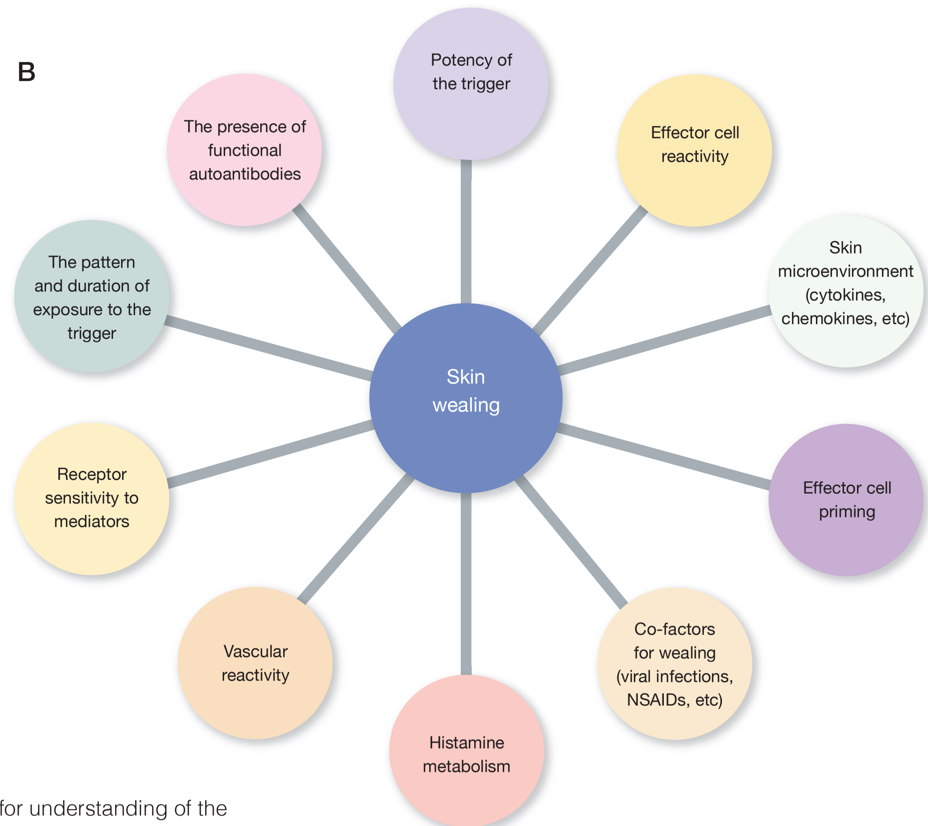


Figure 52. The concept of skin threshold for wealing in health and urticaria (Figure 52A) was suggested by Grattan for understanding of the development of urticaria (Figure 52A). There is limited evidence suggesting that some individuals may be predisposed for urticaria. Asero et al showed that acute urticaria due to NSAID intolerance may precede the development of CSU (Asero et al, 2003). Also, Cohen & Rosenstreich, (1986) showed that patients with active wealing and individuals with a past history of urticaria without any present wealing were more sensitive to skin testing with codeine than patients with asthma or healthy individuals (Cohen & Rosenstreich, 1986). These observations demonstrate increased sensitivity to triggers (NSAIDs, codeine) in individuals prone to urticaria, but without any active symptoms, suggesting a lower threshold for wealing in the predisposed individuals. Putative factors contributing to fluctuating in ski threshold for wealing could relate to the exposure to non-specific trigger (the potency of the trigger, the presence of co-factors, the pattern and duration of the exposure), sensitivity and reactivity of effector cells and receptors for proinflammatory mediators, skin microenvironment (cytokine expression) and biochemistry (histamine metabolism). It remains unclear whether functional autoantibodies can cause wealing, prime effector cells for enhanced response to relevant triggers or both and under which conditions these effects can occur in CSU (Figure 52B).

Abbrevations:
CSU - Chronic spontaneous urticaria
NSAID - nonsteroidal anti-inflammatory drug

Figure 53. Chemotaxis versus Degranulation of Human Basophils

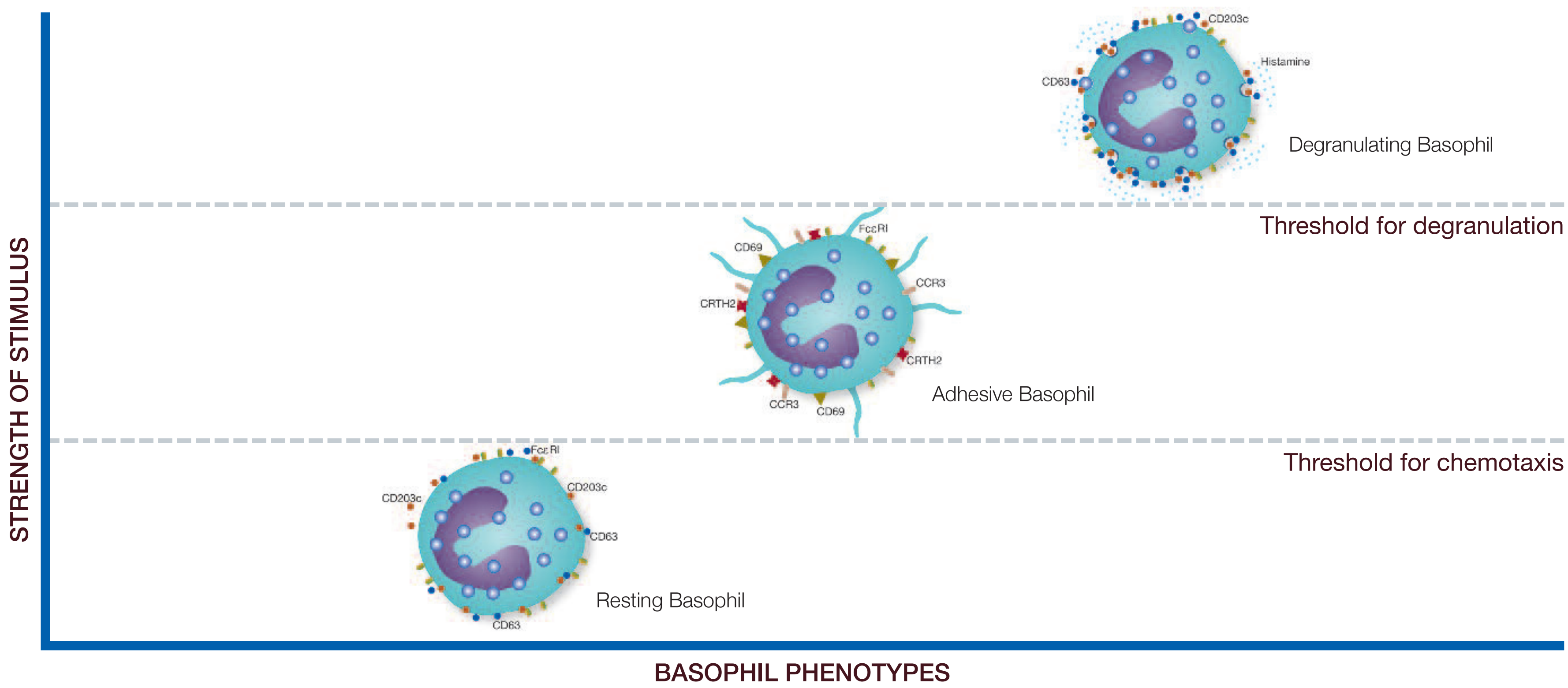


Figure 53. For some stimuli (anti-IgE antibodies, fMLP), phenotypic changes of human basophils may depend on the strength of the stimulation. Low grade stimulation may result in inducing a chemotactic phenotype in human basophils while high intensity stimulation would lead to basophil degranulation. As basophil with a chemotactic phenotype migrates into the tissue, a concentration gradient for these stimuli may increase causing basophil degranulation at the site of the local inflammation. These phenotypic changes may represent a graded process of basophil activation depending on the strength of the stimulation. There may be a threshold control or a signaling machinery in basophils that may help differentiate between the strength of the stimuli to evoke an appropriate response. Targeting chemotactic basophil subpopulations can be a promising strategy for early therapeutic intervention in allergic and chronic inflammatory conditions characterized by marked local inflammation.

Abbrevations:
fMLP - Formyl-Methionyl-Leucyl-Phenylalanine
CRTH2 - Chemoattractant receptor-homologous molecule expressed on Th2 cells
CCR3 - CC-chemokine receptor

Figure 54. Schematic Representation of Basophil Chemotactic Events in Skin Inflammation

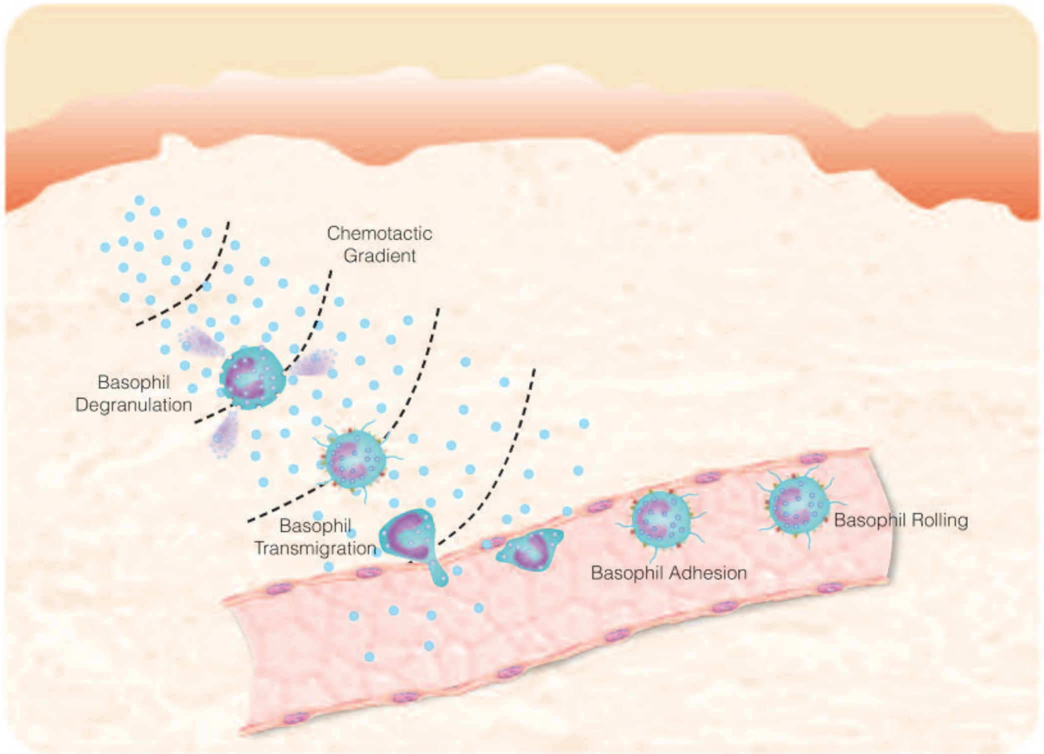


Figure 54. Basophil accumulation may contribute to skin inflammation in CSU. Basophils are capable of directed locomotion (chemotaxis) under chemotactic stimulation (Suzukawa et al, 2006). Some of the stimuli (anti-IgE or fMLP) may induce basophil degranulation or basophil migration depending on the concentration of the stimulus. For example, low-grade FcεRI cross-linking enhances basophil migration (Suzukawa et al, 2007). The concentration-dependent effect of stimuli on basophil function may be important for an induction of basophil chemotaxis at lower concentrations in the bloodstream but causing basophil degranulation at higher concentrations at the tissue site. Cytokine priming (IL-3, GM-CSF, IL-5) is known to regulate basophil chemotaxis. Thus, IL-3 exposure is crucial for basophil transmigration through the baseline membrane (Suzukawa et al, 2006) which may suggest the stimulating effect of skin microenvironment (IL-3, TNF-α) in CSU (Hermes et al, 1999).

Abbrevations:
CSU - Chronic spontaneous urticaria
GM-CSF - Granulocyte macrophage colony-stimulating factor
TNF-α - Tumor necrosis factor α

An observation of several aspects of abnormal basophil releasability to the stimulation with anti-IgE in different pathophysiological subsets in CSU calls for future studies into basophil activation by anti-IgE and other stimulu as well as non-specific basophil hyperreleasability to delineate the basophil activation pathways affected by the disease. Effects of different patterns of basophil pre-stimulation with serum histamine-releasing factors in CSU may define basophils responses to the subsequent stimulation. Further research into basophil responsiveness to stimuli after a prolonged stimulation with anti-FcεRIα autoantibodies and other stimuli may provide more valuable information related to the pathophysiology in CSU. This data are in keeping with the recent tendencies in the research into allergic diseases suggesting the use of prolonged, continuous or repeated allergen challenge to model the events occurring *in vivo* in the natural course of the disease (De Bruin-Weller et al, 1999; Schultze et al, 2012; Ong et al, 2005).

Finally, research into histopathological staging in CSU and urticarial vasculitis brought up new questions about the pathophysiology and the natural history of these two conditions. It is of paramount interest to look at the mechnanisms underlying leukocytoclasis in urticarial vasculitis with the formation of the neutrophil extracellular traps (Figure 55) being one of the possibilities. Also, the perspective of targeted treatment to the histological presentations in lesional skin biopsies was explored in the work of Criado et al (2008) and would highlight the future directions for this histological research in CSU and urticarial vasculitis.

In general, the field of urticaria research and clinical practice is witnessing the advent of diagnostic, therapeutic innovations and awaiting personalised medicine. This will inevitably re-design our current approaches to patient work-up and management. Research into urticaria is on the verge of an introduction of new powerful approaches like systems biology, biobank research and pharmacogenetics which will enhance our capabilities for identifying and targeting molecular defects in the disease with innovative and individualized therapeutic approaches. These changes are on the horizon and make urticaria a fascinating area of research and clinical practice for years to come!

Statistical statement

For this thesis, the data analysis was applied to three interlinked research projects including a microdialysis study in CSU patients and healthy subjects ***(Chapter 2)***, a prospective observational study in CSU patients ***(Chapter 3)*** and a retrospective histological study in patients with CSU and UV ***(Chapter 5)***.

Throughout the thesis, data were visualized by scatterplots or individual dot plots and summary statistics of the data were presented by medians and interquartile ranges.

For the microdialysis study ***(Chapter 2)***, the research hypothesis stated that weal formation, as assessed by an experimental model of ASST, was mediated by circulating serum factors, aberrant skin mast cell releasability and altered skin microenvironment. To test this hypothesis, we firstly addressed the statistical question as to whether there is a difference in the baseline concentrations of histamine, tryptase and IL-6 in skin dialysates between CSU patients and healthy subjects. For this analysis, we employed a non-parametric Mann-Whitney U test to compare the distribution of the variables between the two groups. A non-parametric test was chosen due to the non-normality of the distributions and the sample size. Correlation analysis between two variables was carried out using Pearson's correlation coefficient for variables in which a linear relationship was demonstrated from the scatterplot and by Spearman correlation coefficient for variables with a non-linear relationships.

To assess the importance of serum histamine-releasing factors, we carried out a subgroup analysis of CSU patients stratified by serum histamine-releasing activity (HRA) and healthy subjects. For a comparison between three groups (CSU patients with or without serum HRA and healthy controls), we employed Kruskal-Wallis test. This test was selected as it is a non-parametric ANOVA that can be used to compare a non-normally distributed variable between three groups. For variables demonstrated a statistically significant difference by Kruskal-Wallis test, we applied Mann-Whitney U test for pairwise comparisons to evaluate which two of three groups were different for a given variable. We chose Mann-Whitney U test for the reasons given above.

To assess the noise element in this study, we decided to compare the variance in the outcome due to the patients, that is the between patient variation, and the variance in outcome due to the order of measurement, that is the within patient variation. If the measurement is unreliable then a large amount of variation will be the within patient variation whereas if the measurement is reliable then only small amount of variation will be due to the within patient variation. In order to estimate these parameters, we applied a two-way ANOVA to estimate the variability due to the two factors (patients' ID and the order of measurements). This was the most appropriate way to measure the variability due to these two factors. In general, two-way ANOVA test is a parametric test which is used for the assessment of the effect of two factors on an outcome. However, for our study, we chose this test because i) it estimates the parameter of interest; and ii) there is no clear non-parametric alternative for the estimation of variability due to each factor. Therefore, we appreciate the limitations of this approach, as the assumption of normality may not hold for our dataset and hence the p-values may not be correct. However, the definition of the variation terms does not depend on the assumption of normality and is can be used regardless of the distribution. The rationale for using this method lied in the fact that we were interested in a relative contribution of variance due to the disease process or the measurement error rather than an exact variance estimate. The interpretation of the results warrants a brief comment that the between-patient variability was higher than the inter-patient variability due to the measurement error although the exact contribution of each factor cannot be established in the dataset of this sample size.

For the observational study ***(Chapter 3)***, we hypothesized that disease severity and a persistent course of the disease in CSU patients are associated with serum histamine-releasing activity, aberrant basophil releasability to anti-IgE stimulation and phenotypic changes in peripheral blood basophils. To test this hypothesis, we first tabulated the number of patients according to two parameters (serum HRA and basophil releasability to anti-IgE stimulation). For this analysis, we categorized these variables (Serum HRA: positive or negative; Anti-IgE-induced BHR: Basophil responder, basophil non-responder, total cellular histamine below the LOD). We employed a chi-squared test to compare the patient distribution between these categories. Following this Kruskall-Wallis test (a nonparametric ANOVA test) was chosen to compare clinical scores and biomarkers between CSU patients across the anti-IgE-induced BHR categories because this test does not assume a normal distribution of the data and is suitable for comparison of three independent groups. For example, we used Kruskall-Wallis test to compare the difference in UAS7 score between three groups according to anti-IgE induced BHR (basophil responders, basophil non-responders and those with total cellular histamine below the LOD). If the difference was statistically significant by Kruskall-Wallis test, this justified further use of Mann-Whitney U test for a pairwise comparisons between the groups in order to detect which two groups differ for UAS7. For comparison of UAS7 between CSU patients with or without serum HRA, we chose Mann-Whitney U test as a non-parametric test for comparison of two independent groups based on small sample size of the study and, therefore, a non-normal distribution of the data. For analysis of longitudinal data, we coded the patients with persistent disease if their UAS7 at visit 3 was greater or equal than at the baseline visit. Conversely, patients with baseline UAS7 greater than that at the visit 3 were classified as patients with improving disease. Correlation analysis was carried out by Spearman's correlation coefficient when a linear relationship between the variables could not be ascertained.

Following this, we employed ROC (receiver-operating curve) analysis to estimate the cut-off UAS7 score with optimal sensitivity and specificity to differentiate between the patients with persistent or improving disease. We appreciate the small size of our sample and, therefore, the limitations of this approach such as uncertainty in the cut-point in a small sample size. Further comparisons between the patient groups with persistent or improving CSU were carried out using Mann-Whitney U test as a non-parametric test for comparing numeric variables of non-normal distribution between two independent groups. To compare absolute basophil counts in peripheral blood of CSU patients as determined by three gating strategies on the same sample from each patient, we employed Wilcoxon signed-rank test based on the matched samples (same patient samples) used for analysis and a non-normal distribution of absolute basophil counts in the patient group of this size. The Wilcoxon test was chosen to account for the fact that the three samples were measured on the same individuals. An alternative approach would have been to have used a non-parametric two-analysis of variance (with factors for ID and test used) however, this would have tested for any difference between the three tests whereas we were interested in how the tests compared to each other.

The hypothesis of the retrospective histological study ***(Chapter 5)*** stated that there was a difference in the density and the composition of the inflammatory infiltrate in the dermis between patients with ASU and CSU and between those with CSU and UV. To test this hypothesis, we compared the numbers of eosinophils and neutrophils per HPF in the histological skin specimens between patients with ASU and CSU and between CSU and UV. We used Mann-Whitney U test to compare the neutrophil counts between two independent groups (ASU vs CSU; CSU vs UV). The small sizes of patient groups could not justify a normal distribution within the groups, therefore, we used Mann-Whitney U test for a non-parametric analysis of two patient groups.

For inter-observer agreement, we employed inter-class correlation to detect the difference and its statistical significance for the cell counts carried out on the same histological specimens by two independent observers. The choice of inter-class correlation for this analysis was made by exclusion of the alternatives such as Kappa coefficient or Bland-Altman method. Bland-Altman test is used for comparison of two different approaches to measurement which is not strictly applicable for our study where we compared the performance of two observers using histological samples analysed by the same technique. Kappa-coefficient is used for inter-rater agreement for a categorical variables which could not be used for numeric variables such as cell counts in our study. Therefore, inter-class correlation coefficient was used as the appropriate test for inter-rater agreement for comparison of numeric variables such as cell counts per HPF in our histological study whilst we acknowledge that this assumes a normal distribution of the outcome for confidence intervals, the estimation of the ICC itself does not make this assumption.

Throughout the thesis, the data are presented as medians and interquartile interval. The distribution of the data in our studies was considered non-normal due to small sample sizes. The limitations of the small sample size studies were underscored to avoid an over-statement of the results.

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Zuberbier, T., Asero, R., Bindslev-Jensen, C., Canonica, G.W., Church, M.K., Gimenez-Arnau, A.M., Grattan, C.E.H., Kapp, A., Maurer, M., Merk, H.F., Rogala, B., Saini, S., Sanchez-Borges, M., Schmid-Grendelmeier, P., Schunemann, H., Staubach, P., Vena, G.A., Wedi, B.; Dermatology Section of the European Academy of Allergology and Clinical Immunology; Global Allergy and Asthma European Network; European Dermatology Forum; World Allergy Organization (2009) EAACI/GA^{2}LEN/EDF/WAO guideline: management of urticaria. *Allergy,* 64, 1427-1443.

Zuberbier, T., Asero, R., Bindslev-Jensen, C., Canonica, G.W., Church, M.K., Gimenez- Arnau, A., Grattan, C.E., Kapp, A., Merk, H.F., Rogala, B., Saini, S., Sanchez-Borges, M., Schmid-Grendelmeier, P., Schunemann, H., Staubach, P., Vena, G.A., Wedi, B., Maurer, M. (2009). EAACI/GA(2)LEN/EDF/WAO guideline: definition, classification and diagnosis of urticaria. *Allergy,* 64, 1417-1426.

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Zweiman, B. (2006) Skin chamber techniques. In: J.Serup, G.B.E. Jemec, Grove G.L. *Handbook of Non-Invasive Methods and the Skin.* Boca Raton, CRC press, pp. 433-441.

Zweiman, B., Valenzano, M., Atkins, P.C., Tanus, T., Getsy, J.A. (1996) Characteristics of histamine-releasing activity in the sera of patients with chronic idiopathic urticaria. *J Allergy Clin Immunol,* 98, 89-98.

References

Appendix 1. Ethics Approval Documents for the Research Projects

Appendix 1. BSF Grant Award Letter and Ethics Committee Approval for the Retrospective Study in Diagnostic Criteria for Urticaria and Urticarial Vasculitis

BRITISH SKIN FOUNDATION

Dr Clive Grattan
Dermatology Department
Norfolk & Norwich University Hospitals NHS Foundation Trust
Colney Lane
Norwich
Norfolk
NR4 7UY

23rd July 2009

Project: S527 **Amount: £9,854**

Title: Re-evaluation of diagnostic criteria for chronic ordinary urticaria and urticarial vasculitis: a new look at mast cell signal transduction and autoimmune chronic urticaria

Dear Dr Grattan,

It gives me great pleasure on behalf of the Trustees of the British Skin Foundation to inform you that your application for a 2009 British Skin Foundation Small Grant has been successful.

The grant can be claimed with a single invoice by the finance department of your research institute. It must be activated and the funds claimed by **Friday 6th August 2010**. Any funds not claimed by this date will be redeposited back into the BSF's reserves.

Please note that the project amount quoted above is final and can not be increased for any reason whatsoever.

Where appropriate and before proceeding any further, we ask that you submit written evidence that you have received ethical committee approval for the work.

The British Skin Foundation should be informed of any papers or public presentations resulting from the work and duly credited. The Foundation will also require a written scientific report of approximately 2 sides of A4 at the end of the project as well as 200-word summary suitable for a lay audience. We may also request a mid-term lay report for fundraising purposes.

Once again congratulations on your successful small grant application. Please complete and return the enclosed response form. Should you need to contact the office of the British Skin Foundation, please quote your assigned project number as written at the top of this letter.

Yours sincerely,

Matthew Patey
Chief Executive
British Skin Foundation

4 Fitzroy Square
London W1T 5HQ
Tel: 020-7391 6341
Fax: 020-7391 6099
bsf@bad.org.uk
www.britishskinfoundation.org.uk

East Norfolk and Waveney Research Governance Committee **NHS**

Please reply to: Research Governance Committee Office
Research and Development Department
Level 3, East Block, Room 032
Norfolk & Norwich University Hospitals NHS Foundation Trust
Colney Lane
Norwich
NR4 7UY
Direct Dial: 01603 287408
Internal: 3408
Direct Fax: 01603 289800
e-mail: rdoffice@nnuh.nhs.uk
Website: www.norfolkhealthresearch.nhs.uk

Dr Clive Grattan
Consultant Dermatologist
Norfolk & Norwich University Hospital
Colney Lane
Norwich
NR4 7UY

15 June 2009

Dear Dr Grattan

Re: 2009DERM05L (69-04-09) Re-evaluation of diagnostic criteria for chronic ordinary urticaria and urticarial vasculitis: a histological audit.

Following confirmation of a favourable Ethical opinion I am pleased to confirm that your project has been given full approval from the East Norfolk and Waveney Research Governance Committee and Research Management Team and you may start your research.

Please note that this approval applies to the following sites:

- Norfolk & Norwich University Hospitals NHS Foundation Trust

I have enclosed two copies of the Standard Terms and Conditions of Approval. Please sign and return one copy to the Research Governance Committee office. Failure to return the standard terms and conditions may affect the conditions of approval.

Please note, under the agreed standard terms and conditions of approval you must inform this Committee of any proposed changes to this study and to keep the Committee updated on progress.

If you have any queries regarding this or any other study please contact Julie Dawson, Research Governance Administrator, at the above address. Please note, your reference number is **2009DERM05L (69-04-09)** and this should be quoted on all correspondence.

The Committee would like to take this opportunity to wish you every success with this project.

Yours sincerely

Dr Richard Reading
Chair
Consultant Paediatrician – NHS Norfolk

Encs – Standard terms and conditions
Guidance for screening of patient notes

Ethics Committee Approval for the Prospective Study in Chronic Urticaria

East Norfolk and Waveney Research Governance Committee

Please reply to: Research Governance Committee Office
Research and Development Department
Level 3, East Block, Room 032
Norfolk and Norwich University Hospital NHS Trust
Colney Lane
Norwich
NR4 7UY

Direct Dial: 01603 287408
Internal: 3408
Direct Fax: 01603 289800

e-mail: rdoffice@nnuh.nhs.uk
Website: www.norfolkhealthresearch nhs uk

Dr Clive Grattan
Dermatology Department
Norfolk & Norwich University Hospital
Colney Lane
Norwich
NR4 7UY

06 March 2008

Dear Dr Grattan

Re: 2008DERM01L (21-03-08) Pathophysiological subtypes in chronic ordinary urticaria and their biomarkers: a prospective observational study.

Thank you for submitting the above project to the East Norfolk and Waveney Research Governance Committee for scientific peer review and Research Management approval. The following comments were made as part of the scientific peer review and you are required to address these points before you make a submission to the Ethics Committee.

- Members queried why approval was only being sought for phase I of the study. It was agreed that approval could be given for both phases subject to the required funding being obtained.
- The response to question A23 regarding exclusion criteria is contradicted in question A25 and needs clarifying.
- Members queried whether three time points would be sufficient for the study but acknowledged that more would have an impact on funding.
- The address for PPIRes given on the participant information sheet is incorrect. The new address is: Lakeside 400, Old Chapel Way, Broadland Business Park, Thorpe St Andrew, Norwich NR7 0WG.

When you have had the opportunity to address these points and make the necessary changes please send your response to the Research Governance Committee office at the above address.

When submitting a response, please send revised documentation where appropriate highlighting the changes you have made either by underlining them or using an italic font and giving revised version numbers and dates. You should also note that the original text should not be deleted from the revised document but should be 'struck out'.

The Committee has delegated authority to the Chair to approve these amendments once they have been received. Subject to the Chair's agreement a formal approval letter will then be issued.

If you have any queries regarding this or any other project please contact Julie Dawson, Research Governance Administrator, at the above address. Please note, the reference number for this study is **2008DERM01L (21-03-08)** and this should be quoted on all correspondence.

Yours sincerely

Dr Richard Reading
Chair
Consultant Paediatrician – Norfolk PCT

Cc. Dr Elena Borzova, Dermatology Department, NNUH

NHS

National Research Ethics Service
Norfolk Research Ethics Committee
c/o The Norfolk & Norwich University Hospital NHS Trust
First Floor, Aldwych House
57 Bethel Street
NORWICH
NR2 1NR

Telephone: 01603 286 397
Facsimile: 01603 286 573

23 May 2008

Dr Clive Grattan
Consultant Dermatologist
Department of Dermatology
Norfolk & Norwich University Hospital
Colney Lane
Norwich
NR4 7UZ

Dear Dr Grattan

Full title of study: **Pathophysiological subtypes in chronic ordinary urticaria and their biomarkers: a prospective observational study**

REC reference number: **08/H0310/53**

The Research Ethics Committee reviewed the above application at the meeting held on 12 May 2008. Thank you and Dr Borzova for attending to discuss the study.

Documents reviewed

The documents reviewed at the meeting were:

Document	Version	Date
Application	5.5; Parts A&B Sections 1,5,8	22 April 2008
Investigator CV	Dr Clive Grattan	22 April 2008
Protocol	3	15 April 2008
Covering Letter	From Dr C Grattan	22 April 2008
Peer Review	ENWRGC: 2008DERM01L	16 April 2008
Interview Schedules/Topic Guides	1	14 February 2008
Questionnaire: Visual Analogue Scale	1	14 February 2008
GP/Consultant Information Sheets	3 (Letter 1)	11 April 2008
Participant Information Sheet:	3	15 April 2008
Participant Consent Form:	3	15 April 2008
Protocol Appendix 2	2	15 April 2008
Protocol Appendix 1		
SSI Application: NNUH	5.5	22 April 2008
Applicant's Checklist	5.5	22 April 2008
Correspondence to ENWRGC	From Dr Grattan	04 April 2008
Correspondence from ENWRGC	To Dr Grattan	09 April 2008
Correspondence from ENWRGC	To Dr Grattan	06 March 2008
Responsibilities of Investigators	1	12 February 2008
Letter from Dr C Grattan regarding Funding for		22 April 2008

the study		
Urticaria Assessment Sheet	1	14 February 2008
GP/Consultant Information Sheets	3 (Letter 2)	11 April 2008
Letter from EN&W RGC	2008DERM01L	21 April 2008

Provisional opinion

The Committee would be content to give a favourable ethical opinion of the research, subject to receiving a complete response to the request for further information set out below.

The Committee delegated authority to confirm its final opinion on the application to the Vice Chair Dr Lund in consultation with Dr Langdon and Mr Mathur.

Further information or clarification required

Informed consent process

1. Members requested an additional clause on the consent form to record that permission has been obtained to take blood samples for the purpose of the study and to store the blood.

2. Members considered that the term 'marker' used on the Participant Information Sheet would be not be readily understood and should be rephrased in layman's terms e.g. 'indicator'.

As your research involves the storage of tissue samples, we should like to draw your attention to the following specific points in relation to the Human Tissue Act 2004 and the REC SOPs on human tissue *:

- There is no problem where individual projects are ethically approved and on-going, ie the tissue would be exempt from requiring a licence if being used as per the approval given.
- The problem will arise at the end of the study if storing tissue. Even if the participant's consent has been sought to store the tissue at the end of the trial, it cannot continue to be stored without gaining an HTA (Human Tissue Authority) licence.
- An investigator would therefore have the following choices:
  - To destroy the tissue/sample
  - To apply to the REC for a further specific project before the end of the current research project
  - To transfer the tissue/samples to a licensed tissue bank *
  - Obtain a licence for themselves.

  * Organisations responsible for the management of research tissue banks anywhere in the UK may apply for ethical review of their arrangements for collection, storage, use and distributions of tissue.
- Existing holdings, surplus tissue and imported tissue does not now require the consent of the individual; however, existing holdings would need to gain an HTA licence.
- It is the responsibility of the chief investigator to ensure full compliance with the Human Tissue Act.

* Web sites: www.hta.gov.uk www.nres.npsa.nhs.uk

When submitting your response to the Committee, please send revised documentation where appropriate underlining or otherwise highlighting the changes you have made and giving revised version numbers and dates.

The Committee will confirm the final ethical opinion within a maximum of 60 days from the date of initial receipt of the application, excluding the time taken by you to respond fully to the above points. A response should be submitted by no later than 19 September 2008.

Membership of the Committee

The members of the Committee who were present at the meeting are listed on the attached sheet.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

08/H0310/53	**Please quote this number on all correspondence**

Yours sincerely

EKL

Dr Elizabeth Lund BSc MMSc PGCE PhD
Vice Chair

Email: katheriner.norton@nnuh.nhs.uk

Enclosures: *List of names and professions of members who were present at the meeting*

Copy to: *Sponsor: Mrs Kath Andrews, R&D Manager NNUH*
R&D Department for NNUH Ref: 2008DERM01L

Norfolk Research Ethics Committee

Attendance at Committee meeting on 12 May 2008

Committee Members:

Name	*Profession*	*Present*	*Notes*
Dr Elizabeth Lund	Principal Research Scientist	Yes	
The Reverend Bill Bazely	Senior Chaplain	Yes	
Miss Kim Clipsham	Senior Research Nurse	Yes	
Mr Michael Flowerdew	Acupuncture Practitioner and Writer	Yes	
Miss Sheila Ginty	Senior Sister - Surgery	Yes	
Mrs Janette Guymer	REC Manager	No	
Mrs Belinda Hoste	Senior Housing Law Adviser	Yes	
Miss Rosemary Jackson	Midwife	No	
Mrs Pamela Keeley	East Anglian Eye Bank Nurse Manager	Yes	
Dr Peter Langdon	Clinical Lecturer and Clinical Psychologist	Yes	
Mr Azad Mathur	Consultant Paediatric Surgeon	Yes	
Dr Michael Sheldon	Retired - Clinical Psychologist	Yes	
Dr Robert Stone	General Practitioner	Yes	

Also in attendance:

Name	*Position (or reason for attending)*
Miss Katherine Norton	Minuting Secretary

NHS

National Research Ethics Service
Norfolk Research Ethics Committee
c/o The Norfolk & Norwich University Hospital NHS Trust
First Floor
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NR2 1NR

Telephone: 01603 286 397
Facsimile: 01603 286 573

19 June 2008

Dr Clive Grattan
Consultant Dermatologist
Department of Dermatology
Norfolk & Norwich University Hospital
Colney Lane
NORWICH
NR4 7UZ

Dear Dr Grattan

Full title of study:	**Pathophysiological subtypes in chronic ordinary urticaria and their biomarkers: a prospective observational study**
REC reference number:	**08/H0310/53**

Thank you for your letter of 30 May 2008, responding to the Committee's request for further information on the above research and submitting revised documentation, subject to the conditions specified below.

A sub-committee chaired by Dr Lund has considered the further information on behalf of the Committee.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Ethical review of research sites

The favourable opinion applies to the research sites listed on the attached form.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission at NHS sites ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Application: Parts A&B Sections 1,5,8	5.5	22 April 2008
SSI Application: NNUH	5.5	22 April 2008
Protocol	3	15 April 2008
Investigator CV	Dr Clive Grattan	22 April 2008
Covering Letter	From Dr C Grattan	22 April 2008
Peer Review: EN&W Researcher Governance Committee	2008DERM01L	16 April 2008
Interview Schedules/Topic Guides	1	14 February 2008
Questionnaire: Visual Analogue Scale	1	14 February 2008
GP/Consultant Information Sheets	3 (Letter 1)	11 April 2008
Participant Information Sheet	4	28 May 2008
Participant Consent Form	4	28 May 2008
Response to Request for Further Information	Dr E Borzova	28 May 2008
Response to Request for Further Information	email Dr Grattan	03 June 2008
Correspondence to EN&W RGC	From Dr Grattan	04 April 2008
Correspondence from EN&W RGC	To Dr Grattan	09 April 2008
Letter from EN&W RGC	2008DERM01L	21 April 2008
Reimbursement confirmation form for study participants	1	14 February 2008
Participant Feedback Form	1	14 February 2008
Reimbursement form for study participants	2	02 April 2008
Applicant's Checklist	5.5	22 April 2008
Protocol: Project time line [Appendix 2]	2	15 April 2008
Protocol: The time line for the study [Appendix 1]		
Correspondence from EN&W RGC	To Dr Grattan	06 March 2008
Responsibilities of Investigators	1	12 February 2008
Letter from Dr C Grattan regarding Funding for the study		22 April 2008
Urticaria Assessment Sheet	1	14 February 2008
GP/Consultant Information Sheets	3 (Letter 2)	11 April 2008

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk.

08/H0310/53	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

Katherine K Norton
Assistant Administrator.

pp **Dr Elizabeth Lund BSc MMSc PGCE PhD**
Vice Chair

Email: katheriner.norton@nnuh.nhs.uk

Enclosures:	"After ethical review – guidance for researchers" *SL- AR2* *Site approval form issue 1*
Copy to:	*Sponsor, Mrs Kath Andrews, R&D Manager NNUH* *R&D office for NNUH Ref: 2008DERM01L*

Norfolk Research Ethics Committee

LIST OF SITES WITH A FAVOURABLE ETHICAL OPINION

For all studies requiring site-specific assessment, this form is issued by the main REC to the Chief Investigator and sponsor with the favourable opinion letter and following subsequent notifications from site assessors. For issue 2 onwards, all sites with a favourable opinion are listed, adding the new sites approved.

REC reference number:	08/H0310/53	**Issue number:**	1	**Date of issue:**	19 June 2008
Chief Investigator:	Dr Clive Grattan				
Full title of study:	Pathophysiological subtypes in chronic ordinary urticaria and their biomarkers: a prospective observational study				

This study was given a favourable ethical opinion by Norfolk Research Ethics Committee on 17 June 2008. The favourable opinion is extended to each of the sites listed below. The research may commence at each NHS site when management approval from the relevant NHS care organisation has been confirmed.

Principal Investigator	*Post*	*Research site*	*Site assessor*	*Date of favourable opinion for this site*	*Notes* [1]
Dr Clive Grattan	Consultant Dermatologist	Norfolk & Norwich University Hospital NHS Trust	Norfolk Research Ethics Committee	19/06/2008	

Approved by the Chair on behalf of the REC:

Catherine Enoren ... (Signature of Chair/~~Co-ordinator~~) Assistant Administrator

(delete as applicable)

K E NORTON ... (Name)

(1) *The notes column may be used by the main REC to record the early closure or withdrawal of a site (where notified by the Chief Investigator or sponsor), the suspension of termination of the favourable opinion for an individual site, or any other relevant development. The date should be recorded.*

NHS
National Research Ethics Service
Norfolk Research Ethics Committee
c/o The Norfolk & Norwich University Hospitals NHS Foundation Trust
East of England REC Office [2]
Room 2.08 First Floor
Aldwych House
57 Bethel Street
NORWICH
NR2 1NR

Tel: 01603 289813
Fax: 01603 286573

10 June 2009

Dr Clive Grattan
Consultant Dermatologist
Department of Dermatology
The Norfolk & Norwich University Hospitals NHS Foundation Trust
Colney Lane
Norwich
NR4 7UZ

Dear Dr Grattan

Study title:	**Pathophysiological subtypes in chronic ordinary urticaria and their biomarkers: a prospective observational study**
REC reference:	**08/H0310/53**
Amendment number:	**1**
Amendment date:	**12 May 2009**
Amendment detail:	**1) Addition of co-investigator Dr Roy Bongaerts of IFR.** **2) Addition of second phase of patient recruitment subject to funding by GlaxoSmithKline.** **3) Extension of period between visits 1 and 2.** **4) Introduction of cell sorting step prior to tests.**

The above amendment was reviewed by the Sub-Committee in correspondence.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation. During the course of the review you provided an updated Participant Information Sheet for clarification, which was accepted for review by the Chair.

Approved documents

The documents reviewed and approved at the meeting were:

Document	Version	Date
Protocol	4	12 May 2009
Participant Information Sheet	6	10 June 2009
Notice of Substantial Amendment (non-CTIMPs)	1	12 May 2009

Membership of the Committee

The members of the Committee who took part in the review are

Chair Dr Michael Sheldon, retired Chartered Clinical Psychologist (Lay Member)
Alternate Vice-Chair Dr Elizabeth Lund, Research Scientist (Lay+ Member)

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

08/H0310/53:	**Please quote this number on all correspondence**

Yours sincerely

Mrs Janette Guymer
Committee Co-ordinator

E-mail: janette.guymer@nnuh.nhs.uk

Copy to: *Mrs Kathryn Andrews, R&D office for NHS care organisation at lead site: NNUH ref 2008DERM01L*

NHS
National Research Ethics Service
Norfolk Research Ethics Committee
c/o The Norfolk & Norwich University Hospitals NHS Foundation Trust
East of England REC Office [2]
Room 2.08 First Floor
Aldwych House
57 Bethel Street
NORWICH
NR2 1NR

Tel: 01603 289813
Fax: 01603 286573

12 November 2009

Dr Clive Grattan
Consultant Dermatologist
Department of Dermatology
Room 28.1.000
Centre Block / Level 1
Norfolk & Norwich University Hospital NHS Foundation Trust
Colney Lane, Norwich NR4 7UY

Dear Dr Grattan

Study title:	**Pathophysiological subtypes in chronic ordinary urticaria and their biomarkers: a prospective observational study**
REC reference:	**08/H0310/53**
Amendment number:	**2**
Amendment date:	**01 November 2009**
Amendment detail:	**1. Additional Co-investigator Dr Darren Sexton, UEA** **2. Recruitment of healthy volunteers to allow assessment of performance of assays.** **3. Modification of assay protocols.** **4. Extension of observation period between visits, 2, 3 and 4 to 10 weeks instead of 6 weeks. Total duration of study will therefore be 21 weeks.** **5. Revised consent forms for patients involved in second phase of study and healthy volunteers to allow researchers to be aware of test results at different sites.**

The above amendment was reviewed at the meeting of the Sub-Committee held on 09 November 2009.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Document	Version	Date
Advertisement	1	01 November 2009
Participant Consent Form: Volunteer	1	01 November 2009
Participant Consent Form: Patient	4	01 November 2009
Participant Information Sheet: Volunteer	1	01 November 2009
Participant Information Sheet: Patient	7	01 November 2009
Protocol	5	01 November 2009
Notice of Substantial Amendment (non-CTIMPs)	2	01 November 2009
Covering Letter	Dr Elena Borzova	01 November 2009

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

08/H0310/53:	**Please quote this number on all correspondence**

Yours sincerely

Mrs Janette Guymer
Committee Co-ordinator

E-mail: janette.guymer@nnuh.nhs.uk

Enclosures: *List of names and professions of members who took part in the review*

Copy to: *Mrs Kathryn Andrews: R&D office for NHS care organisation at lead site: NNUH*

Ethics Committee Approval For the Microdialysis Study in Chronic Urticaria

East Norfolk and Waveney Research Governance Committee NHS

Please reply to: Research Governance Committee Office
Research and Development Department
Level 3, East Block, Room 032
Norfolk and Norwich University Hospital NHS Trust
Colney Lane
Norwich
NR4 7UY

Direct Dial: 01603 287408
Internal: 3408
Direct Fax: 01603 289800

e-mail: rdoffice@nnuh.nhs.uk
Website: www.norfolkhealthresearch.nhs.uk

Dr Clive Grattan
Dermatology Department
Norfolk & Norwich University Hospital
Colney Lane
Norwich
Norfolk
NR4 7UY

30 April 2007

Dear Dr Grattan

Re: 2006DERM02L (198-12-06) Microdialysis study of inflammatory mediators and cytokines in the early and late phase of dermal response to phosphate buffered saline, codeine and autologous serum injections in chronic ordinary urticaria and healthy controls.

Further to your submission of the above project, I am pleased to confirm that your project has been given full approval from the East Norfolk and Waveney Research Governance Committee and Research Management Team and you may start your study.

Please note that this approval applies to the following sites:

- Norfolk & Norwich University Hospital NHS Trust

I have enclosed two copies of the Standard Terms and Conditions of Approval. Please sign and return one copy to the Research Governance Committee office. Failure to return the standard terms and conditions may affect the conditions of approval.

Please note, under the agreed standard terms and conditions of approval it is your responsibility to inform this Committee of any proposed changes to this study and to keep the Committee updated on progress.

If you have any queries regarding this or any other study please contact Julie Dawson, Research Governance Administrator, at the above address. Please note, your reference number is **2006DERM02L (198-12-06)** and this should be quoted on all correspondence.

The Committee would like to take this opportunity to wish you every success with this project.

Yours sincerely

Dr Richard Reading
Chair
Consultant Paediatrician – Norfolk PCT

Enc

East Norfolk & Waveney Research Governance Committee – a partnership between:
James Paget University Hospitals NHS Foundation Trust. Norfolk & Norwich University Hospital NHS Trust.
Norfolk & Waveney Mental Health Partnership NHS Trust. Norfolk Primary Care Trust.

Norfolk Research Ethics Committee
c/o The Norfolk & Norwich University Hospital NHS Trust
First Floor
Aldwych House
57 Bethel Street
NORWICH
NR2 1NR

Telephone: 01603 286 397
Facsimile: 01603 286 573

26 April 2007

Dr Clive Grattan
Dermatology Consultant
Department of Dermatology,
Norfolk & Norwich University Hospital
Colney Road
NORWICH
Norfolk
NR4 7UZ

Dear Dr Grattan

Full title of study:	**Microdialysis study of inflammatory mediators and cytokines in the early and late phase of dermal response to phosphate buffered saline, codeine and autologous serum injections in chronic ordinary urticaria and healthy controls (Version 3, 15.03.2007)**
REC reference number:	**07/Q0101/42**

Thank you for your letter of 25 April 2007, responding to the Committee's request for further information on the above research and submitting revised documentation.

The Chairman the Reverend Walter Currie together with Mr Michael Flowerdew and Dr Robert Stone has considered the further information on behalf of the Committee.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Ethical review of research sites

The favourable opinion applies to the research sites listed on the attached form.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Application	5.3; Checklist	15 March 2007
Application	5.3; Parts A&B	15 March 2007
Application	5.3; SSI for NNUH	15 March 2007
Investigator CV	Dr C. Grattan	16 March 2007
Protocol	4	11 April 2007
Covering Letter	From Dr's Grattan & Borzova	19 March 2007
Summary/Synopsis: Research Flow Chart	2	25 January 2007
Summary/Synopsis: Times lines of Microdialysis	2	25 January 2007
Summary/Synopsis: Scheme of Microdialysis	2	25 January 2007
Peer Review: East Norfolk & Waveney Research Governance Committee [EN&W RGC]	2006DERM02L	20 March 2007
Statistician Comments	2; From Dr Gill Price	19 March 2007
Interview Schedules/Topic Guides	2	25 January 2007
Advertisement	4; Healthy Volunteers	11 April 2007
GP/Consultant Information Sheets	3; Letter 1	11 April 2007
GP/Consultant Information Sheets	3; Letter 2 (Results)	11 April 2007
Participant Information Sheet: Patient	4	11 April 2007
Participant Information Sheet: Healthy Volunteer	3	11 April 2007
Participant Consent Form: Patient	3	11 April 2007
Participant Consent Form: Healthy Volunteer	3	11 April 2007
Participant Consent Form: Healthy Volunteer Photographs	2	25 January 2007
Participant Consent Form: Patient - Photographs	2	25 January 2007
Response to Request for Further Information	E-mail of support/approval from Dr C Grattan	25 April 2007
Participant Feedback form	2	20 January 2007
Visual Anologue Scale (VAS)	2	25 January 2007
Reimbursement confirmation form for volunteers	2	25 January 2007
Reimbursement confirmation form for patients	2	25 February 2007
Reimbursement form for participants	2	25 January 2007
Participant Study Instruction	2	25 January 2007
Correspondence to Dr Grattan	From EN&W RGC	09 March 2007
Correspondence to EN&W RGC	From Drs Grattan & Borzova	15 March 2007
Email Correspondence from Dr E Borzova	to J. Romero Norfolk PCT	14 February 2007
Letter confirming Funding	From Dr C Grattan	09 February 2007

R&D approval

The study should not commence at any NHS site until the local Principal Investigator has obtained final approval from the R&D office for the relevant NHS care organisation.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

07/Q0101/42	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

The Reverend Walter Currie
Chairman

Email: janette.guymer@nnuh.nhs.uk

Enclosures: *Standard approval conditions SL-AC2*
Site approval form

Copy to: Sponsor: Norfolk & Norwich University Hospitals NHS Trust
EN&W RGC Ref: 2006DERM02L
Email copy to Dr E Borzova

Appendix 1B. Table 1. Classification of Urticaria Subtypes as recommended by EAACI/GA²LEN/EDF/WAO guideline on Definition, Classification and Diagnosis of Urticaria in 2009

Types	Subtypes	Definition
Spontaneous urticaria	Acute spontaneous urticaria	Spontaneous wheals and/or angioedema < 6 weeks
	Chronic spontaneous urticaria	Spontaneous wheals and/or angioedema > 6 weeks
Physical urticaria	Cold contact urticaria	Eliciting factor: cold objects/air/fluids/wind
	Delayed pressure urticaria	Eliciting factor: vertical pressure (wheals arising with a 3–12 h latency)
	Heat contact urticaria	Eliciting factor: localized heat
	Solar urticaria	Eliciting factor: UV and/or visible light
	Urticaria factitia/dermographic urticaria	Eliciting factor: mechanical shearing forces (wheals arising after 1–5 min)
	Vibratory urticaria/angioedema	Eliciting factor: vibratory forces, e.g. pneumatic hammer
Other urticaria types	Aquagenic urticaria	Eliciting factor: water
	Cholinergic urticaria	Elicitation by increase of body core temperature due to physical exercises, spicy food
	Contact urticaria	Elicitation by contact with urticariogenic substance
	Exercise induced anaphylaxis/urticaria	Eliciting factor: physical exercise

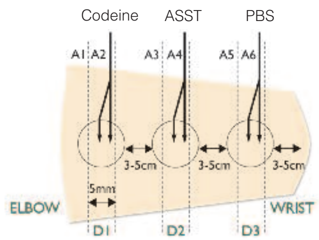
Abbrevations:
EAACI - European Academy of Allergy and Clinical Immunology
GA²LEN - Global Allergy and Asthma European Network
EDF - European Dermatology Forum
WAO - World Allergy Organization

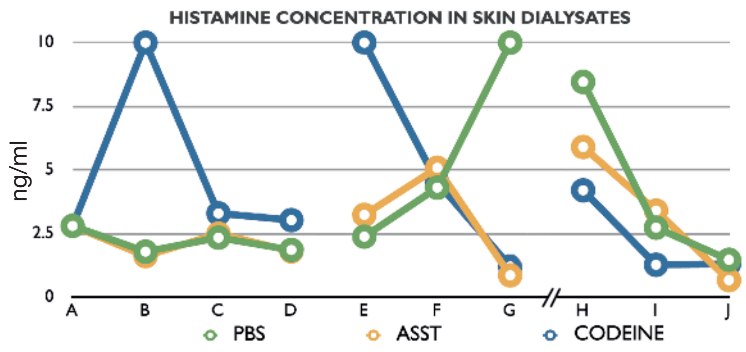
Table 1. The clinical classification of urticaria subtypes was suggested by the EAACI/GA²LEN/EDF/WAO guideline at the 3rd International Meeting on Urticaria Urticaria 2008 which was held as a joint initiative of the EAACI Dermatology Section, GA²LEN, EDF and WAO (Zuberbier T. et al, 2009). According to this classification, three urticaria subtypes included spontaneous urticaria, physical urticaria and other urticaria types. Spontaneous urticaria was subdivided into acute and chronic depending on the duration of the disease. Physical urticarias included cold contact urticaria, delayed pressure urticaria, heat contact urticaria, solar urticaria, urticaria factitia or dermographic urticaria. Aquagenic, cholinergic, contact urticarias and exercise-induced anaphylaxis/urticaria were considered as other urticaria types in the classification. The guideline referred to some inconsistencies in this classification. For example, physical urticarias, although chronic conditions, they were grouped separately due to eliciting physical factors as opposed to acute and chronic spontaneous urticarias that occur spontaneously without physical triggers.

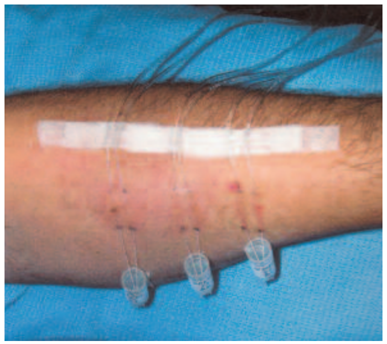
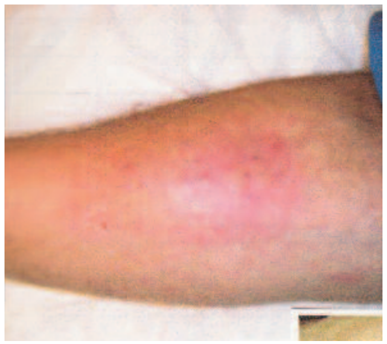
Appendix 3. Figure 1. Microdialysis Experiment - Patient DPP01

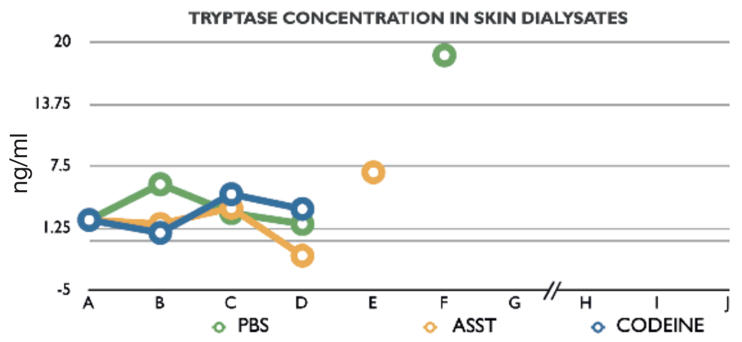
Protocol 1 - Patient	
Date:	1/10/2007
Patient:	DPP01
VAS for itching/24hrs:	76mm
VAS for wealing:	54mm

Microdialysis Sites: Skin Testing





Baseline	Microdialysis	Skin Testing
		
Probes in	Dialysate collection	30 minutes after testing



Histamine Concentration In Skin Dialysates

	A	B	C	D	E	F	G	H	I	J
PBS	2.806	1.794	2.344	1.859	2.385	4.306	10	8.460	2.737	1.474
ASST	2.806	1.620	2.502	1.807	3.239	5.086	0.864	5.904	3.404	0.675
CODEINE	2.806	10	3.297	3.033	10	4.518	1.211	4.202	1.278	1.311

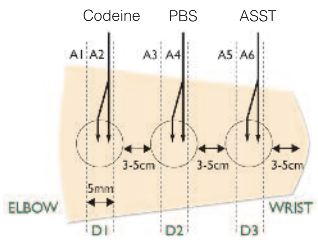
Tryptase Concentration In Skin Dialysates

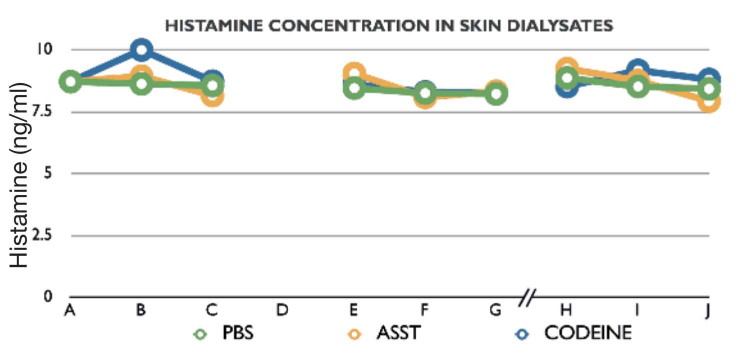
	A	B	C	D	E	F	G	H	I	J
PBS	2.1	5.7	2.8	1.7		18.70				
ASST	2.1	1.70	3.30	-1.5	6.9					
CODEINE	2.1	0.80	4.70	3.20						

Appendix 3. Figure 2. Microdialysis Experiment - Patient MHP03

Protocol 1 - Patient	
Date:	1/12/2007
Patient:	MHP03
VAS for itching/24hrs:	18mm
VAS for wealing:	17mm

Microdialysis Sites: Skin Testing

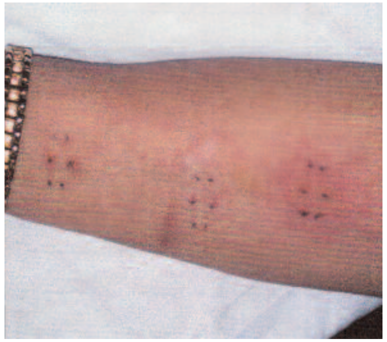




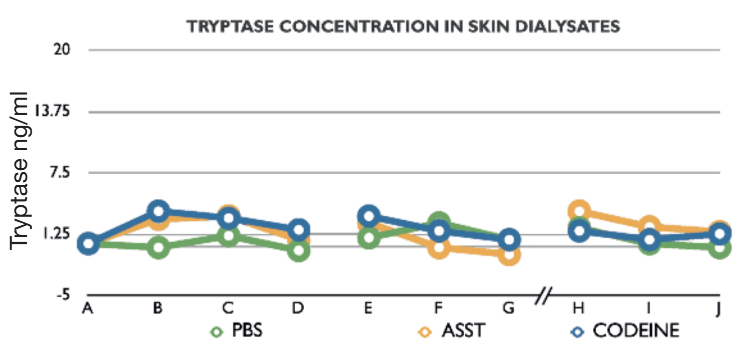
Baseline	Microdialysis	Skin Testing

Probes in

Dialysate collection



30 minutes after testing



Histamine Concentration In Skin Dialysates

	A	B	C	D	E	F	G	H	I	J
PBS	8.721	8.630	8.561		8.458	8.258	8.225	8.866	8.522	8.431
ASST	8.721	8.940	8.161		9.048	8.087	8.333	9.244	8.762	7.927
CODEINE	8.721	10	8.736		8.616	8.301	8.286	8.521	9.174	8.807

Tryptase Concentration In Skin Dialysates

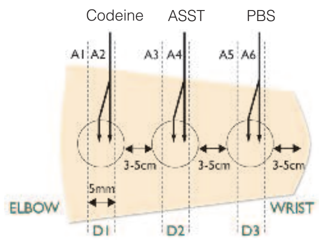
	A	B	C	D	E	F	G	H	I	J
PBS	0.30	2.80	3.10	0.70	2.40	-0.10	-0.80	3.60	2	1.50
ASST	0.3	-0.1	1.10	-0.40	0.90	2.40	0.70	1.90	0.30	-0.10
CODEINE	0.3	3.6	2.90	1.7	3.10	1.60	0.70	1.60	0.70	1.30

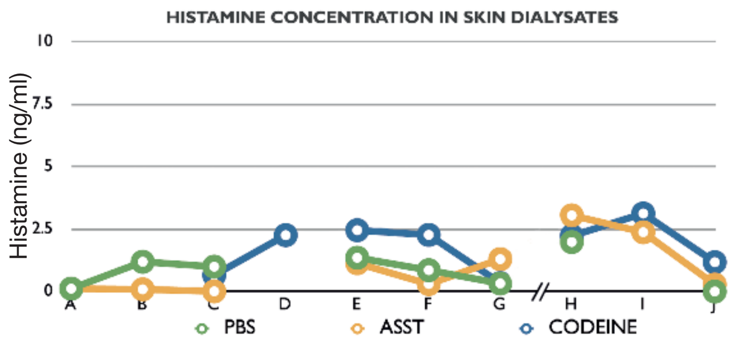
Appendix 3. Figure 3. Microdialysis Experiment - Patient EBP04

Protocol 1 - Patient

Date:	20/02/2008
Patient:	EBP04
VAS for itching/24hrs:	19mm
VAS for wealing:	11mm

Microdialysis Sites: Skin Testing





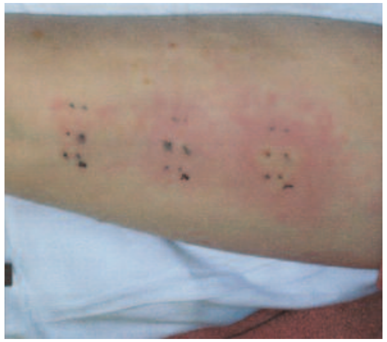
Baseline

Probes in

Microdialysis

Dialysate collection

Skin Testing



30 minutes after testing

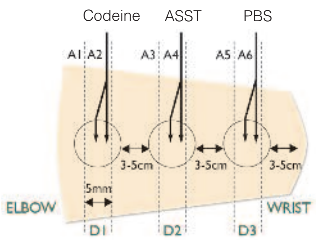
Histamine Concentration In Skin Dialysates

	A	B	C	D	E	F	G	H	I	J
PBS	0.115	1.189	0.994		1.352	0.855	0.322	1.982		0
ASST	0.115	0.087	0.007		1.131	0.297	1.295	3.048	2.374	0.283
CODEINE	0.115		0.654	2.256	2.454	2.266	0.34	2.225	3.123	1.173

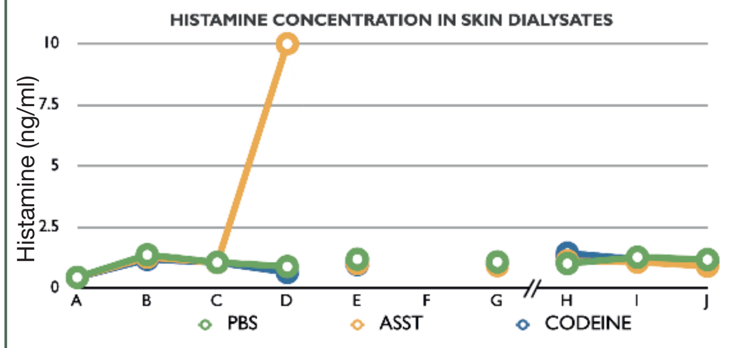
Appendix 3. Figure 4. Microdialysis Experiment - Patient DMP05

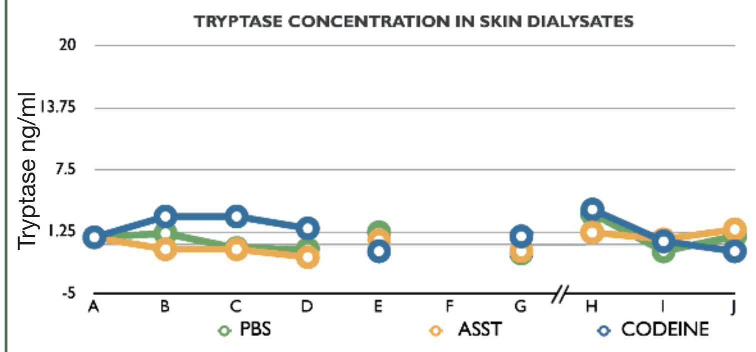
Protocol 1 - Patient	
Date:	13/02/2008
Patient:	DMP05
VAS for itching/24hrs:	48mm
VAS for wealing:	3mm

Microdialysis Sites: Skin Testing



Baseline	Microdialysis	Skin Testing
		
Probes in	Dialysate collection	30 minutes after testing





Histamine Concentration In Skin Dialysates

	A	B	C	D	E	F	G	H	I	J
PBS	0.444	1.367	1.057	0.885	1.188		1.077	1.013	1.269	1.172
ASST	0.444	1.268	1.053	10	1.003		0.927	1.121	1.072	0.909
CODEINE	0.444	1.183	1.059	0.649	0.971		0.978	1.432	1.181	1.061

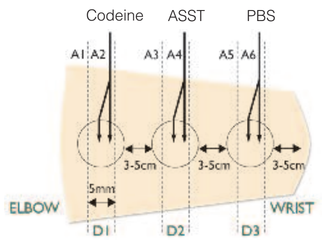
Tryptase Concentration In Skin Dialysates

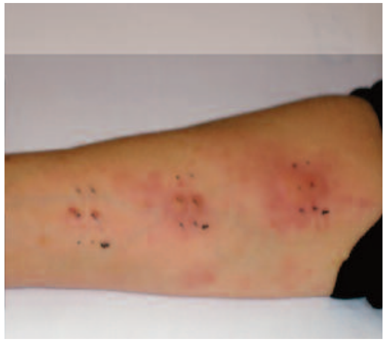
	A	B	C	D	E	F	G	H	I	J
PBS	0.7	1.10	-0.4	-0.5	1.2		-0.90	3.1	-0.7	0.80
ASST	0.7	-0.5	-0.5	-1.30	0.4		-0.7	1.20	0.50	1.50
CODEINE	0.7	2.8	2.8	1.6	-0.7		0.8	3.50	0.3	-0.7

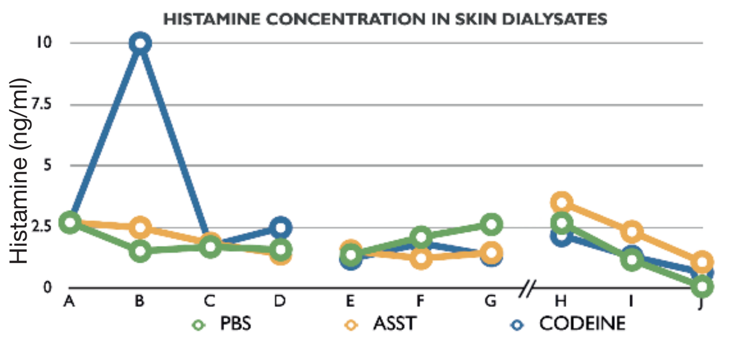
Appendix 3. Figure 5. Microdialysis Experiment - Patient DBP06

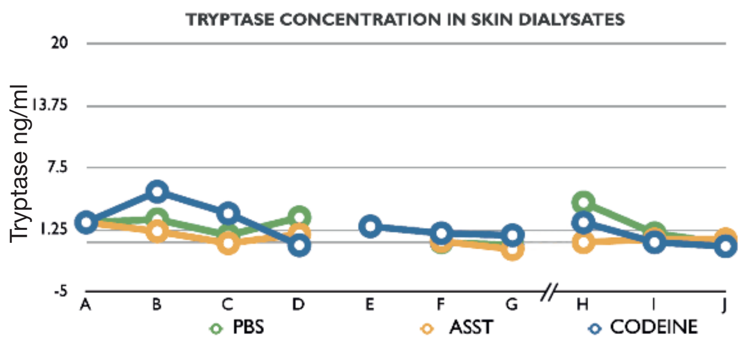
Protocol 1 - Patient	
Date:	05/03/2008
Patient:	DBP06
VAS for itching/24hrs:	93mm
VAS for wealing:	91mm

Microdialysis Sites: Skin Testing



Baseline	Microdialysis	Skin Testing
		
Probes in	Dialysate collection	30 minutes after testing





Histamine Concentration In Skin Dialysates

	A	B	C	D	E	F	G	H	I	J
PBS	2.692	1.528	1.698	1.586	1.372	2.095	2.616	2.675	1.171	0.081
ASST	2.692	2.483	1.843	1.413	1.535	1.233	1.460	3.501	2.314	1.076
CODEINE	2.692	10	1.733	2.476	1.213	1.841	1.345	2.161	1.303	0.653

Tryptase Concentration In Skin Dialysates

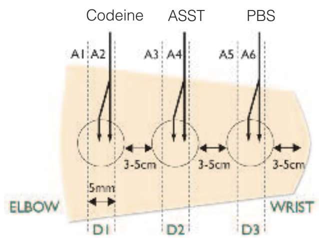
	A	B	C	D	E	F	G	H	I	J
PBS	2	2.3	0.7	2.5		0	-0.3	4	0.9	-0.1
ASST	2	1.1	-0.1	0.8		0.1	-0.7	0	0.3	0.3
CODEINE	2	5.1	2.9	-0.3	1.6	0.9	0.7	2	0	-0.4

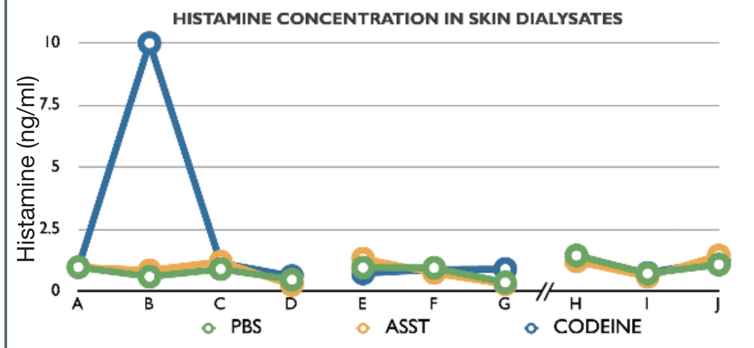
Appendix 3. Figure 6. Microdialysis Experiment - Patient NMCP07

Protocol 1 - Patient

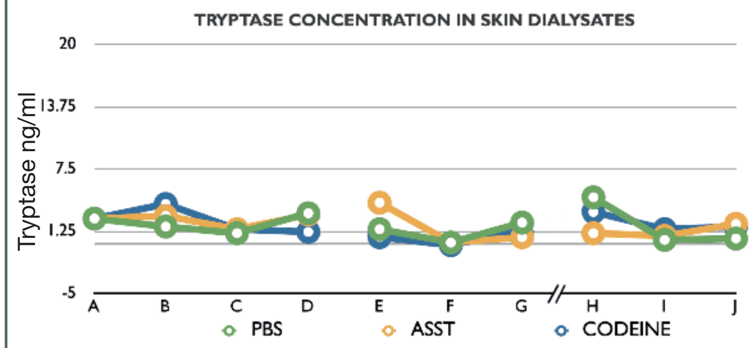
Date:	31/07/2008
Patient:	NMCP07
VAS for itching/24hrs:	80mm
VAS for wealing:	81mm

Microdialysis Sites: Skin Testing





Baseline	Microdialysis	Skin Testing
		
Probes in	Dialysate collection	30 minutes after testing



Histamine Concentration In Skin Dialysates

	A	B	C	D	E	F	G	H	I	J
PBS	0.973	0.593	0.898	0.469	0.956	0.944	0.36	1.444	0.720	1.079
ASST	0.973	0.844	1.197	0.258	1.316	0.740	0.285	1.209	0.591	1.441
CODEINE	0.973	10	1.142	0.611	0.722	0.880	0.897	1.298	0.742	1.248

Tryptase Concentration In Skin Dialysates

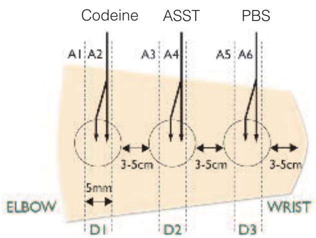
	A	B	C	D	E	F	G	H	I	J
PBS	2.53	1.73	1.07	3.07	1.47	0.13	2.13	4.67	0.40	0.53
ASST	2.53	2.80	1.47	2.93	4.13	0.13	0.67	1.07	0.80	2
CODEINE	2.53	4	1.47	1.20	0.67	-0.13	1.33	3.20	1.47	1.73

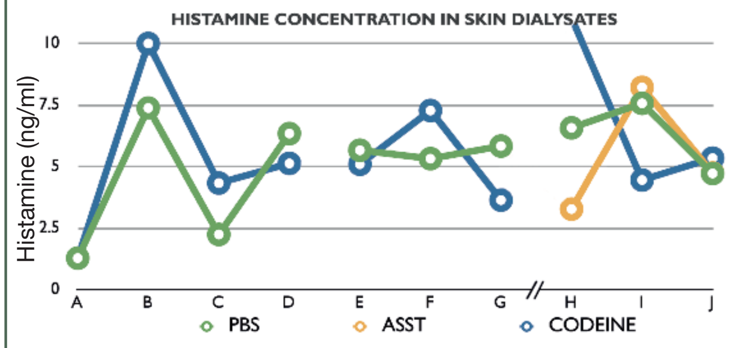
Appendix 3. Figure 7. Microdialysis Experiment - Healthy Control NLV04

Protocol 1 - Healthy Control

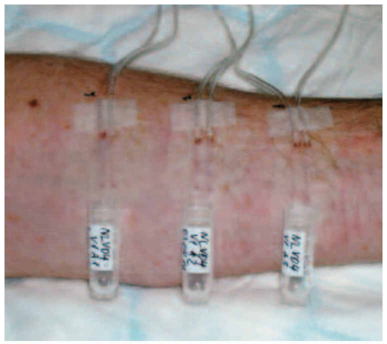
Date:	03/04/2008
Patient:	NLV04
VAS for itching/24hrs:	N/A
VAS for wealing:	N/A

Microdialysis Sites: Skin Testing

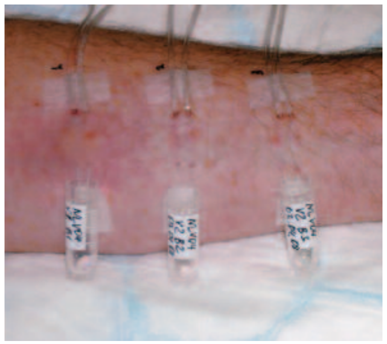




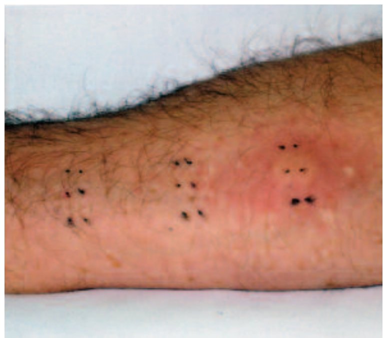
Baseline	Microdialysis	Skin Testing



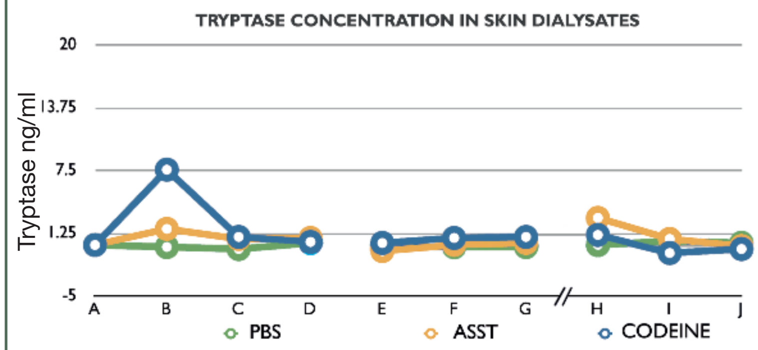
Probes in



Dialysate collection



30 minutes after testing



Histamine Concentration In Skin Dialysates

	A	B	C	D	E	F	G	H	I	J
PBS	1.286	7.378	2.246	6.342	5.665	5.323	5.835	6.574	7.570	4.723
ASST	1.286	7.378	2.246	6.342	5.665	5.323	5.835	3.277	8.219	4.743
CODEINE	1.286	10	4.332	5.13	5.097	7.278	3.641	11.041	4.456	5.345

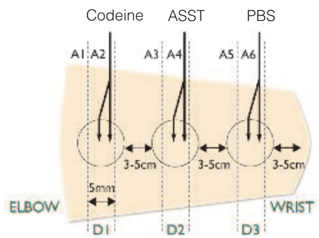
Tryptase Concentration In Skin Dialysates

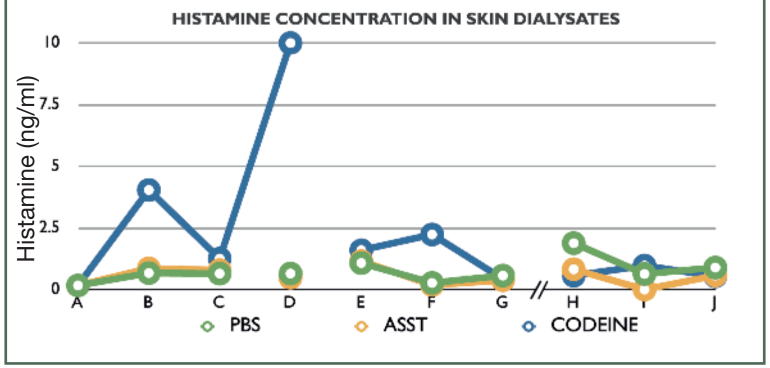
	A	B	C	D	E	F	G	H	I	J
PBS	0.1	-0.10	-0.3	0.3	-0.1	-0.1	-0.1	0.1	0.5	0.3
ASST	0.1	1.70	0.70	0.80	-0.50	0.1	0.3	2.80	0.70	0
CODEINE	0.1	7.60	0.90	0.40	0.30	0.80	0.90	1.10	-0.7	-0.3

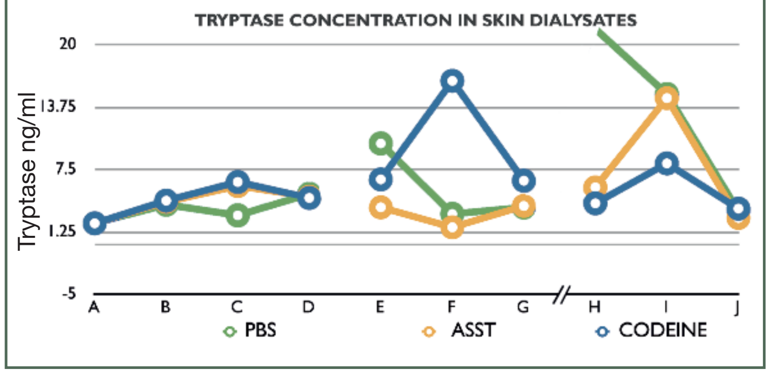
Appendix 3. Figure 8. Microdialysis Experiment - Healthy Control RAV05

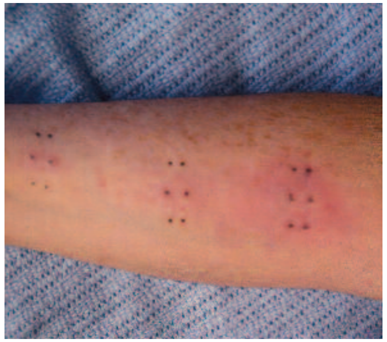
Protocol 1 - Healthy Control	
Date:	28/11/2007
Patient:	RAV05
VAS for itching/24hrs:	N/A
VAS for wealing:	N/A

Microdialysis Sites: Skin Testing







Baseline	Microdialysis	Skin Testing
		
EMLA off	Dialysate collection	30 minutes after testing

Histamine Concentration In Skin Dialysates

	A	B	C	D	E	F	G	H	I	J
PBS	0.172	0.675	0.653	0.655	1.081	0.275	0.573	1.887	0.639	0.898
ASST	0.172	0.865	0.792	0.484	1.161	0.190	0.384	0.829	0.007	0.573
CODEINE	0.172	4.042	1.261	10	1.602	2.239	0.435	0.569	0.977	0.542

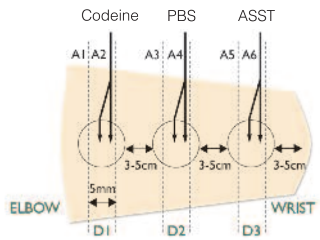
Tryptase Concentration In Skin Dialysates

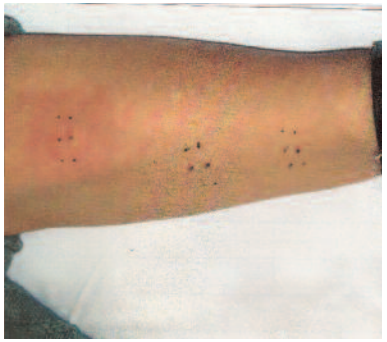
	A	B	C	D	E	F	G	H	I	J
PBS	2.13	4	2.93	5.07	10.13	3.07	3.73	21.60	15.07	3.33
ASST	2.13	4.27	5.87	4.80	3.73	1.73	3.87	5.73	14.67	2.67
CODEINE	2.13	4.4	6.27	4.67	6.53	16.40	6.4	4.13	8.13	3.60

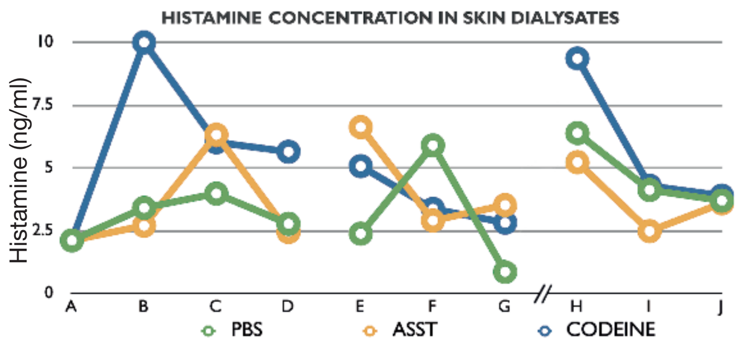
Appendix 3. Figure 9. Microdialysis Experiment - Healthy Control LCV06

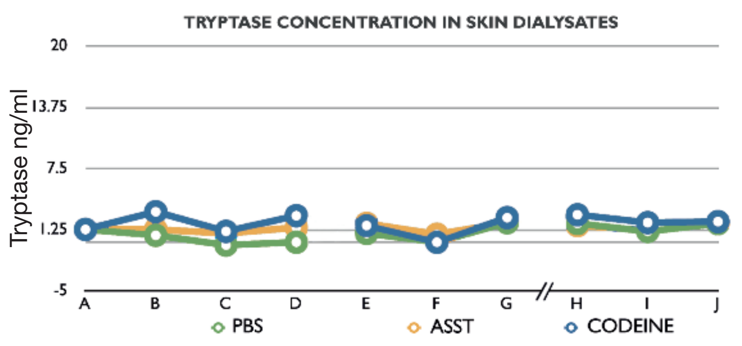
Protocol 1 - Healthy Control	
Date:	06/02/2008
Patient:	LCV06
VAS for itching/24hrs:	N/A
VAS for wealing:	N/A

Microdialysis Sites: Skin Testing



Baseline	Microdialysis	Skin Testing
		
Probes in	Dialysate collection	30 minutes after testing





Histamine Concentration In Skin Dialysates

	A	B	C	D	E	F	G	H	I	J
PBS	2.119	3.417	3.984	2.780	2.390	5.903	0.869	6.388	4.128	3.703
ASST	2.119	2.707	6.324	2.457	6.631	2.910	3.525	5.232	2.489	3.595
CODEINE	2.119	10	6.027	5.649	5.075	3.374	2.825	9.363	4.293	3.901

Tryptase Concentration In Skin Dialysates

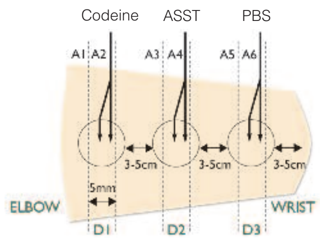
	A	B	C	D	E	F	G	H	I	J
PBS	1.3	1.3	0.9	1.5	1.9	0.8	2	1.7	1.2	
ASST	1.3	0.7	-0.3	0	0.9	0	2	1.9	1.1	1.9
CODEINE	1.3	3.10	1.1	2.7	1.7	0	2.50	2.8	2	2.1

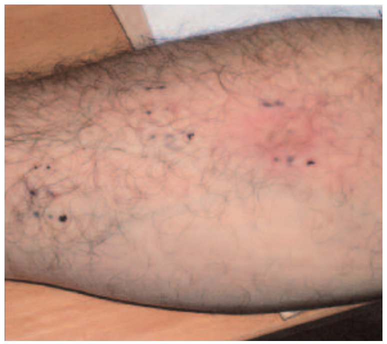
Appendix 3. Figure 10. Microdialysis Experiment - Healthy Control APV07

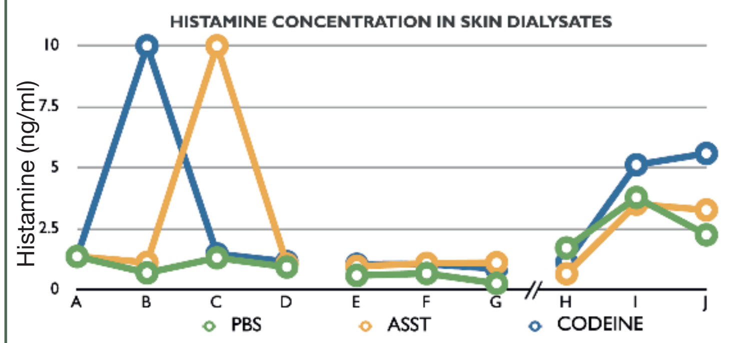
Protocol 1 - Healthy Control

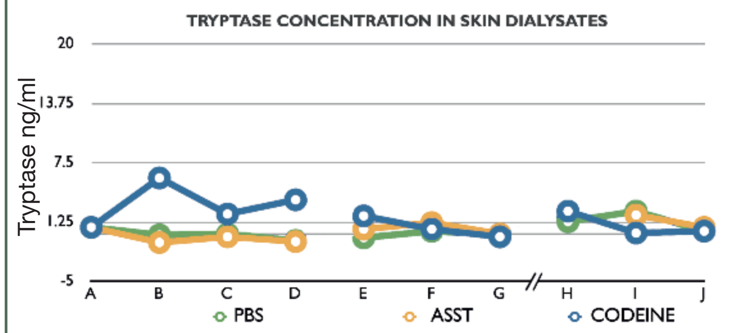
Date:	01/12/2007
Patient:	APV07
VAS for itching/24hrs:	N/A
VAS for wealing:	N/A

Microdialysis Sites: Skin Testing



Baseline	Microdialysis	Skin Testing
 Probes in	 Dialysate collection	 30 minutes after testing





Histamine Concentration In Skin Dialysates

	A	B	C	D	E	F	G	H	I	J
PBS	1.367	0.694	1.312	0.935	0.590	0.670	0.263	1.717	3.785	2.254
ASST	1.367	1.122	10	1.054	0.970	1.085	1.111	0.661	3.508	3.274
CODEINE	1.367	10	1.473	1.154	1.046	1.059	0.864	1.128	5.125	5.588

Tryptase Concentration In Skin Dialysates

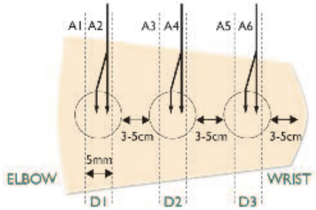
	A	B	C	D	E	F	G	H	I	J
PBS	0.7	-0.10	0	-0.70	-0.40	0.30	-0.10	1.30	2.40	0.30
ASST	0.7	-0.90	-0.30	-0.80	0.50	1.20	0		2	0.70
CODEINE	0.7	5.9	2.10	3.60	1.90	0.50	-0.30	2.40	0.10	0.30

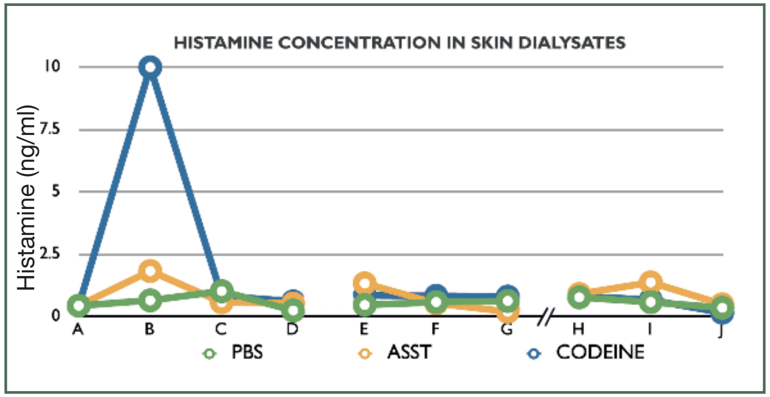
Appendix 3. Figure 11. Microdialysis Experiment - Healthy Control ASV09

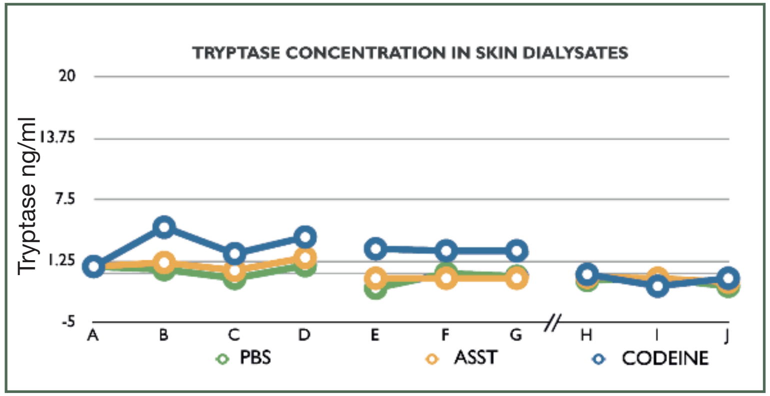
Protocol 1 - Healthy Control

Date:	22/11/2007
Patient:	ASV09
VAS for itching/24hrs:	N/A
VAS for wealing:	N/A

Microdialysis Sites: Skin Testing

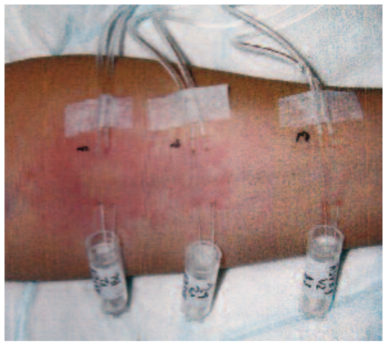




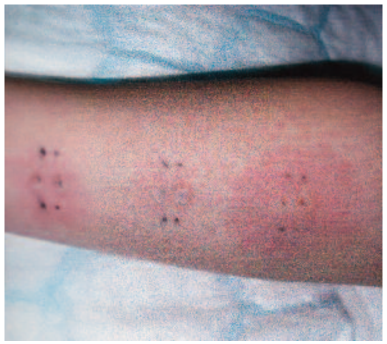


Baseline	Microdialysis	Skin Testing

Probes in



Dialysate collection



30 minutes after testing

Histamine Concentration In Skin Dialysates

	A	B	C	D	E	F	G	H	I	J
PBS	0.436	0.651	1.019	0.243	0.465	0.581	0.627	0.772	0.583	0.352
ASST	0.436	1.827	0.573	0.526	1.330	0.533	0.196	0.907	1.372	0.492
CODEINE	0.436	10	0.827	0.607	0.855	0.829	0.800	0.822	0.668	0.156

Tryptase Concentration In Skin Dialysates

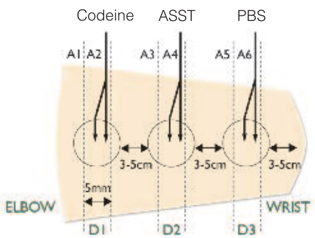
	A	B	C	D	E	F	G	H	I	J
PBS	0.7	0.4	-0.5	0.8	-1.5	0	-0.3	-0.7	-0.5	-1.3
ASST	0.7	1.10	0.30	1.6	-0.5	-0.5	-0.5	-0.4	-0.5	-0.9
CODEINE	0.7	4.70	2	3.70	2.50	2.30	2.3	-0.1	-1.3	-0.5

Appendix 3. Figure 12. Microdialysis Experiment - Healthy Control JEV10

Protocol 1 - Healthy Control

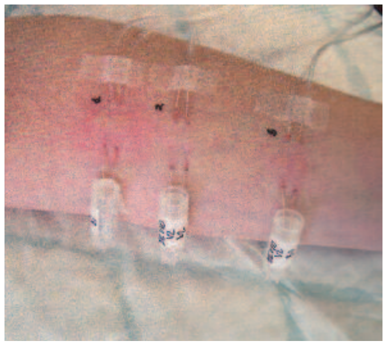
Date:	20/11/2007
Patient:	JEV10
VAS for itching/24hrs:	N/A
VAS for wealing:	N/A

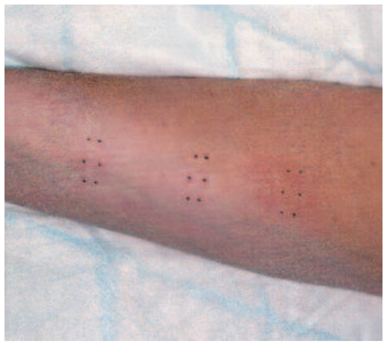
Microdialysis Sites: Skin Testing

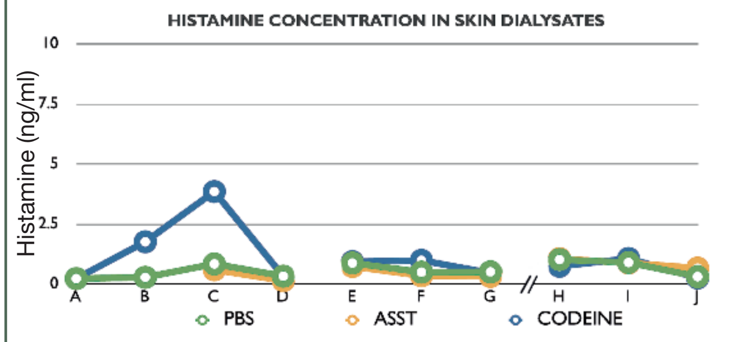


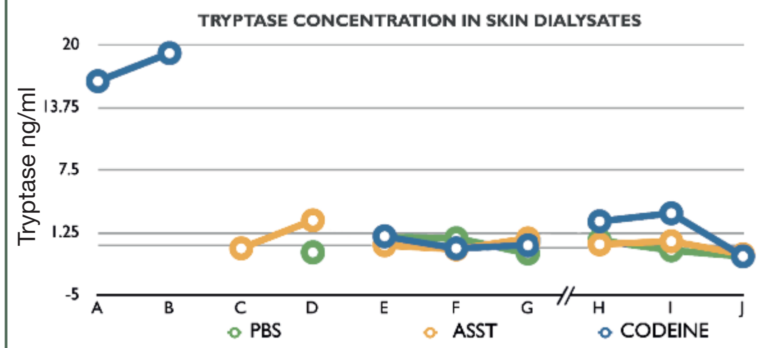
Baseline	Microdialysis	Skin Testing


Probes in


Dialysate collection


30 minutes after testing





Histamine Concentration In Skin Dialysates

	A	B	C	D	E	F	G	H	I	J
PBS	0.236	0.287	0.846	0.336	0.885	0.504	0.515	1.028	0.906	0.315
ASST	0.236		0.603	0.149	0.741	0.356	0.325	1.064	0.886	0.672
CODEINE	0.236	1.768	3.859	0.326	0.940	0.994	0.466	0.748	1.072	0.260

Tryptase Concentration In Skin Dialysates

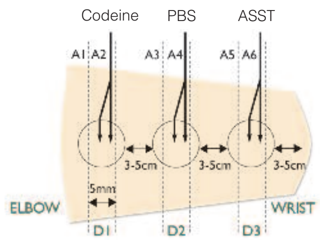
	A	B	C	D	E	F	G	H	I	J
PBS	16.4			-0.7	0.80	0.7	-0.8	0.5	-0.5	-1.1
ASST	16.4		-0.30	2.5	0	-0.4	0.7	0.1	0.4	-0.9
CODEINE	16.4	19.2			0.90	-0.30	0	2.40	3.20	-1.10

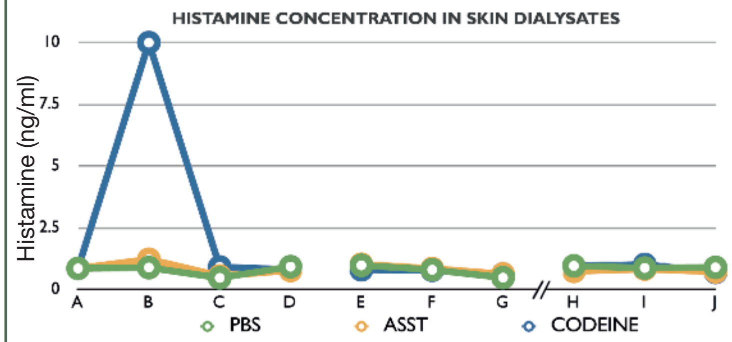
Appendix 3. Figure 13. Microdialysis Experiment - Healthy Control AMSV11

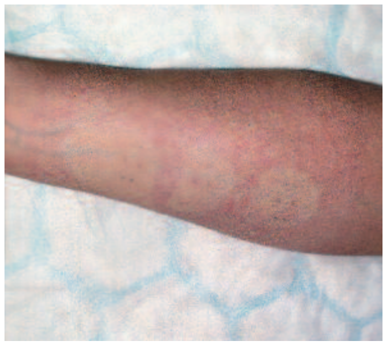
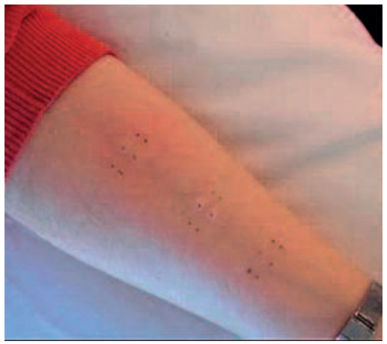
Protocol 1 - Healthy Control

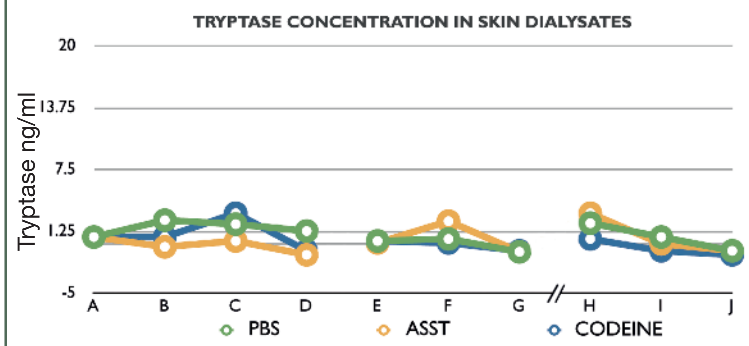
Date:	13/12/2007
Patient:	AMSV11
VAS for itching/24hrs:	N/A
VAS for wealing:	N/A

Microdialysis Sites: Skin Testing





Baseline	Microdialysis	Skin Testing
		
Probes in	Dialysate collection	30 minutes after testing



Histamine Concentration In Skin Dialysates

	A	B	C	D	E	F	G	H	I	J
PBS	0.859	0.892	0.476	0.930	0.983	0.804	0.485	0.960	0.878	0.900
ASST	0.859	1.229	0.555	0.776	1.026	0.853	0.632	0.742	0.834	0.723
CODEINE	0.859	10	0.920	0.808	0.805	0.784	0.635	0.951	1.018	0.697

Tryptase Concentration In Skin Dialysates

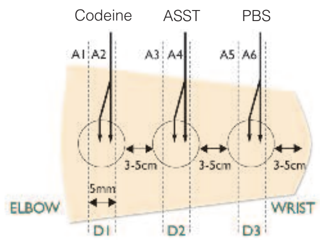
	A	B	C	D	E	F	G	H	I	J
PBS	0.7	2.40	2	1.30	0.30	0.50	-0.80	2.10	0.70	-0.70
ASST	0.7	-0.30	0.30	-1.10	0.10	2.30	-0.80	3.10	0	-0.70
CODEINE	0.7	0.70	3.10	-0.70	0.30	0.10	-0.70	0.50	-0.70	-1.10

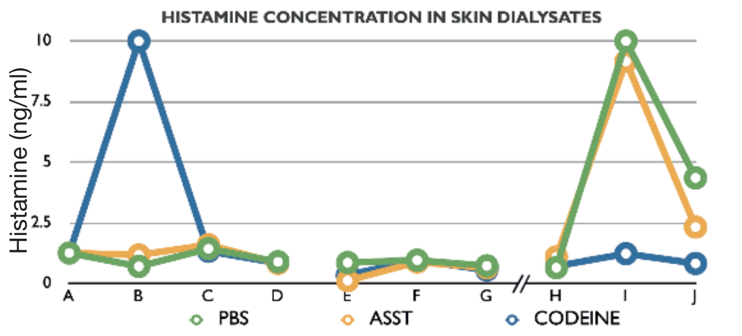
Appendix 3. Figure 14. Microdialysis Experiment - Healthy Control ODV12

Protocol 1 - Healthy Control

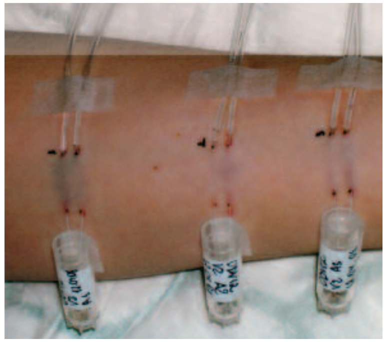
Date:	12/04/2008
Patient:	ODV12
VAS for itching/24hrs:	N/A
VAS for wealing:	N/A

Microdialysis Sites: Skin Testing

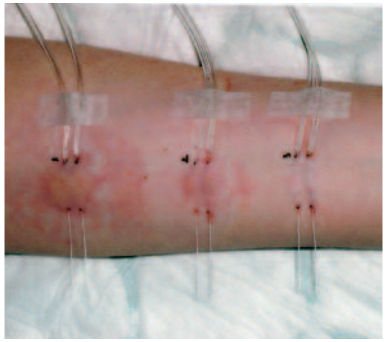




Baseline	Microdialysis	Skin Testing

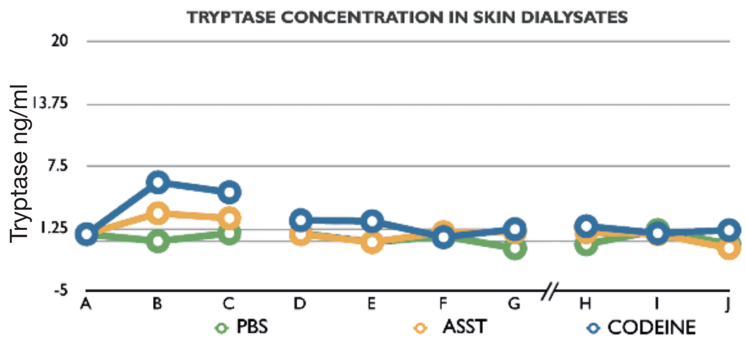


Probes in



Dialysate collection

30 minutes after testing



Histamine Concentration In Skin Dialysates

	A	B	C	D	E	F	G	H	I	J
PBS	1.264	0.711	1.439	0.901	0.867	0.967	0.734	0.658	10	4.360
ASST	1.264	1.182	1.605	0.840	0.126	0.894	0.646	1.106	9.167	2.334
CODEINE	1.264	10	1.337	0.846	0.365	0.954	0.563	0.723	1.236	0.832

Tryptase Concentration In Skin Dialysates

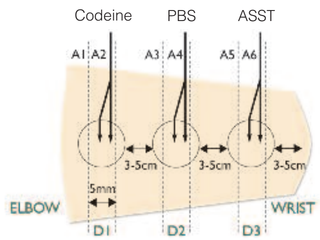
	A	B	C	D	E	F	G	H	I	J
PBS	0.7	0	0.8	0.8	-0.1	0.5	-0.7	-0.3	1.1	-0.3
ASST	0.7	2.8	2.3	0.7	-0.1	0.9	0.9	0.9	0.7	-0.7
CODEINE	0.7	5.9	4.9	2.1	2	0.4	1.2	1.5	0.8	1.1

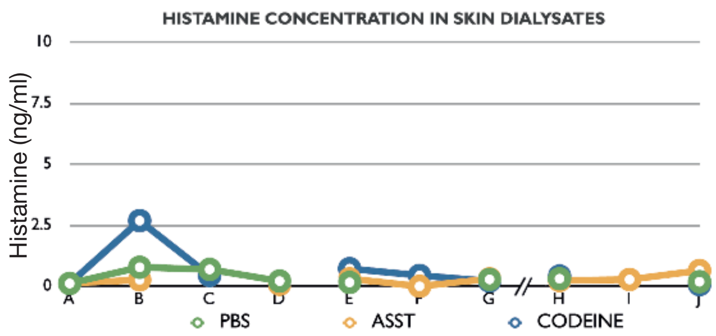
Appendix 3. Figure 15. Microdialysis Experiment - Healthy Control SCV08

Protocol 1 - Healthy Control

Date:	18/11/2007
Patient:	SCV08
VAS for itching/24hrs:	N/A
VAS for wealing:	N/A

Microdialysis Sites: Skin Testing

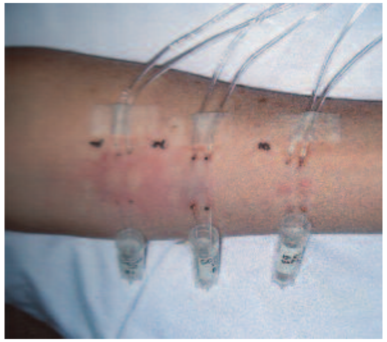




Baseline

Probes in

Microdialysis



Dialysate collection

Skin Testing

30 minutes after testing

Histamine Concentration In Skin Dialysates

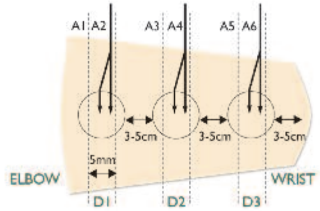
	A	B	C	D	E	F	G	H	I	J
PBS	0.118	0.782	0.693	0.215	0.161		0.284	0.314		0.189
ASST	0.118	0.266		0.085	0.309	0.001	0.308	0.239	0.279	0.646
CODEINE	0.118	2.692	0.444		0.726	0.445	0.217	0.421		0.073

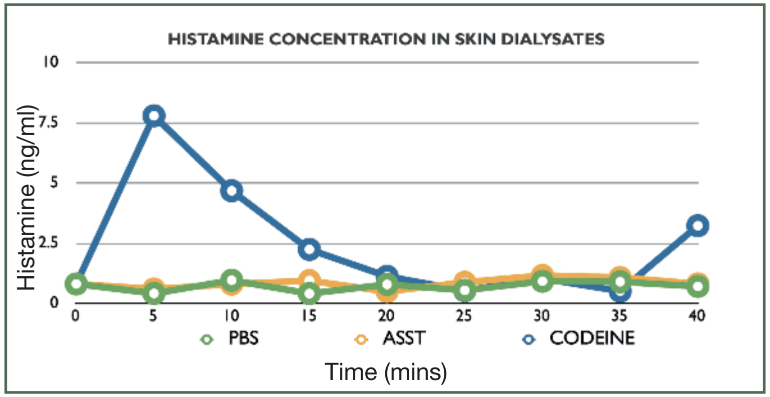
Appendix 3. Figure 16. Microdialysis Experiment - Patient NMP09

Protocol 2 - Patient

Date:	04/09/2008
Patient:	NMP09
VAS for itching/24hrs:	71mm
VAS for wealing:	35mm

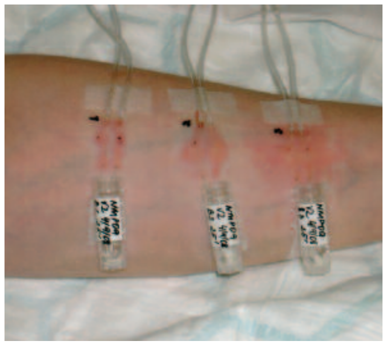
Microdialysis Sites: Skin Testing



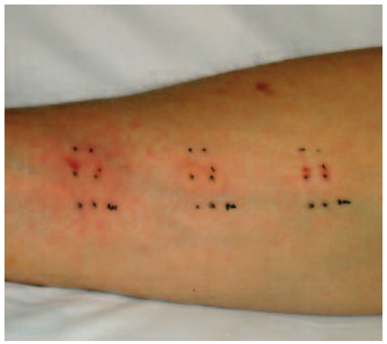


Baseline	Microdialysis	Skin Testing

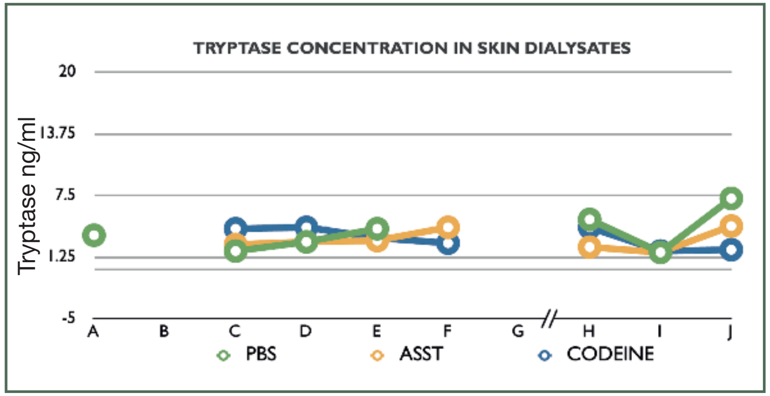
Probes in



Dialysate collection



30 minutes after testing



Histamine Concentration In Skin Dialysates

	0	5	10	15	20	25	30	35	40
PBS	0.816	0.420	0.967	0.415	0.796	0.551	0.927	0.906	0.713
ASST	0.816	0.603	0.808	0.953	0.5	0.885	1.167	1.088	0.818
CODEINE	0.816	7.799	4.685	2.248	1.130	0.536	1.042	0.516	3.234

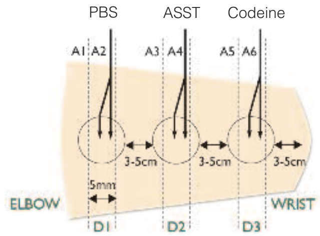
Tryptase Concentration In Skin Dialysates

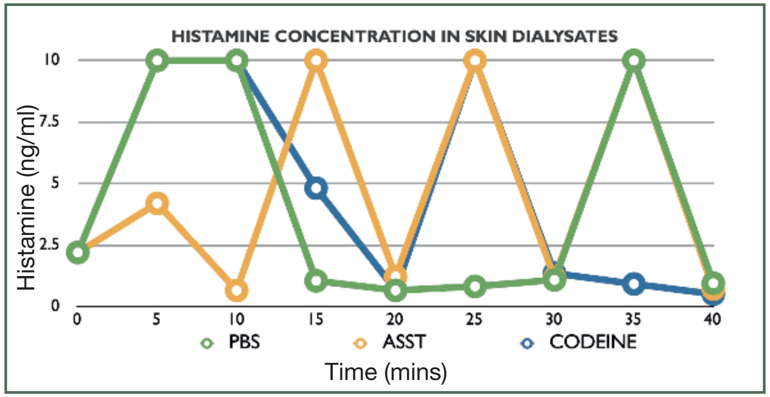
	A	B	C	D	E	F	G	H	I	J
PBS	3.47		1.87	2.8	4.13			5.07	1.73	7.20
ASST	3.47		2.53	2.8	2.93	4.27		2.27	1.73	4.40
CODEINE	3.47		4.13	4.27	3.20	2.67		4.27	1.87	2

Appendix 3. Figure 17. Microdialysis Experiment - Patient JKP10

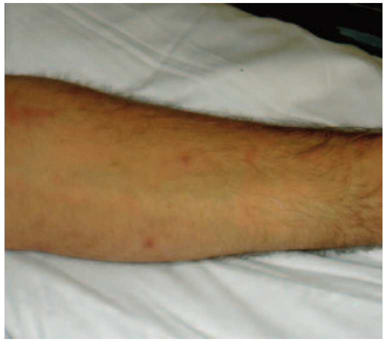
Protocol 2 - Patient	
Date:	17/10/2008
Patient:	JKP10
VAS for itching/24hrs:	85mm
VAS for wealing:	60mm

Microdialysis Sites: Skin Testing

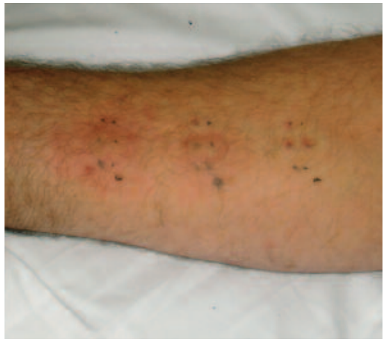


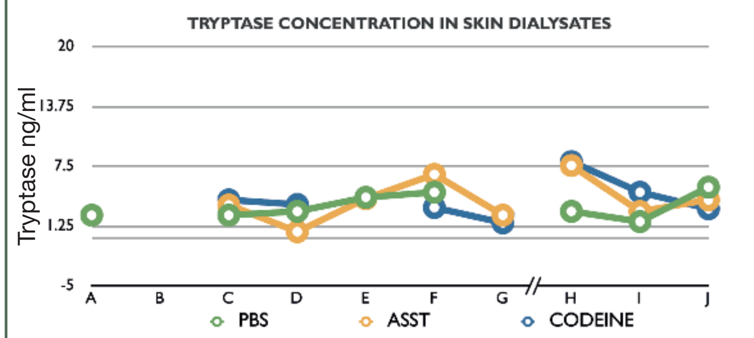


Baseline	Microdialysis	Skin Testing


Probes in


Dialysate collection


30 minutes after testing



Histamine Concentration In Skin Dialysates

	0	5	10	15	20	25	30	35	40
PBS	2.203	10	10	1.054	0.665	0.825	1.091	10	0.956
ASST	2.203	4.199	0.663	10	1.230	10	1.181	10	0.713
CODEINE	2.203	10	10	4.815	0.680	10	1.359	0.927	0.513

Tryptase Concentration In Skin Dialysates

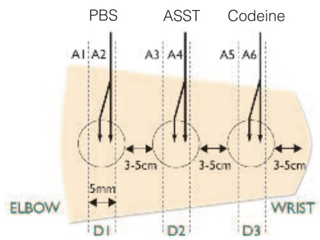
	A	B	C	D	E	F	G	H	I	J
PBS	2.40		2.40	2.80	4.27	4.8		2.80	1.73	5.33
ASST	2.40		3.47	0.67	4.13	6.67	2.40	7.60	2.80	4
CODEINE	2.40		4	3.52		3.2	1.60	8	4.80	3.07

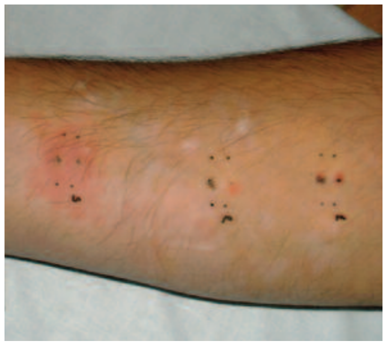
Appendix 3. Figure 18. Microdialysis Experiment - Patient JRP11

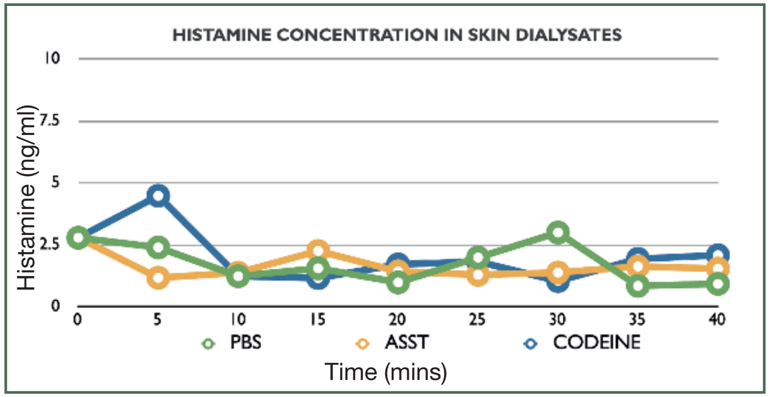
Protocol 2 - Patient

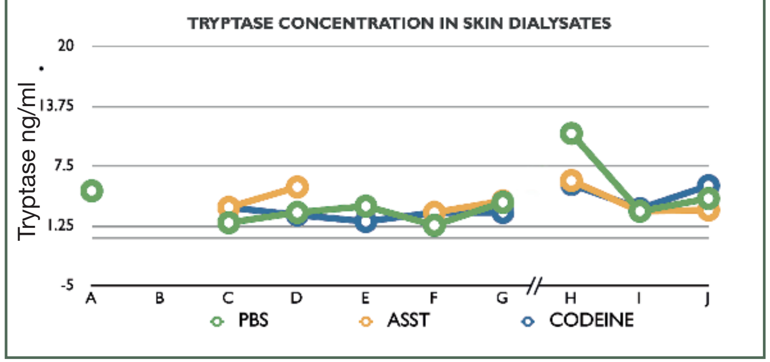
Date:	15/10/2008
Patient:	JRP11
VAS for itching/24hrs:	50mm
VAS for wealing:	32mm

Microdialysis Sites: Skin Testing



Baseline	Microdialysis	Skin Testing
		
Probes in	Dialysate collection	30 minutes after testing





Histamine Concentration In Skin Dialysates

	0	5	10	15	20	25	30	35	40
PBS	2.792	2.400	1.241	1.558	0.989	1.994	3.002	0.850	0.931
ASST	2.792	1.178	1.382	2.259	1.431	1.296	1.386	1.635	1.545
CODEINE	2.792	4.485	1.244	1.164	1.716	1.828	1.043	1.939	2.088

Tryptase Concentration In Skin Dialysates

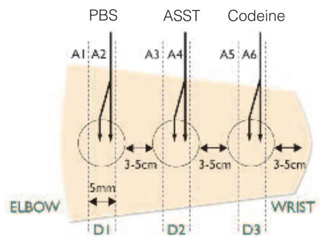
	A	B	C	D	E	F	G	H	I	J
PBS	4.93		1.60	2.67	3.33	1.33	3.73	10.93	2.80	4.13
ASST	4.93		3.20	5.33		2.67	3.87	6	2.80	2.93
CODEINE	4.93		3.20	2.40	1.73	2.67	2.67	5.60	3.07	5.47

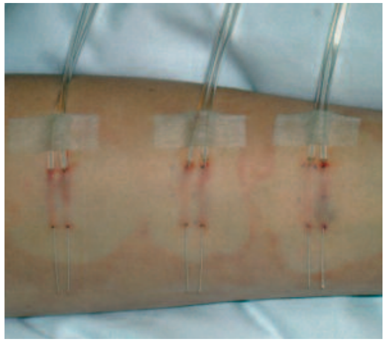
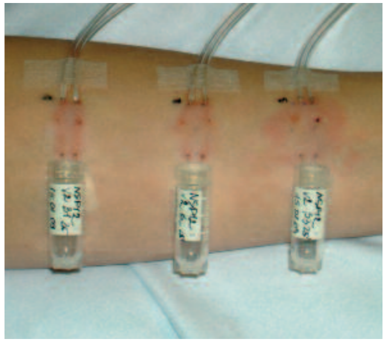
Appendix 3. Figure 19. Microdialysis Experiment - Patient NSP12

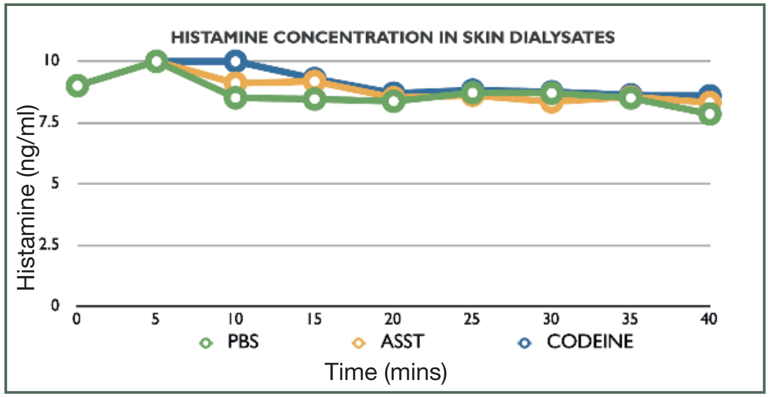
Protocol 2 - Patient

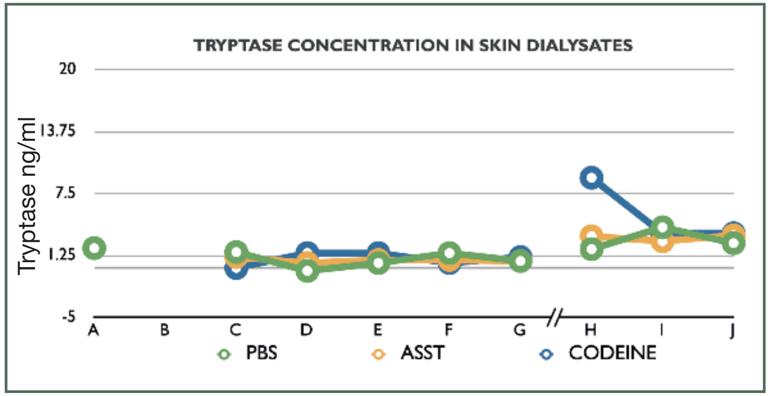
Date:	15/01/2009
Patient:	NSP12
VAS for itching/24hrs:	28mm
VAS for wealing:	27mm

Microdialysis Sites: Skin Testing



Baseline	Microdialysis	Skin Testing
		
Probes in	Dialysate collection	30 minutes after testing





Histamine Concentration In Skin Dialysates

	0	5	10	15	20	25	30	35	40
PBS	9.007	10	8.512	8.461	8.371	8.721	8.708	8.507	7.856
ASST	9.007	10	9.109	9.185	8.518	8.615	8.349	8.552	8.324
CODEINE	9.007	10	10	9.296	8.699	8.824	8.757	8.632	8.596

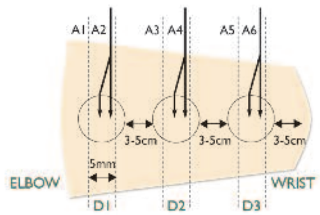
Tryptase Concentration In Skin Dialysates

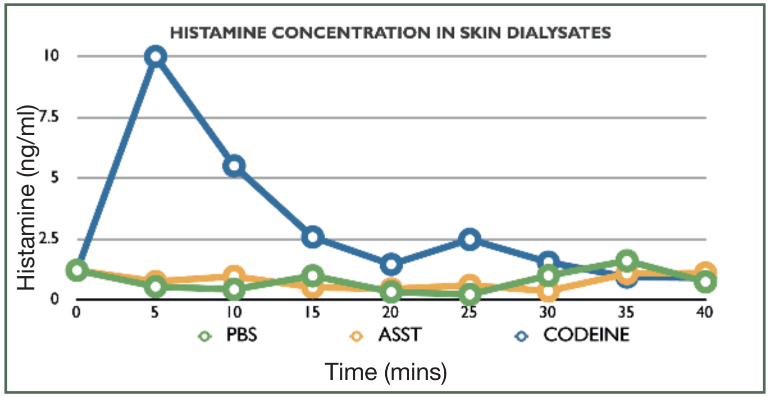
	A	B	C	D	E	F	G	H	I	J
PBS	2		1.60	-0.30	0.5	1.5	0.70	1.90	4.10	2.5
ASST	2		1.10	0.5	0.8	0.8	0.7	3.2	2.7	3.3
CODEINE	2		0	1.5	1.5	0.5	1.1	9.1	3.5	3.5

Appendix 3. Figure 20. Microdialysis Experiment - Healthy Control CGV02

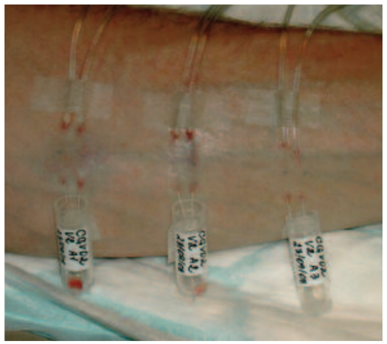
Protocol 2 - Healthy Control	
Date:	23/09/2008
Patient:	CGV02
VAS for itching/24hrs:	N/A
VAS for wealing:	N/A

Microdialysis Sites: Skin Testing

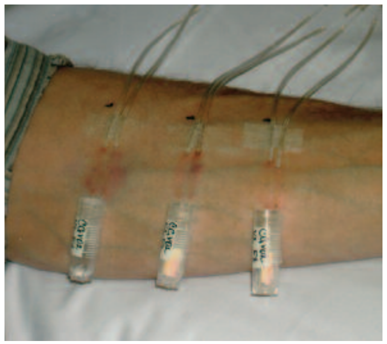




Baseline	Microdialysis	Skin Testing

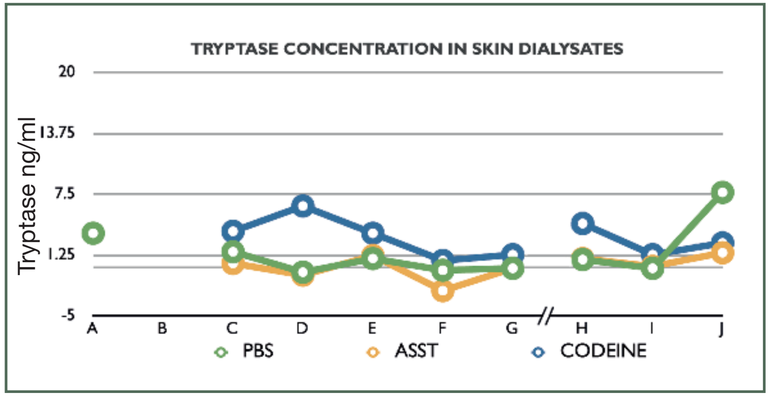


Probes in



Dialysate collection

30 minutes after testing



Histamine Concentration In Skin Dialysates

	0	5	10	15	20	25	30	35	40
PBS	1.218	0.546	0.431	0.994	0.33	0.224	1.002	1.610	0.744
ASST	1.218	0.757	0.970	0.522	0.451	0.601	0.368	1.085	1.107
CODEINE	1.218	10	5.509	2.584	1.461	2.486	1.55	0.941	0.909

Tryptase Concentration In Skin Dialysates

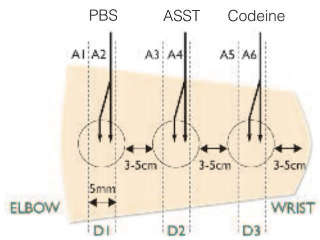
	A	B	C	D	E	F	G	H	I	J
PBS	3.5		1.60	-0.50	0.90	-0.30	-0.10	0.80	-0.10	7.70
ASST	3.5		0.40	-0.80	1.20	-2.40	-0.10	0.90	0.10	1.50
CODEINE	3.5		3.70	6.30	3.50	0.70	1.30	4.50	1.30	2.50

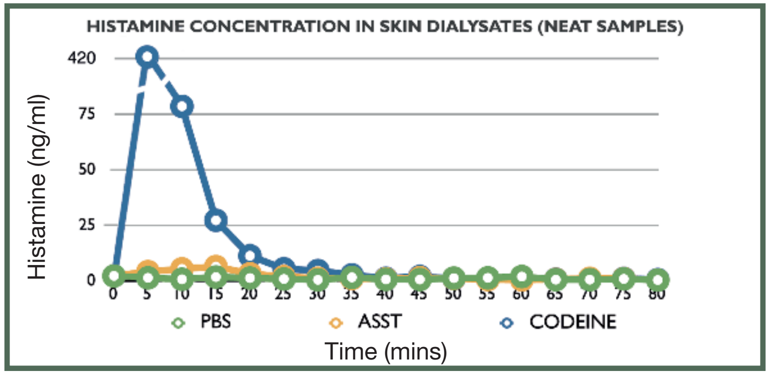
Appendix 3. Figure 21. Microdialysis Experiment - Patient MLP12

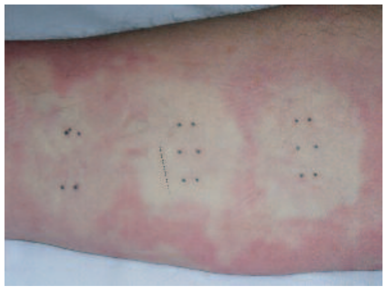
Protocol 3 - Patient

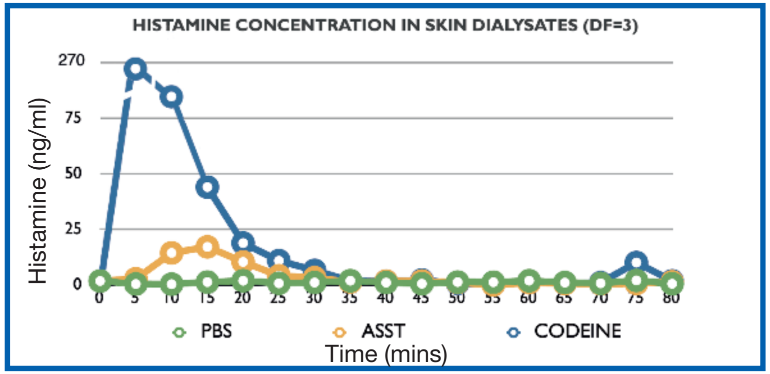
Date:	02/06/2011
Patient:	MLP12
VAS for itching/24hrs:	35mm
VAS for wealing:	42mm

Microdialysis Sites: Skin Testing





Baseline	Microdialysis	Skin Testing
		
Probes in	Dialysate collection	30 minutes after testing



Histamine Concentration In Skin Dialysates (Neat Samples)

	0	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80
PBS	2.439	1.565	0.938	1.944	1.486	1.050	0.687	1.776	0.864	0.825	1.458	1.551	2.138	0.760	0.760	1.092	0.566
ASST	2.439	4.346	5.591	6.621	3.419	2.104	1.389	1.282	1.110	1.691	1.315	0.825	0.671	0.870	1.342	1.211	0.585
CODEINE	2.439	420.5	78.71	27.440	11.470	5.611	4.834	2.876	1.382	2.173	1.198	1.382	1.558	0.961	0.932	1.302	0.755

Histamine Concentration In Skin Dialysates (DF=3)

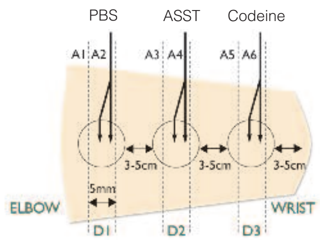
	0	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80
PBS	1.906	0.553	0.481	1.404	2.028	0.853	1.210	2.041	1.174	0.658	1.416	1.354	2.041	1.186	0.875	2.152	0.658
ASST	1.906	2.890	14.540	17.250	10.490	4.293	3.457	1.466	1.879	1.946	1.127	0.134	1.104	0.754	0.605	0.765	1.579
CODEINE	1.906	269.800	100.900	44.160	18.980	11.030	6.689	1.932	1.892	2.365	0.831	0.331	1.416	0.887	1.139	10.210	1.973

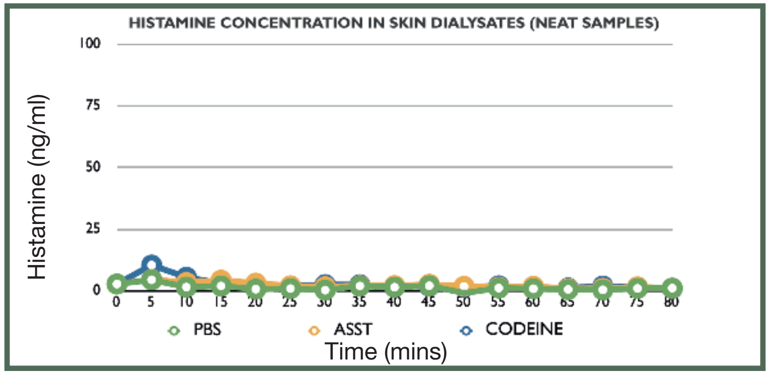
Appendix 3. Figure 22. Microdialysis Experiment - Patient RMP20

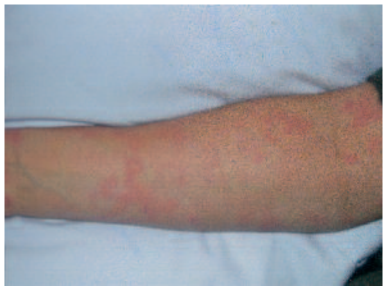
Protocol 3 - Patient

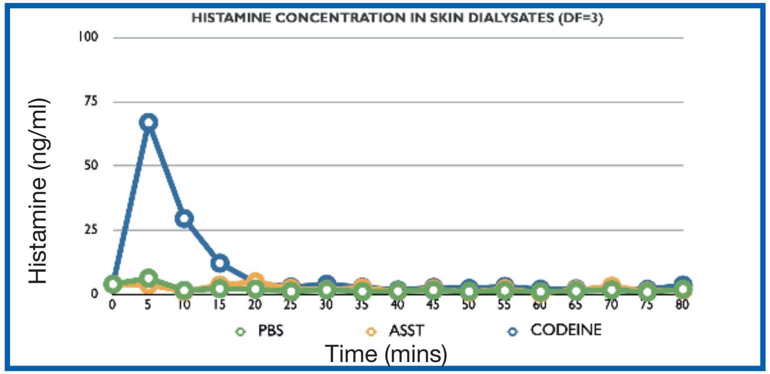
Date:	21/03/2011
Patient:	RMP20
VAS for itching/24hrs:	92mm
VAS for wealing:	91mm

Microdialysis Sites: Skin Testing





Baseline	Microdialysis	Skin Testing
		
Probes in	Dialysate collection	30 minutes after testing



Histamine Concentration In Skin Dialysates (Neat Samples)

	0	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80
PBS	2.759	4.451	1.502	1.964	0.678	0.909	0.263	1.955	1.444	2.048	-0.895	1.12	0.836	0.543	0.396	0.976	1.089
ASST	2.759	4.613	3.361	4.294	3.103	2.039	1.697	2.133	2.095	2.650	1.784	1.510	1.837	0.939	1.013	1.611	1.066
CODEINE	2.759	10.430	5.473	3.032	1.910	2.001	2.523	2.481	1.654	1.882	1.510	2.039	1.315	1.174	1.937	1.387	0.954

Histamine Concentration In Skin Dialysates (DF=3)

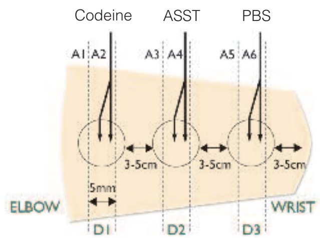
	0	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80
PBS	3.989	6.324	1.586	2.222	2.042	1.214	1.782	1.162	1.318	1.641	1.214	1.477	1.099	1.344	1.767	0.95	1.998
ASST	3.989	3.779	1.188	3.649	4.863	2.376	1.911	2.662	1.214	2.207	0.926	2.268	0.608	1.767	3.217	1.252	1.725
CODEINE	3.989	66.920	29.560	12.190	4.527	2.892	3.969	2.825	1.697	2.727	2.314	3.130	1.998	2.177	1.896	1.954	3.630

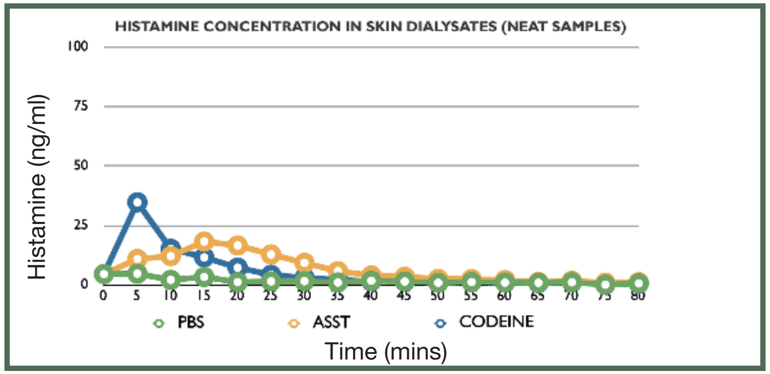
Appendix 3. Figure 23. Microdialysis Experiment - Patient RPP25

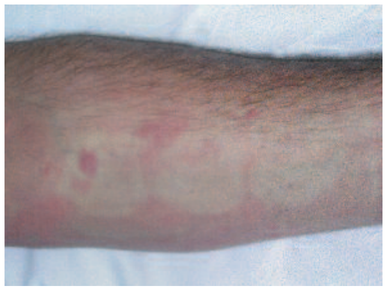
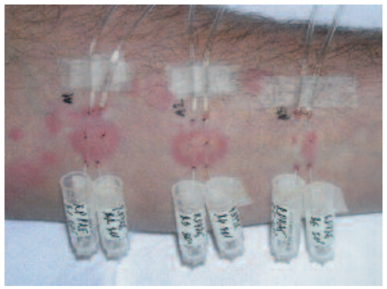
Protocol 3 - Patient

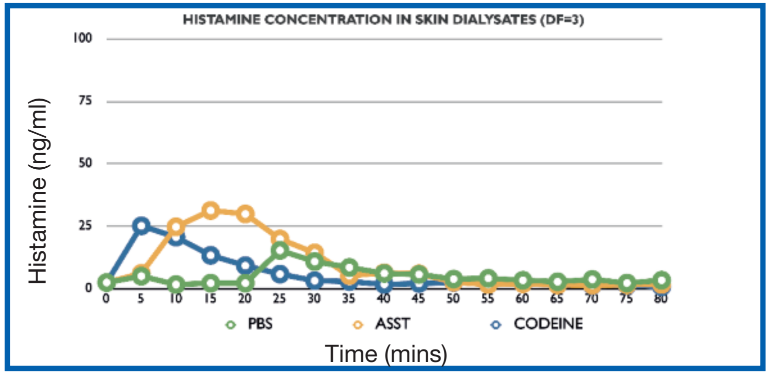
Date:	15/07/2011
Patient:	RPP25
VAS for itching/24hrs:	78mm
VAS for wealing:	77mm

Microdialysis Sites: Skin Testing





Baseline	Microdialysis	Skin Testing
		
Probes in	Dialysate collection	30 minutes after testing



Histamine Concentration In Skin Dialysates (Neat Samples)

	0	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80
PBS	4.573	4.714	2.091	3.35	1.149	1.49	1.576	1.01	1.747	1.407	0.895	1.198	0.971	0.889	1.095	0.067	0.63
ASST	4.573	10.87	12.12	18.220	16.590	12.820	9.265	5.769	3.987	3.443	2.506	2.453	2.072	1.198	1.705	0.927	1.143
CODEINE	4.573	34.740	15.170	11.580	7.256	4.238	2.951	2.119	1.537	1.806	1.069	1.490	1.042	0.774	1.219	0.505	0.597

Histamine Concentration In Skin Dialysates (DF=3)

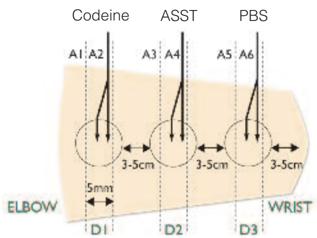
	0	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80
PBS	2.456	4.995	1.643	2.196	2.210	15.3	10.840	8.490	6.104	5.679	3.836	4.115	3.344	2.760	3.674	2.238	3.361
ASST	2.456	6.128	24.730	31.260	29.860	19.850	14.30	5.153	6.349	5.936	2.546	1.630	1.960	1.466	1.319	1.478	1.643
CODEINE	2.456	25.120	20.600	13.320	9.197	5.819	3.177	2.838	1.694	2.056	2.471	1.812	2.014	1.503	1.165	1.295	0.712

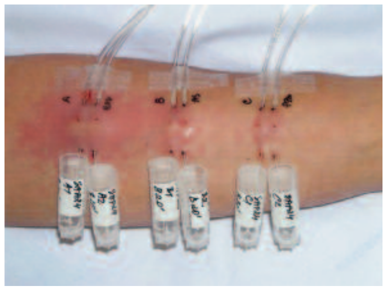
Appendix 3. Figure 24. Microdialysis Experiment - Patient SAP24

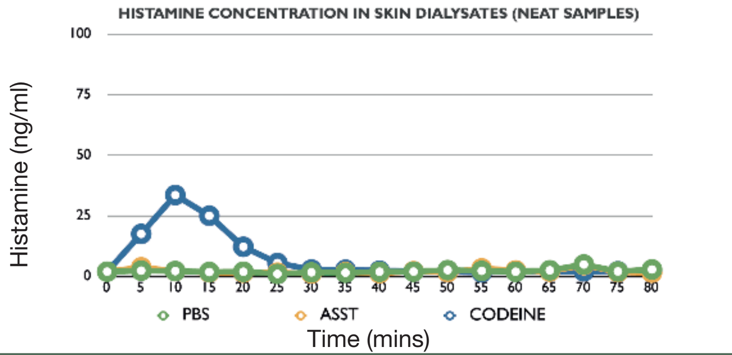
Protocol 3 - Patient

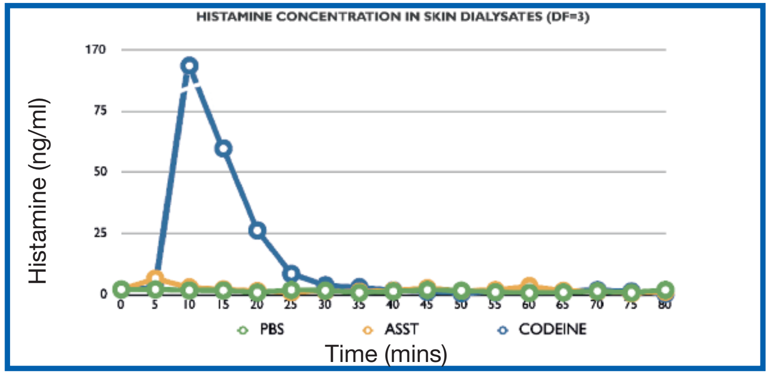
Date:	22/09/2011
Patient:	SAP24
VAS for itching/24hrs:	56mm
VAS for wealing:	64mm

Microdialysis Sites: Skin Testing



Baseline	Microdialysis	Skin Testing
		
Probes in	Dialysate collection	30 minutes after testing





Histamine Concentration In Skin Dialysates (Neat Samples)

	0	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80
PBS	1.963	2.368	2.197	1.804	1.925	1.013	1.659	1.5	1.94	1.963	2.586	2.265	1.902	2.512	4.901	2.042	2.857
ASST	1.963	3.736	2.164	1.638	1.507	1.541	1.146	1.782	1.293	2.123	1.716	3.295	2.468	1.995	4.646	1.723	1.487
CODEINE	1.963	17.55	33.590	25.040	12.220	5.513	2.807	2.748	2.368	1.971	1.917	1.617	2.248	2.058	2.090	2.123	1.811

Histamine Concentration In Skin Dialysates (DF=3)

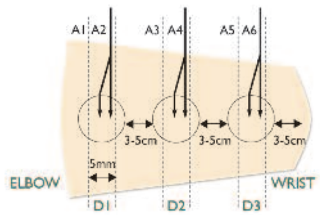
	0	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80
PBS	2.205	2.219	1.841	1.800	0.929	2.006	1.733	0.884	1.434	1.978	1.706	1.069	0.783	0.987	1.485	0.873	1.992
ASST	2.205	6.580	3.021	2.234	1.536	1.069	1.562	1.359	1.733	2.732	1.614	1.950	3.515	1.654	1.359	0.599	1.248
CODEINE	2.205	2.875	135.200	59.730	26.270	8.575	3.858	3.071	1.841	1.261	0.641	0.987	1.8	1.524	1.950	1.397	0.463

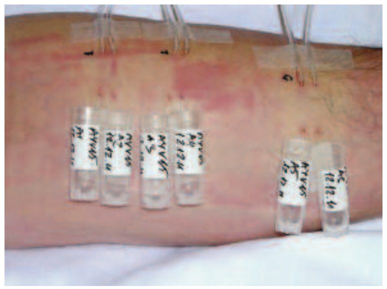
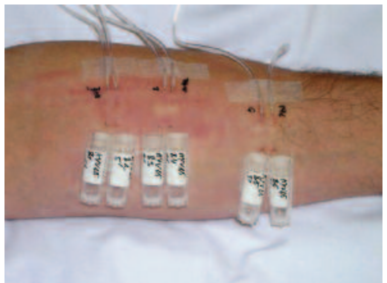
Appendix 3. Figure 25. Microdialysis Experiment - Healthy Control AYV15

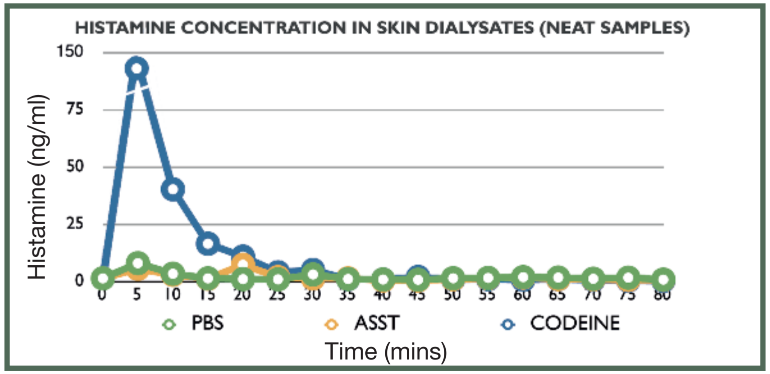
Protocol 3 - Healthy Control

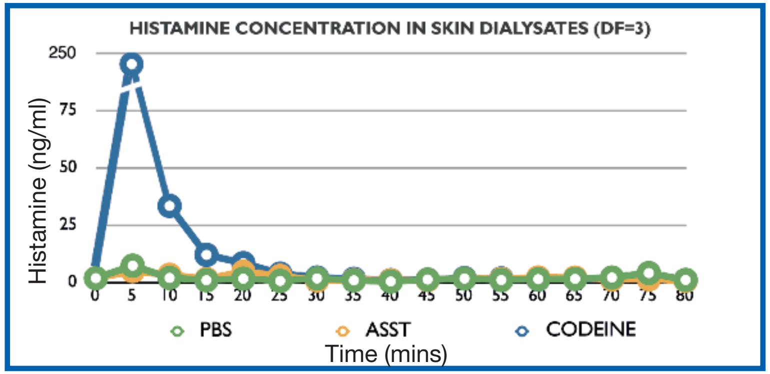
Date:	12/12/2011
Patient:	AYV15
VAS for itching/24hrs:	N/A
VAS for wealing:	N/A

Microdialysis Sites: Skin Testing



Baseline	Microdialysis	Skin Testing
		
Probes in	Dialysate collection	30 minutes after testing





Histamine Concentration In Skin Dialysates (Neat Samples)

	0	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80
PBS	1.404	8.007	3.410	1.395	1.071	0.969	3.103	1.116	0.879	0.788	1.430	1.567	1.931	1.733	1.194	1.673	0.833
ASST	1.404	5.533	2.936	1.226	7.314	1.964	1.309	1.386	0.477	0.482	1.005	1.369	1.693	1.078	0.920	0.660	0.690
CODEINE	1.404	123.9	40.270	16.520	10.890	4.023	5.010	1.041	0.906	2.008	0.833	1.019	0.757	1.012	0.672	0.372	0.294

Histamine Concentration In Skin Dialysates (DF=3)

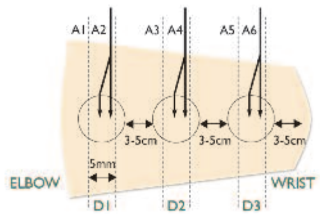
	0	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80
PBS	1.876	7.330	1.979	0.986	1.647	0.680	1.760	0.929	0.416	1.304	1.710	1.056	1.425	1.535	2.138	4.096	1.103
ASST	1.876	4.883	3.363	1.474	4.698	2.923	0.883	1.091	0.929	1.044	1.710	1.535	2.178	2.125	1.316	1.352	0.759
CODEINE	1.876	248.100	33.380	12.030	8.422	3.844	2.138	1.572	1.01	1.15	1.889	1.76	1.34	1.56	1.647	1.850	1.021

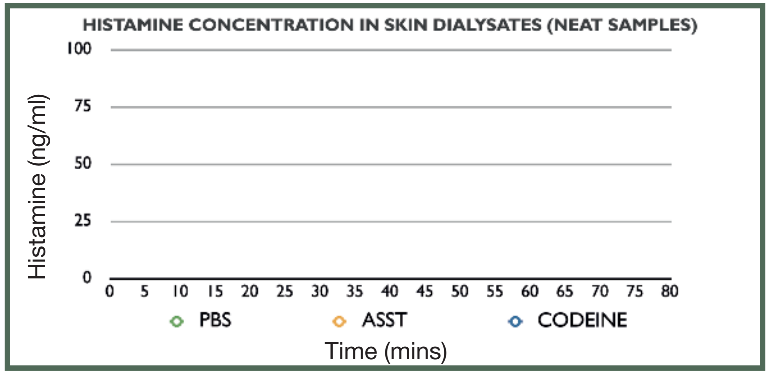
Appendix 3. Figure 26. Microdialysis Experiment - Healthy Control IPV14

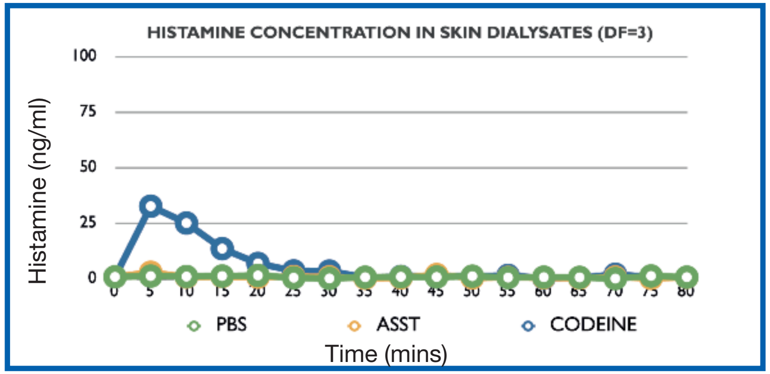
Protocol 3 - Healthy Control

Date:	14/12/2011
Patient:	IPV14
VAS for itching/24hrs:	N/A
VAS for wealing:	N/A

Microdialysis Sites: Skin Testing







Baseline	Microdialysis	Skin Testing
		
Probes in	Dialysate collection	30 minutes after testing

Histamine Concentration In Skin Dialysates (Neat Samples)

	0	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80
PBS																	
ASST																	
CODEINE																	

Histamine Concentration In Skin Dialysates (DF=3)

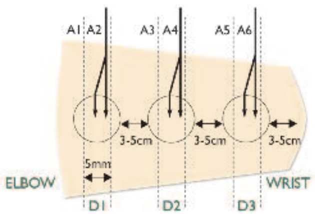
	0	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80
PBS	0.802	1.154	0.94	1.179	1.429	0.423	0.109	0.646	0.836	0.746	1.106	0.496	0.668	0.570	0.046	1.203	0.668
ASST	0.802	2.869	0.701	0.975	0.646	0.940	0.791	0.193	0.371	1.840	0.311	0.592	0.382	0.232	0.723	0.155	0.592
CODEINE	0.802	32.620	25.030	13.510	6.860	3.492	3.440	0.528	0.975	1.455	1.010	1.480	0.193	0.291	1.965	0.679	0.613

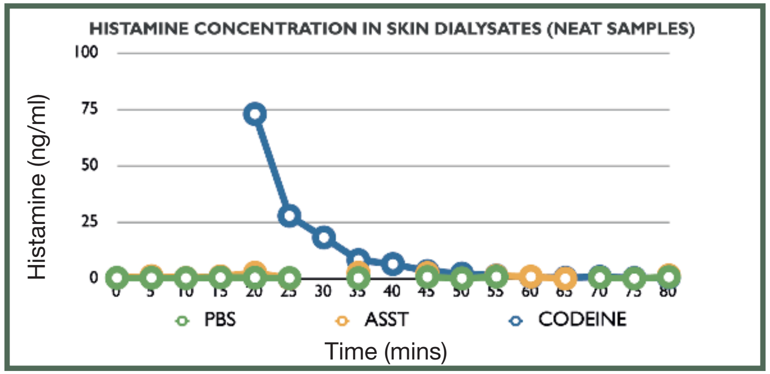
Appendix 3. Figure 27. Microdialysis Experiment - Healthy Control UBV16

Protocol 3 - Healthy Control

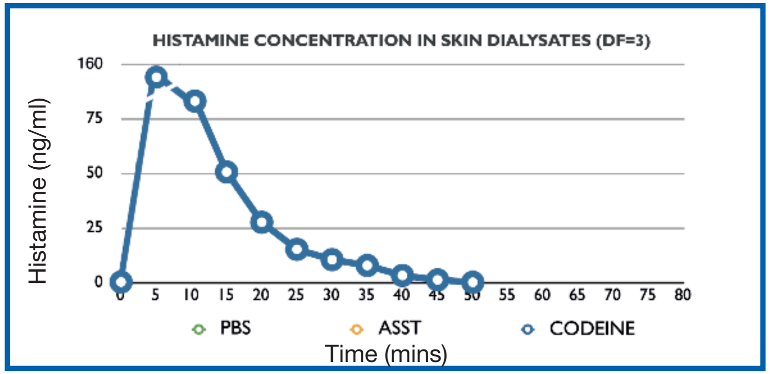
Date:	16/12/2011
Patient:	UBV16
VAS for itching/24hrs:	N/A
VAS for wealing:	N/A

Microdialysis Sites: Skin Testing





Baseline	Microdialysis	Skin Testing
		
Probes in	Dialysate collection	30 minutes after testing



Histamine Concentration In Skin Dialysates (Neat Samples)

	0	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80
PBS	0.315	0.332	0.232	0.518	0.390	0.232		0.215		0.740	0.115	0.846			0.526	0.082	0.684
ASST	0.315	1.129	0.323	0.985	2.630	0.124		2.603		2.510		1.298	0.855	0.039			1.431
CODEINE	0.315				72.990	27.840	18.220	8.198	6.458	3.364	2.227	1.389	0.684	0.365	0.837	0.282	0.441

Histamine Concentration In Skin Dialysates (DF=3)

	0	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80
PBS																	
ASST																	
CODEINE	0.315	158.900	87.450	50.670	27.660	15.270	10.540	7.894	3.320	1.305	0.198						

Appendix 3. Figure 28. Microdialysis Experiment - Technical Aspects (6)

The definition of non-specific skin reactivity to microdialysis procedures

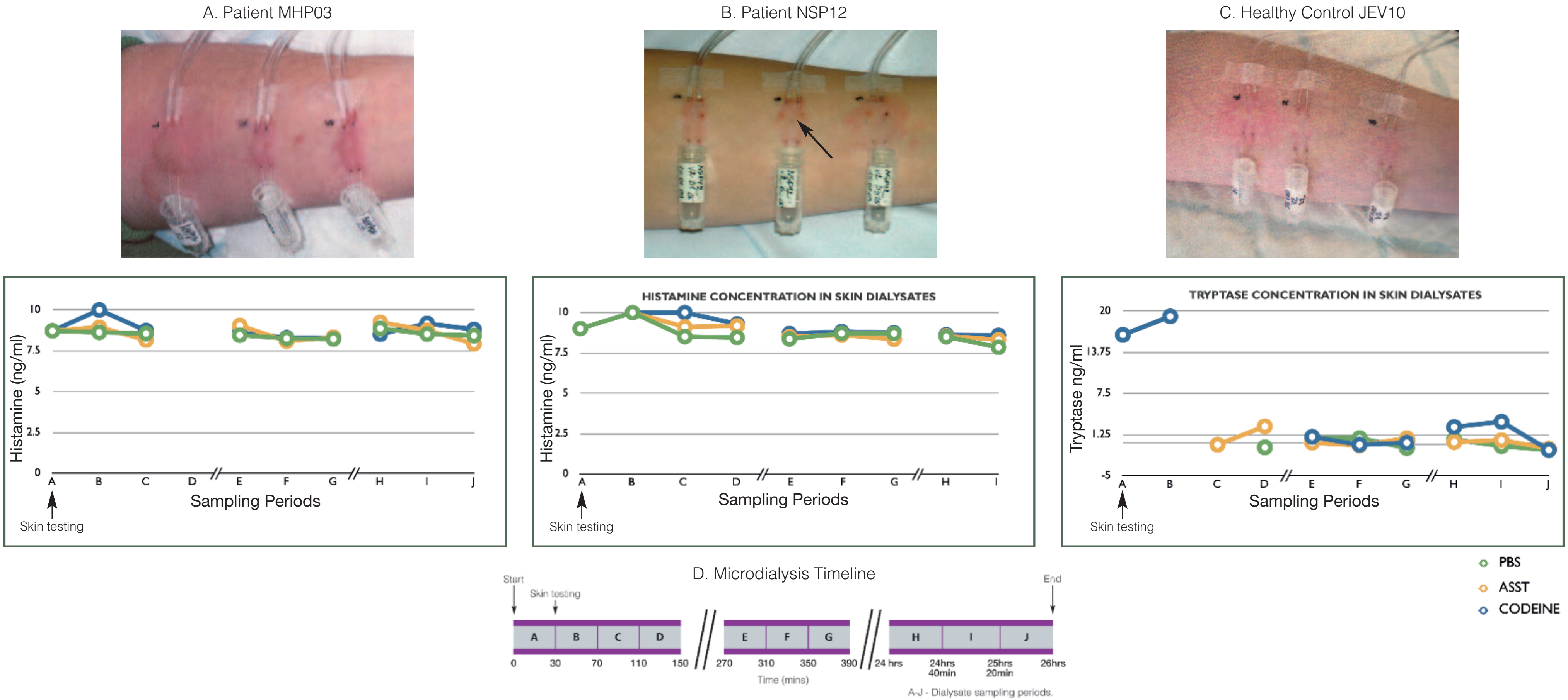


Figure 28. Non-specific skin reactivity to microdialysis procedures. In our study, two patients with CSU (Figures 28A-B) and one healthy subject (Figure 28C) displayed the elevated dermal histamine (Figures 28A-B) or tryptase (Figure 28C) concentration at the baseline comparable to that after skin testing with codeine. This may suggest that this dermal response may occur as a result of the insertion or the presence of the microdialysis probes in the dermis. It is important to be aware of the possibility of these reactions in few individuals. Whether this dermal response can be predicted using high resolution laser Doppler perfusion imaging needs to be established in the future studies. The corresponding time intervals to sampling periods in the microdialysis studies are presented in Figure 28D.

Abbrevations:
ASST - Autologous serum skin test
CSU - Chronic spontaneous urticaria
PBS - Phosphate buffered saline

Appendix 4. Figure 1. Pathophysiological Phenotyping in CSU at Baseline

Figure 1. Based on the results of basophil releasability assays, we could differentiate three subsets of CSU patients:
- responders to anti-IgE stimulation (BHR over 10% of total cellular histamine to anti-IgE stimulation) (n=7);
- non-responders to anti-IgE stimulation (BHR below 10% of total cellular histamine to anti-IgE stimulation) (n=8);
- total cellular histamine below the LOD of spectrofluorimetry (n=7).

Based on serum-induced BHR assay, CSU patients were grouped into CSU patients with (n=8) and without (n=14) serum histamine-releasing activity.

Anti-IgE induced BHR from peripheral blood basophils of CSU patients was carried out by Dr Bernhard Gibbs at the Medway School of Pharmacy (Chatham Maritime, UK). For basophil releasability assays, cells were stimulated with anti-IgE antibodies (Sigma-Aldridge, UK) at 0.1ng/ml. Serum-induced BHR was assessed on peripheral blood basophils from healthy donors at the RefLab (University of Copenhagen, Denmark). For serum-induced BHR assay, a diagnostic cut-off of 16.5% was used to detect serum histamine-releasing activity in CSU patients (Platzer M. et al, 2005).

Abbrevations:
BHR - Basophil histamine release
CSU - Chronic spontaneous urticaria
LOD - Level of detection

Table 1 . CSU patient classification related to serum histamine-releasing activity

Patient	Serum-induced BHR (% of total cellular histamine)	Serum histamine-releasing activity
KDP01	41	positive
MHP02	5	negative
TBP03	39	positive
RHP04	9	negative
SRP05	0	negative
RWP06	0	negative
MMP07	0	negative
MLP08	6	negative
ASP09	36	positive
ISTP11	11	negative
MWP12	34	positive
JHP14	0	negative
JCP15	0	negative
DPP16	0	negative
IHP17	0	negative
BSP19	47	positive
GHP20	44	positive
DMP21	0	negative
TMP21	0	negative
DBP23	50	positive
BHP24	45	positive
DMP25	0	negative

Table 2 . CSU patient classification related to basophil releasability to anti-IgE stimulation

Patient	Anti-IgE-induced BHR (%) (% of total cellular histamine)	CSU Subset
KDP01	4.2	non-responder
MHP02	7.4	non-responder
TBP03	0	histamine below the LOD
RHP04	-10.3	non-responder
SRP05	0.4	non-responder
RWP06	58.1	responder
MMP07	53.9	responder
MLP08	9.5	responder
ASP09	0	histamine below the LOD
ISTP11	48	responder
MWP12	0	histamine below the LOD
JHP14	-0.7	non-responder
JCP15	13.5	responder
DPP16	55.9	responder
IHP17	0	non-responder
BSP19	14.8	responder
GHP20	0	histamine below the LOD
DMP21	68.1	responder
TMP21	0	histamine below the LOD
DBP23	0	histamine below the LOD
BHP24	0	histamine below the LOD
DMP25	-1.1	non-responder

Appendix 4. Table 3. Disease Course Profiles in CSU Patients

Patient	UAS7 Visit 1	UAS7 Visit 3	CSU course profile
KDP01	17	0	improving CSU
MHP02	20	4	improving CSU
TBP03	25	35	persistent CSU
SRP05	2	0	improving CSU
RWP06	0	0	improving CSU
MMP07	0	0	improving CSU
MLP08	32	35	persistent CSU
ISTP11	12	28	persistent CSU
MWP12	22	3	improving CSU
JHP14	20	32	persistent CSU
JCP15	2	0	improving CSU
DPP16	19	25	persistent CSU
IHP17	16	23	persistent CSU
BSP19	28	30	persistent CSU
DMP21	5	0	improving CSU
TMP21	24	37	persistent CSU
DBP23	29	27	persistent CSU
BHP24	36	44	persistent CSU
DMP25	27	0	improving CSU

Table 3. Patients with persistent CSU were defined if their UAS7 score at Visit 3 was greater or equal than that at Visit 1. Patients with improving CSU were defined if their UAS7 score at Visit 3 was less than that at Visit 1. UAS7 with score range of 0 to 49 was used in this study.

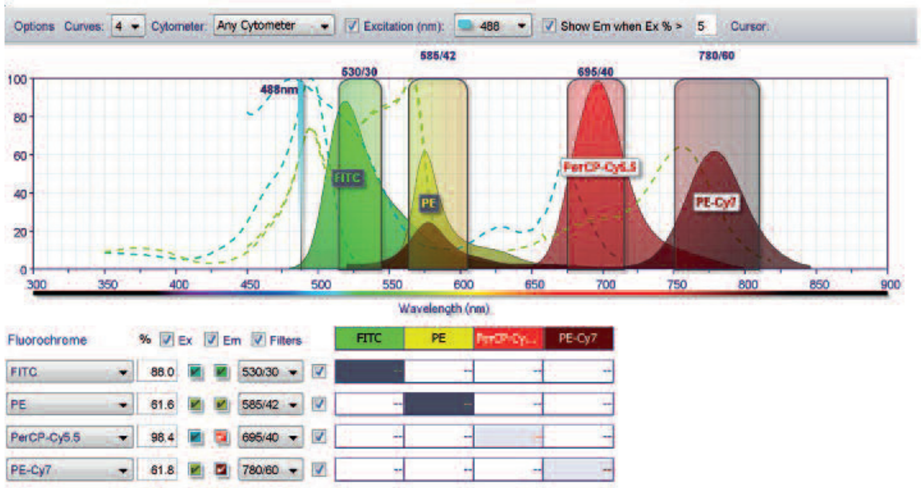
Abbrevations:
CSU - Chronic spontaneous urticaria
UAS7 - Urticaria activity score over 7 days

Appendix 4. Figure 2. The Antibody Panel for Basophil Studies in CSU Patients using Multicolour Flow Cytometry

A. Multicolour Panel used for Flow Cytometry Studies at the Norfolk & Norwich University Hospital

Laser	488nm	488nm	488nm	488nm	633nm	633nm
Fluorescent Dyes	FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-Cy7
Cell Suface Marker	CD63	CCR3	CD123	HLA-DR	CD203c	CD45

B. BD Flourescence Spectrum Viewer - Blue Laser (488 nm)



C. BD Flourescence Spectrum Viewer - Red Laser (633 nm)

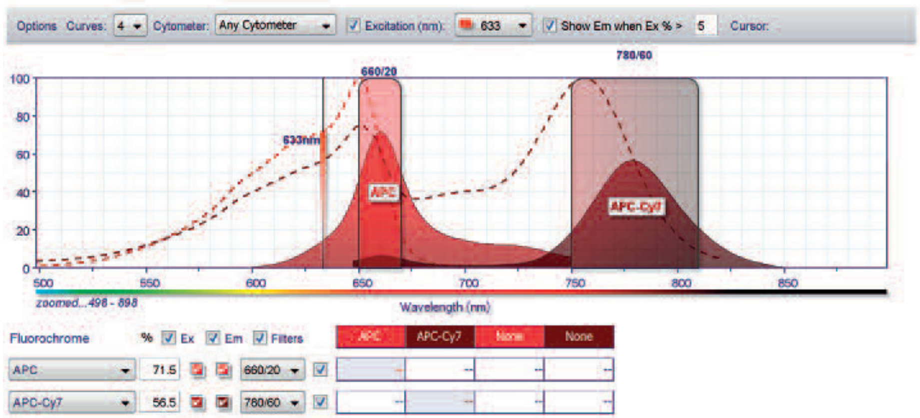


Figure 2. For antibody conjugates, a combination of fluorochromes excited by blue and red lasers (Figure 2A) were selected to ensure a minimal spillover between the channels for BD FACSCanto ™ II instrument configuration as demonstrated by BD Fluorescence Spectrum Viewer output in Figures 2B and C (taken from www.bdbiosciences.com/spectra). Flow cytometry studies were carried out using BD FACSCanto™ II Flow cytometer by Miss Cheryl Barker at the Pathology Department, Norfolk & Norwich University Hospital (Norwich, UK).

Abbrevations:
CSU - Chronic spontaneous urticaria
FITC - Fluorescein isothianate
PE - Phycoerythrin
PerCP - Peridin Chlorophyll Protein Complex
Cy5.5 - Cyanine 5.5
Cy7 - Cyanine 7
CCR3 - Chemokine (C-C motif) receptor type 3
HLA-DR - D-related human leukocyte antigen (related to the D-locus on the chromosome 6)
APC - Allophycocyanin

Appendix 4. Figure 3. Fluorescence-Minus-One Gating Controls and Gate Construction for Peripheral Blood Basophils in Flow Cytometric Studies

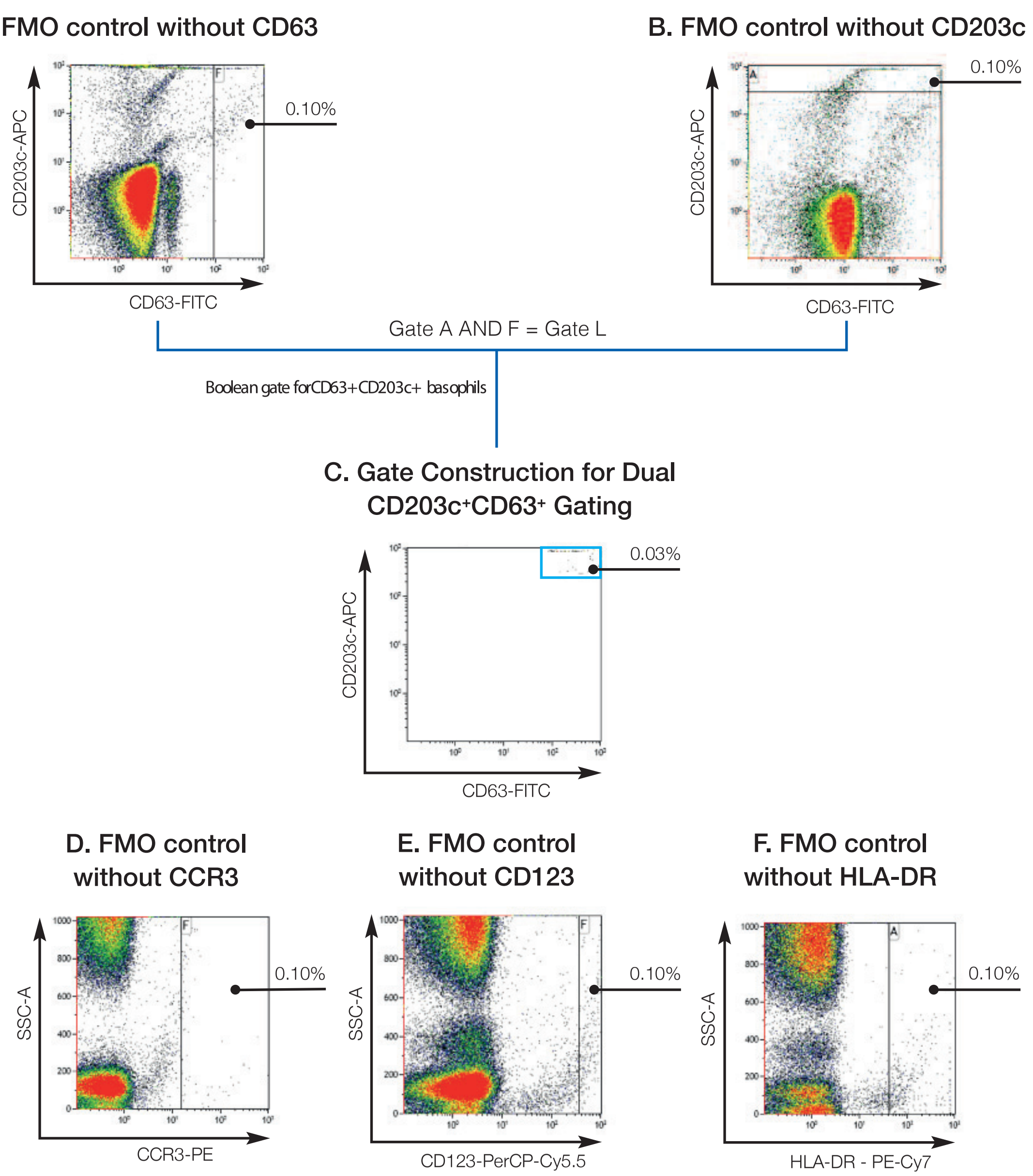


Figure 3. In this sample experiment, fluorescence-minus-one controls were used to identify positive versus negative events and to optimise the gates for peripheral blood basophils. The FMO gate for CD203c was defined by peripheral blood sample stained by a full panel of antibodies (Appendix 3, Figure 1A) except CD203c-APC (Appendix 3, Figure 2A). The FMO gate for CD63 was defined by peripheral blood sample stained by a full panel of antibodies except CD63-FITC (Appendix 3, Figure 2B). The Boolean gate was constructed as a combination of FMO gates for CD63-APC and CD63-FITC for dual positive cells (Appendix 3, Figure 2C). The FMO gates for CCR3-PE, CD123-PerCP-Cy5.5 and HLA-DR-PE-Cy7 presented in the Figures 2D-F. The FMO gates were set at 99.9% percentile. The data were acquired on a BD FACS Canto II™ flow cytometer by Miss Cheryl Barker at the Pathology Department, Norfolk & Norwich University Hospital (Norwich, UK). Data analysis was carried out using Kaluza r analysis software Version 1.1. (Beckman Coulter, Inc.).

Abbrevations:
APC - Allophycocyanin
CCR3 - Chemokine (C-C motif) receptor type 3
CSU - Chronic spontaneous urticaria
Cy5.5 - Cyanine 5.5
Cy7 - Cyanine 7
FITC - Fluorescein isothianate
FMO - Fluorescence-Minus-One
HLA-DR - D-related human leukocyte antigen (related to the D-locus on the chromosome 6)
PE - Phycoerythrin
PerCP - Peridin Chlorophyll Protein Complex
SSC - Side scatter

Appendix 4. Figure 4. Basophil Identification by Three Gating Strategies in Flow Cytometric Studies in CSU Patients

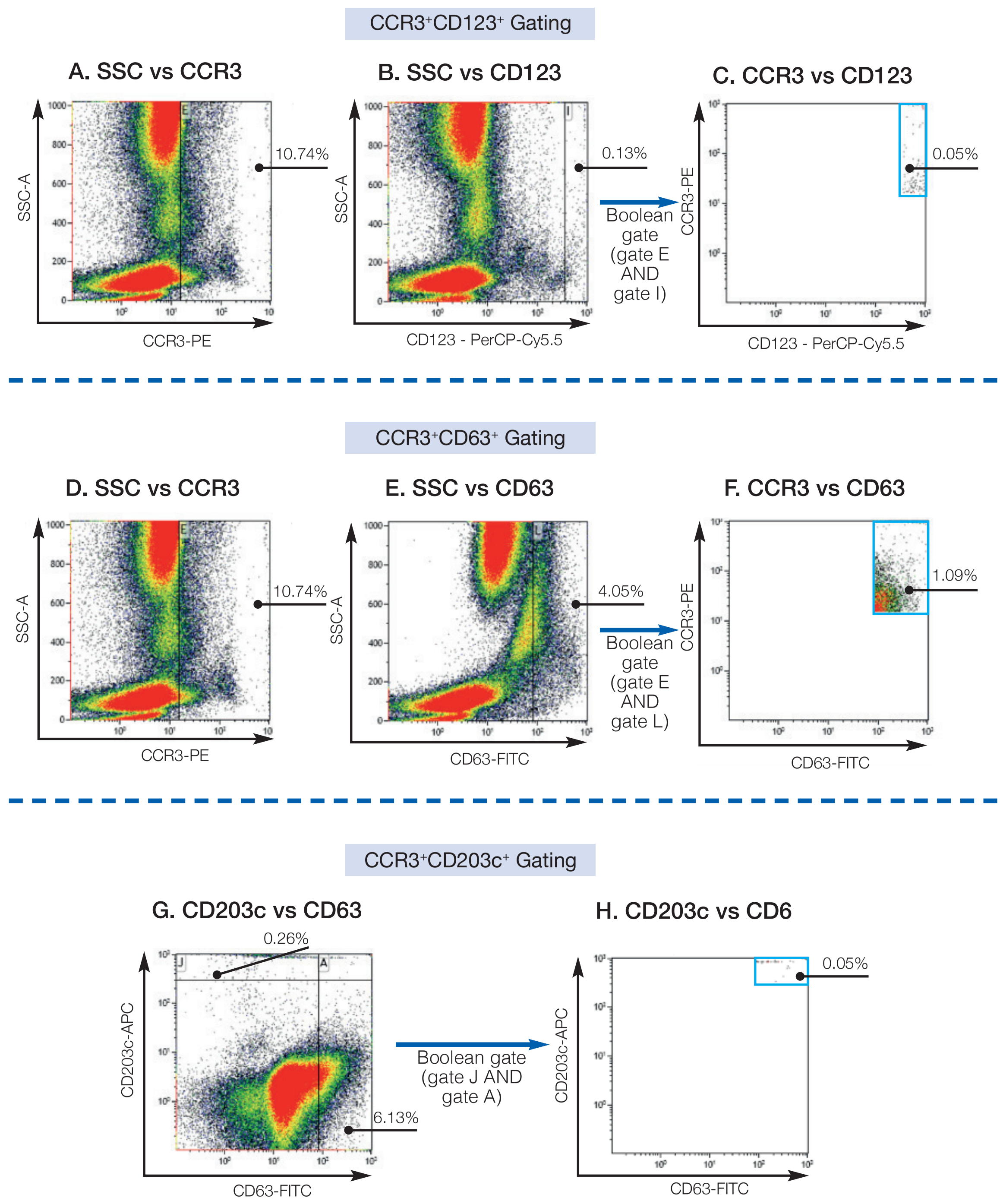
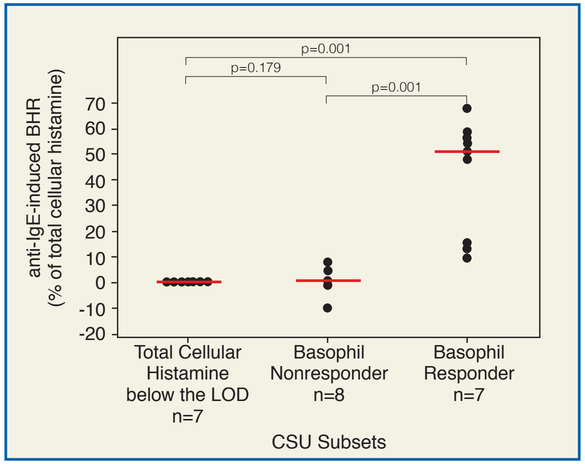


Figure 4. The gates for double-positive cell populations for each gating strategy were constructed by combining FMO gates for each marker using an AND Boolean logic. To isolate CCR3⁺CD123⁺ cell, a Boolean gate (Figure 4C) was constructed by a combination of FMO gates for CCR3 (Figure 4A) and CD123 (Figure 4B) markers. CCR3⁺CD63⁺ cells were selected by a Boolean gating (Figure 4F) for double-positive cells for each marker (Figures 4D-E). Boolean gating for CD203c⁺CD63⁺cells (Figure 4H) was achieved by a combination of FMO gates for CD203c and CD63 markers (Figure 4G). The data were acquired on a BD FACS Canto II™ flow cytometer by Miss Cheryl Barker at the Pathology Department, Norfolk & Norwich University Hospital (Norwich, UK). Data analysis was carried out using Kaluza r analysis software Version 1.1. (Beckman Coulter, Inc.).

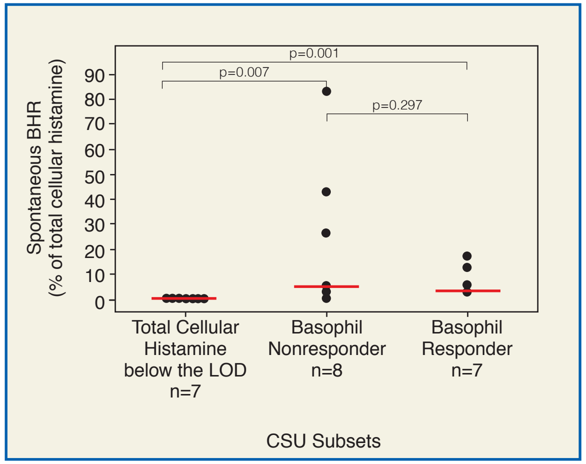
Abbrevations:
APC - Allophycocyanin
CCR3 - Chemokine (C-C motif) receptor type 3
CSU - Chronic spontaneous urticaria
Cy5.5 - Cyanine 5.5
Cy7 - Cyanine 7
FITC - Fluorescein isothianate
FMO - Fluorescence-Minus-One
PE - Phycoerythrin
PerCP - Peridin Chlorophyll Protein Complex
SSC - Side scatter

Appendix 4. Figure 5. Spontaneous and Anti-IgE-induced BHR in CSU Patient Subsets Related to Basophil Releasability to Anti-IgE Stimulation

A. Anti-IgE-induced BHR across the pathophysiological subsets in CSU at baseline



B. Spontaneous BHR across the pathophysiological subsets in CSU at baseline



Red bar represents median values.
Pairwise comparisons between the pathophysiological subsets in CSU were carried out by Mann-Whitney U test.
UAS7 score ranged from 0 to 49.

Figure 5. Pathophysiological phenotyping in CSU was carried out based on patient's BHR to anti-IgE stimulation and serum histamine-releasing activity. (Appendix 3, Table 1).

As a result of the pathophysiological phenotyping, we could differentiate three pathophysiological subsets of CSU patients. Seven CSU patients were grouped into the pathophysiological subset 1 (responders) based on BHR over 10% of total cellular histamine to anti-IgE stimulation (Figure 5A). 8 CSU patients were assigned to the pathophysiological subset 2 (non-responders) based on anti-IgE-induced BHR below 10% of total cellular histamine (Figure 5A). Pathophysiological subset 3 included 7 patients and was characterised by total cellular histamine below the detection limit of spectrofluorimetry (Figure 5A). There was no statistically significant difference in spontaneous BHR between responders and non-responders to anti-IgE stimulation (Figure 5B).

Anti-IgE-induced BHR from peripheral blood basophils of CSU patients was carried out by Dr Bernhard Gibbs at the Medway School of Pharmacy (Chatham Maritime, UK). For basophil releasability assays, cells were stimulated with anti-IgE antibodies (Sigma-Aldridge, UK) at 0.1ng/ml. Serum-induced BHR was assessed on peripheral blood basophils from healthy donors at Reflab (University of Copenhagen, Denmark). UAS7 with the score range of 0 to 49 was used in this study.

Abbrevations:
CSU - Chronic spontaneous urticaria
BHR - Basophil histamine release
UAS7 - Urticaria activity score over 7 days
LOD - Level of detection
fMLP - Formyl-Methionyl-Leucyl-Phenylalanine
IL-3 - Interleukin 3
IL-5 - Interleukin 5

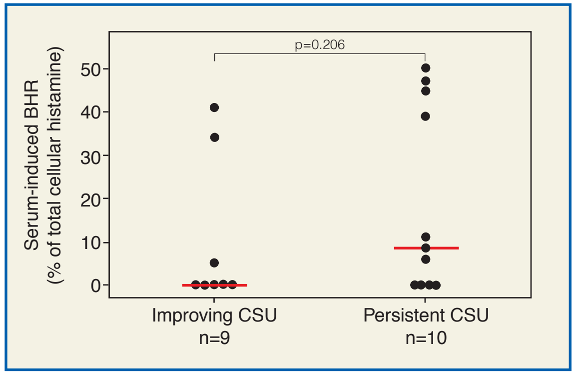
Appendix 4. Figure 6. Baseline Characteristics in Subgroups of CSU Patients related to the Clinical Course of Disease

Figure 6. In our study, 19 CSU completed three visits in the observational study and were included in the longitudinal analysis of the clinical course of the disease. Of these, 9 patients had improving CSU and 10 patients had persistent CSU.

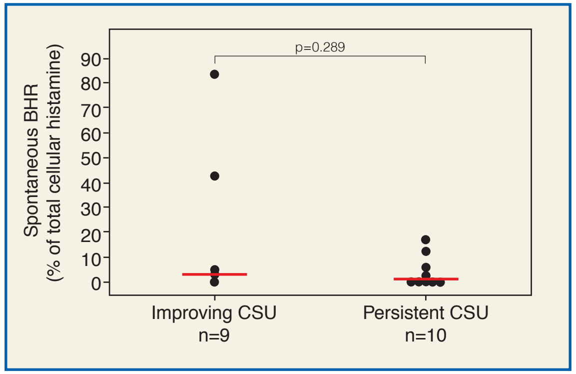
Serum histamine-releasing activity, spontaneous and anti-IgE induced BHR at baseline did not differ between CSU subgroups in relation to the clinical course of the disease (Figure 6A-C).

BHR assays from peripheral blood basophils of CSU patients were carried out at the Medway School of Pharmacy (Chatham Maritime, UK). For basophil releasability assays, cells were stimulated with anti-IgE antibodies (Sigma-Aldridge, UK) at 0.1ng/ml.

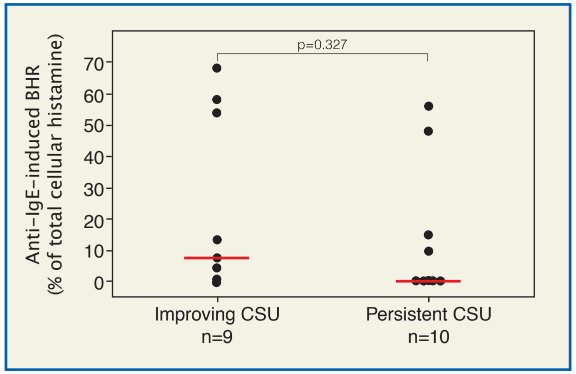
A. Baseline serum histamine-releasing activity in patients with persistent or improving CSU



B. Baseline spontaneous BHR in patients with persistent or improving CSU



C. Baseline anti-IgE-induced BHR from peripheral blood basophils in patients with persistent or improving CSU



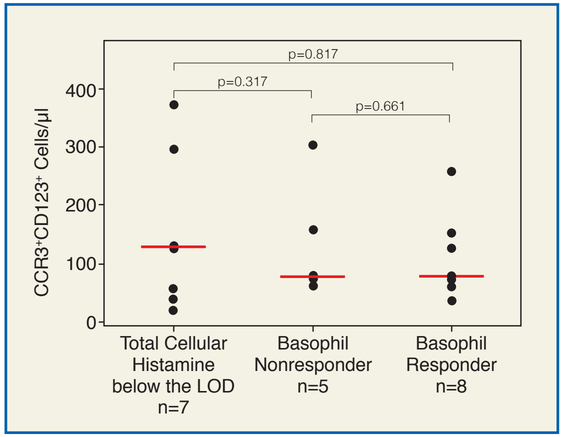
Red bar represents median values.

Pairwise comparisons between CSU subgroups in relation to the clinical course of disease was carried out by Mann-Whitney U test.

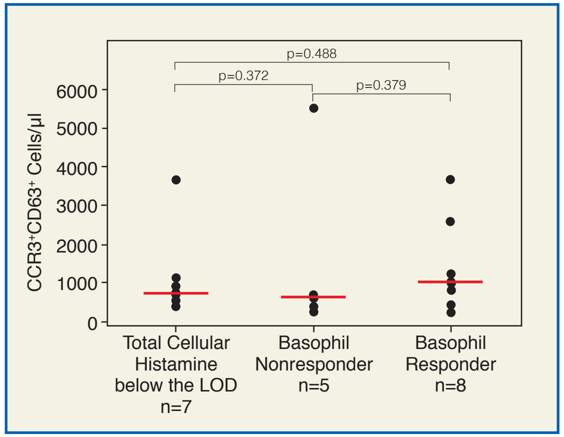
Abbrevations:
BHR - Basophil histamine release
CSU - Chronic spontaneous urticaria

Appendix 4. Figure 7. Flow Cytometric Basophil Subpopulations in CSU Patient Subsets Related to Basophil Releasability to Anti-IgE Stimulation

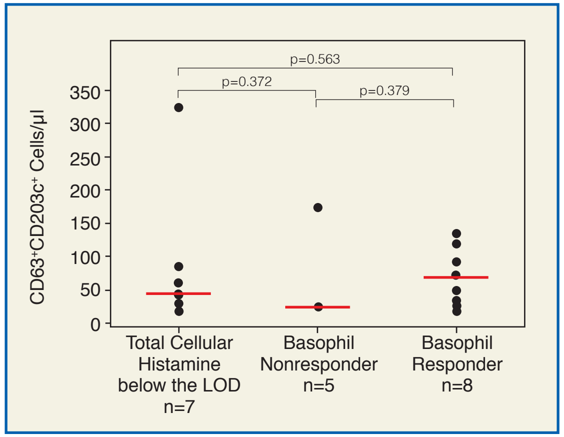
A. Baseline CCR3⁺CD123⁺ cell counts in the peripheral blood in relation to basophil releasability subsets in CSU patients



B. Baseline CCR3⁺CD63⁺ cell counts in the peripheral blood in relation to basophil releasability subsets in CSU patients



C. Baseline CD63⁺CD203c⁺ cell counts in the peripheral blood in relation to basophil releasability subsets in CSU patients



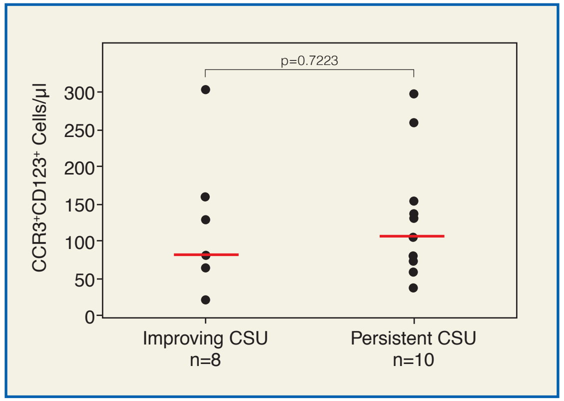
Red bar represents median values.
Pairwise comparisons between the pathophysiological subsets were carried out using Mann-Whitney U test.

Figure 7. Flow cytometric basophil studies revealed that there was no statistically significant difference in absolute basophil counts determined by three gating strategies across the pathophysiological subsets of CSU patients (Figures 7A-C). In flow cytometric analysis, basophil gating was carried out using CCR3⁺CD123⁺, CCR3⁺CD63⁺ and CD63⁺CD203c⁺ gating strategies on the same sample for each CSU patient. Absolute basophil counts were quantified using BD Trucount beads. Flow cytometric data were acquired by Miss Cheryl Barker using BD FACS Canto™ II instrument at the Norfolk & Norwich University Hospital (Norwich, UK). 22 CSU patients were included in the analysis, 2 patients were excluded for technical reasons related to data acquisition.

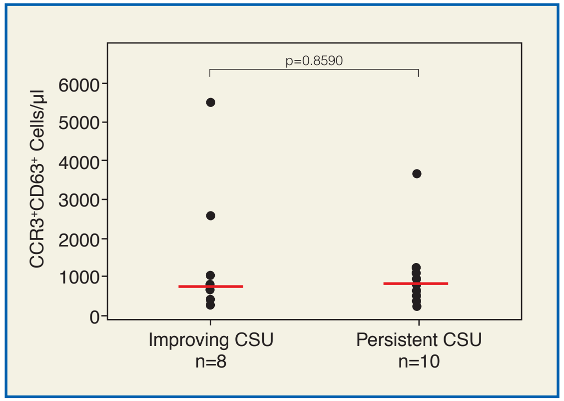
Abbrevations:
CSU - Chronic spontaneous urticaria

Appendix 4. Figure 8. Baseline Absolute Basophil Counts in Peripheral Blood of CSU Patients in relation to the Clinical Course of the Disease

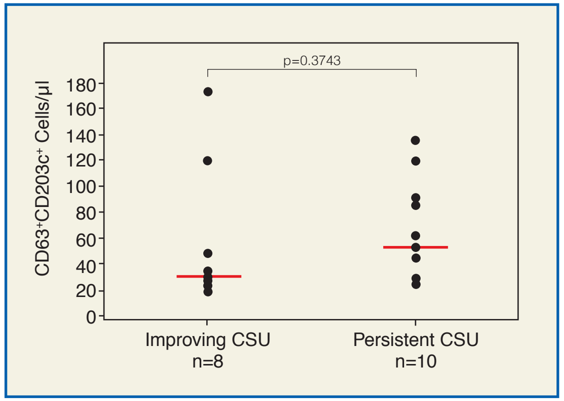
A. CCR3⁺CD123⁺ cell counts in the peripheral blood of CSU patients in relation to the course of the disease



B. CCR3⁺CD63⁺ cell counts in the peripheral blood of CSU patients in relation to the course of the disease



C. CD63⁺CD203c⁺ cell counts in the peripheral blood of CSU patients in relation to the course of the disease



Red bar represents median values.
Pairwise comparisons were carried out using Mann-Whitney U test.

Figure 8. Flow cytometric basophil studies revealed that there was no statistically significant difference in absolute basophil counts determined by three gating strategies between CSU subgroups related to the clinical course of disease (Figure 8A-C). In flow cytometric analysis, basophil gating was carried out using $CCR3^+CD123^+$, $CCR3^+CD63^+$ and $CD63^+CD203c^+$ gating strategies on the same sample for each CSU patient. Absolute basophil counts were quantified using BD Trucount beads. Flow cytometric data were acquired by Miss Cheryl Barker using BD FACS Canto™ II instrument at the Norfolk & Norwich University Hospital (Norwich, UK). 18 CSU patients who completed three visits in the observational study were included in the analysis, one patient was excluded for technical reasons related to data acquisition.

Abbrevations:
CSU - Chronic spontaneous urticaria
CCR3 - Chemokine (C-C motif) receptor type 3

Figure 9. Subgroup Analysis of CSU Patients with or without Serum Histamine-Releasing Activity

A. Baseline characteristics in CSU patients with or without serum histamine-releasing activity

Baseline characteristics	CSU patients without serum histamine-releasing activity (n=14)	CSU patients with serum histamine-releasing activity (n=8)	p-value*
VAS for itch (mm)	53 (21,59)	39.5 (28,58.5)	0.7326
VAS for wealing (mm)	33.5 (4,52)	43 (37,62)	0.3054
UAS7	14 (2,20)	25.5 (20.5,28.5)	0.0152
Number of weals on examination	2 (0,47)	21.5 (2,33)	0.4442
Serum-induced BHR (% of total cellular histamine content)	0 (0,5)	42.5 (37.5,46)	0.0001
Spontaneous BHR (% of total cellular histamine content)	3.15 (2.4,5.5)	0 (0,6.25)	0.0804
Anti-IgE-induced BHR (% of total cellular histamine content)	8.45 (0,53.9)	0 (0,2.1)	0.2345

B. Absolute counts of peripheral blood basophils identified by three gating strategies on the same same in CSU patients

Baseline characteristics	CSU patients without serum histamine-releasing activity (n=12)	CSU patients with serum histamine-releasing activity (n=8)	p-value*
CCR3+CD123+ cells/µl	77.63 (66.86,128.12)	145.78 (48.92,277.54)	0.4875
CCR3+CD63+ cells/µl	916.17 (672.45,1890.5)	604.2 (332.42,1011.62)	0.1649
CD63+CD203c+ cells/µl	40.86 (25.39,105.08)	36.95 (23.67,102.41)	0.7576

Pairwise comparisons were carried out using Mann-Whitney U test.

Data are presented as median (IQR).

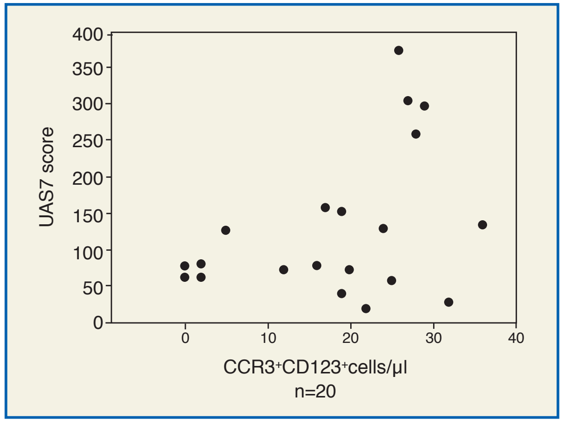
In our study, UAS7 score ranged from 0 to 49.

Figure 9. Data analysis revealed a statistically significant difference in disease severity between CSU patients with and without serum histamine-releasing activity (Mann-Whitney U test, p=0.0152). Serum histamine-releasing activity was measured by a serum-induced BHR at the RefLab (University of Copenhagen, Denmark). For serum-induced BHR assay, a diagnostic cut-off of 16.5% was used to detect serum histamine-releasing activity in CSU patients (Platzer M. et al, 2005). Data analysis for biomarkers was carried out in 22 CSU patients, flow cytometry data analysis included 20 CSU patients. Two patients were excluded from the flow cytometry data analysis due to the technical reasons with data acquisition.

Abbrevations:
BHR - Basophil histamine release
CSU - Chronic spontaneous urticaria
IQR - Interquartile range
UAS7 - Urticaria activity score over 7 days
VAS - Visual analogue scale

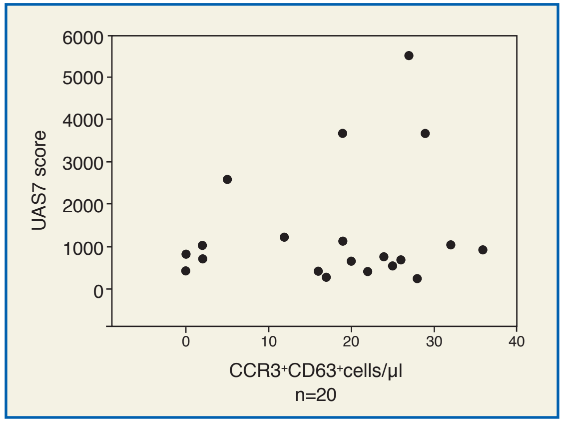
Appendix 4. Figure 10. The Relationship between Disease Severity and Basophil Subpopulations Gated by Three Different Gating Strategies in CSU Patients at Baseline

A. The relation between UAS7 and absolute counts of $CCR3^{+}CD123^{+}$ cells in peripheral blood in CSU patients



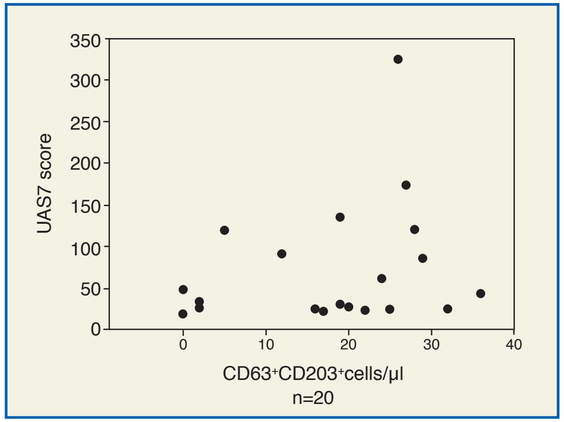
Spearman correlation r=0.30, p=0.1935

B. The relation between UAS7 and absolute counts of $CCR3^{+}CD63^{+}$ cells in peripheral blood in CSU patients



Spearman correlation r = 0.09; p=0.6908

C. The relation between UAS7 and absolute counts of $CD63^{+}CD203c^{+}$ cells in peripheral blood in CSU patient



Spearman correlation r = 0.27; p = 0.2492

Figure 10. Three gating strategies for peripheral blood basophils were applied on the same sample from each CSU patient. These gating strategies included dual gating based on CCR3 and CD123, CCR3 and CD63, CD63 and CD203c. There was no correlation between UAS7 and absolute counts of basophil subpopulations selected by three gating strategies in CSU patients (Figure 10A-C). Absolute basophil counts were enumerated using BD Trucount beads. Flow cytometric data were acquired on BD FACS Canto^{TM} II instrument by Miss Cheryl Barker at the Norfolk & Norwich University Hospital (Norwich, UK). Data analysis included 20 CSU patients, two CSU patients were excluded from the analysis for technical reasons with flow cytometry data acquisition.

Abbrevations:
CSU - Chronic spontaneous urticaria
CCR3 - chemokine (C-C motif) receptor type 3

Appendix 4. Figure 11. Biomarker Prospective Study - Patient KDP01

Period of Observation: 21.01.2009 - 29.04.2009   ASST: Positive

WEEK 0 (21.01.2009)

CLINICAL ASSESSMENT

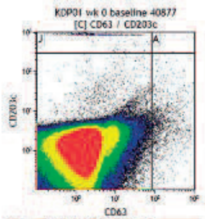
VAS for itching/24hrs:	12mm
VAS for wealing:	20mm
UAS7:	17
Number of weals:	24
Treatment:	Cetirizine 10mg/day Ranitidine 150mg/day

SERUM-INDUCED BHR: 41%

ANTI-IGE-INDUCED BHR:

Basophil count/ml of blood:	809.14
Basophil purity:	0.067%
Spontaneous BHR:	83.3%
Optimal anti-IgE Concentration:	1µg/ml
Anti-IgE induced HR (1µg/ml):	8.3%

FLOW CYTOMETRY STUDIES



Gate	Number	%Total	%Gated
All	2,665,423	99.50	100.00
A	1,724	0.06	0.06
J	720	0.03	0.03

Baseline

CCR3+CD123+ basophils - 157 cells/µL

CCR3+CD63+ basophils - 263 cells/µL

CD63+CD203c+ basophils - 22 cells/µL

WEEK 6 (02.03.2009)

CLINICAL ASSESSMENT

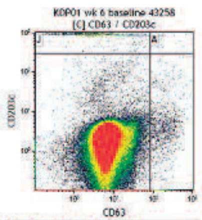
VAS for itching/24hrs:	0mm
VAS for wealing:	0mm
UAS7:	0
Number of weals:	0
Treatment:	Cetirizine 10mg/day

SERUM-INDUCED BHR: 10%

ANTI-IGE-INDUCED BHR:

Basophil count/ml of blood:	666.35
Basophil purity:	0.057%
Spontaneous BHR:	5.9%
Optimal anti-IgE Concentration:	0.1µg/ml
Anti-IgE induced HR (1µg/ml):	5.2%

FLOW CYTOMETRY STUDIES



Gate	Number	%Total	%Gated
All	463,966	92.78	100.00
A	1,472	0.29	0.32
J	1,792	0.36	0.39

Baseline

CCR3+CD123+basophils - 102 cells/µL

CCR3+CD63+ basophils - 96 cells/µL

CD63+CD203c+ basophils - 22 cells/µL

WEEK 12 (29.04.2009)

CLINICAL ASSESSMENT

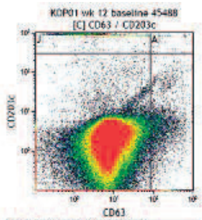
VAS for itching/24hrs:	0mm
VAS for wealing:	0mm
UAS7:	0
Number of weals:	0
Treatment:	Cetirizine 10mg/day

SERUM-INDUCED BHR: 4%

ANTI-IGE-INDUCED BHR:

Basophil count/ml of blood:	3777.77
Basophil purity:	0.33%
Spontaneous BHR:	5.6%
Optimal anti-IgE Concentration:	1µg/ml
Anti-IgE induced HR (1µg/ml):	2.9%

FLOW CYTOMETRY STUDIES



Gate	Number	%Total	%Gated
All	457,147	91.43	100.00
A	4,977	1.00	1.09
J	3,666	0.73	0.80

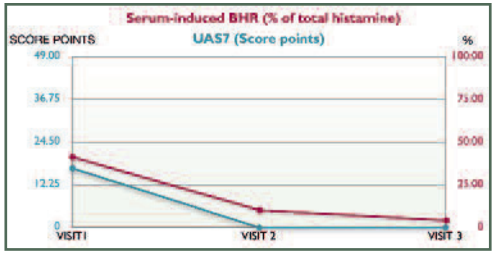
Baseline

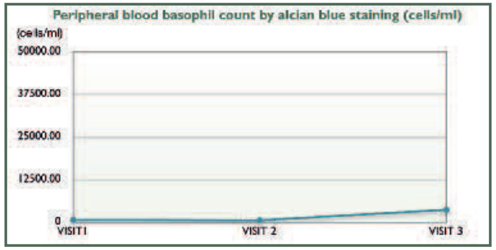
CCR3+CD123+basophils - 250 cells/µL

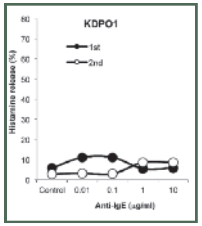
CCR3+CD63+ basophils - 252 cells/µL

CD63+CD203c+ basophils - 34 cells/µL

SUMMARY







Appendix 4. Figure 12. Biomarker Prospective Study - Patient MHP02

Period of Observation: 28.01.2009 - 18.05.2009   ASST: Positive

WEEK 0 (28.01.2009)

CLINICAL ASSESSMENT

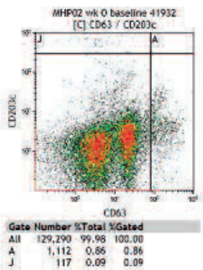
VAS for itching/24hrs:	15mm
VAS for wealing:	27mm
UAS7:	20
Number of weals:	2
Treatment:	Fexofenadine 180mg/day Montelukast 10mg/day

SERUM-INDUCED BHR: 5%

ANTI-IGE-INDUCED BHR:

Basophil count/ml of blood:	16828.57
Basophil purity:	0.78%
Spontaneous BHR:	42.6%
Optimal anti-IgE Concentration:	0.1μg/ml
Anti-IgE induced HR (1μg/ml):	7.4%

FLOW CYTOMETRY STUDIES



Baseline

$CD63^{+}CD203c^{+}$cells/μL 0.95%

CD63+CD203c+CCR3+ cells/μL

0.29% (30.78%)

WEEK 6 (05.03.2009)

CLINICAL ASSESSMENT

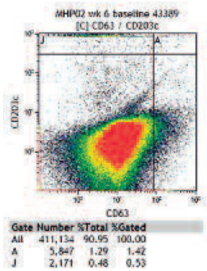
VAS for itching/24hrs:	97mm
VAS for wealing:	86mm
UAS7:	34
Number of weals:	4
Treatment:	Fexofenadine 180mg/day Montelukast 10mg/day Acrivastine 8mg PRN

SERUM-INDUCED BHR: 0%

ANTI-IGE-INDUCED BHR:

Basophil count/ml of blood:	346.11
Basophil purity:	0%
Spontaneous BHR:	6.1%
Optimal anti-IgE Concentration:	10μg/ml
Anti-IgE induced HR (1μg/ml):	12.3%

FLOW CYTOMETRY STUDIES



Baseline

CCR3+CD123+basophils - 92 cells/μL

CCR3+CD63+ basophils - 299 cells/μL

CD63+CD203c+ basophils - 27 cells/μL

WEEK 12 (18.05.2009)

CLINICAL ASSESSMENT

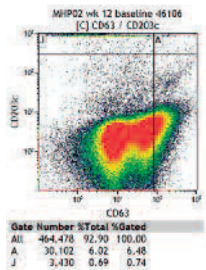
VAS for itching/24hrs:	18mm
VAS for wealing:	14mm
UAS7:	4
Number of weals:	
Treatment:	Fexofenadine 180mg/day

SERUM-INDUCED BHR: 1%

ANTI-IGE-INDUCED BHR:

Basophil count/ml of blood:	5034.72
Basophil purity:	0.16%
Spontaneous BHR:	4%
Optimal anti-IgE Concentration:	1μg/ml
Anti-IgE induced HR (1μg/ml):	2.5%

FLOW CYTOMETRY STUDIES



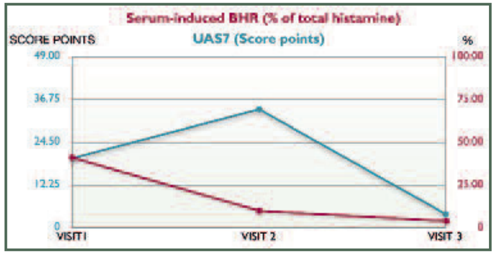
Baseline

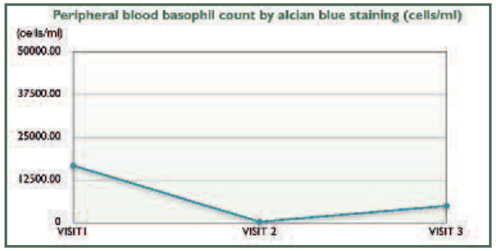
CCR3+CD123+basophils - 249 cells/μL

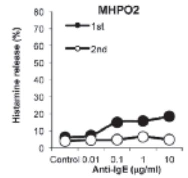
CCR3+CD63+ basophils - 2860 cells/μL

CD63+CD203c+ basophils - 56 cells/μL

SUMMARY







Appendix 4. Figure 13. Biomarker Prospective Study - Patient TBP03

Period of Observation: 13.03.2009 - 07.09.2009   ASST: Positive

WEEK 0 (13.03.2009)

CLINICAL ASSESSMENT

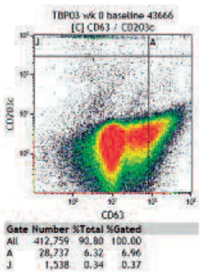
VAS for itching/24hrs: 18mm
VAS for wealing: 36mm
UAS7: 25
Number of weals: 2
Treatment: Cetirizine 10mg BD
Fexofenadine 180mg BD
Montelukast 10mg/day

SERUM-INDUCED BHR: 39%

ANTI-IGE-INDUCED BHR:

Basophil count/ml of blood: 0
Basophil purity: 0%
Spontaneous BHR: 0%
Optimal anti-IgE Concentration: N/A
Anti-IgE induced HR (1μg/ml): 0%

FLOW CYTOMETRY STUDIES



Baseline

CCR3+CD123+ basophils - 57 cells/μL
CCR3+CD63+ basophils - 531 cells/μL
CD63+CD203c+ basophils - 24 cells/μL

WEEK 6 (18.05.2009)

CLINICAL ASSESSMENT

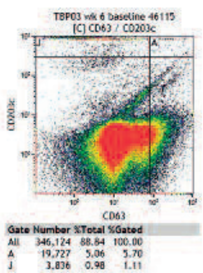
VAS for itching/24hrs: 83mm
VAS for wealing: 80mm
UAS7: 47
Number of weals: 40
Treatment: Cetirizine 10mg BD
Fexofenadine 180mg 4 tabs per day

SERUM-INDUCED BHR: 39%

ANTI-IGE-INDUCED BHR:

Basophil count/ml of blood: 0
Basophil purity: 0%
Spontaneous BHR: 0%
Optimal anti-IgE Concentration: N/A
Anti-IgE induced HR (1μg/ml): 0%

FLOW CYTOMETRY STUDIES



Baseline

CCR3+CD123+basophils - 223 cells/μL
CCR3+CD63+ basophils - 600 cells/μL
CD63+CD203c+ basophils - 46 cells/μL

WEEK 12 (07.09.2009)

CLINICAL ASSESSMENT

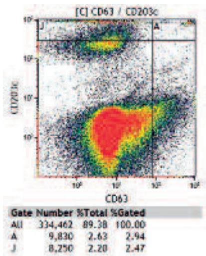
VAS for itching/24hrs: 52mm
VAS for wealing: 47mm
UAS7: 35
Number of weals: 28
Treatment: Cetirizine 10mg BD
Fexofenadine 180mg 4 tabs per day
Montelukast 10mg/day

SERUM-INDUCED BHR: 46%

ANTI-IGE-INDUCED BHR:

Basophil count/ml of blood: 0
Basophil purity: 0%
Spontaneous BHR: 0%
Optimal anti-IgE Concentration: N/A
Anti-IgE induced HR (1μg/ml): 0%

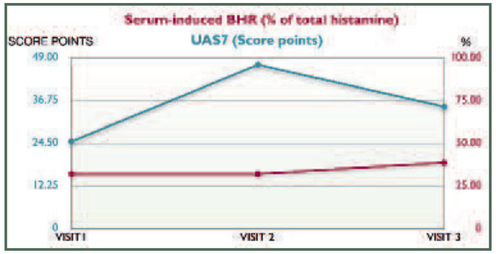
FLOW CYTOMETRY STUDIES

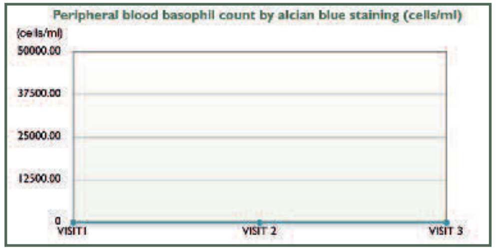


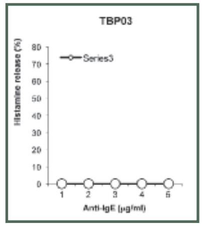
Baseline

CCR3+CD123+basophils - 373 cells/μL
CCR3+CD63+ basophils - 714 cells/μL
CD63+CD203c+ basophils - 189 cells/μL

SUMMARY







Appendix 4. Figure 14. Biomarker Prospective Study - Patient RHP04

Period of Observation: 02.02.2009	ASST: Positive

WEEK 0 (02.02.2009)

CLINICAL ASSESSMENT

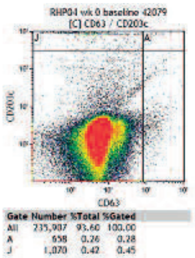
VAS for itching/24hrs:	6mm
VAS for wealing:	4mm
UAS7:	2
Number of weals:	0
Treatment:	Fexofenadine 180mg/day

SERUM-INDUCED BHR: 9%

ANTI-IGE-INDUCED BHR:

Basophil count/ml of blood:	333.72
Basophil purity:	0.027%
Spontaneous BHR:	26.3%
Optimal anti-IgE Concentration:	N/A
Anti-IgE induced HR (1 μg/ml):	0%

FLOW CYTOMETRY STUDIES



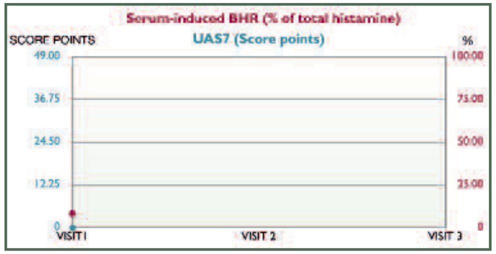
Baseline

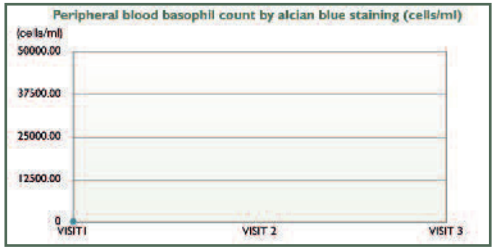
$CD63^+CD203c^+$cells/μL 0.67%

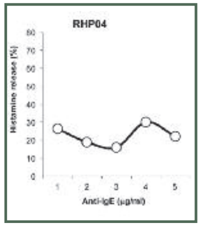
CD63+CD203c+CCR3+ cells/μL

0.16% (23.37%)

SUMMARY







Appendix 4. Figure 15. Biomarker Prospective Study - Patient SRP05

Period of Observation: 09.03.2009 - 08.09.2009 ASST: Positive

NR = Non-responder

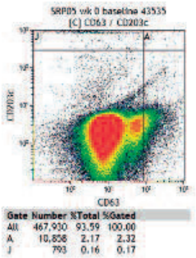
WEEK 0 (09.03.2009)

CLINICAL ASSESSMENT

VAS for itching/24hrs:	26mm
VAS for wealing:	28mm
UAS7:	2
Number of weals:	2
Treatment:	No Medication

SERUM-INDUCED BHR:	0%
ANTI-IGE-INDUCED BHR:	
Basophil count/ml of blood:	21142.85
Basophil purity:	1.21%
Spontaneous BHR:	4.9%
Optimal anti-IgE Concentration:	NR
Anti-IgE induced HR (1μg/ml):	N/A

FLOW CYTOMETRY STUDIES



Baseline

CCR3+CD123+ basophils - 61 cells/μL

CCR3+CD63+ basophils - 697 cells/μL

CD63+CD203c+ basophils - 26 cells/μL

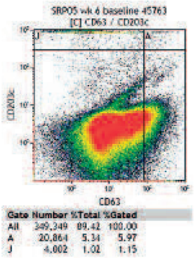
WEEK 6 (08.05.2009)

CLINICAL ASSESSMENT

VAS for itching/24hrs:	0mm
VAS for wealing:	0mm
UAS7:	0
Number of weals:	0
Treatment:	No Medication

SERUM-INDUCED BHR:	0%
ANTI-IGE-INDUCED BHR:	
Basophil count/ml of blood:	23888.88
Basophil purity:	3.28%
Spontaneous BHR:	2.2%
Optimal anti-IgE Concentration:	NR
Anti-IgE induced HR (1μg/ml):	N/A

FLOW CYTOMETRY STUDIES



Baseline

CCR3+CD123+basophils - 217 cells/μL

CCR3+CD63+ basophils - 1037 cells/μL

CD63+CD203c+ basophils - 55 cells/μL

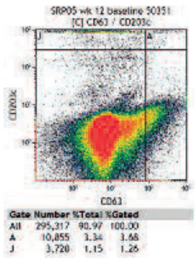
WEEK 12 (08.09.2009)

CLINICAL ASSESSMENT

VAS for itching/24hrs:	0mm
VAS for wealing:	0mm
UAS7:	0
Number of weals:	0
Treatment:	

SERUM-INDUCED BHR:	0%
ANTI-IGE-INDUCED BHR:	
Basophil count/ml of blood:	30894.84
Basophil purity:	1.65%
Spontaneous BHR:	1.5%
Optimal anti-IgE Concentration:	NR
Anti-IgE induced HR (1μg/ml):	N/A

FLOW CYTOMETRY STUDIES



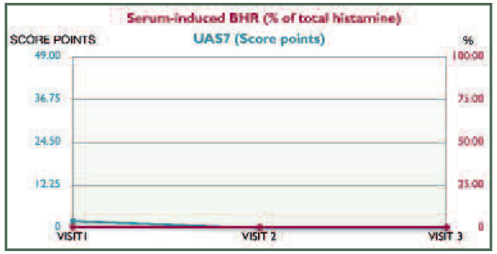
Baseline

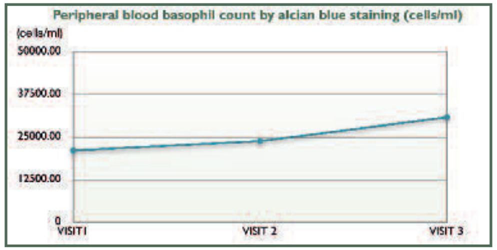
CCR3+CD123+basophils - 320 cells/μL

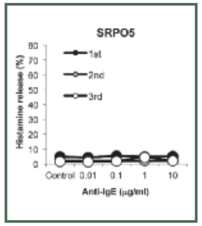
CCR3+CD63+ basophils - 1136 cells/μL

CD63+CD203c+ basophils - 136 cells/μL

SUMMARY







Appendix 4. Figure 16. Biomarker Prospective Study - Patient RWP06

Period of Observation: 11.03.2009 - 19.10.2009 ASST: Positive

WEEK 0 (11.03.2009)

CLINICAL ASSESSMENT

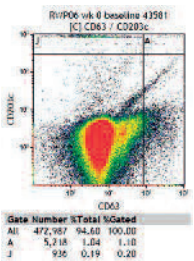
VAS for itching/24hrs:	0mm
VAS for wealing:	0mm
UAS7:	0
Number of weals:	0
Treatment:	Fexofenadine 180mg/day

SERUM-INDUCED BHR: 0%

ANTI-IGE-INDUCED BHR:

Basophil count/ml of blood:	2265.6
Basophil purity:	0.143%
Spontaneous BHR:	2.4%
Optimal anti-IgE Concentration:	0.1μg/ml
Anti-IgE induced HR (1μg/ml):	58.1%

FLOW CYTOMETRY STUDIES



Baseline

$CD63^+CD203c^+$cells/μL 1.22%

CD63+CD203c+CCR3+ cells/μL

0.41% (33.25%)

WEEK 6 (06.07.2009)

CLINICAL ASSESSMENT

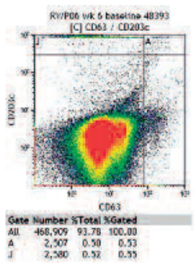
VAS for itching/24hrs:	0mm
VAS for wealing:	0mm
UAS7:	0
Number of weals:	0
Treatment:	Fexofenadine 180mg/day

SERUM-INDUCED BHR: 0%

ANTI-IGE-INDUCED BHR:

Basophil count/ml of blood:	27200
Basophil purity:	1%
Spontaneous BHR:	1.1%
Optimal anti-IgE Concentration:	0.1μg/ml
Anti-IgE induced HR (1μg/ml):	38.4%

FLOW CYTOMETRY STUDIES



Baseline

CCR3+CD123+basophils - 244 cells/μL

CCR3+CD63+ basophils - 412 cells/μL

CD63+CD203c+ basophils - 65 cells/μL

WEEK 12 (19.10.2009)

CLINICAL ASSESSMENT

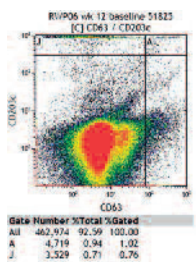
VAS for itching/24hrs:	0mm
VAS for wealing:	0mm
UAS7:	0
Number of weals:	0
Treatment:	Loratadine 10mg/day

SERUM-INDUCED BHR: 0%

ANTI-IGE-INDUCED BHR:

Basophil count/ml of blood:	21988.23
Basophil purity:	1.15%
Spontaneous BHR:	7.7%
Optimal anti-IgE Concentration:	0.1μg/ml
Anti-IgE induced HR (1μg/ml):	31.3%

FLOW CYTOMETRY STUDIES



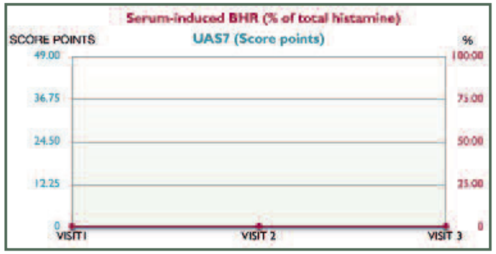
Baseline

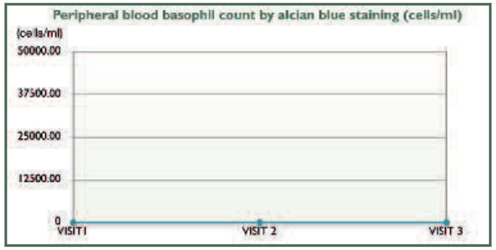
CCR3+CD123+basophils - 168 cells/μL

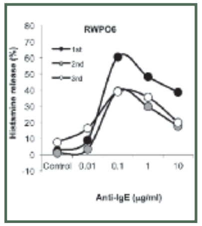
CCR3+CD63+ basophils - 454 cells/μL

CD63+CD203c+ basophils - 70 cells/μL

SUMMARY







Appendix 4. Figure 17. Biomarker Prospective Study - Patient MMP07

Period of Observation: 16.03.2009 - 23.10.2009 ASST: Positive

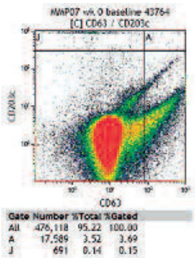
WEEK 0 (16.03.2009)

CLINICAL ASSESSMENT

VAS for itching/24hrs: 0mm
VAS for wealing: 0mm
UAS7: 0
Number of weals: 0
Treatment: Fexofenadine 180mg BD

SERUM-INDUCED BHR: 0%
ANTI-IGE-INDUCED BHR:
Basophil count/ml of blood: 20657.14
Basophil purity: 1.45%
Spontaneous BHR: 2.7%
Optimal anti-IgE Concentration: 0.1µg/ml
Anti-IgE induced HR (1µg/ml): 53.9%

FLOW CYTOMETRY STUDIES



Baseline
CCR3+CD123+ basophils - 61 cells/µL
CCR3+CD63+ basophils - 813 cells/µL
CD63+CD203c+ basophils - 48 cells/µL

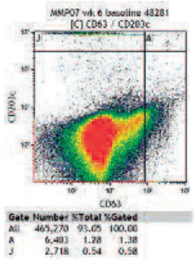
WEEK 6 (02.07.2009)

CLINICAL ASSESSMENT

VAS for itching/24hrs: 0mm
VAS for wealing: 0mm
UAS7: 0
Number of weals: 0
Treatment: Fexofenadine 180mg BD

SERUM-INDUCED BHR: 0%
ANTI-IGE-INDUCED BHR:
Basophil count/ml of blood: 51000
Basophil purity: 2.5%
Spontaneous BHR: 6.5%
Optimal anti-IgE Concentration: 0.1µg/ml
Anti-IgE induced HR (1µg/ml): 34.7%

FLOW CYTOMETRY STUDIES



Baseline
CCR3+CD123+basophils - 242 cells/µL
CCR3+CD63+ basophils - 1079 cells/µL
CD63+CD203c+ basophils - 41 cells/µL

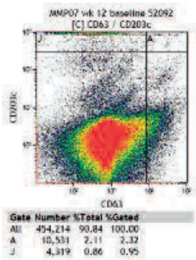
WEEK 12 (23.10.2009)

CLINICAL ASSESSMENT

VAS for itching/24hrs: 0mm
VAS for wealing: 0mm
UAS7: 0
Number of weals: 0
Treatment: Fexofenadine 180mg BD

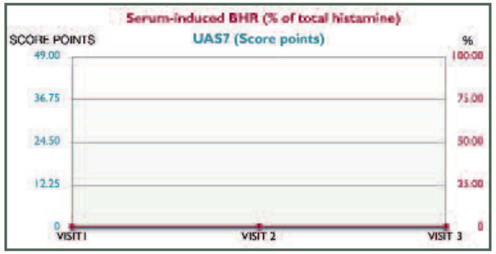
SERUM-INDUCED BHR: 0%
ANTI-IGE-INDUCED BHR:
Basophil count/ml of blood: 34000
Basophil purity: 2.3%
Spontaneous BHR: 5.3%
Optimal anti-IgE Concentration: 10µg/ml
Anti-IgE induced HR (1µg/ml): 19.1%

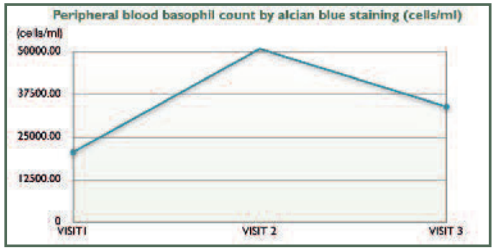
FLOW CYTOMETRY STUDIES

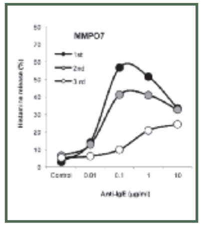


Baseline
CCR3+CD123+basophils - 214 cells/µL
CCR3+CD63+ basophils - 568 cells/µL
CD63+CD203c+ basophils - 38 cells/µL

SUMMARY







Appendix 4. Figure 18. Biomarker Prospective Study - Patient MLP08

Period of Observation: 18.03.2009 - 09.09.2009 ASST: Positive

LR = Low responder

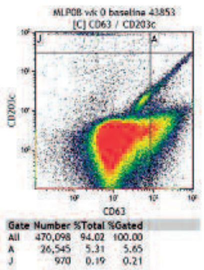
WEEK 0 (18.03.2009)

CLINICAL ASSESSMENT

VAS for itching/24hrs:	87mm
VAS for wealing:	89mm
UAS7:	32
Number of weals:	33
Treatment:	Fexofenadine 180mg BD Ranitidine 150mg BD

SERUM-INDUCED BHR:	6%
ANTI-IGE-INDUCED BHR:	
Basophil count/ml of blood:	11142.85
Basophil purity:	0.6%
Spontaneous BHR:	5.5%
Optimal anti-IgE Concentration:	LR
Anti-IgE induced HR (0.1μg/ml):	9.5%

FLOW CYTOMETRY STUDIES



Baseline

CCR3+CD123+ basophils - 36 cells/μL

CCR3+CD63+ basophils - 1024 cells/μL

CD63+CD203c+ basophils - 24 cells/μL

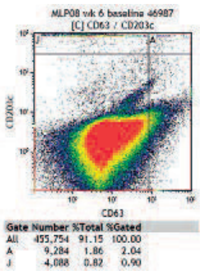
WEEK 6 (04.06.2009)

CLINICAL ASSESSMENT

VAS for itching/24hrs:	56mm
VAS for wealing:	66mm
UAS7:	26
Number of weals:	18
Treatment:	Fexofenadine 180mg BD Ranitidine 150mg BD

SERUM-INDUCED BHR:	0%
ANTI-IGE-INDUCED BHR:	
Basophil count/ml of blood:	6676.05
Basophil purity:	0.41%
Spontaneous BHR:	5.1%
Optimal anti-IgE Concentration:	1μg/ml
Anti-IgE induced HR (1μg/ml):	41.9%

FLOW CYTOMETRY STUDIES



Baseline

CCR3+CD123+basophils - 231 cells/μL

CCR3+CD63+ basophils - 1718 cells/μL

CD63+CD203c+ basophils - 68 cells/μL

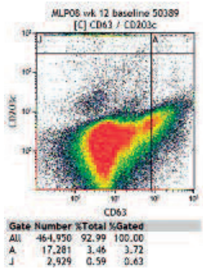
WEEK 12 (09.09.2009)

CLINICAL ASSESSMENT

VAS for itching/24hrs:	54mm
VAS for wealing:	62mm
UAS7:	35
Number of weals:	32
Treatment:	Fexofenadine 180mg BD Ranitidine 150mg BD

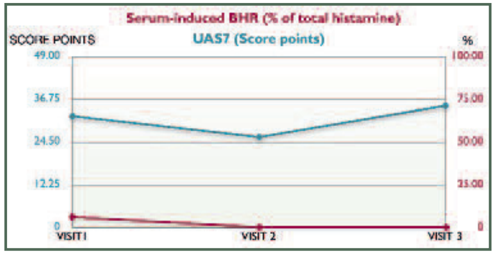
SERUM-INDUCED BHR:	0%
ANTI-IGE-INDUCED BHR:	
Basophil count/ml of blood:	17600
Basophil purity:	0.84%
Spontaneous BHR:	6.2%
Optimal anti-IgE Concentration:	0.1μg/ml
Anti-IgE induced HR (1μg/ml):	38.1%

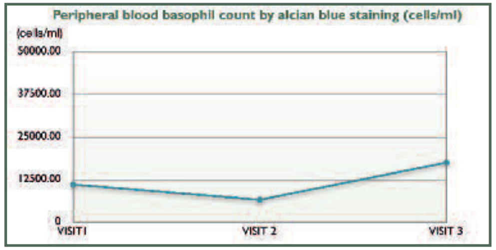
FLOW CYTOMETRY STUDIES

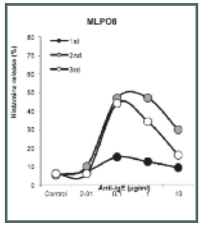


Baseline

SUMMARY







Appendix 4. Figure 19. Biomarker Prospective Study - Patient ASP09

Period of Observation: 20.03.2009 ASST: Positive

WEEK 0 (20.03.2009)

CLINICAL ASSESSMENT

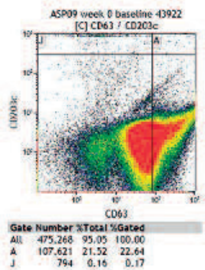
VAS for itching/24hrs:	84mm
VAS for wealing:	70mm
UAS7:	19
Number of weals:	2
Treatment:	Fexofenadine 180mg BD

SERUM-INDUCED BHR: 36%

ANTI-IGE-INDUCED BHR:

Basophil count/ml of blood:	629.33
Basophil purity:	0.078%
Spontaneous BHR:	0%
Optimal anti-IgE Concentration:	N/A
Anti-IgE induced HR (1μg/ml):	N/A

FLOW CYTOMETRY STUDIES



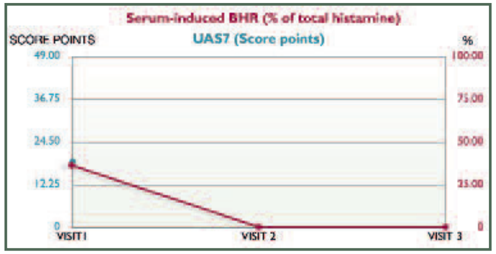
Baseline

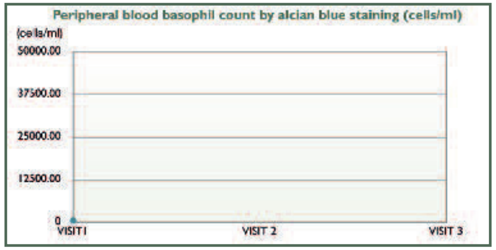
CCR3+CD123+ basophils - 39 cells/μL

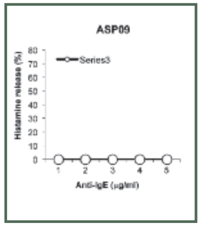
CCR3+CD63+ basophils - 1113 cells/μL

CD63+CD203c+ basophils - 30 cells/μL

SUMMARY







Appendix 4. Figure 20. Biomarker Prospective Study - Patient ISTP11

Period of Observation: 23.03.2009 - 17.09.2009 ASST: Positive

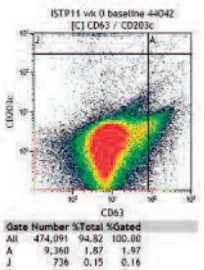
WEEK 0 (23.03.2009)

CLINICAL ASSESSMENT

VAS for itching/24hrs:	21mm
VAS for wealing:	39mm
UAS7:	12
Number of weals:	0
Treatment:	Cetirizine 10mg BD

SERUM-INDUCED BHR:	11%
ANTI-IGE-INDUCED BHR:	
Basophil count/ml of blood:	16314.28
Basophil purity:	1.023%
Spontaneous BHR:	17.3%
Optimal anti-IgE Concentration:	0.1µg/ml
Anti-IgE induced HR (1µg/ml):	48%

FLOW CYTOMETRY STUDIES



Baseline

CCR3+CD123+ basophils - 72 cells/µL

CCR3+CD63+ basophils - 1212 cells/µL

CD63+CD203c+ basophils - 90 cells/µL

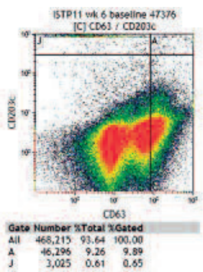
WEEK 6 (11.07.2009)

CLINICAL ASSESSMENT

VAS for itching/24hrs:	0mm
VAS for wealing:	6mm
UAS7:	12
Number of weals:	2
Treatment:	Cetirizine 10mg BD

SERUM-INDUCED BHR:	18%
ANTI-IGE-INDUCED BHR:	
Basophil count/ml of blood:	39666.66
Basophil purity:	2.5%
Spontaneous BHR:	6.3%
Optimal anti-IgE Concentration:	0.1µg/ml
Anti-IgE induced HR (1µg/ml):	63.8%

FLOW CYTOMETRY STUDIES



Baseline

CCR3+CD123+basophils - 245 cells/µL

CCR3+CD63+ basophils - 2990 cells/µL

CD63+CD203c+ basophils - 101 cells/µL

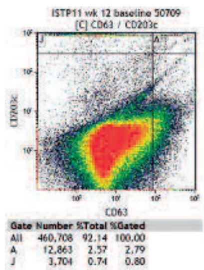
WEEK 12 (17.09.2009)

CLINICAL ASSESSMENT

VAS for itching/24hrs:	53mm
VAS for wealing:	74mm
UAS7:	28
Number of weals:	22
Treatment:	Cetirizine 10mg BD

SERUM-INDUCED BHR:	18%
ANTI-IGE-INDUCED BHR:	
Basophil count/ml of blood:	14875
Basophil purity:	1%
Spontaneous BHR:	8.8%
Optimal anti-IgE Concentration:	0.1µg/ml
Anti-IgE induced HR (1µg/ml):	66.5%

FLOW CYTOMETRY STUDIES



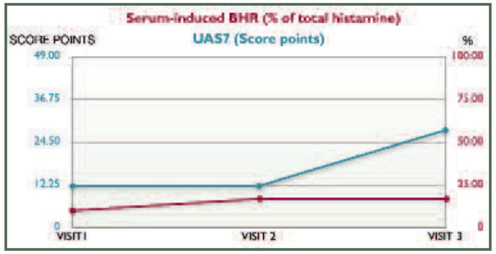
Baseline

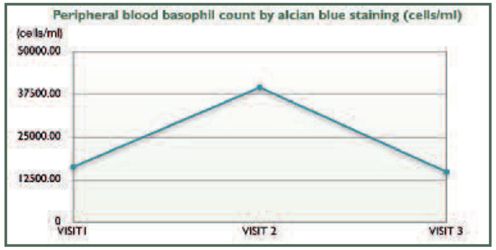
CCR3+CD123+basophils - 202 cells/µL

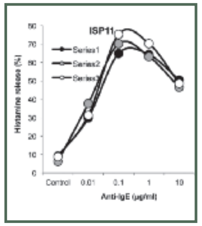
CCR3+CD63+ basophils - 857 cells/µL

CD63+CD203c+ basophils - 87 cells/µL

SUMMARY







Appendix 4. Figure 21. Biomarker Prospective Study - Patient MWP12

Period of Observation: 22.04.2009 - 08.10.2009   ASST: Positive

WEEK 0 (22.04.2009)

CLINICAL ASSESSMENT

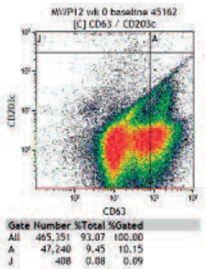
VAS for itching/24hrs:	38mm
VAS for wealing:	54mm
UAS7:	22
Number of weals:	32
Treatment:	Fexofenadine 180mg BD

SERUM-INDUCED BHR: 34%

ANTI-IGE-INDUCED BHR:

Basophil count/ml of blood:	0
Basophil purity:	0%
Spontaneous BHR:	0%
Optimal anti-IgE Concentration:	N/A
Anti-IgE induced HR (1µg/ml):	N/A

FLOW CYTOMETRY STUDIES



Baseline

CCR3+CD123+ basophils - 19 cells/µL

CCR3+CD63+ basophils - 401 cells/µL

CD63+CD203c+ basophils - 23 cells/µL

WEEK 6 (18.06.2009)

CLINICAL ASSESSMENT

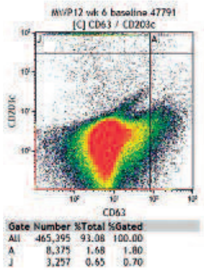
VAS for itching/24hrs:	25mm
VAS for wealing:	38mm
UAS7:	22
Number of weals:	50
Treatment:	Fexofenadine 180mg BD

SERUM-INDUCED BHR: 46%

ANTI-IGE-INDUCED BHR:

Basophil count/ml of blood:	0
Basophil purity:	0%
Spontaneous BHR:	0%
Optimal anti-IgE Concentration:	N/A
Anti-IgE induced HR (1µg/ml):	N/A

FLOW CYTOMETRY STUDIES



Baseline

CCR3+CD123+basophils - 207 cells/µL

CCR3+CD63+ basophils - 497 cells/µL

CD63+CD203c+ basophils - 47 cells/µL

WEEK 12 (08.10.2009)

CLINICAL ASSESSMENT

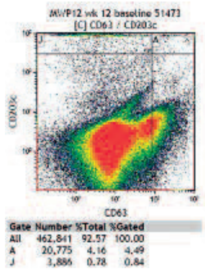
VAS for itching/24hrs:	0mm
VAS for wealing:	2mm
UAS7:	3
Number of weals:	0
Treatment:	Fexofenadine 180mg BD

SERUM-INDUCED BHR: ND

ANTI-IGE-INDUCED BHR:

Basophil count/ml of blood:	0
Basophil purity:	0%
Spontaneous BHR:	22.2%
Optimal anti-IgE Concentration:	N/A
Anti-IgE induced HR (1µg/ml):	N/A

FLOW CYTOMETRY STUDIES



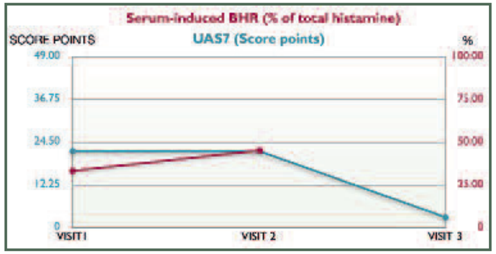
Baseline

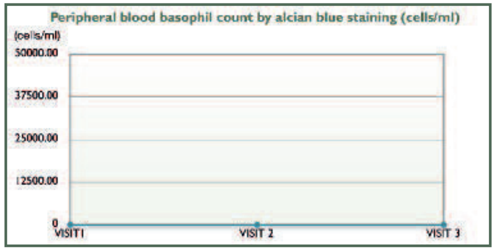
CCR3+CD123+basophils - 188 cells/µL

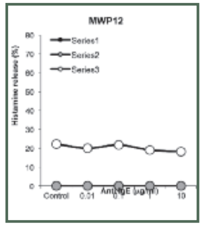
CCR3+CD63+ basophils - 2057 cells/µL

CD63+CD203c+ basophils - 95 cells/µL

SUMMARY







Appendix 4. Figure 22. Biomarker Prospective Study - Patient JHP14

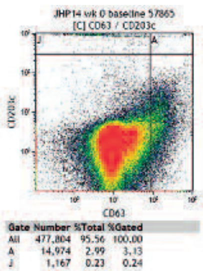
Period of Observation: 16.04.2010 - 30.09.2010 ASST: Positive

WEEK 0 (16.04.2010)

CLINICAL ASSESSMENT

VAS for itching/24hrs:	59mm
VAS for wealing:	48mm
UAS7:	20
Number of weals:	51
Treatment:	Levocetirizine 5mg/day Loratadine 10mg BD Montelukast 10mg/day
SERUM-INDUCED BHR:	0%
ANTI-IGE-INDUCED BHR:	
Basophil count/ml of blood:	30466.66
Basophil purity:	1.3%
Spontaneous BHR:	2.9%
Optimal anti-IgE Concentration:	NR
Anti-IgE induced HR (1µg/ml):	N/A

FLOW CYTOMETRY STUDIES



Baseline

CCR3+CD123+ basophils - 71 cells/µL

CCR3+CD63+ basophils - 646 cells/µL

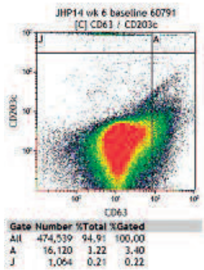
CD63+CD203c+ basophils - 27 cells/µL

WEEK 6 (05.07.2010)

CLINICAL ASSESSMENT

VAS for itching/24hrs:	94mm
VAS for wealing:	88mm
UAS7:	49
Number of weals:	25
Treatment:	Levocetirizine 5mg/day Loratadine 10mg BD Montelukast 10mg/day
SERUM-INDUCED BHR:	0%
ANTI-IGE-INDUCED BHR:	
Basophil count/ml of blood:	0
Basophil purity:	0%
Spontaneous BHR:	19.5%
Optimal anti-IgE Concentration:	NR
Anti-IgE induced HR (1µg/ml):	N/A

FLOW CYTOMETRY STUDIES



Baseline

CCR3+CD123+basophils - 134 cells/µL

CCR3+CD63+ basophils - 1221 cells/µL

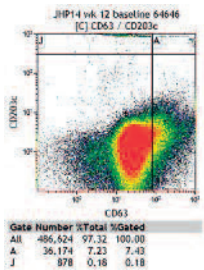
CD63+CD203c+ basophils - 80 cells/µL

WEEK 12 (30.09.2010)

CLINICAL ASSESSMENT

VAS for itching/24hrs:	74mm
VAS for wealing:	76mm
UAS7:	32
Number of weals:	56
Treatment:	Levocetirizine 5mg/day Loratadine 10mg BD Montelukast 10mg/day
SERUM-INDUCED BHR:	0%
ANTI-IGE-INDUCED BHR:	
Basophil count/ml of blood:	11941.17
Basophil purity:	0.9%
Spontaneous BHR:	6.7%
Optimal anti-IgE Concentration:	NR
Anti-IgE induced HR (1µg/ml):	N/A

FLOW CYTOMETRY STUDIES



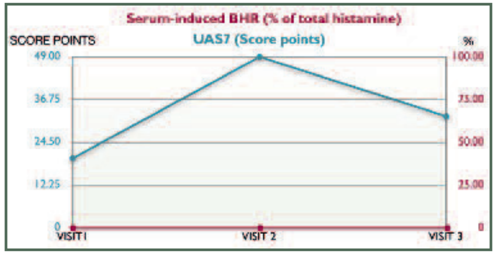
Baseline

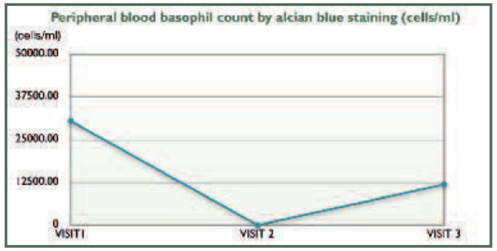
CCR3+CD123+basophils - 146 cells/µL

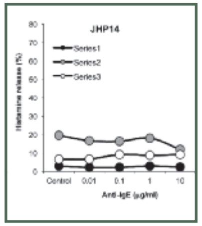
CCR3+CD63+ basophils - 3794 cells/µL

CD63+CD203c+ basophils - 96 cells/µL

SUMMARY







Appendix 4. Figure 23. Biomarker Prospective Study - Patient JCP15

Period of Observation: 09.04.2010 - 06.10.2010 ASST: Positive

WEEK 0 (09.04.2010)

CLINICAL ASSESSMENT

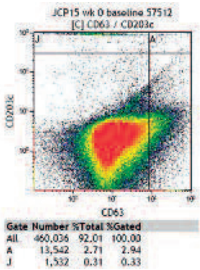
VAS for itching/24hrs:	59mm
VAS for wealing:	0mm
UAS7:	2
Number of weals:	0
Treatment:	Cetirizine 10mg PRN

SERUM-INDUCED BHR: 0%

ANTI-IGE-INDUCED BHR:

Basophil count/ml of blood:	30166.66
Basophil purity:	1.4%
Spontaneous BHR:	2.1%
Optimal anti-IgE Concentration:	0.1μg/ml
Anti-IgE induced HR (1μg/ml):	13.5%

FLOW CYTOMETRY STUDIES



Baseline

CCR3+CD123+ basophils - 79 cells/μL

CCR3+CD63+ basophils - 1018 cells/μL

CD63+CD203c+ basophils - 33 cells/μL

WEEK 6 (23.06.2010)

CLINICAL ASSESSMENT

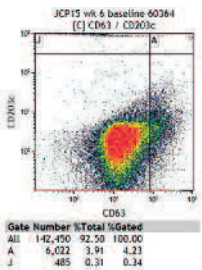
VAS for itching/24hrs:	68mm
VAS for wealing:	0mm
UAS7:	2
Number of weals:	0
Treatment:	Cetirizine 10mg PRN

SERUM-INDUCED BHR: 0%

ANTI-IGE-INDUCED BHR:

Basophil count/ml of blood:	21294.11
Basophil purity:	1.53%
Spontaneous BHR:	3.6%
Optimal anti-IgE Concentration:	0.1μg/ml
Anti-IgE induced HR (1μg/ml):	10.1%

FLOW CYTOMETRY STUDIES



Baseline

CCR3+CD123+basophils - 82 cells/μL

CCR3+CD63+ basophils - 1064 cells/μL

CD63+CD203c+ basophils - 24 cells/μL

WEEK 12 (06.10.2010)

CLINICAL ASSESSMENT

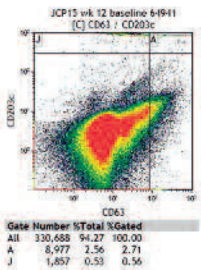
VAS for itching/24hrs:	41mm
VAS for wealing:	0mm
UAS7:	0
Number of weals:	0
Treatment:	Cetirizine 10mg PRN Acrivastine 8mg PRN

SERUM-INDUCED BHR: 0%

ANTI-IGE-INDUCED BHR:

Basophil count/ml of blood:	26941.17
Basophil purity:	2.5%
Spontaneous BHR:	2.4%
Optimal anti-IgE Concentration:	0.1μg/ml
Anti-IgE induced HR (1μg/ml):	9.8%

FLOW CYTOMETRY STUDIES



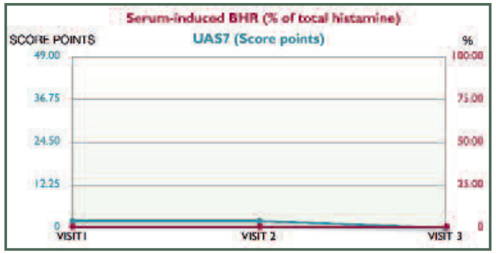
Baseline

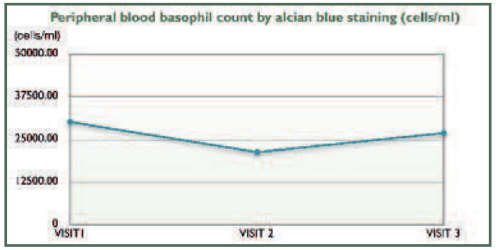
CCR3+CD123+basophils - 263 cells/μL

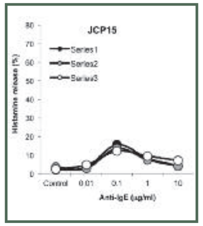
CCR3+CD63+ basophils - 694 cells/μL

CD63+CD203c+ basophils - 161 cells/μL

SUMMARY







Appendix 4. Figure 24. Biomarker Prospective Study - Patient DPP16

Period of Observation: 29.09.2010 - 06.04.2011   ASST: Positive

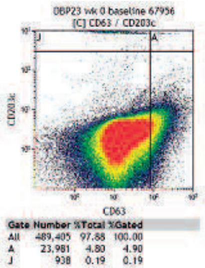
WEEK 0 (29.09.2010)

CLINICAL ASSESSMENT

VAS for itching/24hrs:	78mm
VAS for wealing:	73mm
UAS7:	19
Number of weals:	47
Treatment:	Fexofenadine 180mg BD Montelukast 10mg/day

SERUM-INDUCED BHR:	0%
ANTI-IGE-INDUCED BHR:	
Basophil count/ml of blood:	16000
Basophil purity:	1.4%
Spontaneous BHR:	3%
Optimal anti-IgE Concentration:	1μg/ml
Anti-IgE induced HR (1μg/ml):	58.7%

FLOW CYTOMETRY STUDIES



Baseline

CCR3+CD123+ basophils - 152 cells/μL

CCR3+CD63+ basophils - 3659 cells/μL

CD63+CD203c+ basophils - 135 cells/μL

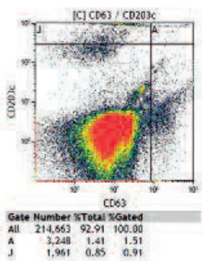
WEEK 6 (13.01.2011)

CLINICAL ASSESSMENT

VAS for itching/24hrs:	38mm
VAS for wealing:	43mm
UAS7:	28
Number of weals:	29
Treatment:	Fexofenadine 180mg QD Montelukast 10mg/day

SERUM-INDUCED BHR:	20%
ANTI-IGE-INDUCED BHR:	
Basophil count/ml of blood:	29687.5
Basophil purity:	1.7%
Spontaneous BHR:	9.5%
Optimal anti-IgE Concentration:	1μg/ml
Anti-IgE induced HR (1μg/ml):	44.9%

FLOW CYTOMETRY STUDIES



Baseline

CCR3+CD123+basophils - 218 cells/μL

CCR3+CD63+ basophils - 333 cells/μL

CD63+CD203c+ basophils - 58 cells/μL

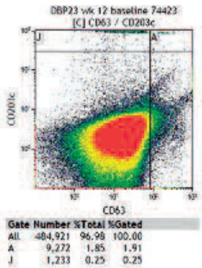
WEEK 12 (06.04.2011)

CLINICAL ASSESSMENT

VAS for itching/24hrs:	43mm
VAS for wealing:	47mm
UAS7:	25
Number of weals:	25
Treatment:	Fexofenadine 180mg QD Montelukast 10mg/day

SERUM-INDUCED BHR:	0%
ANTI-IGE-INDUCED BHR:	
Basophil count/ml of blood:	11357.14
Basophil purity:	0.77%
Spontaneous BHR:	1.2%
Optimal anti-IgE Concentration:	0.1μg/ml
Anti-IgE induced HR (1μg/ml):	29.7%

FLOW CYTOMETRY STUDIES



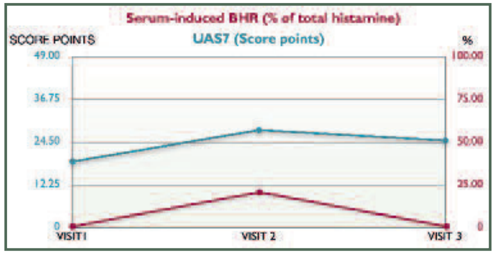
Baseline

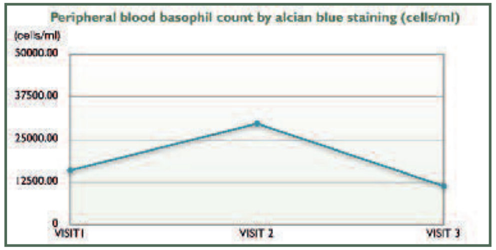
CCR3+CD123+basophils - 184 cells/μL

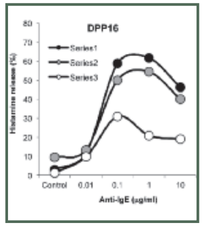
CCR3+CD63+ basophils - 1584 cells/μL

CD63+CD203c+ basophils - 81 cells/μL

SUMMARY







Appendix 4. Figure 25. Biomarker Prospective Study - Patient IHP17

Period of Observation: 29.03.2010 - 28.09.2010 ASST: Positive

WEEK 0 (29.03.2010)

CLINICAL ASSESSMENT

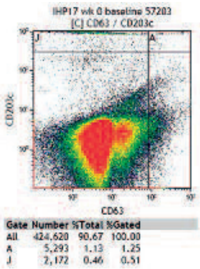
VAS for itching/24hrs:	52mm
VAS for wealing:	52mm
UAS7:	16
Number of weals:	62
Treatment:	Cetirizine 10mg/day

SERUM-INDUCED BHR: 0%

ANTI-IGE-INDUCED BHR:

Basophil count/ml of blood:	9912.5
Basophil purity:	0.5%
Spontaneous BHR:	0%
Optimal anti-IgE Concentration:	N/A
Anti-IgE induced HR (1μg/ml):	NR

FLOW CYTOMETRY STUDIES



Baseline

CCR3+CD123+ basophils - 78 cells/μL

CCR3+CD63+ basophils - 398 cells/μL

CD63+CD203c+ basophils - 24 cells/μL

WEEK 6 (24.06.2010)

CLINICAL ASSESSMENT

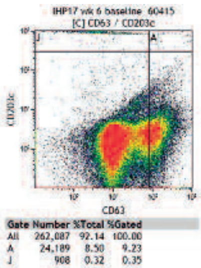
VAS for itching/24hrs:	38mm
VAS for wealing:	44mm
UAS7:	28
Number of weals:	23
Treatment:	Cetirizine 10mg/day

SERUM-INDUCED BHR: 0%

ANTI-IGE-INDUCED BHR:

Basophil count/ml of blood:	10588.24
Basophil purity:	0.94%
Spontaneous BHR:	15.8%
Optimal anti-IgE Concentration:	1μg/ml
Anti-IgE induced HR (1μg/ml):	18.8%

FLOW CYTOMETRY STUDIES



Baseline

CCR3+CD123+basophils - 93 cells/μL

CCR3+CD63+ basophils - 3100 cells/μL

CD63+CD203c+ basophils - 33 cells/μL

WEEK 12 (28.09.2010)

CLINICAL ASSESSMENT

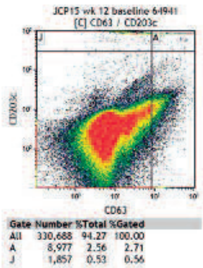
VAS for itching/24hrs:	32mm
VAS for wealing:	42mm
UAS7:	23
Number of weals:	43
Treatment:	Cetirizine 10mg/day

SERUM-INDUCED BHR: 0%

ANTI-IGE-INDUCED BHR:

Basophil count/ml of blood:	10000
Basophil purity:	1%
Spontaneous BHR:	2.3%
Optimal anti-IgE Concentration:	1μg/ml
Anti-IgE induced HR (1μg/ml):	18.1%

FLOW CYTOMETRY STUDIES



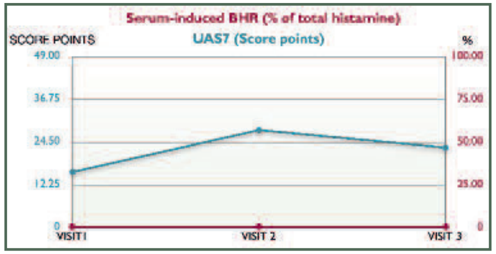
Baseline

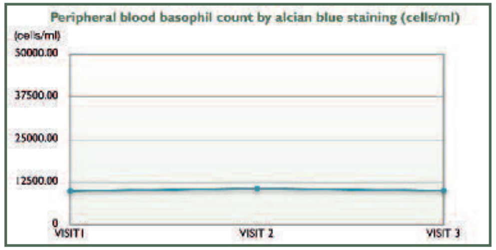
CCR3+CD123+basophils - 356 cells/μL

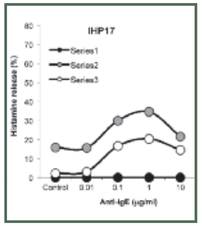
CCR3+CD63+ basophils - 8835 cells/μL

CD63+CD203c+ basophils - 109 cells/μL

SUMMARY







Appendix 4. Figure 26. Biomarker Prospective Study - Patient BSP19

Period of Observation: 05.10.2010 - 11.04.2011 ASST: Positive

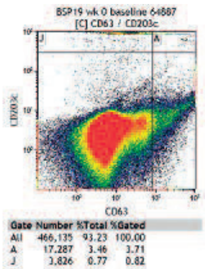
WEEK 0 (05.10.2010)

CLINICAL ASSESSMENT

VAS for itching/24hrs:	44mm
VAS for wealing:	42mm
UAS7:	28
Number of weals:	19
Treatment:	Cetirizine 10mg BD Montelukast 10mg/day

SERUM-INDUCED BHR:	47%
ANTI-IGE-INDUCED BHR:	
Basophil count/ml of blood:	2000
Basophil purity:	0.14%
Spontaneous BHR:	12.5%
Optimal anti-IgE Concentration:	0.1μg/ml
Anti-IgE induced HR (1μg/ml):	14.8%

FLOW CYTOMETRY STUDIES



Baseline

CCR3+CD123+ basophils - 258 cells/μL

CCR3+CD63+ basophils - 238 cells/μL

CD63+CD203c+ basophils - 119 cells/μL

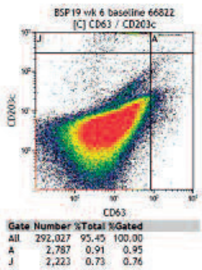
WEEK 6 (02.12.2010)

CLINICAL ASSESSMENT

VAS for itching/24hrs:	6mm
VAS for wealing:	11mm
UAS7:	4
Number of weals:	1
Treatment:	Cetirizine 10mg BD Montelukast 10mg/day

SERUM-INDUCED BHR:	0%
ANTI-IGE-INDUCED BHR:	
Basophil count/ml of blood:	1800
Basophil purity:	0.09%
Spontaneous BHR:	10%
Optimal anti-IgE Concentration:	0.1μg/ml
Anti-IgE induced HR (1μg/ml):	17.3%

FLOW CYTOMETRY STUDIES



Baseline

CCR3+CD123+basophils - 656 cells/μL

CCR3+CD63+ basophils - 717 cells/μL

CD63+CD203c+ basophils - 365 cells/μL

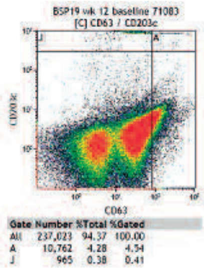
WEEK 12 (11.04.2011)

CLINICAL ASSESSMENT

VAS for itching/24hrs:	38mm
VAS for wealing:	44mm
UAS7:	30
Number of weals:	22
Treatment:	Cetirizine 10mg BD Montelukast 10mg/day

SERUM-INDUCED BHR:	21%
ANTI-IGE-INDUCED BHR:	
Basophil count/ml of blood:	3020.8
Basophil purity:	0.45%
Spontaneous BHR:	0%
Optimal anti-IgE Concentration:	1μg/ml
Anti-IgE induced HR (1μg/ml):	25%

FLOW CYTOMETRY STUDIES



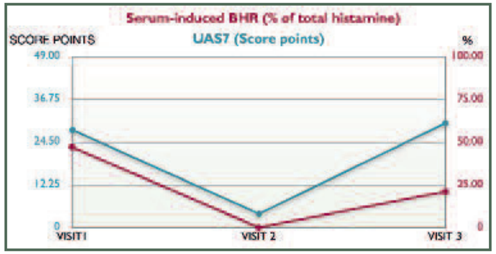
Baseline

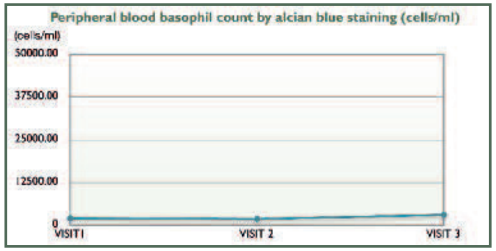
CCR3+CD123+basophils - 276 cells/μL

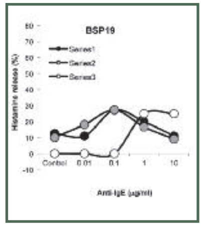
CCR3+CD63+ basophils - 1344 cells/μL

CD63+CD203c+ basophils - 274 cells/μL

SUMMARY







Appendix 4. Figure 27. Biomarker Prospective Study - Patient GHP20

Period of Observation: 04.10.2010 ASST: Positive

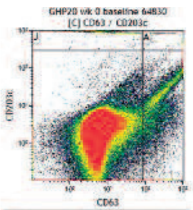
WEEK 0 (04.10.2010)

CLINICAL ASSESSMENT

VAS for itching/24hrs:	41mm
VAS for wealing:	38mm
UAS7:	26
Number of weals:	34
Treatment:	Fexofenadine 180mg BD Doxepine 50mg nocte

SERUM-INDUCED BHR:	44%
ANTI-IGE-INDUCED BHR:	
Basophil count/ml of blood:	4000
Basophil purity:	0.34%
Spontaneous BHR:	0%
Optimal anti-IgE Concentration:	N/A
Anti-IgE induced HR (1µg/ml):	NR

FLOW CYTOMETRY STUDIES



Gate	Number	%Total	%Gated
All	485,168	97.03	100.00
A	19,249	3.85	3.97
J	2,649	0.53	0.55

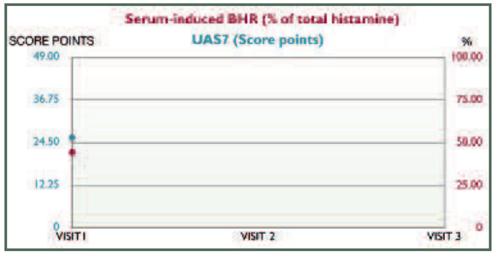
Baseline

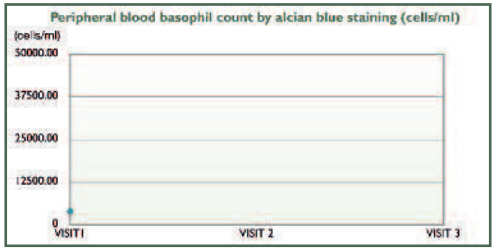
CCR3+CD123+ basophils - 372 cells/µL

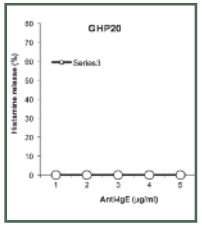
CCR3+CD63+ basophils - 677 cells/µL

CD63+CD203c+ basophils - 324 cells/µL

SUMMARY







Appendix 4. Figure 28. Biomarker Prospective Study - Patient DMP21

Period of Observation: 12.01.2011 - 18.08.2011 ASST: Positive

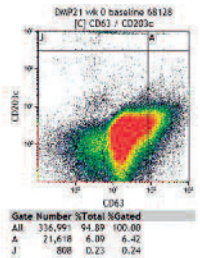
WEEK 0 (12.01.2011)

CLINICAL ASSESSMENT

VAS for itching/24hrs:	54mm
VAS for wealing:	16mm
UAS7:	5
Number of weals:	0
Treatment:	

SERUM-INDUCED BHR:	0%
ANTI-IGE-INDUCED BHR:	
Basophil count/ml of blood:	19125
Basophil purity:	0.93%
Spontaneous BHR:	3.3%
Optimal anti-IgE Concentration:	0.1μg/ml
Anti-IgE induced HR (1μg/ml):	68.1%

FLOW CYTOMETRY STUDIES



Baseline

CCR3+CD123+ basophils - 127 cells/μL

CCR3+CD63+ basophils - 2568 cells/μL

CD63+CD203c+ basophils - 119 cells/μL

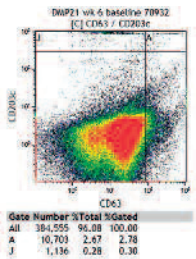
WEEK 6 (07.04.2011)

CLINICAL ASSESSMENT

VAS for itching/24hrs:	9mm
VAS for wealing:	0mm
UAS7:	0
Number of weals:	0
Treatment:	

SERUM-INDUCED BHR:	0%
ANTI-IGE-INDUCED BHR:	
Basophil count/ml of blood:	24921.8
Basophil purity:	1.15%
Spontaneous BHR:	2.5%
Optimal anti-IgE Concentration:	0.1μg/ml
Anti-IgE induced HR (1μg/ml):	47.6%

FLOW CYTOMETRY STUDIES



Baseline

CCR3+CD123+basophils - 170 cells/μL

CCR3+CD63+ basophils - 1566 cells/μL

CD63+CD203c+ basophils - 97 cells/μL

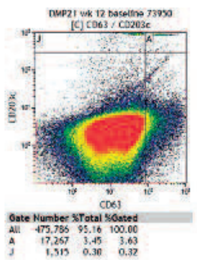
WEEK 12 (18.08.2011)

CLINICAL ASSESSMENT

VAS for itching/24hrs:	49mm
VAS for wealing:	28mm
UAS7:	0
Number of weals:	0
Treatment:	

SERUM-INDUCED BHR:	33%
ANTI-IGE-INDUCED BHR:	
Basophil count/ml of blood:	14263.15
Basophil purity:	1.3%
Spontaneous BHR:	5.7%
Optimal anti-IgE Concentration:	0.1μg/ml
Anti-IgE induced HR (1μg/ml):	49.2%

FLOW CYTOMETRY STUDIES



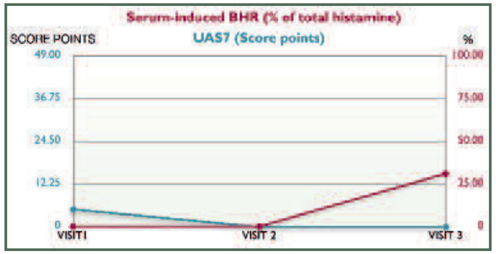
Baseline

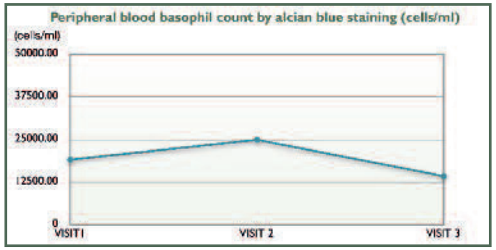
CCR3+CD123+basophils - 518 cells/μL

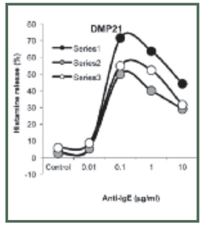
CCR3+CD63+ basophils - 3037 cells/μL

CD63+CD203c+ basophils - 231 cells/μL

SUMMARY







Appendix 4. Figure 29. Biomarker Prospective Study - Patient TMP21

Period of Observation: 12.01.2011 - 18.08.2011 ASST: Positive

WEEK 0 (12.01.2011)

CLINICAL ASSESSMENT

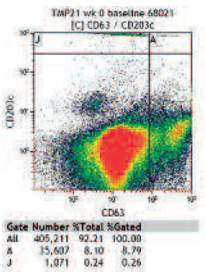
VAS for itching/24hrs:	54mm
VAS for wealing:	51mm
UAS7:	24
Number of weals:	16
Treatment:	No Medication

SERUM-INDUCED BHR: 0%

ANTI-IGE-INDUCED BHR:

Basophil count/ml of blood:	6577.61
Basophil purity:	0.3%
Spontaneous BHR:	0%
Optimal anti-IgE Concentration:	1 µg/ml
Anti-IgE induced HR (1 µg/ml):	NR

FLOW CYTOMETRY STUDIES



Baseline

CCR3+CD123+ basophils - 129 cells/µL

CCR3+CD63+ basophils - 744 cells/µL

CD63+CD203c+ basophils - 61 cells/µL

WEEK 6 (07.04.2011)

CLINICAL ASSESSMENT

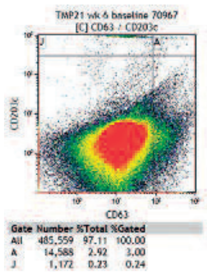
VAS for itching/24hrs:	99mm
VAS for wealing:	97mm
UAS7:	42
Number of weals:	108
Treatment:	Cetirizine 10mg/day

SERUM-INDUCED BHR: 0%

ANTI-IGE-INDUCED BHR:

Basophil count/ml of blood:	7250
Basophil purity:	0.6%
Spontaneous BHR:	0%
Optimal anti-IgE Concentration:	1 µg/ml
Anti-IgE induced HR (1 µg/ml):	40%

FLOW CYTOMETRY STUDIES



Baseline

CCR3+CD123+basophils - 266 cells/µL

CCR3+CD63+ basophils - 1939 cells/µL

CD63+CD203c+ basophils - 141 cells/µL

WEEK 12 (18.08.2011)

CLINICAL ASSESSMENT

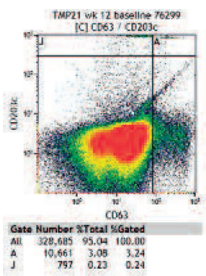
VAS for itching/24hrs:	81mm
VAS for wealing:	83mm
UAS7:	37
Number of weals:	51
Treatment:	Cetirizine 10mg/day

SERUM-INDUCED BHR: 0%

ANTI-IGE-INDUCED BHR:

Basophil count/ml of blood:	0
Basophil purity:	0%
Spontaneous BHR:	0%
Optimal anti-IgE Concentration:	N/A
Anti-IgE induced HR (1 µg/ml):	NR

FLOW CYTOMETRY STUDIES



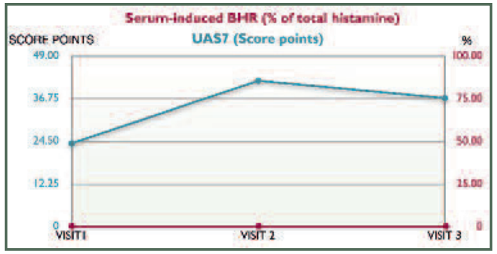
Baseline

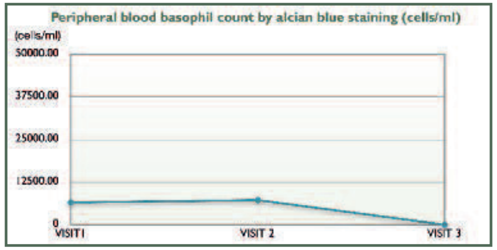
CCR3+CD123+basophils - 365 cells/µL

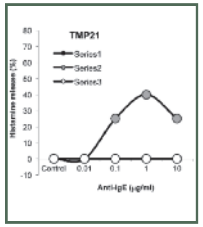
CCR3+CD63+ basophils - 1211 cells/µL

CD63+CD203c+ basophils - 189 cells/µL

SUMMARY







Appendix 4. Figure 30. Biomarker Prospective Study - Patient DBP23

Period of Observation: 11.01.2011 - 11.07.2011 ASST: Positive

WEEK 0 (11.01.2011)

CLINICAL ASSESSMENT

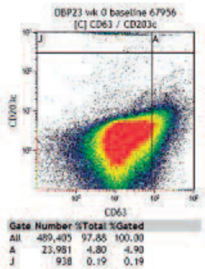
VAS for itching/24hrs:	38mm
VAS for wealing:	44mm
UAS7:	29
Number of weals:	0
Treatment:	Cetirizine 10mg/day

SERUM-INDUCED BHR: 50%

ANTI-IGE-INDUCED BHR:

Basophil count/ml of blood:	2125
Basophil purity:	0.14%
Spontaneous BHR:	0%
Optimal anti-IgE Concentration:	N/A
Anti-IgE induced HR (1μg/ml):	NR

FLOW CYTOMETRY STUDIES



Baseline

CCR3+CD123+ basophils - 296 cells/μL

CCR3+CD63+ basophils - 3652 cells/μL

CD63+CD203c+ basophils - 85 cells/μL

WEEK 6 (08.04.2011)

CLINICAL ASSESSMENT

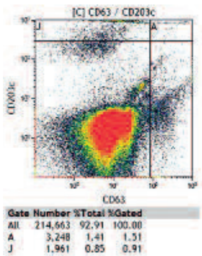
VAS for itching/24hrs:	73mm
VAS for wealing:	68mm
UAS7:	36
Number of weals:	12
Treatment:	Cetirizine 10mg/day

SERUM-INDUCED BHR: 38%

ANTI-IGE-INDUCED BHR:

Basophil count/ml of blood:	1510.4
Basophil purity:	0.1%
Spontaneous BHR:	0%
Optimal anti-IgE Concentration:	N/A
Anti-IgE induced HR (1μg/ml):	NR

FLOW CYTOMETRY STUDIES



Baseline

CCR3+CD123+basophils - 192 cells/μL

CCR3+CD63+ basophils - 950 cells/μL

CD63+CD203c+ basophils - 82 cells/μL

WEEK 12 (11.07.2011)

CLINICAL ASSESSMENT

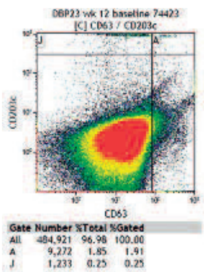
VAS for itching/24hrs:	81mm
VAS for wealing:	78mm
UAS7:	27
Number of weals:	47
Treatment:	Cetirizine 10mg/day

SERUM-INDUCED BHR: 46%

ANTI-IGE-INDUCED BHR:

Basophil count/ml of blood:	7533.33
Basophil purity:	0.4%
Spontaneous BHR:	33.3%
Optimal anti-IgE Concentration:	N/A
Anti-IgE induced HR (1μg/ml):	NR

FLOW CYTOMETRY STUDIES



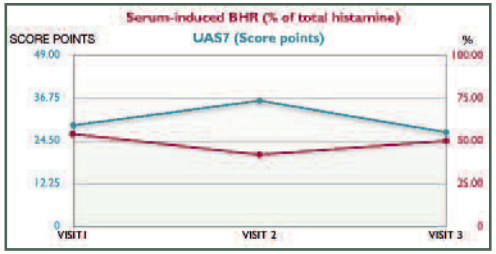
Baseline

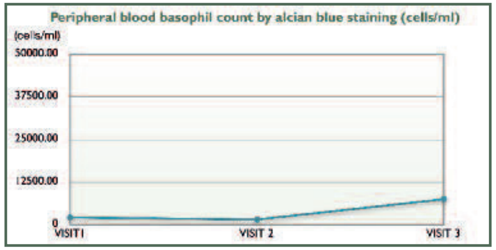
CCR3+CD123+basophils - 541 cells/μL

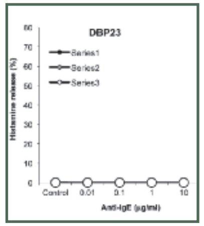
CCR3+CD63+ basophils - 3320 cells/μL

CD63+CD203c+ basophils - 205 cells/μL

SUMMARY







Appendix 4. Figure 31. Biomarker Prospective Study - Patient BHP24

Period of Observation: 10.01.2011 - 28.06.2011   ASST: Positive

WEEK 0 (10.01.2011)

CLINICAL ASSESSMENT

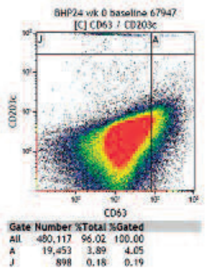
VAS for itching/24hrs:	73mm
VAS for wealing:	75mm
UAS7:	36
Number of weals:	58
Treatment:	Fexofenadine 180mg QD Piriton Montelukast 10mg/day Ranitidine 150mg/day

SERUM-INDUCED BHR: 45%

ANTI-IGE-INDUCED BHR:

Basophil count/ml of blood:	0
Basophil purity:	0%
Spontaneous BHR:	0%
Optimal anti-IgE Concentration:	N/A
Anti-IgE induced HR (1µg/ml):	NR

FLOW CYTOMETRY STUDIES



Baseline

CCR3+CD123+ basophils - 133 cells/µL

CCR3+CD63+ basophils - 909 cells/µL

CD63+CD203c+ basophils - 43 cells/µL

WEEK 6 (08.04.2011)

CLINICAL ASSESSMENT

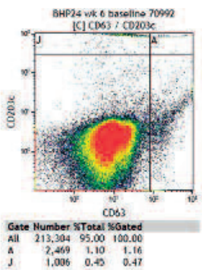
VAS for itching/24hrs:	67mm
VAS for wealing:	64mm
UAS7:	34
Number of weals:	52
Treatment:	Fexofenadine 180mg QD Piriton Montelukast 10mg/day Ranitidine 150mg/day

SERUM-INDUCED BHR: 45%

ANTI-IGE-INDUCED BHR:

Basophil count/ml of blood:	4531.2
Basophil purity:	0.24%
Spontaneous BHR:	0%
Optimal anti-IgE Concentration:	N/A
Anti-IgE induced HR (1µg/ml):	NR

FLOW CYTOMETRY STUDIES



Baseline

CCR3+CD123+basophils - 267 cells/µL

CCR3+CD63+ basophils - 883 cells/µL

CD63+CD203c+ basophils - 116 cells/µL

WEEK 12 (28.06.2011)

CLINICAL ASSESSMENT

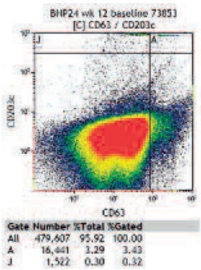
VAS for itching/24hrs:	88mm
VAS for wealing:	74mm
UAS7:	44
Number of weals:	27
Treatment:	Fexofenadine 180mg QD Piriton Montelukast 10mg/day Ranitidine 150mg/day

SERUM-INDUCED BHR: 48%

ANTI-IGE-INDUCED BHR:

Basophil count/ml of blood:	4250
Basophil purity:	0.3%
Spontaneous BHR:	0%
Optimal anti-IgE Concentration:	1µg/ml
Anti-IgE induced HR (1µg/ml):	20%

FLOW CYTOMETRY STUDIES



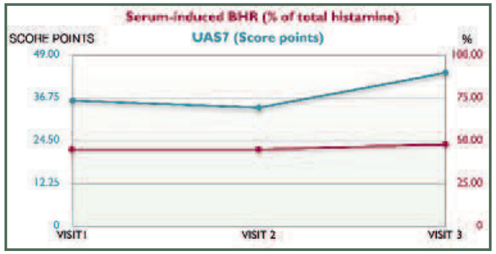
Baseline

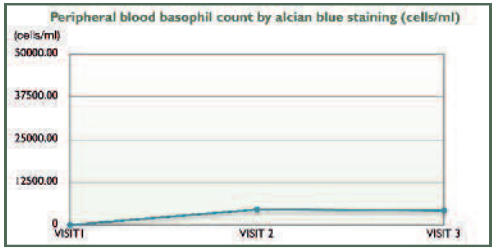
CCR3+CD123+basophils - 741 cells/µL

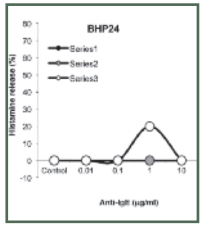
CCR3+CD63+ basophils - 3037 cells/µL

CD63+CD203c+ basophils - 295 cells/µL

SUMMARY







Appendix 4. Figure 32. Biomarker Prospective Study - Patient DMP25

Period of Observation: 15.02.2011 - 27.06.2011 ASST: Positive

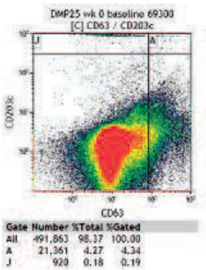
WEEK 0 (15.02.2011)

CLINICAL ASSESSMENT

VAS for itching/24hrs:	66mm
VAS for wealing:	56mm
UAS7:	27
Number of weals:	54
Treatment:	Fexofenadine 180mg TD Montelukast 10mg/day

SERUM-INDUCED BHR:	0%
ANTI-IGE-INDUCED BHR:	
Basophil count/ml of blood:	14666.66
Basophil purity:	0.75%
Spontaneous BHR:	5.1%
Optimal anti-IgE Concentration:	N/A
Anti-IgE induced HR (1μg/ml):	NR

FLOW CYTOMETRY STUDIES



Baseline

CCR3+CD123+ basophils - 303 cells/μL

CCR3+CD63+ basophils - 5480 cells/μL

CD63+CD203c+ basophils - 173 cells/μL

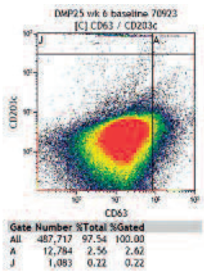
WEEK 6 (06.04.2011)

CLINICAL ASSESSMENT

VAS for itching/24hrs:	38mm
VAS for wealing:	44mm
UAS7:	14
Number of weals:	9
Treatment:	Fexofenadine 180mg QD Montelukast 10mg/day

SERUM-INDUCED BHR:	0%
ANTI-IGE-INDUCED BHR:	
Basophil count/ml of blood:	2265.6
Basophil purity:	0.39%
Spontaneous BHR:	0%
Optimal anti-IgE Concentration:	1μg/ml
Anti-IgE induced HR (1μg/ml):	25%

FLOW CYTOMETRY STUDIES



Baseline

CCR3+CD123+basophils - 227 cells/μL

CCR3+CD63+ basophils - 3479 cells/μL

CD63+CD203c+ basophils - 89 cells/μL

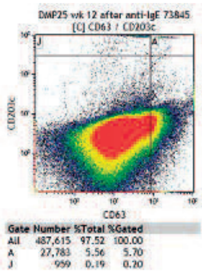
WEEK 12 (27.06.2011)

CLINICAL ASSESSMENT

VAS for itching/24hrs:	0mm
VAS for wealing:	0mm
UAS7:	0
Number of weals:	0
Treatment:	Fexofenadine 180mg QD Montelukast 10mg/day

SERUM-INDUCED BHR:	0%
ANTI-IGE-INDUCED BHR:	
Basophil count/ml of blood:	35789.47
Basophil purity:	2%
Spontaneous BHR:	4%
Optimal anti-IgE Concentration:	0.1μg/ml
Anti-IgE induced HR (1μg/ml):	10.3%

FLOW CYTOMETRY STUDIES



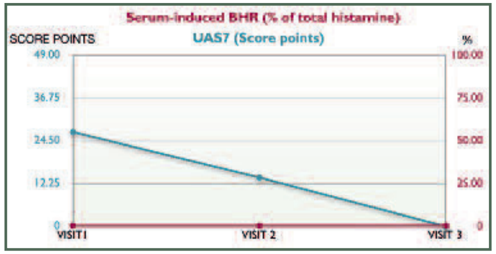
Baseline

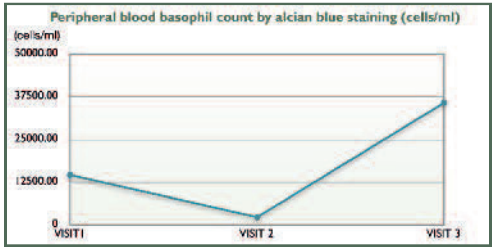
CCR3+CD123+basophils - 786 cells/μL

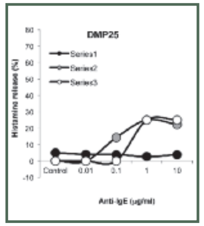
CCR3+CD63+ basophils - 17558 cells/μL

CD63+CD203c+ basophils - 227 cells/μL

SUMMARY

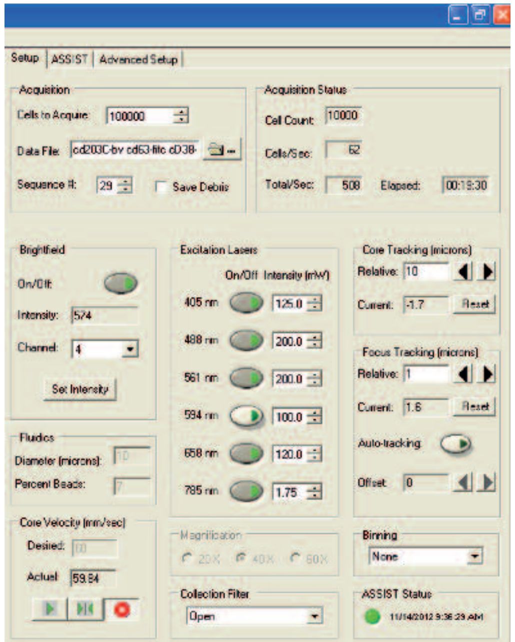




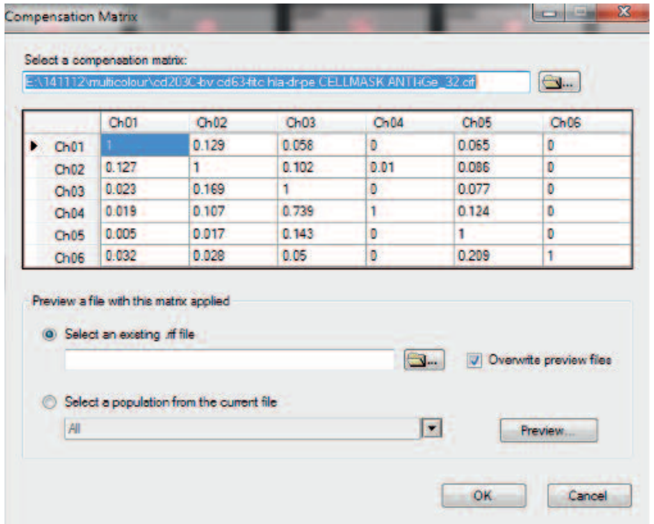


Appendix 5. Figure 1. Experimental Settings and a Compensation Matrix for Imaging Flow Cytometry Studies using Imagestream^{X} Imaging Flow Cytometer

A. Laser Power Settings



B. The Compensation Matrix for a Fluorochrome Combination: BV421, FITC, PE, CellMask Deep Red



	Ch01	Ch02	Ch03	Ch04	Ch05	Ch06
Ch01	1	0.129	0.058	0	0.065	0
Ch02	0.127	1	0.102	0.01	0.086	0
Ch03	0.023	0.169	1	0	0.077	0
Ch04	0.019	0.107	0.739	1	0.124	0
Ch05	0.005	0.017	0.143	0	1	0
Ch06	0.032	0.028	0.05	0	0.209	1

Figure 1. Data acquisition was performed using five excitation lasers (405nm, 488nm, 561nm, 658nm and 785nm) (Figure 1A). The laser power adjustment was carried out using single stained controls. The spectral overlap for this multicolour panel is presented in Figure 1B. All samples were acquired on an ImageStream^{X} imaging flow cytometer at X40 magnification using INSPIRE software.

Abbrevations:
FITC - Fluorescein isothiocyanate
PE - Phycoerythrin
BV - Brilliant Violet

Appendix 5. Figure 2. Multicolour Staining Panels used in Imaging Flow Cytometry Studies

A. Excitation Lasers, Fluorescent Dyes and Cell Surface Markers used in Flow cytometry Studies for Human Basophils

Panel 1

Laser	405nm	488nm	561nm	658nm	785nm
Fluorescent Dyes	Brilliant violet 421	FITC	PE	CellMask Deep Red	
Cell Suface Marker	CD203c	CD63	CCR3		SSC

Panel 2

Laser	405nm	488nm	561nm	658nm	785nm
Fluorescent Dyes	Brilliant violet 421	FITC	PE	CellMask Deep Red	
Cell Suface Marker	CD203c	CD63	CRTH2		SSC

Panel 3

Laser	405nm	488nm	561nm	658nm	785nm
Fluorescent Dyes	Brilliant violet 421	FITC	PE	CellMask Deep Red	
Cell Suface Marker	CD203c	CD63	CD69		SSC

Figure 2. Three multicolour staining panels were used for imaging flow cytometry studies in human basophils (Appendix 4, Figure 3A). Our main panel of antibodies (Panel 1) included anti-CD203c-Brillian Violet 421, anti-CD63-FITC and anti-CCR3-PE. Panel 1 was used for comparative studies between gating strategies for peripheral blood basophils. Panels 2 and 3 were used for basophil immunophenotyping using surface basophil markers outlined in Table 5. Anti-CD123-Brilliant Violet 421, anti-CD45-FITC, anti-CCR3-PE and CellMask™ Deep Red Plasma membrane stain were used for generating a fluorescence compensation matrix. Anti-CD61-PE and anti-PAC1-FITC were used to detect platelet markers (Table 2).

Table 1. Antibody specificities, clones and suppliers for antibody conjugates used in imaging flow cytometry studies for human basophils

Catalogue No.	Clone	Antibody Conjugates	Manufacturer
324611	NP4D6	anti-CD203c Brilliant Violet 421	Biolegend
557288	H5C6	anti-CD63-FITC	BD Bioscience
310706	5E8	anti-CCR3-PE	Biolegend
C10046	N/A	CellMask™ Deep Red Plasma membrane stain	Invitrogen
306017	6M6	anti-CD123 - Brilliant Violet 421	Biolegend
345808	2DI	anti-CD45-FITC	BD Bioscience
120-001-698	BM16	anti-CRTH2-PE	MACS Miltenyi
12-0699-71	FN5D	anti-CD69-PE	e-Bioscience
336405	V1 - PL2	anti-CD61-PE	Biolegend
340507	PAC-1	anti-PAC-1-FITC	BD Bioscience

Table 2. Surface cellular markers used in Imaging flow cytometry studies

Marker	Biological Family	Biological Function
CD203c	E-NNP3 (family of ectoenzymes)	Involved in hydrolysis of extracellular nucleotides
CD63	TM4 family (tetraspanin)	Expressed in late endosomes, role as an intracellular transport regulator
CCR3	Seven-transmembrane G-protein coupled receptor	C-C chemokine receptor for eotaxin, eotaxin 2, RANTES, MCP-2, -3 and -4
CD123	IL-3 receptor α-chain	Receptor for IL-3, IL-5, GM-CSF
CD45	protein tyrosine phosphatase	Leukocyte common antigen
CRTH2	Seven-transmembrane receptor	PGD_2 receptor
CD69	Type II transmembrane C-type lectin protein	Early leukocyte activation antigen, early T cell activation antigen
CD61	integrin β-3	A cluster of differentiation on thrombocytes
PAC-1	epitope on glycoprotein Iib/IIIa complex of activated platelets	Fibrinogen binding site on activated platelets

Abbrevations:
FITC - Fluorescein isothiocyanate
PE - Phycoerythrin
CCR3 - Chemokine (C-C motif) receptor type 3
CRTH2 - Chemoattractant receptor-homologous molecule expressed on Th2 cells
SSC - Side scatter
PAC-1 - Platelet Activation Complex -1
IL-3 - Interleukin 3
IL-5 - Interleukin 5
GM-CSF - Granulocyte macrophage colony-stimulating factor
PGD_2 - Prostaglandin D2
TM4 - Transmembrane 4 superfamily (tetraspanin family)
E-NNP3 - Ectonucleotide pyrophosphatase/phosphodiesterase 3

Appendix 5. Figure 3. Data analysis with IDEAS® software for Imaging Flow Cytometry

Data Analysis with IDEAS® Software

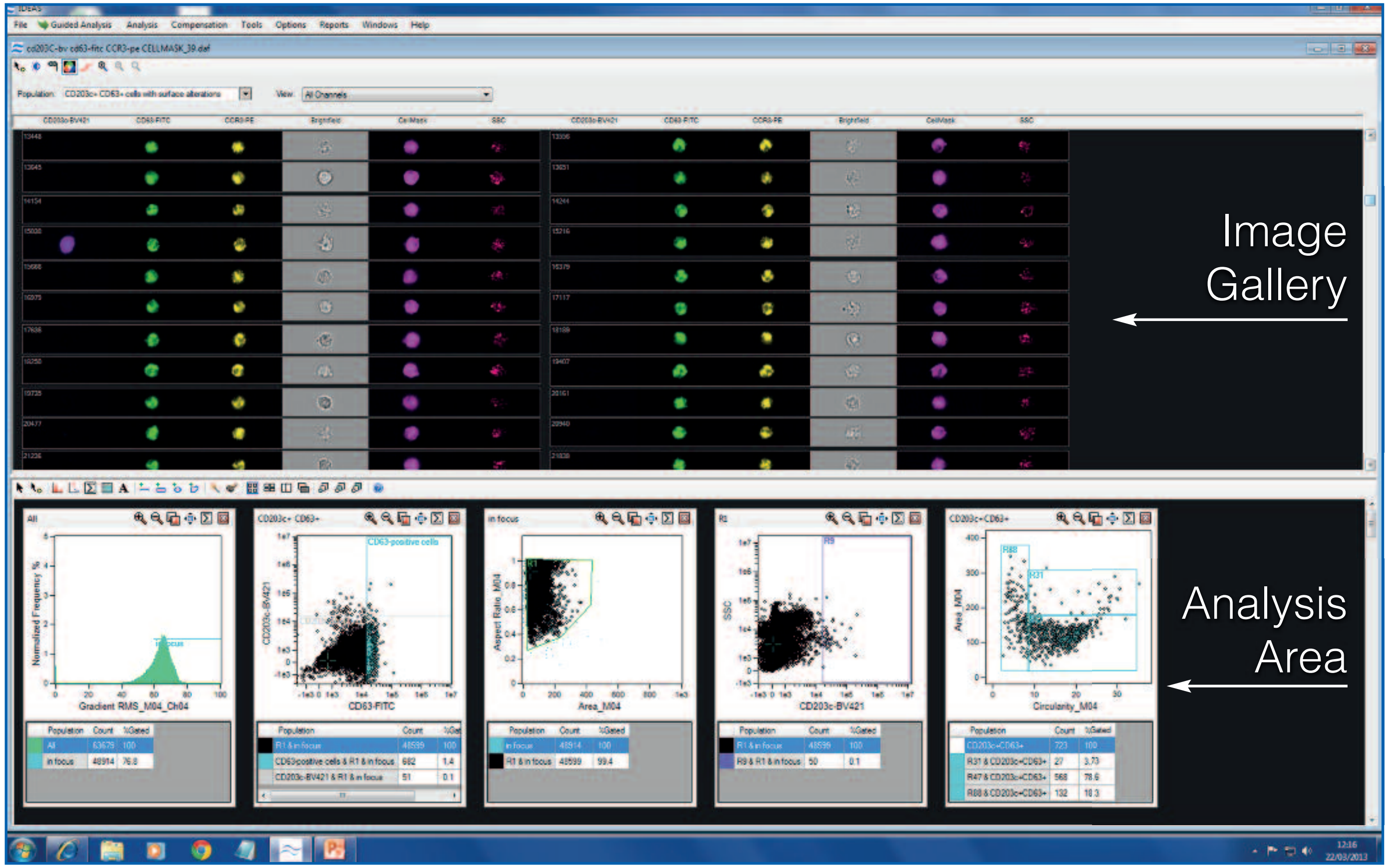


Figure 3. Data exploration and analysis were carried out using IDEAS® software version 4.0 (Amnis Corporation).

Appendix 5. Figure 4. Fixation and Single Stained Controls for Imaging Flow Cytometry Basophil Studies in Healthy Subjects

Figure 4. For the Image Stream studies the samples were fixed with 0.0025% glutaraldehyde. Fixed and unfixed unstained controls were used to assess the background fluorescence of cells due to autofluorescence or fixation-induced fluorescence (Figures 4A-B).

The laser power was set-up using single stained controls for basophil markers used in our fluorochrome panel. Spectral compensation matrix for our four colour- fluorochrome panel was defined by using single stained controls with the cell surface markers characterised by marked expression on human basophils (Figures 4C-F).

Spectral compensation was successfully applied to single stained control samples (Figures 4C-E).

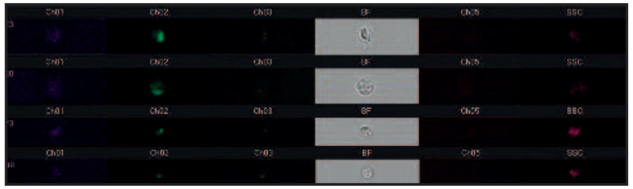
Enriched basophil preparations were prepared by density gradient centrifugation using Ficoll-Paque PREMIUM medium with density of 1.084g/ml (GE Healthcare, UK).

Abbrevations:
BV - Brilliant Violet
FITC - Fluorescein isothiocyanate
PE- Phycoerythrin

Fixation Controls

Unstained Unfixed Sample

A



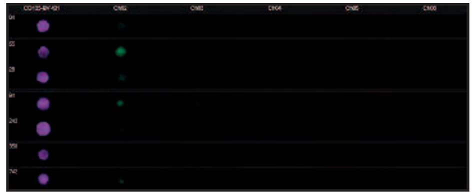
Unstained Fixed Sample

B

Single Stained Controls

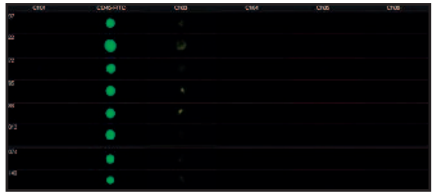
CD123-BV 421

C



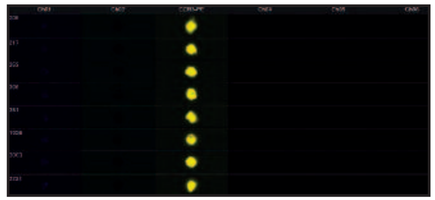
CD45-FITC

D



CCR3-PE

E



CellMask

F

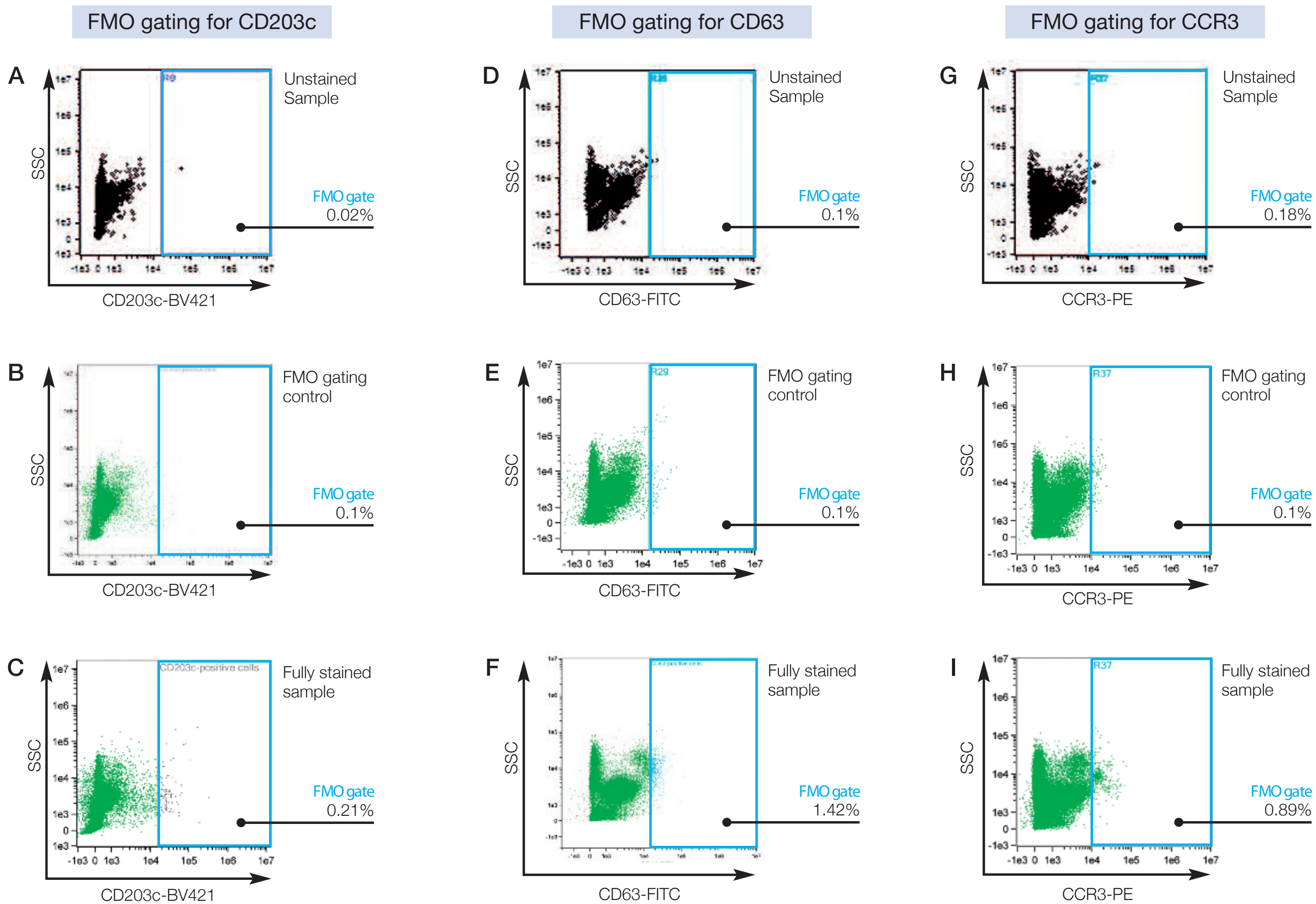
Appendix 5. Figure 5. FMO Gating for Peripheral Blood Basophils in Imaging Flow Cytometry Studies

Figure 5. Three cellular surface markers (CD63, CD203c and CCR3) were used for gating for peripheral blood basophils in CSU patients. The gates for cells expressing each of these markers were defined by FMO gating. The gates on the dot plots B, E and H were set at the 99th percentile of the intensity of CD203c, CD63 and CCR3 to include 0.1% of cells in FMO gating controls (Figures 5B, 5E, 5F).

Cells were considered positive for expression of these markers if they displayed fluorescence intensity above the level of the 99th percentile of FMO gating controls. The percentages of positive cells for each marker were assessed in fully stained samples (Figures 5C, 5F, 5I) using a 5-colour flow cytometric panel (Figure 10A, Appendix 3). These gates were also verified on unstained samples (Figures 5A, 5D, 5G).

Enriched basophil preparations were prepared by density gradient centrifugation using Ficoll-Paque PREMIUM medium with density of 1.084g/ml (GE Healthcare, UK).

Abbrevations:
BV - Brilliant Violet
FITC - Fluorescein isothiocyanate
PE - Phycoerythrin
FMO - Fluorescence minus one control
SSC - Side scatter



Appendix 5. Table 3. Glossary of Morphometric Features of IDEAS® Software used in Imaging Flow Cytometry Studies in Human Basophils

Feature	Feature Category	Definition
Area	Size	A measure of the size of the cell in square microns
Width	Size	Based on a bounding rectangle, the Width is the smaller side and the Height is the longer side of the rectangle
Thickness Max	Size	A measure of the longest width of the cell image
Aspect Ratio	Shape	The ratio of the Minor Axis divided by the Major Axis
Circularity	Shape	The degree of the cell image deviation from a circle
Shape Ratio	Shape	The ratio of Thickness Min/ Length features
Gradient RMS	Texture	A measure of changes of pixel values in the image to measure the focus quality of an image
Intensity	Signal Strength	A sum of the pixel intensities in the cell image, background subtracted.

Table 3. The Features are defined according to the IDEAS Image Data Exploration and Analysis Software User's Manual (Amnis Corporation, Version 5,0, September 2011).

Appendix 5. Figure 6. Imaging Flow Cytometry Studies with KU812 Cell Line

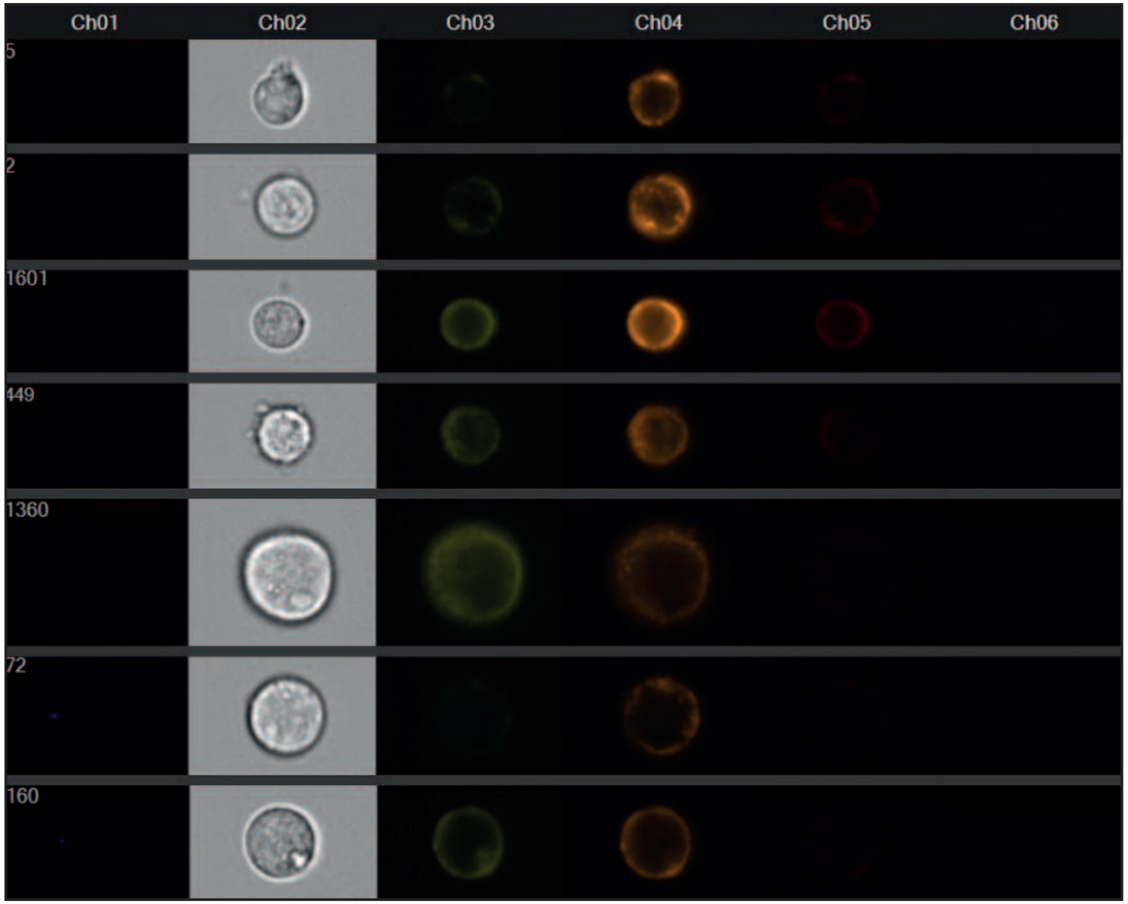


Figure 6. The Image Stream imagery of unstained KU812 cell line fixed with glutaraldehyde. The brightfield (Channel 2) imagery demonstrates morphology of human leukaemic KU812 cell line. KU812 cell line was derived from a patient with chronic myelogenous leukaemia in 1985 (Kishi K, 1985). In our studies, KU812 cells were used to study autofluorescence (Channels 3-4) induced by glutaraldehyde fixation.

Appendix 5. Figure 7. Basophil Subpopulation with Surface Alterations in Imaging Flow Cytometry Studies in a Healthy Subject using different Pre-analytical Sample Handling Protocols

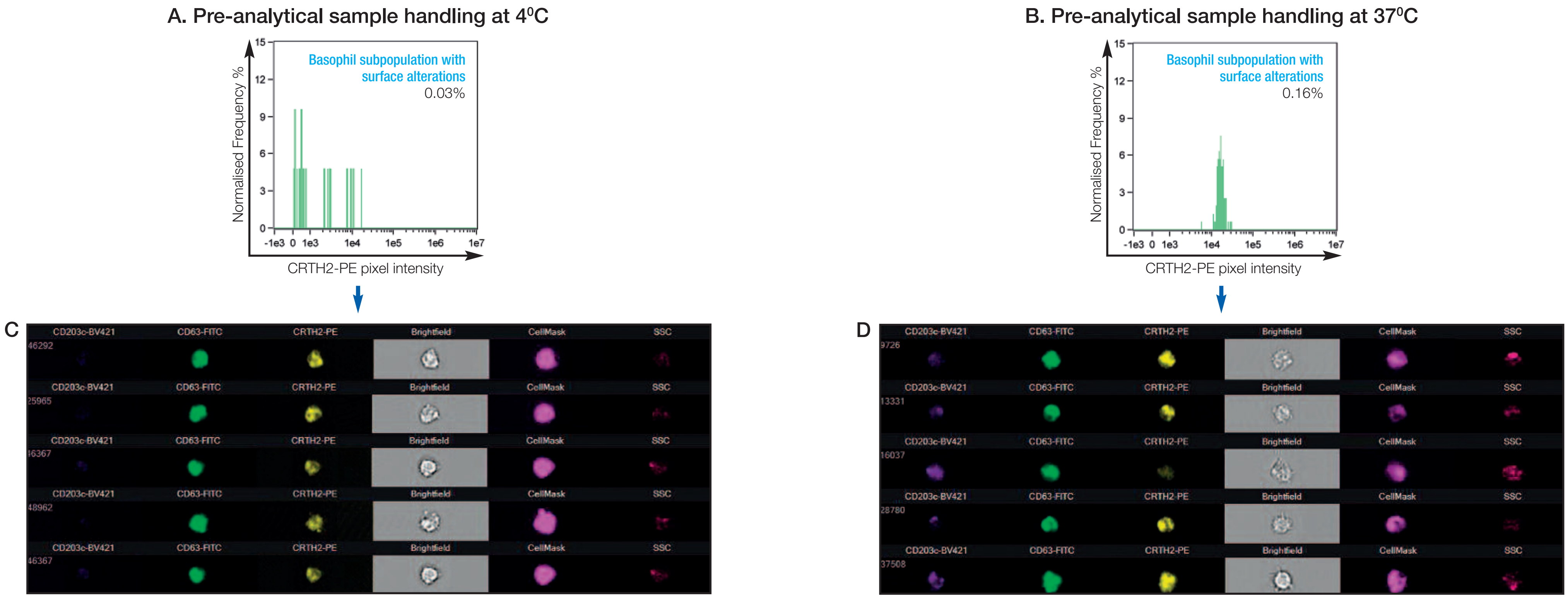


Figure 7. Sample handling protocols: the effects of sample pre-warming. After Ficoll density centrifugation, basophil-enriched leukocyte suspensions were subjected to different sample handling protocols depending on the type of the experiment. For basophil immunophenotyping, sample handling and staining was performed at 4°C (Figures 7A, C). By contrast, optimal conditions for basophil functional studies include pre-warming of samples at 37°C before *in vitro* stimulation (Figures 7B, D). Sample pre-warming at 37°C resulted in the increased percentage of basophil subpopulations with surface alterations which are morphologically identified as cells with surface alterations. Basophil-enriched samples were prepared for analysis by density gradient centrifugation using Ficoll-Paque PREMIUM medium with density of 1.084g/ml (GE Healthcare, UK). All samples were acquired on an ImageStream^{X} flow cytometer at x40 magnification using INSPIRE software. Data analysis was carried out using IDEAS® software version 4.0 (Amnis Corporation).

Abbrevations:
CRTH2 - Chemoattractant receptor-homologous molecule expressed on Th2 cells

Appendix 5. Figure 8. Basophils with Surface Alterations in Cellular Aggregates in Imaging Flow Cytometry Studies

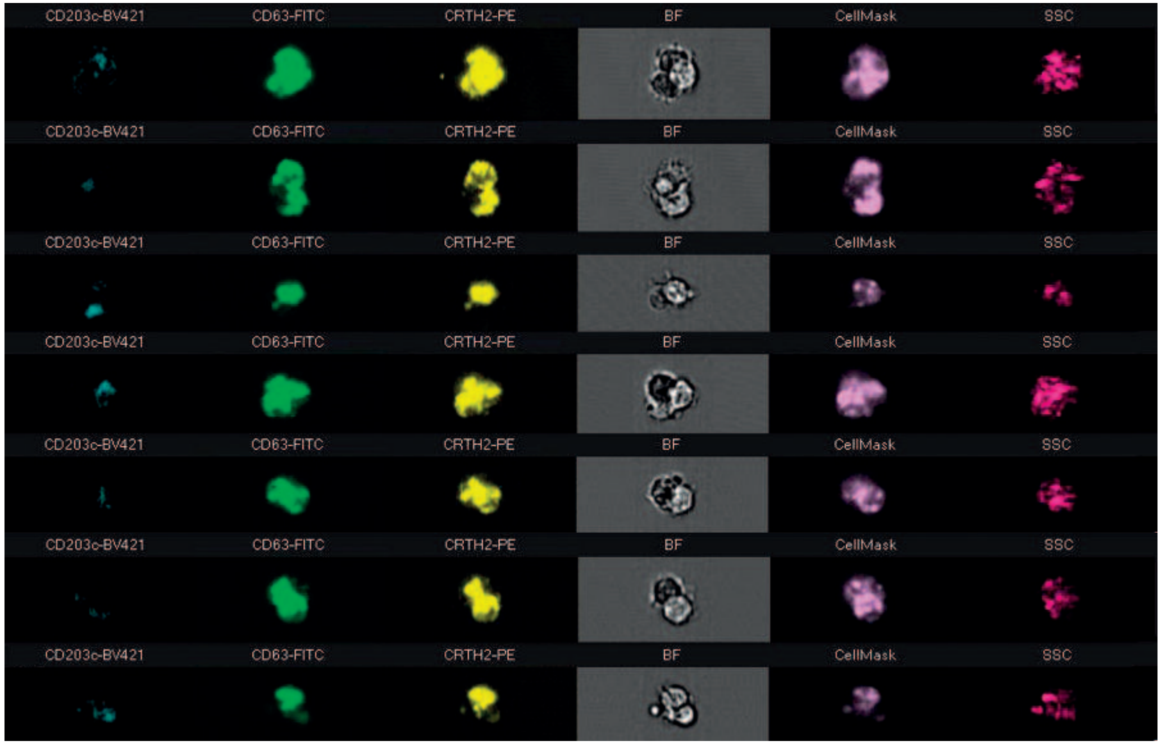


Figure 8. Visual inspection of cellular aggregates revealed enhanced formation of cellular aggregates by basophil subpopulation with surface alterations. Basophil-enriched samples were prepared for analysis by density gradient centrifugation using Ficoll-Paque PREMIUM medium with density of 1.084g/ml (GE Healthcare, UK). All samples were acquired on an ImageStream^X flow cytometer at x40 magnification using INSPIRE software. Data analysis was carried out using IDEAS® software version 4.0 (Amnis Corporation).

Abbrevations:
CRTH2 - Chemoattractant receptor-homologous molecule expressed on Th2 cells
BF - Brightfield
BV - Brilliant Violet
FITC - Fluorescein isothiocyanate
PE- Phycoerythrin
SSC - Side Scatter