The Impact of Diet on Immunosenescence

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Abstract

Introduction: The population is ageing but this accompanies increased susceptibility to infection and age-associated diseases, as well as reduced vaccination responses; potentially attributable to reduced immune function. Immunosenescence describes the deleterious effects of ageing on the immune system and is associated with a chronic, low-grade, inflammatory state; inflammaging. Habitants of Mediterranean regions maintain good health into old age; often attributed to Mediterranean (MED)-diets.

Hypothesis: Adoption of a MED-diet by elderly subjects, in Norfolk, may improve immune responses of these individuals; particularly in terms of dendritic cell (DC) function and antibody diversity.

Experimental approach**:** Elderly subjects recruited onto the Nu-AGE study were randomised to the control or MED-diet groups, for one year. Blood samples were compared from pre- and post-intervention, and to blood samples from young subjects. Study compliance was assessed using high performance liquid chromatography-with tandem mass spectrometry (HPLC-MS/MS) analysis of urine samples. Immune cell subset numbers and concentrations of secreted proteins were determined by flow cytometry, after staining for surface markers and intracellular proteins. Age and dietary impact on antibody diversity was quantitated using a novel-polymerase chain reaction (PCR)-based technique developed by the Babraham Institute.

Results**:** The MED-diet group had higher urinary hydroxytyrosol sulphate post-intervention but self-reported diet diary analyses showed no difference in MED-diet scores. Reduced myeloid DC numbers were observed in blood samples from elderly subjects compared to young. The elevated secretion of the adipokine, resistin, after *ex vivo* stimulation of peripheral blood mononuclear cells (PBMCs) from elderly subjects, was significantly reduced after MED-diet intervention, but this change from baseline was not significantly different to the control group. Antibody diversity was reduced with age, dietary intervention may prevent further reductions in unique clonotypes.

Conclusions**:** Further evidence of numerical and functional effects of ageing on DCs, are shown. The MED-diet showed potential to impact on the ageing immune cells investigated and could provide an economical approach to address problems associated with our ageing population.

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I dedicate this thesis to my late Nan, Kath Kirk, the woman I always looked up to and strive to be like; I hope you would be very proud of me.

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Abbreviations

Ab	Antibody
ABCB1	ATP-binding-cassette-B1 transporter
ADCC	Antibody-dependent cell-mediated cytotoxicity
Ag	Antigen
AgR	Antigen receptor
AID	Activation-induced deaminase
AMP	Antimicrobial peptide
-APC	Allophycocyanin
APC	Antigen presenting cell
ATP	Adenosine triphosphate
BDCA	Blood dendritic cell antigen
BCR	B cell receptor
B-GOS	Beta-galactooligosaccharide
BM	Bone marrow
BMD	Bone mineral density
BMI	Body mass index
BMR	Basal metabolic rate
CAD	Coronary artery disease
CCR	CC chemokine receptor
CD	Cluster of differentiation
CDCs	Conventional dendritic cells
CDP	Common dendritic cell precursor
CDR	Complementarity determining region

CFSE	Carboxyfluorescein succinimidyl ester
CHD	Coronary heart disease
CI	Confidence interval
CLA	Conjugated linoleic acid
CLnA	Conjugated linolenic acid
CLP	Common lymphoid progenitor
CM	Chylomicron
CMV	Cytomegalovirus
ConA	Conconavalin A
CpG ODN	CpG Oligodeoxynucleotides
Cpn	*Chlamydophila pneumoniae*
CRP	C-reactive protein
CRTU	Clinical research and Trials Unit
CSR	Class switch recombination
CT	Computed tomography
CV	Coefficient of variance
CVD	Cardiovascular disease
CXCL	Cys-X-Cys motif (CXC) chemokine ligand
DAMP	Damage associated molecular pattern
DC	Dendritic cell
7DD	7-day diet diary
DHA	Docosahexanoic acid
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid

DRV	Dietary reference value
DTH	Delayed-type hypersensitivity
DXA	Dual energy x-ray absorbtiometry
EBV	Epstein-barr herpes virus
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentanoic acid
ER	Endoplasmic reticulum
FA	Fatty acid
FBS	Foetal bovine serum
FFA	Free fatty acid
FFM	Fat free mass
FFQ	Food frequency diary
-FITC	Fluorescein
FM	Fat mass
FMD	Flow mediated dilation
FOS	Fructo-oloigosaccharide
FR	Framework region
FSH	Follicle stimulating hormone
G-CSF	Granulocyte colony stimulating factor
GERD	Gastroesophageal Reflux Disease
GI	Gastrointestinal
GM-CSF	Granulocyte macrophage colony stimulating factor
GOS	Galacto-oligosaccharide
GP	General Practice

GWAS	Genome wide association study
HB	Hepatitis B
HDL	High density lipooprotein
HI	Hemagglutination inhibition
HPLC-MS	High Performance Liquid Chromatography-Mass Spectrometry
HSC	Haematopoietic stem cell
HT	Hydroxytyrosol
HTS	Hydroxytyrosol sulphate
ICAM	Intercellular adhesion marker
IECs	Intestinal epithelial cells
IFN	Interferon
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IgH	Immunoglobulin heavy chain
IGHV	Immunoglobulin heavy chain variable region
IgL	Immunoglobulin light chain
IL-6	Interleukin-6
IMGT	International ImMunoGeneTics
IP-10	IFN-γ-inducing protein 10
IRAK	IL-1R-associated kinase
IRF	Interferon regulatory factor
IRP	Immune risk profile
ITT	Intention-to-treat
Kg	Kilogram

KIR	Killer cell Immunoglobulin-like receptor
LDL	Low density lipoprotein
LN	Lymph node
LTA	Lipoteichoic acid
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
M-CSF	Macrophage colony stimulating factor
mDC	Myeloid dendritic cell
MDP	Macrophage and dendritic cell precursor
MDS	Mediterranean diet score
MED diet	Mediterranean diet
MetS	Metabolic syndrome
MFI	Mean fluorescence intensity
2-MG	2-Mono-glycerides
MHC	Major histocompatibility complex
MI	Myocardial Infarction
MLN	Mesenteric lymph node
MLR	Mixed leucocyte reaction
MNC	Mononuclear cell
MoDC	Monocyte derived dendritic cell
MRI	Magnetic resonance imaging
mRNA	micro ribonucleic acid

MSC	Mesenchymal stem cells
MUFA	Monounsaturated fatty acid
MyD88	Myeloid differentiation factor 88
NADPH	Nicotinamide adenine dinucleotide phosphate
NAFLD	Non-alcoholic fatty liver disease
NDNS	National diet and nutrition survey
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK cell	Natural killer cell
NMES	Non-milk extrinsic sugars
NND	New Nordic diet
NNUH	Norfolk and Norwich University Hospital
NO	Nitric oxide
NSP	Non-starch polysaccharide
OTC	Over the counter
OVA	Ovalbumin
P	Probability value
PAMP	Pathogen associated molecular pattern
PBDCs	Peripheral blood dendritic cells
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCA	Principle component analysis
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell

PE	Phycoerythrin
PE-Cy5	Phycoerythrin-cyan dye 5
PE-Cy7	Phycoerythrin-cyan dye 7
PGE	Prostaglandin
PHA	Phytohaemagglutinin
PI 3-K/AkT	Phosphatidylinositol 3-kinase/ protein kinase B
PKC	Protein kinase C
PLS-LDA	Partial least squares-linear discriminant analysis
PMA	Phorbol myristate acetate
PMN	Polymorphonuclear
PPARγ	Peroxisome proliferator-activated receptor gamma
PPS	Pneumonia polysaccharide
PRR	Pattern recognition receptor
P38SAPK	p38 stress-activated protein kinase
PUFA	Polyunsaturated fatty acid
R848	Resiquimod
RA	Retinoic acid
RBP4	Retinoic binding protein 4
RDA	Recommended daily allowance
RMR	Resting metabolic rate
RNA	Ribonucleic acid
RNI	Required nutrient intake
RNS	Reactive nitrogen species
ROS	Reactive oxygen species

rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute buffer
SCFA	Short chain fatty acid
SEM	Standard error of the mean
SFA	Saturated fatty acid
SHM	Somatic hypermutation
Sir2	Sirtuin protein 2
SLE	Systemic lupus erythematosus
ssRNA	Single stranded ribonucleic acid
T1D	Type I diabetes mellitus
T2D	Type II diabetes mellitus
TCR	T cell receptor
Th	T helper
TLR	Toll like receptor
TNF	Tumour necrosis factor
T_{reg} cell	T regulatory cell
UEA	University of East Anglia
UK	United Kingdom
US	United States
UTI	Urinary tract infection
VDJ	Variable, diversity, joining regions
VLDL	Very low density lipoprotein
VZV	Varicella-Zoster herpes virus
XOS	Xylooligosaccharide

Chapter 1

Introduction & literature review

1.1.1 Ageing

Ageing is often defined very differently in studies of ageing (Mysliwska, 1999; Shodell and Siegal, 2001), with some including “elderly” participants as young as 50 years. The United Nation’s report of World Ageing (United Nations, 2015) classified older people as those aged 60 years or older, with some studies categorising those aged over 85 years as being the “oldest old” (Forsey et al., 2003; Wikby et al., 2002). It is important to recognise that ageing does not refer solely to chronological age (Kirkwood and Mathers, 2009) as biological ageing is a key contributing factor. Biological ageing is strongly influenced by genetic and environmental factors that can also influence the expected lifespan (Balcombe and Sinclair, 2001) as a result of accumulating cellular damage due to an imbalance between damage and repair mechanisms (Adams and White, 2004) and increased susceptibility to disease and mortality. Therefore, there is uncertainty when defining ageing, aged and elderly, especially since environmental factors play a role in the ageing process, with geographical location also affecting life expectancy and thus the definition of aged and elderly by country (Reques, 2008; Wilson et al., 2011; Wilson, 2014). Hereafter, this thesis will refer to the UN classification, 2015, of all elderly people as those aged 60 years and older, unless otherwise stated.

We have an ageing population which can be vastly attributed to improvements in public health and vaccination. Adverse consequences of which are apparent in the East of England, where a 13.2% increase in people aged 85 years and older were residing in care homes between 2001 and 2011 (Smith, 2014). Problems of the ageing population include increasing demands on social care, healthcare and the economy (Tinker, 2002); between 2014 and 2015 72% of UK social care requests were from those aged 65 years and over (Buttery, 2015), highlighting that while people may be living longer, they may not be doing so in good health.

1.1.2 Impact of ageing on physiology and organ function

Biological ageing impacts on numerous organ systems and has been associated with various diseases, including cancer and atherosclerosis (Adams and White, 2004). The musculoskeletal system becomes impaired with increased age as the body loses muscle and bone mass and bone mineral density (BMD) which are implicated in the risk of osteoporosis and increased fracture risk in the elderly (Hannan et al., 2000; Janssen et al., 2002; Jones et al., 1994; Svedbom et al., 2013). Cardiovascular

disease (CVD) risk factors, such as high systolic blood pressure, high levels of total and low density lipoprotein (LDL) cholesterol and low levels of high density lipoprotein (HDL) cholesterol, accumulate with increased age and elderly subjects demonstrate impaired endothelial function (Black et al., 2009; Gates et al., 2007; Thijssen et al., 2006). Cognitive decline is a common feature of ageing and Alzheimer's disease deaths increased 71% from 2000–2013 (Alzheimer's Association, 2016) with reductions in hippocampal volumes and poorer recall (Marquis et al., 2002) and an associated inflammatory state (elevated plasma levels of IL-1β, TNF-α and IL-6) (De Luigi et al., 2002; Licastro et al., 2000). With age the respiratory system experiences reduced respiratory muscle function (Janssens, 2005; Watsford et al., 2007). The menopause represents a key alteration of endocrine function in ageing women, resulting from reduced serum oestrogen and oestradiol and elevated follicle stimulating hormone (FSH) and has a conflicting association with CVD and osteoporosis risk (Cauley et al., 2001; Muka et al., 2016; WGWHII, 2002). While, low circulatory levels of insulin-like growth factor-1 (IGF-1) and gene mutations in the insulin/IGF-1 pathway have been observed in humans with exceptional longevity (>90 years) (Deelen et al., 2013; Milman et al., 2014; van der Spoel et al., 2015). The volume and blood flow to the liver are reduced, with potential reductions in phase I metabolism, and an increased risk of non-alcoholic fatty liver disease (NAFLD) occurring with increased age (Schmucker, 2005; Tajiri and Shimizu, 2013) which may influence clearance of prescription medications from the liver (Klotz, 2009); intake of which is typically high in the elderly (Hubbard et al., 2015). Urinary tract infections (UTI), one of the most common infections in the elderly, often necessitate hospital admission and catheterisation, further increasing UTI risk (Foxman, 2003; Ginde et al., 2004; Juthani-Mehta et al., 2009). Body composition and the gastrointestinal and immune systems will be discussed in more detail below, with discussion of the implications of increased age.

Body composition

Body composition is defined by the proportions of fat and lean tissue within the body, and the various measurable areas include lean body mass (LBM), the sum of body water, total body protein, carbohydrates, non-fat lipids and soft tissue minerals, free fat mass (FFM), which comprises skeletal and non-skeletal muscle, organs, connective tissue and bone, and fat mass (FM) which comprises triglycerides and makes up 80% of adipose tissue (Prado and Heymsfield, 2014). The body mass index (BMI) uses weight in kilograms (kg) and height in meters (m) and is classified as normal (18.50–24.99 kg/ m^2), overweight (pre-obese) (25.00–29.99 kg/ m^2) and

obese (≥30 kg/ m^2) (WHO, 2016). Adipose tissue, a connective tissue formed by adipocytes, elastic and collagen derived fibres, fibroblasts and capillaries (Prado and Heymsfield, 2014), secretes numerous hormones and signalling molecules, with roles including modulation of appetite, insulin sensitivity, energy expenditure, inflammation and immunity (Makki et al., 2013). These secreted proteins are known collectively as adipokines and include adiponectin, leptin, resistin and visfatin, which are cytokines produced by adipose tissue (but not solely), in addition to cytokines such as TNF-α, IL-6, IL-1, MCP-1 and certain complement factors (Tilg and Moschen, 2006). Resistin has pro-inflammatory properties while adiponectin has anti-inflammatory properties, important in regulating the pathophysiology of atherosclerosis, while resistin appears to induce inflammatory diseases, which may be associated with insulin-resistance (Tilg and Moschen, 2006). The function of resistin in humans in not confirmed, however there are reported correlations with obesity and insulin resistance, and increasing resistin levels (Koerner et al., 2005).

Body composition changes with increased age, assessment of the general UK population (45–69 years), showed 64% were overweight or obese, as determined by BMI, while FFM was positively associated with BMI and decreased with age (Franssen et al., 2014). In the obese state, low level systemic inflammation is observed (Makki et al., 2013), which is also observed with increased age (Bartlett et al., 2012; Franceschi et al., 2007). While, in an elderly cohort (≥75 years), the risk of mortality was significantly higher in those with lower BMIs (≤24.6 kg/m^2) and fat mass over a 17-year follow-up period (Rolland et al., 2014).

Gastrointestinal function

The gastrointestinal (GI) tract is a tubular tract consisting of the oesophagus, stomach, small and large intestines, anus and rectum (Furness et al., 2015). Food enters the body through the mouth where digestion begins via mechanical breakdown and mastication which involves the mixture of food with salvia to aid swallowing and entry of food into the oesophagus, before reaching the stomach (DeSesso and Jacobson, 2001). In the stomach muscular contractions and enzyme secretions result in the formation of a semifluid mixture of solutes, suspended material and emulsion particles, called chyme, which enters the small intestine, where most digestion and absorption occurs (DeSesso and Jacobson, 2001). The GI tract is essential for the break down and digestion of food into simple molecules, such as free fatty acids (FAs), monosaccharides and amino acids, which are subsequently absorbed to provide energy, vitamins and minerals (Furness et al., 2015). Any resulting waste

matter passes through the large intestine and leaves the body by excretion via the rectum, while water and electrolytes are re-absorbed (DeSesso and Jacobson, 2001).

Ageing is associated with pathophysiology of the GI tract, with significant increases in gastroesophageal reflux disease (GERD), assessed by a self-reported, frequency of symptoms, GERD Questionnaire in Japan (Okimoto et al., 2015). GERD is the most common chronic disease in Iranian individuals aged 52.1 ± 9 years (Ahmadi et al., 2016). Additionally, the swallowing disorder, dysphagia, reported in 11.4% of an elderly cohort (n=633), was positively correlated with age (Holland et al., 2011). These pathologies could result from reduced peristaltic pressure in the oesophagus (Rayner and Horowitz, 2013). Undernutrition is also common in the elderly, with loss of dentition and reduced salivary production contributing, which impact on mastication and chewing ability, while appetite and satiation are influenced by alterations in production of gut hormones (Dunn-Walters et al., 2004).

The small intestine is thought to be relatively unaffected by age, with minimal effects on structural integrity (Britton and McLaughlin, 2013), with duodenal biopsies from subjects aged 46–89 years showing no significant histological differences in brush border or enterocytes, nor were crypt area or depth, villus height, crypt: villus ratio or number of intraepithelial cells influenced by subject age (Lipski et al., 1992). Small intestinal absorption is also considered to be unaffected by age, since no correlation between age (19–91 years) and faecal fat excretion was observed after supervised intake of a high-fat meal by 114 healthy volunteers (Arora et al., 1989). While, urinary levels of xylose significantly decreased five hours post-consumption of 25 g D-xylose by older subjects (56–86 years), after prior fasting, which the authors attributed to decreased renal function (n=54) (Arora et al., 1989). However, constipation is often a problem for the elderly and is typically attributed to reduced mobility, dehydration, dietary intake and medication use (Leung, 2007). Heathy older individuals (74–85 years) displayed slower colonic transit than the younger subjects (n=16), post ingestion of radiolabelled markers within a mixed liquid and solid meal, using a gamma camera to acquire anterior and posterior images (Madsen and Graff, 2004), however no effect of age on gastric emptying or small intestinal transit rate was observed. Similarly, small bowel video capsule endoscope investigations found no effect of age on small intestinal transit time (Fischer and Fadda, 2016).

Immunological function

The first line of defence against invasion of pathogens, toxins or allergens are the anatomical and physiological barriers which include the intact skin, mucociliary

clearance mechanisms, low stomach pH and the presence of lysozyme in secretions such as saliva and tears (Turvey and Broide, 2010). The immune system can be divided into the innate and adaptive immune responses. Innate and adaptive immune cells are collectively termed leukocytes or white blood cells and originate from haematopoietic stem cells (HSC) that are present within the bone marrow (BM) (Weiskopf et al., 2016). Leukocytes can be of lymphoid or myeloid origin, with macrophages, granulocytes, mast cells and dendritic cells (DCs) derived from the common myeloid progenitor, while T and B lymphocytes, and natural killer (NK) cells are derived from the common lymphoid progenitor (CLP) (Janeway Jr et al., 2012; Weiskopf et al., 2016). Sensors of the innate immune response are present in anatomically distinct locations of tissue and cellular origin, since location of detection informs the host of the severity of the threat (Iwasaki and Medzhitov, 2015). Pathogens entering the GI tract are recognised by epithelial cells in the epithelial barrier, beneath which DCs, macrophages and mast cells reside within the lamina propria to detect any cells that have crossed the epithelial barrier (Iwasaki and Medzhitov, 2015). The lymphatic system comprises the capillaries, collecting vessels, lymph nodes, trunks and duct (Swartz, 2001). This enables immune cells to migrate from peripheral blood through lymph nodes, into the lymphatics and back, allowing continual immune surveillance and relocation of DCs to the lymph nodes where large numbers of naïve lymphocytes can attempt to locate their target Ag (Girard et al., 2012).

Innate immune system

The innate immune system is fast acting and the first line of defence against invading microorganisms, parasites, cancer cells and other non-self antigens (Ags); it is non-specific and short-lived (Solana et al., 2012). Innate immune cells comprise the phagocytic cells (DCs, macrophages, monocytes and neutrophils) which detect, engulf and destroy invading pathogens or cancer cells (Janeway Jr et al., 2012), and natural killer (NK) cells which kill virus-infected and tumour cells (Garff-Tavernier et al., 2010). These cells rely on a finite repertoire of receptors known as pattern recognition receptors (PRRs), to detect pathogen- and danger- associated molecular patterns known as PAMPs and DAMPs (Reddick and Alto, 2014). PAMPs are microbial structures such as bacterial and fungal cell wall components and viral nucleic acids (Iwasaki and Medzhitov, 2015) recognised as “non-self” by toll-like receptors (TLRs), while DAMPs are common metabolic consequences of infection and inflammation, often released during cell lysis and tissue damage (Turvey and Broide, 2010). Activation of these PRRs upon binding with PAMPs or DAMPs

activates intracellular signal transduction cascades which in turn induce expression and secretion of cytokines and chemokines, important for eliciting innate and adaptive immune responses (Reddick and Alto, 2014). Cytokines are small proteins secreted by cells in order to induce or impact on the interaction and communication between cells (Zhang and An, 2007). The term cytokine encompasses lymphokines (produced by lymphocytes), monokines (produced by monocytes), chemokines (cytokines which have chemotactic activities), adipokines (produced predominantly by adipocytes), growth factors, tumour necrosis factors (TNFs) and interleukins (produced by one leukocyte and act on another) (Duitman et al., 2011; Zhang and An, 2007).

Antigen presentation

Collaboration between the innate and adaptive immune systems is required for effective host defence via Ag-specific effector responses and this occurs via Ag presentation by antigen presenting cells (APCs): macrophages, B cells and DCs (Turvey and Broide, 2010). APCs respond, via PRRs, upon detection of PAMPs or DAMPs, as well as complement, coagulation factors, self-molecules or inflammatory cytokines (Clark et al., 2000; Guermonprez et al., 2002). Ag is taken up by APCs via the mechanisms phagocytosis, macropinocytosis or receptor-mediated endocytosis (Aderem and Underhill, 1999) and engulfed particles are subjected to progressive degradation within maturing phagosomes such that Ags are broken down to peptides, these phagosomes act as cytokine signalling platforms for PRRs to stimulate presentation of peptides via loading and transport of peptide-MHC class II molecule complexes to the cell surface, using phagosomal tubules (Mantegazza et al., 2014; Roche and Furuta, 2015). Accumulation of major histocompatibility complex class I (MHC I) or MHC II molecules on the cell surface (Guermonprez et al., 2002) enables Ags to be loaded on the MHC molecules for presentation to T (or B) lymphocytes (T cells or B cells) with specificity for that Ag (Bonilla and Oettgen, 2010). MHC I molecules bind peptides generated from exogenous proteins (cross-presentation), whereas MHC II molecules bind peptides derived from endogenous membrane proteins (Blum et al., 2013). Expression of MHC II molecules is only found on APCs, while MHC I is more universally expressed (Neefjes et al., 2011). T cell activation is dependent on three signals: Ag-specific interaction of the T cell receptor (TCR) with the peptide-MHC class II complex (Roche and Furuta, 2015), involvement of co-stimulatory molecules such as CD40 and CD80 which bind CD28 and CTLA-4 on T cells (Guermonprez et al., 2002) and secretion of cytokines (Kambayashi and Laufer, 2014; Mantegazza et al., 2014). Ag presentation is a minor role for macrophages and

B cells (Delamarre et al., 2005; Janeway Jr et al., 2012; Roche and Furuta, 2015) but the major role of DCs.

Adaptive immune system

The adaptive immune system comprises the T and B cells which provide effector and memory responses specific to target Ags, to elicit targeted responses which can act quickly upon re-infection (Bonilla and Oettgen, 2010). B cell precursors develop in the BM (Hystad et al., 2007) while T cells develop as thymocytes within the thymus (Takahama, 2006). The specificity of lymphocyte responses against countless different pathogens is possible due to the generation of numerous different Ag binding regions on TCRs and immunoglobulins (Igs), or antibodies (secreted form of Ig), during early developmental stages (Cooper and Alder, 2006). The antigen receptor (AgR) loci contain hundreds of genes from the variable (V), diversity (D) and joining (J) groups assembled in numerous combinations to create a repertoire of unique sequences via the process of V(D)J recombination (Matheson and Corcoran, 2012). The variable regions of the Ag-binding sites are made up of hypervariable loops which comprise the complementary determining regions (CDR) 1, 2 and 3 (Wang et al., 2007), the lengths of these regions, measured by nucleotide or amino acid number, determine the area for Ag binding (Robins et al., 2009; Rosner et al., 2001).

Functional Igs, which have encountered Ag, also undergo the processes of somatic hypermutation (SHM) and class switch recombination (CSR) in the periphery as mechanisms of further increasing antibody specificity and binding affinity (Meffre and Wardemann, 2008). SHM introduces point mutations and nucleotide insertions and deletions (indels) into the variable region (Teng and Papavasiliou, 2007), while CSR alters the expression of IgM and IgD to IgG, IgE or IgA to improve the efficiency of the antibody response against the pathogen (Stavnezer and Schrader, 2014) by excising and replacing gene segments encoding the constant region (Cμ) within the IgH (Matthews et al., 2014). The constant regions of Ig heavy chains : IgA (α), IgD (δ), IgE (ϵ), IgG (γ) and IgM (μ), determine the different effector functions of the Ig and are responsible for initiating antibody dependent cell cytotoxicities (ADCC), binding to complement to recruit phagocytes and transporting antibodies to mucosal sites, tear ducts and mammary glands (Schroeder Jr and Cavacini, 2010; Wang et al., 2007).

T cell effector functions are initiated after the binding of Ag-MHC I/II complexes with TCR and CD4/CD8 molecules (Bonilla and Oettgen, 2010). The type of T cell that naïve (Th0) T cells differentiate into is dependent on their required function, which is

stipulated by the expression of MHC I or II, the DC subset and the cytokine secretion profile (Bonilla and Oettgen, 2010). Clonal expansion of $CD4^+$ T helper cells leads to the proliferation of specific T cell subsets. These effector cells, typically characterised by their differential expression of cytokines (Raphael et al., 2015), migrate to B cell areas or to inflamed tissues (Sallusto et al., 1999). Clonally expanded naïve $CD8^+$ cytotoxic (killer) T cells elicit their effector functions via the production of cytotoxic molecules such as perforin and granzymes, and effector cytokines (IFN-γ and TNF-α) to destroy specific pathogens (Sarkar et al., 2008).

T and B memory cells provide a rapid response to specific Ags and are divided into subsets according to their required function (Sallusto et al., 2004). Protective memory B cells, the plasma cells, secrete specific antibodies in response to the Ag, while reactive memory B cells proliferate and differentiate into plasma cells upon secondary antigenic stimulation (Sallusto et al., 2004). Effector memory T cells migrate to inflamed tissues and provide immediate effector functions, while central memory T cells home to T cell areas of secondary lymphoid organs where they proliferate and differentiate into effector cells upon antigenic stimulation (Sallusto et al., 2004).

Immunosenescence

With age there is a progressive decline in the functionality of the immune system, termed immunosenescence (Ostan, 2008), and is frequently identified as a reduction in response to vaccinations and greater susceptibility to infection and age-associated disease, such as CVD, rheumatoid arthritis (RA), type II diabetes (T2D) and cancer (Salvioli et al., 2013). Many age-associated diseases are accompanied by dysregulated immune function and excessive inflammation; termed inflammaging (Vasto et al., 2007). “Inflammaging” is a modification of the cytokine network such that pro-inflammatory cytokines are secreted more readily by immune cells and are present at greater levels within plasma, at older ages (Ostan, 2008). Aged cells avoiding apoptosis undergo cellular senescence which may lead to inflammaging (Salvioli et al., 2013). Factors contributing to inflammaging include increases in fat tissue, chronic disorders, genetic background and continual exposure to environmental stressors such as ultraviolet light and pollution (Jacob et al., 2013; Ostan, 2008). Immunosenescence has been associated with the increased production of the pro-inflammatory cytokines IL-6, TNF-α and IL-1β (Salvioli et al., 2013). Frailty, defined by the presence of deficits in health using at least 30–40 variables (Searle et al., 2008), was associated with significant increases in basal IL-6 and TNF-α in PBMCs from very old subjects (>85 years) (Collerton et al., 2012) and IL-6 in unstimulated serum (Qu et al., 2009). Cys-X-Cys chemokine ligand 10

(CXCL10) upregulation has been associated with frailty (Qu et al., 2009). CXCL10, also referred to as IFN-γ-inducing protein 10 (IP-10), is a pro-inflammatory mediator and it is produced by monocytes as well as other cells (Qu et al., 2009). Increased circulating CXCL10 has been suggested as a marker of normal ageing, specifically in women, since increases were observed, despite no elevation of C-reactive protein (CRP), in healthy individuals (39–75 years), with elevations typically seen at 57 years (Antonelli et al., 2006).

While, healthy ageing and longevity are associated with a balance between inflammatory and anti-inflammatory responses (Franceschi et al., 2007) since centenarians had significantly increased plasma levels of the immunosuppressive transforming growth factor β (TGF-β) cytokine, compared to younger subjects (20–60 years) (Carrieri et al., 2004). The maintained balance between pro- and anti-inflammatory cytokine levels is suggested to contribute to the reduction or delayed onset of age-associated disease in centenarians (Franceschi et al., 2007). Observed genetic variations between elderly and centenarian subjects suggest that different alleles for genes encoding pro- or anti-inflammatory cytokines may influence the cytokine profiles in these individual groups; potentially influencing how successfully individuals age (Carrieri et al., 2004; De Martinis et al., 2006; Vasto et al., 2007).

The burden of immunosenescence is becoming more apparent with a projected 2.9 million increase in the number of people with ≥2 long term conditions by 2018, and 70% of UK government spending on health and social care is for older people with long term health conditions (NICE, 2015). The impact of immunosenescence on physiological functions includes the high incidence of CVD and T2D in the elderly, which are associated with inflammation (Dragsbæk et al., 2016). This has therefore led to an increase in the investigation of immunological changes in the elderly. The next section will highlight immune cell specific changes observed with increasing age.

1.1.3 Ageing of innate immune cells

Monocytes/ macrophages

Alterations in the proportions of monocyte subsets have been observed with increasing age, with significant increases in intermediate (Hearps et al., 2012) and non-classical monocytes (Hearps et al., 2012; Sadeghi et al., 1999; Seidler et al., 2010). In addition, expression of MHC II, and the chemokine receptor, CX3CR1, were significantly lower on non-classical monocytes of older subjects compared to young, while, serum levels of MCP-1, which promotes the migration of classical monocytes from the BM, significantly increased with age (Seidler et al., 2010). While, the

differentiation of peripheral blood monocytes into DCs, in response to GM-CSF and IL-4, were comparable between cells from young and elderly subjects (Lung et al., 2000).

In terms of function, monocytes from elderly subjects stimulated *in vitro* with LPS displayed significantly greater production of TNF-α and MCP-1, and lower production of TGF-β (Pinke et al., 2013), in addition to significantly increased spontaneous production of IL-6, IL-1β and IL-1RA compared to monocytes from young subjects (Sadeghi et al., 1999). In contradiction to these findings LPS stimulated monocytes from healthy elderly subjects produced lower concentrations of G-CSF, GM-CSF, IL-1β, IL-8 and MIP-1α compared to those from young subjects (Gon et al., 1996) and when cultured within PBMCs the cytokine secretion profile was comparable between age groups (Pinke et al., 2013). While, respiratory burst reaction, the process of producing highly reactive oxygen species to aid the killing of pathogens and tumour cells, was significantly reduced in monocytes from elderly subjects (65–75 years) compared to young subjects (25–35 years) (Alvarez and Santa María, 1996). Phagocytic ability of monocytes was not significantly different between young and elderly subjects (Gardner et al., 1981).

Few studies have been performed to date using human macrophages as most studies utilise monocytes, the precursor of macrophages, which provide a limited view of tissue macrophages (Sebastián et al., 2005). Human monocyte-derived macrophages from elderly subjects stimulated with LPS showed significantly reduced secretion of IL-1β and greater concentrations of reactive oxygen species (ROS) and nitric oxide (NO) (Suchy et al., 2014). With monocytes, derived from elderly human PBMCs, co-cultured with Dengue virus exhibiting lower secretion of TNF-α, IL-6 and IL-1β in comparison to cells derived from young adults (Valero et al., 2014). While, the ability of monocyte-derived macrophages to produce cytokines, both spontaneously and upon stimulation were comparable between young and elderly subjects (Seidler et al., 2010).

Neutrophils

Numerically, neutrophils appear to remain unaffected by age (Solana et al., 2012), though the expression of CD15 on neutrophils from elderly subjects was significantly higher compared to cells from young controls, in addition to a significantly reduced frequency of neutrophils with more than two lobes (Verschoor et al., 2015). While, the functional impact of ageing on neutrophils is conflicting with observations of impaired phagocytosis of fluorescently labelled *E. coli.* with fewer neutrophils available and

less bacteria ingested within whole blood samples from elderly subjects (Butcher et al., 2001). With ADCC and intracellular killing by neutrophils, assessed by GM-CSF induced specific lysis and chromium release, significantly reduced in the cells from elderly (60–90 years) compared to young subjects (20–25 years) (Seres et al., 1993). Additionally, defective neutrophil extracellular trap (NET) formation by neutrophils from elderly subjects (mean age 69 years) was observed after TNF-α priming and stimulation with IL-8 or LPS, compared to neutrophils from young subjects (mean age 25 years) (Hazeldine et al., 2014). Also, neutrophils from elderly subjects have demonstrated significantly increased levels of respiratory burst and spontaneous ROS generation when compared to cells from young subjects (Butcher et al., 2001; Kovalenko et al., 2014; Ogawa et al., 2008), however this has not been replicated in all investigations (Sauce et al., 2016; Tortorella et al., 1993). With significantly reduced oxidative burst, determined by reduction in ROS generation, and phagocytosis after incubation with *E. coli*, by neutrophils from elderly subjects (70–91 years), which were predominantly $CD16^{bright}/CD62L^{dim}$, compared to young subjects (23–35 years) (Sauce et al., 2016). Neutrophils from nonagenarians displayed similar ROS generation to cells from young subjects but lower than that observed in the 60–89 year group (Kovalenko et al., 2014). GM-CSF priming of neutrophils from elderly subjects was impaired, with no production of the superoxide anion compared to cells from young subjects, this finding was attributed to activation of a different signalling pathway (Seres et al., 1993). The short lifespan of neutrophils has been extended with GM-CSF *in vitro* in cells from young subjects, however, the same finding was not observed in neutrophils from elderly subjects (Fortin et al., 2006; Larbi et al., 2005).

Natural Killer (NK) cells

Total NK cell numbers increase with advanced age (Almeida-Oliveira et al., 2011; Campos et al., 2014; Garff-Tavernier et al., 2010) which may be due to increases observed in the mature cytotoxic effector subsets, $CD56^{-}CD16^{+}$ and $CD56^{dim}$ cells, (Almeida-Oliveira et al., 2011; Borrego et al., 1999; Di Lorenzo et al., 1999; Lutz et al., 2011) and reductions in $CD56^{bright}$ NK cells from elderly subjects (Lutz et al., 2011). Similarly, a progressive increase in the ratio of $CD56^{dim}$: $CD56^{bright}$ cells was observed in NK cells with increasing subject age (20–83 years) (Hayhoe et al., 2010). A gender differentiation was also observed, such that elderly women (≥70 years) had significantly higher $CD56^{bright}$: $CD56^{dim}$ NK cells than elderly men (Al-Attar et al., 2016). Additionally, CD57 expression is a suggested marker of replicative senescence (Lutz et al., 2011), expression of which increased with age (Sansoni et al., 1993).

The cytotoxic ability of NK cells was unaffected between children (≤18 years), adults (19–59 years) and elderly (≥60 years) subjects (Almeida-Oliveira et al., 2011) including centenarians (Sansoni et al., 1993). While, significantly greater expression of killer cell Ig-like receptors (KIRs) was observed on $CD56^{bright}$ NK cells from the elderly, compared to young adults (Almeida-Oliveira et al., 2011). Since KIR expression can be induced by cytokine activation (Romagnani et al., 2007), this suggests a potential effect of inflammaging. While no differences in intracellular levels of perforin and granzyme were observed between resting NK cells from elderly (61–91 years) and young (20–30 years) subjects (Hazeldine et al., 2012). Significantly reduced perforin secretion into the immunological synapse and reduced binding of secreted perforin to the plasma membrane of the target cell, by NK cells from the elderly cohort was observed; which correlated with reduced target cell death in cytotoxicity assays (Hazeldine et al., 2012). Implying that while NK cells continue to produce sufficient quantities of cytotoxic proteins with increased age, their ability to secrete them and the subsequent binding of these proteins appears to be impaired with age. Additionally, proliferation rates of NK cells from elderly subjects were significantly reduced, from 4.3% to 2.5% per day compared to cells from young subjects (Zhang et al., 2007); suggesting that ageing may impair NK cell production from the BM.

Spontaneous production of IL-8 by unstimulated NK cells increased significantly with increasing age of the donor, but IL-2 induced secretion of IL-8 by NK cells was significantly higher in NK cells from young (21–36 years) compared to old donors (91–107 years) (Mariani et al., 2001). Similarly, while purified NK cells from nonagenarians retained the ability to secrete MIP-1α, RANTES and IL-8, in response to IL-12 and IL-2 stimulation, the levels were significantly lower in NK cells from young subjects (mean age 30 years) (Mariani et al., 2002a). IL-15-stimulated PBMCs *in vitro* induced significantly greater secretion of IFN-γ in subjects >60 years old, compared to younger subjects (Hayhoe et al., 2010). While, stimulation of NK cells with cytokines, leukaemia cells or IL-12 and anti-NKp46 mAb, resulted in the significantly elevated secretion of MIP-1β by $CD56^{dim}$ NK cells from elderly females (≥70 years), in addition to spontaneous secretion, compared to cells from elderly males (Al-Attar et al., 2016).

Dendritic cells (DCs)

DCs can be divided into plasmacytoid and myeloid DC subsets (pDC and mDC, respectively), pDCs are a small subset of DCs that express low levels of MHC II and costimulatory molecules, and only express TLRs 7 and 9 but secrete large quantities of type I IFN upon recognition of foreign nucleic acids (Merad et al., 2013). While

mDCs, also referred to as conventional DCs (cDCs), sense tissue injuries and phagocytose and capture Ags for presentation to T cells, and are able to migrate to lymphoid organs to prime naïve T cell responses (Merad et al., 2013). With age, a number of studies have found reductions in the number of plasmacytoid DCs (pDCs), while the numbers of myeloid DCs (mDCs) remains comparable between young and elderly individuals (Jing et al., 2009; Pérez-Cabezas et al., 2007; Shodell and Siegal, 2002). This finding is inconsistent since there have been observations of no changes in numbers of mDCs and pDCs with age (Agrawal et al., 2007) and also a reduction in just mDCs (Della Bella et al., 2007). Upon comparison of frail (had chronic illness and lived in an assisted living facility) and healthy elderly subjects it was observed that the frail elderly also had a reduction in peripheral blood mDC numbers (Jing et al., 2009). The definition of age, however, is rather inconsistent with 50–65 years regarded as middle aged by some (Mysliwska, 1999; Shodell and Siegal, 2002) but was the oldest age group studied by Pérez-Cabezas et al. (2007). Additionally, methodological approaches varied for enumerating DCs such as using either PBMCs or whole blood samples, the antibodies used to distinguish DC subsets, and the use of either count beads of haemocytometer based counting to determine absolute leukocyte counts. This has resulted in an inconclusive overall representation of the effect of age on DC subset proportions.

Induction of T cell responses by DCs involves a number of steps including upregulation of micropinocytosis and phagocytosis, upregulation of MHC II synthesis, subsequent binding of Ag with MHC II, expression of co-stimulatory markers on the DC surface and secretion of cytokines (Vega-Ramos et al., 2014; You et al., 2013b). With age expression of markers of maturation including CD40, CD86, MHC Class II (Wong et al., 2010), CD86, CD80 and CD54 (Agrawal et al., 2007; Lung et al., 2000) were not changed in response to bacterial stimulation with LPS or influenza virus (Saurwein-Teissl, 1998). Other groups however, observed a reduction in HLA-DR expression (a marker of MHC II) in DCs from elderly compared to young subjects (Pietschmann, 2000). Mature monocyte-derived dendritic cells (MoDCs) from elderly subjects displayed reduced expression of the activation marker CD25, as well as intercellular adhesion marker-1 (ICAM-1) after LPS stimulation (Ciaramella et al., 2011). However, impaired ability to secrete both type I and type III IFN was observed in pDCs from aged subjects (Jing et al., 2009) upon stimulation with CpG oligodeoxynucleotides (CpG ODN) and influenza virus, compared to pDCs from young subjects, in addition to significantly reduced CD4 and CD8 T cell proliferation (Sridharan et al., 2011). However, TNF-α and IFN-γ secretion by HLA-DR^{+} low density

cells from elderly subjects (determined to be DCs) were unresponsive to LPS stimulation compared to cells form young subjects, while unstimulated samples showed significantly elevated IFN-γ compared to cells from young subjects (You et al., 2013b). Investigation of TLR function in mDCs, using multiple TLR ligands, showed that with age production of TNF-α, IL-6 and IL-12/23 (IL-12 p40 subunit) was reduced, while in pDCs TNF-α and IFN-γ production was reduced; which were stable over time (Panda et al., 2010). These results were consistent whether the whole PBMC fraction was stimulated or DCs were enriched and then stimulated (Panda et al., 2010). With age DCs appear to be less functional since there are observed reductions in secretion of cytokines and chemokines, which are required to aid T cell priming and are part of DC maturation (Vega-Ramos et al., 2014) and the subsequent initiation of T cell proliferation and cytotoxicity (Bonilla and Oettgen, 2010). The reduced response of DCs, from elderly donors, to TLR ligands (Panda et al., 2010), suggests that TLRs may be impaired with increased age, reducing DC cytokine secretion. However, increased expression of IL-6 and TNF-α in supernatants after LPS or single-stranded RNA (ssRNA) stimulation of MoDCs from elderly subjects, along with impaired phagocytic capacity were observed with age (Agrawal et al., 2007), which the authors attribute to their observed significant impairment in PI3-K/AkT pathway in MoDCs derived from elderly subjects. However, these findings were not consistent across all time points and there were no differences in basal levels (Agrawal et al., 2007).

A key finding that DC functionality is impaired with age was observed after the adoptive transfer of MHC-II labelled $CD4^+$ T cells, isolated from young (2–4 months) or aged mice (20–24 months), into recipient young or aged mice, since aged recipient mice (source of DCs) had significantly reduced $CD4^+$ proliferation after specific-Ag exposure, regardless of the age of the donor mouse (source of T cells) (Pereira et al., 2011). While the transfer of old CD4+ T cells into young mice still reduced proliferation, the effect was 3-fold greater than that for old mice, suggesting that the recipient age (age of donor DCs) is influential in the ability of DCs to present Ag to T cells (Pereira et al., 2011). Similarly in human derived cells, co-culture of a $CD8^+$ T cell line (from a young donor) with HLA-typed influenza-infected MoDCs from elderly subjects (≥65 years) significantly reduced T cell proliferation, IFN-γ production and release of granzyme granules compared to culture with DCs from young subjects (20–40 years) (Liu et al., 2012). These findings were in addition to reduced production of TNF-α by DCs from elderly subjects, a factor which could have impaired the induction of an effective T cell response (Liu et al., 2012). Additionally, *in vitro* mixed

leucocyte reactions (MLR) showed co-culture of DC-enriched low density cells (non-adherent cells, after overnight culture of PBMCs) from elderly subjects (65–75 years) with T cells from young subjects (20–30 years) resulted in reduced T proliferation, similarly co-culture of DCs from young subjects with T cells from elderly subjects, significantly reduced proliferation (You et al., 2013b), highlighting that both cell types are affected by age of the host, and influence T cell proliferation. Recently, MoDCs, derived from monocytes of healthy subjects (29–78 years), infected *in vitro* with human cytomegalovirus (CMV) and co-cultured with autologous $CD4^+$ T cells resulted in production of IFN-γ, expression of CD107a and ability of T cells to prevent viral spread; these effector functions were consistent across all ages (Jackson et al., 2017).

1.1.4 Ageing of primary and secondary lymphoid *organs*

The BM and thymus are key primary lymphoid organs, the BM is responsible for the generation of HSCs and CLPs, and is the primary site of B cell development, while the thymus is the primary site of T cell development (Chinn et al., 2012). However, both organs are highly susceptible to ageing which can influence lymphocyte production in aged subjects (Chinn et al., 2012). Failure of HSCs, within BM, to maintain lymphopoiesis has been observed with ageing (Chinn et al., 2012). High ROS activity within BM with age significantly exhausted HSCs, though HSC function could be restored upon antioxidant treatment (Jang and Sharkis, 2007). Additionally, mesenchymal stem cells (MSCs) differentiate into either osteoblasts or adipocytes within the BM, but upregulation of peroxisome proliferator-activated receptor-γ2 (PPAR-γ2) demonstrated increased adipogenesis-associated gene expression (Shockley et al., 2009). Increased adipogenesis in the BM has been observed with increased age, at the expense of osteoblast production (Tuljapurkar et al., 2011). The thymus begins to decline in size and output during young adulthood, this continues throughout adult life and is termed thymic involution; a key determinant of age-related loss of T cell production (Chinn et al., 2012). This is seen in humans as a reduction in naïve $CD8^+$ T cells and accumulation of oligoclonal memory $CD8^+$ T cells (Czesnikiewicz-Guzik et al., 2008; Khan et al., 2002). Thymic involution has been implicated to shift from stimulatory to suppressive cytokines within the tissue and steady state mRNA expression of stem cell factor (SCF), IL-6 and M-CSF were significantly elevated in thymus tissue from aged humans (obtained after thymectomy for myasthenia gravis or during the course of corrective heart surgery) while IL-2, IL-9, IL-10, IL-13 and IL-14 were not expressed in thymus samples from aged subjects, but were expressed in thymus samples from young subjects (Sempowski et al., 2000).

Additionally, while the absolute size of the thymus remains constant, an expansion of the perivascular space within the thymus was observed with age, such that thymic epithelial space (medulla and cortex) shrunk to less than 10% by 70 years of age (Gruver et al., 2007; Steinmann et al., 1985). Both of which have been associated with increased adiposity within the thymus (Chinn et al., 2012), with the possibility that reducing the accumulation of adipocytes could revert thymic involution (Yang et al., 2009).

Secondary lymphoid organs such as the lymph nodes (LNs) and spleen provide specific areas termed T and B cell zones which are essential for lymphocyte survival and interaction with APCs (Becklund et al., 2016). LN tissue from human donors undergoing pelvic or cervical vascular reconstruction or lymphadenectomy showed a significant age-related loss of $CD8^+$ and $CD45RA^+$ T cells when comparing tissue samples from elderly (67–88 years) and young (1–20 years) subjects (Lazuardi et al., 2005). Additionally, clonally expanded T cells in LNs from young subjects were accompanied by elevated B220+ cells, which was not observed in samples from elderly subjects; implying an impairment in the T:B cell interaction with increased age (Lazuardi et al., 2005). Limited access for naïve $CD4^+$ T cells to secondary lymphoid organs was observed in aged mice with reduced homing of naïve T cells to peripheral LNs and impaired segregation of lymphocytes into T cell and B cell zones (Becklund et al., 2016). Similarly, LNs in aged mice (18–21 months) displayed less defined structural localisation of B cells within follicular regions than that in young mice (7–12 weeks) accompanied by a relative decrease in the T cell population but a relative increase in the B cell population (Turner and Mabbott, 2017). Additionally, reduction of CD4 and CD8 T cells were observed in lymphoid organs in aged mice (22–26 months) compared to young mice (2–6 months) (Martinet et al., 2014). While, there have been no age-associated observations of impaired or enhanced migration of the innate immune cells into or out of the secondary lymphoid organs (Nikolich-Žugich and Davies, 2016).

1.1.5 Ageing of adaptive immune cells

During the 2014/2015 influenza season, predominantly older adults were infected, despite an influenza vaccination uptake of 72.7% by those aged over 65 years (Public Health England, 2015a), suggesting that T and B cell proliferation and effector functions could be effected by increasing age.

Humoral

With increased age B cell production, antibody generation and diversification mechanisms, and effector functions are all potential targets for deterioration (Frasca et al., 2011). As a vast diversity of the B cell repertoire is crucial for protection against infection, this is an area of interest for investigation.

Effect of ageing on B cell production and cellular repertoire

Alterations in B cell subset composition in the BM and the periphery may be due to increased B cell longevity and reduced production of naïve B cells in the BM (Tabibian-Keissar et al., 2016). This is evident in mouse models where B cell production is reduced in aged mice, with changes to the proportions of naïve to Ag-activated B cells, such that activated (e.g. marginal zone B cells) are dominant within the B cell pool (Johnson et al., 2002) in addition to a reduced rate of entry of B cells into the mature B cell population (Kline et al., 1999). While, in humans investigation of circulating B cells within peripheral blood samples from young (17–29 years), elderly (70–83 years) and centenarian (100–106 years) subjects, showed significantly reduced numbers of $CD19^+$ cells in the old compared to young subjects (Paganelli et al., 1992). More recently, while trends for reductions in total and naïve peripheral B cell populations and increased memory B cell populations were observed with increased age of the donor, the average rates of change with age were not statistically significant (Lin et al., 2016). Proliferation and disappearance rates of total peripheral blood B cells, from blood drawn one hour post deuterated glucose consumption, were not significantly different between young (<35 years) and elderly (>65 years) subjects (Macallan et al., 2005). However, proliferation of memory B cells ($CD27^+$) was significantly higher (almost five times) than naïve B cells ($CD27^-$) derived from the elderly, in addition to greater $CD27^+$ proportions in peripheral blood from elderly than young subjects (Macallan et al., 2005). Complemented by the finding that peripheral B cell populations from elderly subjects (75–102 years) had fewer IgD^+CD27^- (naïve B cells) but significantly greater numbers of IgD^-CD27^- cells (IgG^+ memory cells), compared to young subjects (20–55 years) (Colonna-Romano et al., 2009). The IgD^-CD27^- subset of B cells lacked expression of ATP-binding-cassette-B1 transporter (ABCB1), which is observed in classical ($CD27^+$) memory B cells (Colonna-Romano et al., 2009). While, increases in monoclonal B cells in peripheral blood of elderly subjects (65–98 years) were seen, with 19 out of 500 subjects demonstrating increased presence of clones of $CD5^-$ and $CD5^+$ B cells by flow cytometric analysis, which were confirmed by PCR analysis of IgH rearrangements (Ghia et al., 2004). Similarly, B cell monoclonality was observed in 5% of elderly subjects (>60 years)

compared to 2.1% in adults (40–60 years) (Rawstron et al., 2002). The presence of $CD5^-$ B cells is implicated with B cell monoclonality and may be a marker of lymphoid senescence (Ghia et al., 2004).

Plasma cells are not frequently investigated, due to their low circulatory levels, but in addition to memory B cells were reduced with age, but the decrease in plasma cells was more pronounced (Caraux et al., 2010). Replacement of haematopoietic BM with adipocytes (adipogenesis) has been observed (Rosen et al., 2009; Tuljapurkar et al., 2011) with increases from 40% to 68% observed between young and elderly subjects, respectively (Justesen et al., 2001). Additionally, age-associated functional impairments were observed in follicular DC and T cell compartments (Della Bella et al., 2007; Jing et al., 2009; Rosenberg et al., 2013; Silveira-Nunes et al., 2017; Sridharan et al., 2011). Adipogenesis is a normal physiological process, with MSCs acting as precursors to adipocytes, however, this process increases with age, in addition to diabetes mellitus and osteoporosis (Rosen et al., 2009), and the reduction in BM reduces the migratory niches of plasma cell, which support their survival (Caraux et al., 2010).

Serum levels of IgG and IgA increased with advanced age, while IgM was unaffected (Paganelli et al., 1992). Similarly, analysis of serum Ig concentrations in subjects from 20 to 106 years revealed significant positive correlations between age and IgG and IgA concentrations, while IgM and IgD were significantly, negatively correlated with age; the subclasses IgG1 and IgG3 were significantly positively correlated with age (Listì et al., 2006).

Effect of ageing on the functional response of B cells

The response of elderly subjects to vaccinations is often suboptimal (Public Health England, 2015b). While similar concentrations of pneumonia polysaccharide (PPS)-specific IgG and ability to opsonise *S. pneumoniae* were observed in young (23–32 years) and elderly (65–84 years) subjects post-23-valent pneumococcal vaccination, a significant proportion of elderly subjects (20.4%) mounted a 2-fold increase in specific-IgG in < 2 of the 7 serotypes; not observed in the young age group (Rubins et al., 1998). Additionallly, significant age-associated decreases in influenza-specific serum hemagglutination inhibition (HI) were observed after vaccination in young and elderly subjects during the 2008–2009 and 2009–2020 influenza seasons (Frasca et al., 2010), showing that fewer specific antibodies were produced against the vaccine in the elderly subjects since inhibition of this assay requires influenza-specific antibodies. Furthermore, serum IgM response to pneumococcal vaccination was

significantly reduced in elderly subjects (65–89 years) compared to young (18–49 years) 7 and 28 days post immunisation, in addition to a slower increase in serum IgA, peaking at 28 days, compared to 7 days in young subjects (Ademokun et al., 2011). Intrinsic alterations to the B cell, which impact on vaccination responses, include the significant down-regulation of AID mRNA expression in peripheral B cells with increased age (Frasca et al., 2008) which was observed 7 and 28 days after influenza vaccination (Frasca et al., 2010). Since AID is required for effective induction of SHM and CSR these findings imply that the genetic arrangement of the Ig may also be effected. As the information to date from ageing subjects does not solely represent differences in total number of B cells or B cell subsets, it is clear that the impact of age on the B cell repertoire is complex and not simply a shift in the proportions of naïve to memory cells, and that there may be other contributory factors impairing B cell functionality with increasing age.

Cell mediated

The proliferation of numerous cytotoxic effector T cells and the generation of central and effector memory T cells are crucial for an effective cell mediated adaptive immune response to destroy pathogens and respond quickly upon re-encounter (Janeway Jr et al., 2012). Thus, the potential impact of the ageing process on these functions is of interest and importance.

Effect of ageing on T cell production and cellular repertoire

The first results of the Swedish longitudinal OCTO immune study in the very old (86–92 years), showed an association between mortality and high percentages of $CD8^+$ cells, and low percentages of $CD4^+$ and $CD19^+$ cells, in addition to impaired T cell proliferation in response to Concanavalin A (ConA) (Ferguson et al., 1995). Subsequent time points of this study further substantiate these findings, with the additional association with CMV seropositivity (Olsson et al., 2001; Wikby et al., 1998). An extension to the study, with a new sample (Swedish NONA sample) of very old subjects (mean age 90.3 years), also confirmed these results and demonstrated that the predominant $CD8^+$ T cell phenotypes were $CD27^-$, $CD28^-$, $CD56^+$, $CD57^+$ and $CD45RA^+$ (Wikby et al., 2002); typical of cytotoxic T cells. These findings have collectively been termed the immune risk profile (IRP) and has also been investigated in a younger cohort of elderly subjects (66 years), the HEXA cohort, with 14.6% of subjects displaying a CD4/CD8 ratio less than one, in addition to significant reductions in $CD19^+$ B cells, and significantly greater prevalence of CMV seropositivity (Strindhall et al., 2013). Additionally, the proportion of terminally differentiated effector memory cells ($CD45RA^+CCR7^-perforin^+$) was significantly higher in this subgroup (Strindhall

et al., 2013). These findings have been replicated by numerous research groups and the reduction in numbers of naïve T cells in favour of Ag-experienced T cells is mutually agreed (Kang et al., 2004; Kovaiou and Grubeck-Loebenstein, 2006; Ouyang et al., 2003; Saavedra et al., 2017; Saule et al., 2006). Thymic involution partially explains this, since fewer new T cells are produced, however the combined observations of increased CMV seropositively suggests that CMV may induce the generation of terminally differentiated effector memory $CD8^+$ T cells. The role of prior CMV infection has been investigated in young (<60 years) compared to elderly subjects (>60 years), with reduced $CD28^-$ CD4 T cells and increased CD56 and HLA-DR expression not influenced by subject age but by CMV seropositivity (Looney et al., 1999). However, the classification of the two age groups in this study was inappropriate, as thymic atrophy can occur from 50 years (Goronzy and Weyand, 2005), so a more appropriate differentiation of the age groups would have defined younger subjects as < 50 years. While, CMV seropositivity has also been associated with greater oligoclonality of the CD8 T cell repertoire in healthy elderly subjects (60–95 years), compared to young controls (20–55 years); after assessing epitope-specific CD8 T cell frequencies using the two HLA-peptide tetramers (A2-NLV and B7-TPR) and HLA-typing blood samples for MHC I alleles (Khan et al., 2002).

Effect of ageing on the functional response of T cells

Significantly reduced proportions of memory CD4 and CD8 T cells were observed in elderly (65–85 years) compared to young (20–30 years) subjects, in response to whole virus (respiratory syncytial virus; RSV) and an RSV Ag (Cherukuri et al., 2013). In addition to reduced numbers of $CD107a^+$ $CD8^+$ T cells, a degranulating, cytotoxic T cell subset, in PBMC samples from the elderly (Cherukuri et al., 2013). This suggests that the impaired immune response, seen by increased infection with RSV, could be as a result of fewer T cells, as opposed to impaired functional T cell responses. Additionally, human PBMCs, derived from HLA-typed ($HLA\text{-}A2^+$) elderly subjects (>70 years) after vaccination against tick-borne encephalitis virus (TBEv), demonstrated significantly reduced expansion of naïve $CD8^+$ T cells specific for the model Ag upon *in vitro* stimulation with HLA-A2-specific peptide (ELA; model Ag) and pro-inflammatory cytokines; compared to younger subjects (20–50 years) (Briceño et al., 2016). This finding was a suggested consequence of reduced size of the naïve T cell pool and was associated with efficacy of vaccine response; subjects with more $CD8^+$ ELA^+ cells had higher binding and neutralising antibody titres for TBE and greater cellular response (IFN-γ secretion) (Briceño et al., 2016).

Significant increases in secretion of IL-8 by T cells was observed in response to anti-CD3 mAb activation in elderly (91–107 years) subjects, compared to young (21–30 years) (Mariani et al., 2001). Additionally, production of RANTES (CCR5) and MIP-1α by T cells from elderly subjects was significantly increased compared to cells from young subjects in response to anti-CD3 mAb stimulation (Mariani et al., 2002b). CD8 T cells, from elderly subjects, stimulated with PMA-ionomycin produced increased IL-2, IL-4 and IFN-γ in the absence of CMV infection, while CMV seropositivity inhibited the increases in IL-2 and IL-4, but IFN-γ production was further increased in middle-aged (35–60 years) and elderly subjects (>65 years) seropositive for CMV (Almanzar et al., 2005); associated with a reduction in $CD25^{+}CD8^{+}$ T cells. TNF-α producing $CD4^{+}$ T cells increased with age, while TNF-α and IL-4 producing $CD8^{+}$ T cells and IL-10 production by all lymphocytes decreased with increasing age (Silveira-Nunes et al., 2017). CMV-specific CD8 T cells demonstrated increased secretion of IFN-γ and significantly elevated levels of lysis in response to incubation with Ag-specific peptides (Khan et al., 2002).

PBMCs, isolated from older subjects (≥60 years), post influenza vaccination, challenged *in vitro* with A/H3N2-influenza produced significantly more granzyme B in response to influenza virus compared to pre-vaccination (Shahid et al., 2010). However, low granzyme B levels prior to infection strongly correlated with individuals developing a fever and a lack of seroconversion to the infection (T-dependent antibody response) (Shahid et al., 2010), linking poor cell mediated cytolytic effects to severity of influenza illness. Additionally, older subjects (≥55 years) demonstrated reduced Ag-specific T cell proliferation in response to hepatitis B (HB) vaccination, assessed by thymidine incorporation, and a lack of IFN-γ secretion upon HB Ag stimulation, compared to young (≤35 years) subjects (Rosenberg et al., 2013). Expression of the lymph node homing marker, CD62L, significantly increased with greater response to the vaccination in naïve and central memory T cells derived from young subjects, while expression was consistent in cells from the elderly (Rosenberg et al., 2013); suggesting that age may influence availability of this ligand and thus homing of naïve or central memory T cells to secondary lymphoid organs, impairing recall responses to HB Ag. In addition, the reactivation of Varicella-Zoster virus (VZV) to induce Herpes zoster (shingles) infection was associated with lower counts of VZV-specific IFN-γ with increasing age; which was significant between the 60–69 and 70–79 year groups (Shirane et al., 2017).

A diverse TCR repertoire is required to enable the immune system to respond to new infections, however the diversity of TCR repertoire has been observed to decline with

increased age (Britanova et al., 2014; Goronzy and Weyand, 2005). Comparison of $CD8^+$ T cells isolated from young and aged mice showed similar effects of reduced reactivity to viral epitopes to young thymectomised mice, implicating thymic atrophy in this process (Yager et al., 2008); as the TCR develops during thymocyte development in the thymus.

1.1.6 Leukocyte trafficking between the gut and the bloodstream

The luminal content of the GI tract is separated from the bloodstream via the intestinal barrier including physical (epithelial cells), chemical (antimicrobial peptides; AMPs) and immunological (secretory IgA) components (Turner, 2009; Yu et al., 2012). Development and maintenance of the intestinal barrier is influenced by interaction between the gut microbiota and the immune system (Caricilli et al., 2014). The intestinal barrier comprises the mucus layer, intestinal epithelial cells (IECs) and intercellular tight junction proteins which physically separate the microbiota and luminal content from the lamina propria (Yu et al., 2012). Encounter of bacterial products (e.g. LPS or flagellin) can induce epithelial cell neutrophils to secrete AMPs, which are a broad-spectrum class of peptides effective against Gram-negative and Gram-positive bacteria, fungi, yeasts and viruses (Yu et al., 2012). The exposure of the GI tract to dietary Ags on a daily basis requires the immune system to distinguish between these beneficial Ags and potentially harmful, pathogenic organisms; oral tolerance (Forchielli and Allan Walker, 2005). Microfold (M) cells and intestinal DCs, residing within the lamina propria, constantly sample luminal contents to detect and capture harmful pathogens (Cahenzli et al., 2013; Chieppa et al., 2006). Frequency of extension of dendrites into the intestinal lumen depends on bacterial content within the lumen and TLR recognition of PAMPs on IECs and DCs (Chieppa et al., 2006). After pathogen destruction, DCs can present pathogenic peptides to B or T cells to induce an adaptive immune response; including sIgA production by plasma cells translocated to the lamina propria and cytokine secretion by differentiated T helper cells (Yu et al., 2012).

Induction of oral tolerance is vital in generating systemic non-response to ingested Ags, and involves both Ag-nonspecific and Ag-specific modes of response. The Ag-nonspecific mode involves the generation of immunosuppressive conditions within the local (gut) environment, and processing of dietary Ags by the gut associated lymphoid tissue (GALT) system, making the host immunologically unresponsive to them (Tsuji and Kosaka, 2008). While the Ag-specific mode requires the homing marker, chemokine receptor-7 (CCR7), to enable tolerogenic DCs to migrate to the

mesenteric LNs (Worbs et al., 2006) where they interact with and contribute to the functional maturation of Ag-specific regulatory T (T_{reg}) cells (Tsuji and Kosaka, 2008).

Naïve T cells are confined to lymphoid organs, however, once activated by DCs, they are able to enter peripheral tissues due to altered expression of homing markers (Hart et al., 2010). DCs migrate to lymph nodes via lymphatic vessels after encounter with foreign Ags, to maximise chances of presenting Ag to naïve T cells (Randolph et al., 2005). Different origins of DC may determine their functional roles within the intestinal immune system, since DCs which migrate from the gut to mesenteric lymph nodes undertake typical DC functions by initiating adaptive immune responses, while blood DCs which have been habituated to home to the gut, maintain tolerance to dietary and self-Ags (Hart et al., 2010) or act as a first line of defence against invading pathogens (Schulz et al., 2009). Retinoic acid (RA), a metabolite of vitamin A, was shown to be important in the homing of both DCs and IgA-secreting B cells to the gut (Bernardo et al., 2013; Mora and von Andrian, 2009). Dietary components metabolised by members of the gut microbiota have the potential to impact on immune cell responses, which will be discussed further in the sections below.

1.1.7 Microbiota and its effect on the immune system

Commensal microorganisms residing within the GI tract make up the microbiota which differs vastly between anatomical regions, however much less is known about the composition of the small intestinal microbiota composition due to the difficulty in sampling this area (Flint et al., 2012). The colon is the most populated area of the GI tract with in excess of 1×10^{14} bacteria, archaea, viruses and eukaryotic microbes (Wang et al., 2017), with recent observations of 97.6% from bacteria, 2.2% from archaea, 0.2% from viruses and <0.01% from eukaryokes (Zhernakova et al., 2016). At least 1000 different species of bacteria are known, which are thought to be dominated by the phyla Firmicutes and Bacteroidetes (Johnson et al., 2016). Other phyla, up to ten different members, including Actinobacteria, Proteobacteria, Verrucomicrobia and Fusobacteria are also present and thought to have important functional effects (Harakeh et al., 2016; Marchesi et al., 2016; Plé et al., 2015; Wang et al., 2017). While, Actinobacteria abundance was greater than Bacteroidetes in a cohort of healthy Dutch participants (Zhernakova et al., 2016). Very few studies to date have investigated the viral component (virome) or the contribution of eukaryotes (protozoa and fungi) to the microbiota composition (Marchesi et al., 2016), though nucleic acids of viruses from 1200 different virotypes have been reported in faecal samples (Breitbart et al., 2003; Columpsi et al., 2016; Reyes et al., 2010). While it is known that the gut microbiota is vital for maintenance of host health and functioning,

the keystone species of the microbiota, those that have a disproportionately large influence on the community composition and function relative to abundance, are yet to be confirmed (Marchesi et al., 2016) but *Helicobacter pylori*, *Akkermansia municula* and *Lactobacillus johnsonii* are potential keystone species (Karkman et al., 2017). Inter-individual variability in microbiota composition is high, but it is thought that there remains a highly conserved common bacterial core of approximately 57 species which are common to >90% of individuals; of which Bacteroidetes and Firmicutes have the highest abundance (Qin et al., 2010). Recently, (Jeffery et al., 2016) determined *Bacteroides, Alistipes, Parabacteroides, Faecalibacterium* and *Ruminoccocus* to be the core microbiota. This conserved core encodes gene products unique to bacterial genomes which exhibit specific functions within the gut such as transport and degradation of complex plant-derived carbohydrates to produce short chain FAs (SCFAs), amino acids and vitamins (Kolmeder et al., 2012; Qin et al., 2010). With *Ruminococcus bromii* shown to efficiently ferment resistant starch (RS) which the authors suggest as a keystone species required to yield products from RS for use by other bacteria (Ze et al., 2012).

The human intestinal microbiota forms soon after birth (Palmer et al., 2007), with delivery mode impacting on the neonate's dominant bacterial communities (Dominguez-Bello et al., 2010). An absence of organisms in the Bacteroidetes phylum (Azad et al., 2013; Jakobsson et al., 2014) and lower abundance of *Escherichia-Shigella* communities have been observed in infants born by caesarean delivery (Azad et al., 2013). However, more recently it has been suggested that initial colonisation may occur *in utero* via microbial transfer from the placenta and amniotic fluid (Collado et al., 2016) though this research is in its infancy, factors such as maternal diet, antibiotic use and maternal stress may all impact on amniotic or placental microbiome compositions and thus initial colonisation of the infant (Greenhalgh et al., 2016). The intestinal microbiota establishes in parallel with the immune system, typically both the microbiota and the adaptive immune response become fully established within the first decade of life (Adlerberth and Wold, 2009; Palmer et al., 2007). The importance of the intestinal microbiota in health has been demonstrated using sterile, germ-free animal models which have shown nutritional deficiencies, increased permeability of the intestinal barrier and functionally immature immune systems (Al-Asmakh and Zadjali, 2015; Crabbe et al., 1970; Hapfelmeier et al., 2010; Tlaskalova-Hogenova et al., 2011; Yamamoto et al., 2012). Nutrition in early infant life is an important factor in developing the microbiota and immune system, with observations that breast milk consumption aids the establishment of *Bifidobacterium*

species which aid modulation of the immune system (Liu et al., 2016); lysozyme in breast milk is suggested to inhibit the growth of human-non-resident *Bifidobacterium* species (Minami et al., 2016). While, the divergent dietary patterns between children from rural Africa (Burkino Faso) and Europe revealed strikingly different faecal microbiota profiles, associated with the differing daily fibre intakes of 14.2 g and 8.4 g, by Burkino Faso and European children, respectively (De Filippo et al., 2010). The Burkino Faso children were colonised with species including *Xylanibacter, Prevotella, Butyrivibrio* and *Treponema* which can utilise cellulose and xylose present in plant fibres for energy provision via SCFA generation (De Filippo et al., 2010). Similarly, the phylogenetic diversity of the microbiota of Hazda hunter-gatherers (8–70 years) from Tanzania was notably distinct from Italians (20–40 years) with *Prevotella, Eubacteria, Butyricoccus, Sporobacter, Succinivibrio* and *Treponema* most represented at the genus level, while *Bifidobacterium, Bacteroides, Ruminoccocus* and *Faecalibacterium* were depleted in the Hazda microbiota (Schnorr et al., 2014). Actinobacteria were abundant in the Italian gut microbiota but almost completely absent from the Hazda microbiota (Schnorr et al., 2014). These examples demonstrate the dramatic differences in microbial ecology as a result of differing environments and dietary consumption between Western and indigenous populations (Karkman et al., 2017). Recently, it has been shown that inclusion of microbiota accessible carbohydrates within dietary fibre can recover diversity but that low fibre diets over many generations may irreversibly remove taxa (predominantly Bacteroidales) from the microbiota in humanised mice after switching from a high to low fibre diet before returning to a high fibre diet (Sonnenburg et al., 2016).

Striking changes to the microbiota composition occur in the elderly (O'Toole and Brigidi, 2013), with observations of reduced Bacteroides, Clostridia and Lactobacillus and increases in Fusobacteria, Streptococci and Stapylococci genus, while the total number of bacteria remained constant (Woodmansey et al., 2004). The ELDERMET study showed that faecal microbiota profiles in elderly subjects (>65 years) were Bacteroidetes dominant, while younger adults (28–44 years) were Firmicutes dominant (Claesson et al., 2012). In addition, assignment of the core microbiota to phylum, genus and *Clostridium* cluster highlighted substantial differences between the two age groups, with *Clostridium* cluster IV predominant in the elderly, while cluster XIVa predominated in young samples (Claesson et al., 2012). More recent data from the ELDERMET study shows that microbiota diversity of long stay residential care subjects clearly differentiated from elderly subjects residing within the community, with the major genera for the residential care group corresponding to

anaerotruncus, Desulfovibrio and *Coprobacillus* (Jeffery et al., 2016). Frailty was also significantly negatively associated with abundance of *Faecalibacterium prausnitzii* and positively associated with *Coprobacillus* and *Eggerthella* in the Twins UK cohort (42–86 years), a number of which correlated with the ELDERMET subjects (64–102 years) (Jackson et al., 2016). Batch culture and 16S amplicon sequencing of faecal samples from elderly subjects residing within the community (69–81 years) or in residential care units (82–93 years), defined as healthy and frail respectively, showed strikingly different microbiota compositions at baseline with predominance of Firmicutes (68%) and Bacteroidetes (22.5%) in healthy elderly, but Euryarcheaota in the frail elderly with only 22% and 11% abundance of Firmicutes and Bacteroidetes (Ntemiri et al., 2017). The frail elderly also demonstrated reductions in the Bacteroidaceae, Bifidobacteriaceae and an increases in the Methanobacteriaceae and Enterobacteriaceae families compared to healthy elderly subjects (Ntemiri et al., 2017).

The microbiota composition changes with increased age and further with frailty, with changes (dysbiosis) associated with pathologies including reduced diversity correlating with disease duration and autoantibody levels in RA (Chen et al., 2016) and altered harvest and storage of energy from food and lower *Akkermansia muciniphila* abundance in obesity (Bäckhed et al., 2004; Dao et al., 2016; Turnbaugh et al., 2006). Recently, faecal sample analysis using paired-end metagenomics shotgun sequencing predicted that use of antibiotics was associated with significant decreases in *Bifidobacterium* species from the Actinobacteria phylum, in addition to features of a Western-style diet associated with lower species diversity (Zhernakova et al., 2016). Since the microbiota is known to be influenced by dietary intake and has subsequent effects on the immune system, dietary intervention is a key target for influencing immune function.

1.1.8 Diet

Diets are defined as the foods chosen and consumed by individuals in order to meet the body's energy requirements for growth, movement, maintenance of body temperature and survival (Pocock et al., 2013). This energy is provided by the consumption of the macronutrients, carbohydrates, proteins and fats, present in the foods we eat (Pocock et al., 2013). Different foods have different energy and nutrient densities, with nutrient density of foods an important classification of foods, particularly in relation to promoting fruit and vegetable intakes (Di Noia, 2014; Drewnowski, 2005). Energy requirements are dependent on basal metabolic rate (BMR) and physical activity level (DoH, 1991) and since nutrients are continually

utilised during metabolism (molecular, cellular, tissue, organs or whole body) daily intakes are required to replace these losses (Lanham-New et al., 2011). Foods also contain micronutrients, vitamins and minerals which are essential in the diet, in addition to essential amino acids and essential FAs, which the body cannot produce (Pocock et al., 2013). The composition of diets varies depending on numerous factors including geographical location, food availability, socio-economic status, food beliefs and cultural beliefs (Geissler and Powers, 2010).

Carbohydrates

Carbohydrates are an important source of energy in the diet, providing 4 kcal/ g, and include simple sugars termed monosaccharides (glucose and fructose), oligosaccharides (lactose and sucrose) and the more complex polysaccharides (starch, glycogen and cellulose) (Geissler and Powers, 2010). Monosaccharides consist of a straight chain of between three and eight carbon atoms, all but one of which carries a hydroxyl group (CH_2OH), the remaining carbon forms a carbonyl group (C=O), to provide the reducing properties of monosaccharides (Coultate, 2009). Monosaccharides are sweet tasting components occurring in foods and are typically found in honey, fruits and vegetables; this sweetness is lost in longer chain carbohydrates, oligosaccharides and polysaccharides (Geissler and Powers, 2010). Prebiotic carbohydrates are defined as “non-digestible food ingredients which beneficially affect the host by selectively stimulating the growth or activity of one or a limited number of bacteria in the colon, and thus improve host health” (Gibson and Roberfroid, 1995). Examples of prebiotics include inulin-derived fructans (fructo-oligosaccharides (FOS), inulin and oligofructose), galacto-oligosaccharides (GOS) and lactulose (Rastall and Gibson, 2015). Polysaccharides contain large numbers of monosaccharides linked together by α- or β-linkages and chains can be branched or linear (Geissler and Powers, 2010). Polysaccharides are derived from plants and their purpose is to provide an energy reserve, within seeds and tubers, in addition to providing structure to the plant, while they are essential for the moderation of nutrient absorption in the small intestine and the healthy functioning of the large intestine in humans, in addition to providing an energy source (Coultate, 2009). Plant polysaccharides, such as non-starch polysaccharide (NSP), makeup insoluble dietary fibre which is non-digestible by the human GI tract, however upon reaching the large intestine can be fermented by the resident host microbiota into SCFAs (Marchesi et al., 2016). A mutualistic relationship is present between insoluble dietary fibre and prebiotics, and carbohydrate fermenting bacteria since the human host requires bacterial species including those from the Bacteroidetes and Actinobacteria phyla to

break down starch, inulin and oligosaccharides, and *Clostridium* Group XIVa of the Firmicutes phylum to produce SCFAs (Dethlefsen et al., 2006). However, reduction in species diversity within the microbiota has been observed in elderly subjects (≥ 65 years) with the lowest fibre intakes (Claesson et al., 2012), and diets rich in saturated fat and sugar were associated with low diversity profile (Jeffery et al., 2016), typical of elderly subjects in long-term residential care (Claesson et al., 2012). Additionally, downregulated expression of genes associated with methanogenesis and metabolism of glycans and lipids was observed in healthy adults (19–25 years) consuming low fibre diets (10 g/ per day) (Tap et al., 2015). Additionally, a small scale study compared gut microbiota composition between elderly women (68–76 years) residing inland compared to the island area of South Korea, who had greater intakes of dietary fibre (and lower intakes of animal lipids) than island dwellers and distinct bacterial communities including greater abundance of *Butyricimonas* which produce butyric acid (Shin et al., 2016).

Protein

Proteins within the diet provide energy (4 kcal/ g) but consumption is also required for the provision of amino acids, vital for the synthesis of numerous essential proteins within the body such as enzymes, Igs, membrane transport proteins, and structural collagens, in addition to carrying vitamins, oxygen and carbon dioxide around the body (Coultate, 2009; Geissler and Powers, 2010). Proteins are composed of amino acids linked together by peptide linkages, with a typical structure of H_2N-C(R)H-COOH, within which the R represents the side chain which varies depending on the protein structure (Geissler and Powers, 2010). The amino acids isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine are considered essential amino acids since the human body cannot synthesise the amine group (Geissler and Powers, 2010).

The small intestine is the main site of amino acid catabolism and 30–50% of these amino acids do not enter hepatic portal circulation (Lanham-New et al., 2011). The remaining amino acids enter the liver, which is the only organ that contains the enzymatic capacity to catabolise all of the amino acids (Lanham-New et al., 2011). Consumption of protein-rich meals, containing bovine serum albumin, showed that albumin was present in jejunal and ileal fluids four hours after consumption, with increased free and peptide amino acids present in both luminal content and plasma (Adibi and Mercer, 1973). Evidence suggests that dietary peptides, which survive luminal digestion and brush-border membrane hydrolysis, can be detected in the peripheral blood and urine (Picariello et al., 2013). Indigestible protein matter which

reaches the large intestine is fermented by the resident microbiota to produce phenols, ammonia, nitrates and branched chain FAs, in addition to SCFAs and gases, primarily in the distal colon (Blaut and Clavel, 2007). Sulphate reducing bacteria are responsible for the degradation of the amino acids cysteine and methionine into hydrogen sulphide and are predominantly from the *Desulfovibrio* genus (Gibson et al., 1988) while fermentation of the aromatic amino acids tyrosine and tryptophan produces phenols and indoles (Blaut and Clavel, 2007).

Fat

Dietary fat is a crucial energy source since it provides the most energy per gram, 9 kcal/ g, when compared to protein and carbohydrate (Wang et al., 2013). Lipids can be categorised as FAs, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids and polyketides (Ratnayake and Galli, 2009). Dietary fat also provides the fat soluble vitamins A, D, E and K, and the essential FAs (linoleic acid (LA) and α-linolenic acid (ALNA)) (Simopoulos, 2002; Wang et al., 2013). FAs consist of a carboxylic acid (COOH) attached to an aliphatic chain, which is typically straight and can be saturated, or contain double bonds, making the chain unsaturated (Ratnayake and Galli, 2009). FAs with one double bond in the chain are monounsaturated FAs (MUFAs) while more than one double bond denotes a polyunsaturated FA (PUFA) (Ratnayake and Galli, 2009). Chain length can vary from short 4-carbon FAs in dairy fat to much larger 30-carbon marine derived FAs (Ratnayake and Galli, 2009). FAs and glycerol make-up glycerolipids, a key example being triglycerides (TGs), the main constituent of dietary fats and oils, which consist of an ester of three FAs and a glycerol (Wang et al., 2013).

Fat digestion begins in the stomach where gastric lipase aids the breakdown of TGs to diacylglycerol and free FAs (FFAs), emulsification within the stomach aids pancreatic lipase action and the rate of hydrolysis in intestinal digestion (Kindel et al., 2010). Pancreatic lipase cleaves the TG to yield 2-mono-glycerides (2-MG) and FFA, while pancreatic cholesterol esters are hydrolysed completely into FFAs and free cholesterol. The FFA and 2-MG, with phospholipids form mixed micelles which enables the lipids to be absorbed at the microvillus membrane of the small intestine into enterocytes (Ramírez et al., 2001). These absorbed lipids are re-esterified to TGs and phospholipids, and along with cholesterol and apolipoproteins are packaged into chylomicrons (CM) for secretion to the lymph, then the general blood stream via the thoracic duct (D'Aquila et al., 2016). CM remnants are formed inside tissues by cleavage of TGs by lipoprotein lipase, the CM remnants are taken up by the liver (Ramírez et al., 2001). TGs can also be packaged in cytoplasmic lipid droplets (CLDs)

for storage by enterocytes, which typically occurs when dietary TG levels are high, accumulation can result in fat malabsorption and steatorrhea (D'Aquila et al., 2016). HDL transports excess cholesterol from peripheral tissues to the liver, via the lymphatic vessels, for excretion in the bile and faeces (Hellerstein and Turner, 2014; Lim et al., 2013). In contrast LDL transports cholesterol for uptake by cells in order to obtain α-tocopherol (Kayden and Traber, 1993). Chain length is an important factor in digestion and absorption of FAs, since unsaturated and medium chain FAs are absorbed more efficiently than long chain SFAs (Ramírez et al., 2001), while longer chain FAs are emptied from the stomach more slowly (Hunt and Knox, 1968; Mu and Porsgaard, 2005). FAs are stored in the adipose tissue, with approximately 99% comprising TG, 0.3% cholesterol and less than 0.1% phospholipids in healthy humans (Hodson et al., 2008). Since FAs can be released from the adipose tissue into the venous blood flow the concentration of non-esterified FAs in the blood is a suggested useful marker of the FA acid composition of adipose tissue, while plasma TG levels are more indicative of dietary intake of the preceding days (Hodson et al., 2008).

The predominant FA composition of the diet can influence serum lipids and lipoproteins since six week consumption of diets rich in SFAs (lauric acid and palmitic acid) resulted in significantly higher serum total, LDL and HDL cholesterol (Temme et al., 1996) and total LDL and HDL cholesterol, while a MUFA-rich diet increased HDL cholesterol, reduced total and LDL cholesterol and significantly improved (2.1%) insulin sensitivity compared the SFA-rich diets (Vessby et al., 2001). The dietary intake of the essential FAs, LA (18:2 *n*-6) and ALNA (18:3 *n*-3), determines the omega-6: omega-3 (*n*-6: *n*-3) ratio, and since humans lack the omega-3 desaturase converting enzyme, the balance of this ratio is important since they are converted to anti- and pro-inflammatory eicosanoids, respectively (Simopoulos, 2002) (Figure 1.1). LA and ALNA are present in oils, meat, fish and eggs, rich sources of ALNA are walnuts, flaxseed and rapeseed or soyabean oil (Gebauer et al., 2006; Simopoulos, 2006; Sioen et al., 2017; Williams and Burdge, 2006). Consumption of the longer chain *n*-3 FAs, EPA and DHA, from oily fish and fish oil, is also important however, especially since supplementation with ALNA rich capsules (providing 1.1% of energy or 9.5 g/day) did not result in increased DHA within plasma phospholipids compared to control or low-LA groups; while EPA significantly increased (Childs et al., 2014; Goyens et al., 2006). In addition, supplementation with stearidonic acid (SDA, 18:4 *n*-3), an *n*-3 intermediate of ALNA, resulted in ~40–60%

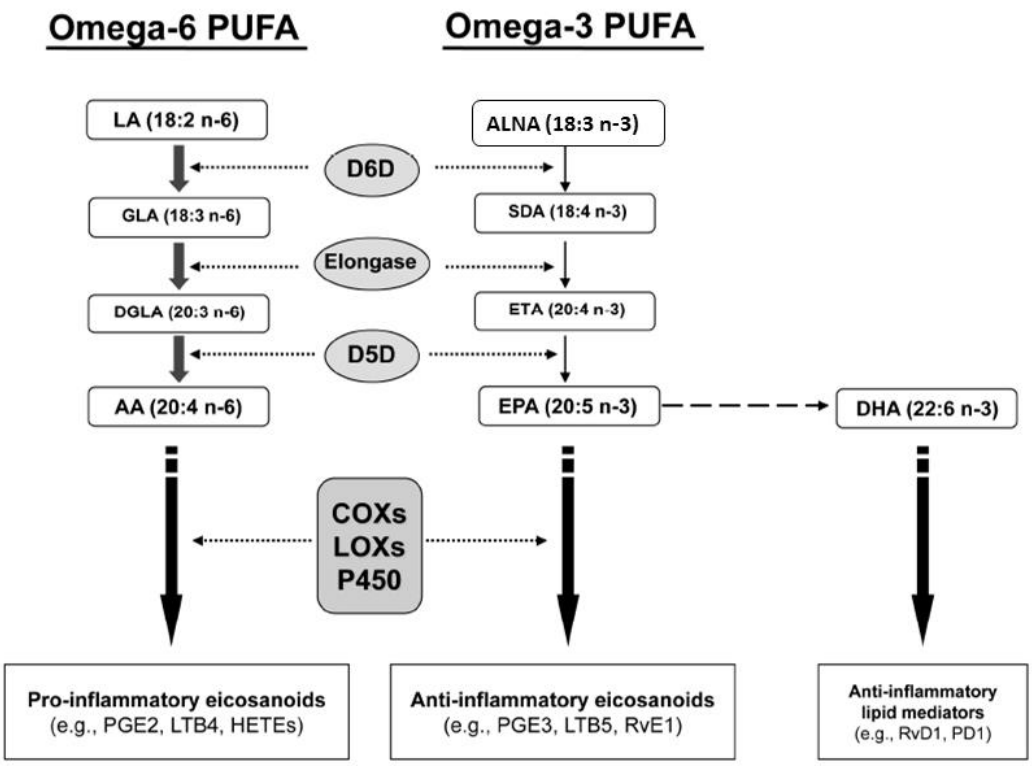


Figure 1.1 n-3 and n-6 desaturation and elongation pathways. Linoleic acid (LA) and α-linolenic acid (ALNA) are converted via desaturation enzymes (D6D and D5D) and elongase before the production of pro-inflammatory (*n*-6) or anti-inflammatory (*n*-3) eicosanoids and lipid mediators; via EPA and DHA production for *n*-3 products. From (Brigandi et al., 2015) with minor modifications.

increases in eicosatetrenoic acid (ETA), EPA and docosapentanoic acid (DPA) in plasma and PBMCs, compared to ALNA supplementation (Kuhnt et al., 2016). These studies suggest the consumption of ALNA rich foods alone may not be sufficient to meet the body's requirements.

TGs or FAs reaching the colon, typically PUFAs, have been shown to be metabolised by the intestinal microbiota to produce conjugated linoleic acid (CLA) isomers (Gorissen et al., 2010). CLAs are eighteen carbon FAs of which there are different geometric isomers dependant on the location and position of the conjugated double bonds (Burdge et al., 2005).

Phytochemicals

Phytochemicals, are compounds present in plants which have no nutritional value but have demonstrated health benefits, and include phenolic compounds, flavonoids, glucosinolates, isothiocyanates and carotenoids (Craig, 1997; Holst and Williamson, 2008). Polyphenols, are typically found in red wine, fruits, vegetables, cereals, tea and cocoa (Bravo, 1998; Del Rio et al., 2010) and may have potential for immune modulation. Polyphenols undergo metabolic conversion mostly in the large intestine by action of the commensal microbiota (Del Rio et al., 2010). Anthocyanins, a class of flavonoids, are metabolised to protocatechuic acid and isothiocyanate sulforaphane which can impair the production of the pro-inflammatory cytokines IL-6, IL-8, IL-12 and IL-23 by LPS-stimulated DCs (Del Cornò et al., 2014; Geisel et al., 2014). These metabolites are thought to interact with the nuclear factor kappa light chain enhaner of activated B cells (NF-κB) signalling pathway since neither DC maturation nor T cell activation were affected by sulforaphane (Geisel et al., 2014), however as this study was carried out using mice the results may not extrapolate to humans.

1.1.9 Changing dietary patterns over time

Current UK dietary recommendations provide dietary reference values (DRVs) for macronutrients and indicate the population average intakes of total fat (≤35%), saturated FAs (≤11%), *trans* FAs (≤2%) (Bates et al., 2014) and the recently updated free sugars (≤5%) and non-starch polysaccharides (NSP; at least 30g/ day) (SACN, 2015). Increased PUFA intake (0.45g/ day) by healthy adults is advised in the UK (SACN, 2004), supported by observations of reduced risk or incidence of stroke, coronary heart disease or myocardial infarction (MI) in healthy and at risk adults (Burr et al., 1989; Daviglus et al., 1997; Gillum et al., 2000; Gillum et al., 1996; He et al., 2002). Adequate vitamin and mineral intakes are advised via age and sex specific

recommendations (Bates et al., 2014) including lower reference nutrient intakes (LRNIs), estimated average requirements (EARs), reference nutrient intakes (RNIs) and safe intakes (DoH, 1991).

Dietary intakes have changed dramatically with evolution, the introduction of agriculture and industrialisation resulting in the Westernised diet of the 21st century (Cordain et al., 2005; Geissler and Powers, 2010; Ströhle and Hahn, 2011). Modelling of *n*-3 and *n*-6 essential FA consumption from 1909 to 1999, using current and 20th century typical nutrient composition data, estimated that most current foods contain more LA and less EPA and DHA compared to 20th century foods with a 1163-fold increase in soybean oil consumption by 1999 (Blasbalg et al., 2011). Data from the National Health and Nutrition Examination Survey (NHANES) of 1988–1994 showed that American adults (≥20 years) derived ~27% of daily energy from energy dense, nutrient poor foods (desserts, sweeteners, salty snacks, visible fat), which was characterised by high BMI and inversely associated with HDL cholesterol and intakes of micronutrients meeting the RDA (Kant, 2000; Kant and Graubard, 2005). However, Western dietary consumption still resembles this, with the inclusion of greater quantities of refined sugars, saturated and *trans* fats, and processed meats (Geissler and Powers, 2010). The most recent UK National Diet and Nutrition Survey (NDNS) of 2008–2012, found that the UK population were consuming saturated fat and non-milk extrinsic sugars (free sugars) at levels greater than dietary recommendations, while fruit, vegetables, NSP and oily fish consumption were below recommendations (Bates et al., 2014). These dietary patterns are associated with increased incidence of T2D, heart diseases and some cancers (Tilman and Clark, 2014).

Studies of energy expenditure in the healthy elderly (>65 years) have shown decreases of 0.69 and 0.43 MJ/ day/ decade for males (average 75 kg) and females (average 65 kg), respectively (Elia et al., 2000), which was attributed to reductions in physical activity, BMR and thermogenesis (Elia et al., 2000; Goran and Poehlman, 1992). While, hospitalised elderly subjects had inadequate energy intakes, only 41% (n=134) met estimated resting energy requirements; associated with poor appetite, required feeding assistance and diagnosis of infection or cancer (Mudge et al., 2011). Additionally, elderly women consuming inadequate protein intakes had significant losses in lean tissue, immune response, muscle function (Castaneda et al., 1995) and increased risk of osteoporotic fractures (Rizzoli et al., 2001). Evidence from the PROT-AGE study suggests that older adults (>65 years) have higher protein requirements than younger adults, from which the authors suggest an average intake of at least 1.0–1.2 g protein/ kg body weight (Bauer et al., 2013). Therefore the elderly

may require specific nutritional recommendations, additionally, when considering the increase of age-associated diseases, the public may also need to accept some of the responsibility for preventative action by adapting their lifestyle choices accordingly; including physical activity, not smoking and dietary intakes (Shlisky et al., 2017).

1.1.10 Influence of diet on the ageing process

Caloric restriction is the reduction in dietary intake to a point which does not prevent the intake of essential nutrients or cause malnutrition (Sohal and Forster, 2014) and has been shown in numerous experimental models to be beneficial for lifespan and slowing the rate of age-associated pathologies (Colman et al., 2009; Sohal et al., 1994; Weindruch and Walford, 1982). Resveratrol has been suggested to act similarly to caloric restriction to extend lifespan (Baur and Sinclair, 2006). Sirtuin proteins, a family of NAD^+ dependant deacetylases named after the *Saccharomyces cerevisiae* silent information regulator 2 (sir2) protein, are thought to be activated during caloric restriction and potentially with resveratrol intake, and have been associated with extended lifespan in yeast, worms and flies (Baur and Sinclair, 2006). Studies investigating the effect of caloric restriction on human ageing are lacking, as long term interventions are unethical, although some short term studies have shown reductions in resting metabolic rate (RMR) (Ravussin et al., 2015) and oxidative damage of DNA and RNA in white blood cells (Hofer et al., 2008). Additionally, the Okinawan's traditional diet demonstrates caloric restriction (Willcox et al., 2007) and has been associated with improved cardiovascular profiles and longevity (4–5% compared to Japan, the highest life expectancy among industrialised countries) (Gavrilova and Gavrilov, 2012; Sho, 2001).

1.1.11 Dietary impact on immune cells in adults and the elderly

Immune cells require energy for cellular proliferation and effector functions and therefore must acquire metabolic substrates from their extracellular environment (Fox et al., 2005). In order to control cellular proliferation, growth factors are required to stimulate cells, in addition to nutrient abundance (Vander Heiden et al., 2009). Metabolite production by the microbiota, including bile acids, lipids, amino acids, vitamins and SCFAs, is a potential way that dietary intake can influence the immune system (Brestoff and Artis, 2013; Clements and Carding, 2017).

Naïve and memory T cells have lower requirements for energy and produce ATP by breaking down glucose, FAs and amino acids, predominantly via oxidative phosphorylation, while proliferating T cells require much more ATP and NADH molecules to sustain their energy requirements and undergo aerobic glycolysis (Fox

et al., 2005). Inappropriate nutrient intake by T cells can, however, inhibit T cell activation and proliferation, and if prolonged can result in apoptosis (Angela et al., 2016). Furthermore, T cells cannot take up the amino acid cystine, but human blood MoDCs promoted the secretion of thioredoxin, which reduced extracellular cystine to cysteine, upon co-culture with T cells, with increased secretion of cysteine by DCs observed (Angelini et al., 2002), this is of significance since cysteine serves as a substrate for glutathione, which regulates T cell proliferation after Ag-receptor engagement (Edinger and Thompson, 2002). With similar observations of elevated secretion of cysteine in the culture supernatant and lymphocyte intracellular glutathione levels in MLRs between macrophages and T cells, after LPS or TNF-α stimulation (Gmünder et al., 1990).

Recent evidence shows that DC and macrophage function are also determined by metabolic reprogramming in response to nutrient alterations, as well as cytokines and danger signals, in which immune cells switch between glycolysis, FA synthesis, oxidative phosphorylation, FA oxidation and the Krebs cycle, dependent of the cell type and required function (O'Neill and Pearce, 2016). In the early stages of DC activation, via TLR agonists, increased glucose consumption and lactic acid production occur producing ATP by oxidative phosphorylation, whereas LPS activation induced rapid commitment of BM derived MoDCs to glycolysis, observed by increased extracellular lactate concentrations and glucose consumption and an abundance of glycolysis metabolic intermediates (Everts et al., 2014). This represents a response to inducible nitric oxide synthase (iNOS), blocking mitochondrial electron transport. While, the uptake of extracellular FAs acids by activated $CD4^+$ T cells from 6–24 hours after TCR stimulation was paralleled with proliferation commencement, while cells treated with a FA inhibitor (TOFA) demonstrated substantial inhibition of proliferation (Angela et al., 2016). Additionally, reductions in citrate concentration and accumulation of FAs were observed alongside expanded endoplasmic reticulum (ER) and golgi apparatus, suggesting a role for FAs, since they act as ligands for transcription factors such as PPARγ, and thus may expand the cellular organelle networks to meet protein synthesis requirements to induce T cell activation (Everts et al., 2014). While, SCFAs may influence innate immune cell function via activation of the FA receptor GPR43 (Brestoff and Artis, 2013; Poulin et al., 2010).

There is developing interest in the influence of nutrition and the immune system, termed immunonutrition. Since, the present evidence suggests that differing metabolites have different effects on immune cell function this may represent a way

of targeting aspects of immunosenescence, via nutritional intake. It should be noted, however, that not all effects of nutrients on the immune system are beneficial.

Effect of carbohydrates on immune function in the elderly

The consumption of a prebiotic GOS mixture (B-GOS; Bi^{2}muno) by a group of elderly subjects (64–79 years) significantly increased IL-10 and decreased IL-6, IL-1β and TNF-α production by PBMCs, in addition to monocyte and neutrophil phagocytic and NK cell effector activity, compared to placebo (maltodextrin) (Vulevic *et al.,* 2008). Similarly, more recently, PBMCs from elderly subjects (65–80 years), produced significantly decreased IL-1β, increased IL-10 and IL-8, elevated numbers of *Bifidobacterium*, which was metabolically linked to elevated lactic acid levels, and significantly increased NK cell activity, compared to placebo (Vulevic et al., 2015). The beneficial effects of prebiotics (GOS and bifidogenic growth stimulator) and heat-treated fermented milk products, administered via enteral feeding to elderly subjects, demonstrated a seroprotective rate (proportion achieving antibody titres ≥40) of 5% against the A/H1N1-like strain six and eight weeks post-influenza vaccination in the intervention group (Nagafuchi et al., 2015). Whereas the initial increase in seroprotective rate in the control group at week six was not maintained at week eight, observed in parallel to significantly higher counts of *Bifidobacterium* in the intervention compared to the control group (Nagafuchi et al., 2015). Significant increases in seroprotective rates against the A/H1N1-like and A/H3N2-like influenza strains were observed after six weeks and maintained after ten weeks (64%) of prebiotic-enteral feeding compared to control (10%) (Akatsu et al., 2016). While, an inulin-type β2-1 fructan consumed by older individuals (45–63 years) improved only antibody responses against the H3N2-like strain of influenza virus (after vaccination with the 2008/2009 vaccine; A/H1H1, A/H3N2 and B-like strains) (Lomax et al., 2015). The combination of probiotics with prebiotics, synbiotics, has recently been trialled in both young and elderly subjects, however no improvement to the age associated impairment to influenza vaccination response was observed (Przemska-Kosicka et al., 2016). The authors indicate that the grouping of the elderly participants was actually uneven since there were more individuals with elevated presence of immunosenescence markers compared to the placebo group, implying that this may have affected the observed results. Consumption of the probiotic drink Actimel® by subjects aged ≥70 years resulted in significantly increased antibody titres against only the B-like strain three weeks post vaccination, after vaccination with three influenza strains (A/H1N1, A/H3N2 and B-like), compared to placebo, with effects still apparent after nine weeks (Boge et al., 2009). Additionally, the increased intake of fruit and

vegetables in an elderly population resulted in improved response to pneumonia vaccination due to significant elevations in antibody reactive to pneumococcal capsular polysaccharide (Gibson et al., 2012).

Effect of protein on immune function in the elderly

The greater protein requirements of the elderly was demonstrated by the provision of protein intake 50% below the RDA (0.92 g per kg body weight) to elderly females for nine weeks, which resulted in reduced delayed-type hypersensitivity (DTH) response, while those meeting their RDA had an almost 50% increase in DTH response to Ags applied to the skin (Castaneda et al., 1995). The RDA used within this study was much greater than the UK RNI of 0.75 g protein per kg weight per day, which is recommended for all adults (BNF, 2015), providing no age limit, highlighting a lack of specific guidelines for the elderly. More recently, small scale studies have determined the protein requirements of elderly subjects and suggested a higher RDA of 0.8 g (>65 years) and 0.85 g per kg per day (80–87 years) but acknowledge that much more evidence is required (Rafii et al., 2015; Tang et al., 2014).

The limited data investigating the effect of protein intake of elderly subjects reveals that increasing the intake of red meat of elderly women when carrying out resistance training significantly reduced levels of serum IL-6 (Daly et al., 2014); with a further confirmatory study currently underway. In addition, amino acid supplementation of endurance athletes or adults undertaking intensive exercise was associated with decreased IL-1β, IFN-γ and CRP, and increased IL-10 (Kraemer et al., 2014), neutrophil numbers and total lymphocytes, compared to the placebo group (Murakami et al., 2009). This data is of interest since, like an ageing population, endurance athletes can have compromised immune function and increased susceptibility to infection.

Effect of FAs on immune function in the elderly

Evidence from prospective studies demonstrated a reduction in risk of pneumonia in elderly men reporting the greatest intakes of ALNA, LA, EPA and DHA (1.53 g ALNA, 15.75 g LA/ day and ≥5 servings fish/ week) (Merchant et al., 2005). Opposing results were seen in elderly women (increased risk of developing pneumonia) (Alperovich et al., 2007), implying a potential gender specific effect of PUFAs. However these data should be considered with caution since they depend on self-reported food frequency questionnaires (FFQs), which are associated with reporter bias (Brunner et al., 2001; Cade et al., 2002).

Administration of PUFA (in the form of oil containing capsules) to elderly women increased lipid peroxidation and impaired secretion of IL-2 and T cell proliferation at high doses (1680 mg EPA and 720 mg DHA/ day) (Meydani et al., 1991), while very low doses (30 mg EPA and 150 mg DHA/ day) of fish oil significantly reduced mitogen (ConA, PHA or OKT3) induced lymphocyte proliferation, by 34–45% (Bechoua et al., 2003). Further implication of detrimental effects of PUFAs in the elderly were demonstrated by the dose-dependent reduction in neutrophil respiratory burst with increased EPA dosage (1.35, 2.7 or 4.05 g EPA /day), only observed in older subjects, in addition to incorporation of EPA into plasma and PBMC phospholipids more readily than in the younger subjects suggesting that the elderly handle PUFAs differently than young subjects (Rees et al., 2006). Significant reductions in ConA induced lymphocyte proliferation were also observed after fish oil capsule consumption (720 mg EPA and 280 mg DHA/ day) compared to supplementation with placebo, ALNA, γ-linolenic acid (GLA), arachidonic acid (ARA) and DHA containing capsules by older adults (Thies et al., 2001). Though this study included subjects from 55 years, so is not representative of the elderly. While, consumption of blackcurrant seed oil, rich in GLA and α-linoleic acid (ALA), by elderly subjects resulted in greater DTH skin response to tetanus toxoid in addition to a significant decrease in the pro-inflammatory serum prostaglandin E_2 (PGE_2), (Wu et al., 1999) which has been shown to regulated by CLAs in cultured macrophages (Stachowska et al., 2009). However, EPA demonstrated no effect on cytokine secretion at any dose trialled (1.35, 2.7 or 4.05 g EPA /day) (Rees et al., 2006), with similar observations with 4 g encapsulated oil/day (Thies et al., 2001). Lower doses of 0.18g EPA and 0.28 g DHA or 1.11 g EPA and 1.72 g DHA per day, achieved by combined consumption of spread and fish oil capsules by healthy adults (25–72 years) showed no impact on cytokine secretion by PBMCs, neutrophil or monocyte phagocytosis, oxidative burst or ConA induced lymphocyte proliferation, compared to the ALNA only placebo groups, after six months of supplementation (Kew et al., 2003). This suggests that these lower dosages are insufficient to effect immune function.

However, the dosages used within some of these studies (Meydani et al., 1991; Rees et al., 2006; Thies et al., 2001) equate to intakes of fish which would be difficult to achieve, since current fish intakes by the UK elderly are 85 g per week (Bates et al., 2012), below the current recommendations of two portions (~280 g for adults) of fish per week, which should provide 0.45g/ day EPA and DHA (SACN, 2004; Weichselbaum et al., 2013). Consumption of large quantities of fish, four portions per week for eight weeks, by healthy older Australians (mean 69.6 years) had no effect

on serum levels of IL-1β or IL-6, though the participants were in good health with no risk factors for CVD (Grieger et al., 2014), implying that in order to see PUFA-mediated effects a baseline inflammatory state may be required. This also suggests that the PUFA dose provided by capsules may not be reflective of the concentration available to the target tissue after oily fish or other *n*-3 PUFA rich foods. This was addressed by Mantzioris et al. (2000) who aimed to increase intakes of n-3 PUFA by providing subjects (n=15) with products naturally high in *n*-3 PUFA or which had been fortified to contain high amounts, making the intervention more biologically relevant. The intervention resulted in significantly increased incorporation of total *n*-3 PUFA within mononuclear cell (MNC) and plasma phospholipids after four weeks (3-fold in EPA and 1.5-fold increase in DHA), in addition to inverse associations with synthesis of thromboxane B2 (TXB_2), PGE_2 and IL-1β by LPS-stimulated, aspirin pre-treated whole blood (Mantzioris et al., 2000).

A key study utilising soybean oils containing varying FA compositions highlighted the beneficial effects of oils rich in MUFA (18.9% MUFA, 2.82% PUFA, 5.76% SFA) and low in SFA (6.19% MUFA, 14.6% PUFA, 4.91% SFA) which increased PHA induced lymphocyte proliferation in cells from older adults (>50 years), with elevated cholesterol (Han et al., 2012). While, low ALNA and LA oil consumption (18.3% LA:ALNA) significantly reduced lymphocyte proliferation (Han et al., 2012); however the study had a very small study population of only 18 subjects. Further to this study consumption of extra virgin olive oil by overweight or obese subjects (≥ 65 years) for three months significantly increased anti-CD3/CD28-stimulated T cell proliferation in whole blood compared to cells from the placebo group (10% corn oil, 90% soybean oil, plus butter to use as spread), a total of 44 subjects completed the study (Rozati et al., 2015). Another very small study (n=10) demonstrated that consumption of 5L / week of an almond (3%) and olive oil (0.6%) based drink (51.7% MUFA, 38.3% PUFA and 9.9% SFA) by athletes resulted in significantly increased plasma IL-6 and TNF-α, from baseline, post exercise in the young subjects (19-26 years) but not in the older subjects (44-47.2 years) (Capó et al., 2016); though this group was not representative of an elderly population.

Currently, most studies have investigated the effects of PUFAs on immune responses, while some (small scale) evidence suggests that increased MUFA intakes may be a more suitable for the elderly, since reduced lymphocyte proliferation, frequently observed with high PUFA intakes may increase susceptibility to infections. This, however, requires much more research to be able to make such recommendations. Immune cells derived from the elderly appear to respond to FAs

differently to healthy adults, and thus the elderly should be considered separately when making dietary recommendations, rather than providing guidance for the general adult population.

Effect of phytochemicals on immune function in the elderly

There has been limited investigation to date into the effect of polyphenols on the immune response of elderly subjects, however a number of reviews have postulated the potential for beneficial effects (Magrone and Jirillo, 2011; Magrone et al., 2008). Resveratrol is a suggested key compound for longevity and controlling inflammation supported by evidence of increased serum adiponectin and reduced thrombogenic plasminogen in resveratrol supplemented coronary artery disease (CAD) patients (mean age of 59± 10 years) (Tomé-Carneiro et al., 2013). *In vitro* observations show administration of olive oil polyphenols (caffeic acid and oleuopein glycoside) to LPS-stimulated human whole blood cultures (subjects 18–25 years) resulted in significantly reduced IL-1β levels compared to stimulated control cultures; responses were correlated inversely with dose (Miles et al., 2005). In addition consumption of cocoa polyphenols (40g/ day) with 500 ml skimmed milk, by subjects at high CVD risk (≥55 years), significantly reduced surface expression of the adhesion molecules CD36 and CD40 on monocytes *ex vivo*, compared to monocytes from the control group (skimmed milk) (Monagas et al., 2009). A small scale (n=23) pilot study has recently shown that daily consumption of 12 green olives, containing oleuropein and hydroxytyrosol, significantly reduced serum IL-6 levels after 30 days of consumption by healthy adults (18–65 years) (Accardi et al., 2016).

1.1.12 Traditional diets in studies of longevity and age-associated disease

Conflicting evidence exists for all food groups (carbohydrates, protein, FAs and phytochemicals) on the ageing immune system with both beneficial and detrimental effects observed. It is noteworthy that the studies discussed so far have investigated dietary components in isolation, or as encapsulated supplements. This ignores the real life response after consumption of whole foods which needs to be considered along with any interactions occurring between different components of the diet. Whole diet studies can provide the most representative and realistic outcomes from which more robust conclusions can be drawn. There has been some interest in traditional diets such as the Mediterranean, Okinawan and Nordic diets, with regard to longevity and perceived health outcomes, predominantly cardiovascular.

Mediterranean diets

Mediterranean diets differ in composition dependent on their originating country within the Mediterranean Basin, since these countries have different religious and cultural beliefs influencing their dietary choice (Simopoulos, 2001). The traditional Mediterranean (MED) diet refers to dietary patterns of people habitant to olive-growing regions within the Mediterranean Basin during the 1950s and 1960s (Trichopoulou and Lagiou, 1997). Which was first described by Ancel Keys (Trichopoulou and Lagiou, 1997) following the Seven countries study (former Yugoslavia, Finland, Italy, The Netherlands, Greece, USA and Japan) with observations that risk of all cause and CHD death were correlated with SFA intake (Keys, 1970). With the follow-up studies showing associations of SFA intake and consuming olive oil with every meal in the 15-, 20- and 25-year CHD mortality rates (Keys et al., 1984; Keys et al., 1986; Menotti et al., 1989; Menotti et al., 1993). The MED diet consists of fruits, vegetables and wholegrain cereals with every main meal, olives, olive oil, nuts, seeds and dairy products every day, while potatoes, meat, (oily) fish or seafood, eggs, and sweet products are consumed less frequently (Figure 1.) (Bach-Faig et al., 2011).

The MED diet has since been investigated in prospective cohort studies and increasing adherence was associated with reduced mortality in CHD patients after 3.78, 6.7 and 10 years of follow-up (Knoops et al., 2004; Trichopoulou et al., 2007; Trichopoulou A, 2005). With 1.3–1.5 times increased risk of MI, coronary bypass, angioplasty and CVD with every one point reduction in MED diet score, in a Turkish population (Hoşcan et al., 2015). Similarly a longitudinal study from six European cities (Helsinki, Stockholm, Augsburg, Rome, Barcelona and Athens), consisting of MI survivors (mean age 62 years), showed reductions (3.1% and 1.9%) in mean CRP and plasma IL-6 for every increased point of adherence (Panagiotakos et al., 2009). Increased MED diet adherence, by participants from the twin heart study, significantly associated with greater heart rate variability, a measure of cardiac autonomic function, which were independent of genes, shared environmental factors and cardiovascular risk factors (Dai et al., 2010). While evaluation of dietary consumption of Spanish institutionalised subjects (65–95 years) demonstrated that consumption of diets with higher composite scores, indicative of MED diet compliance, only reduced mortality risk in those <80 years (Lasheras et al., 2000).

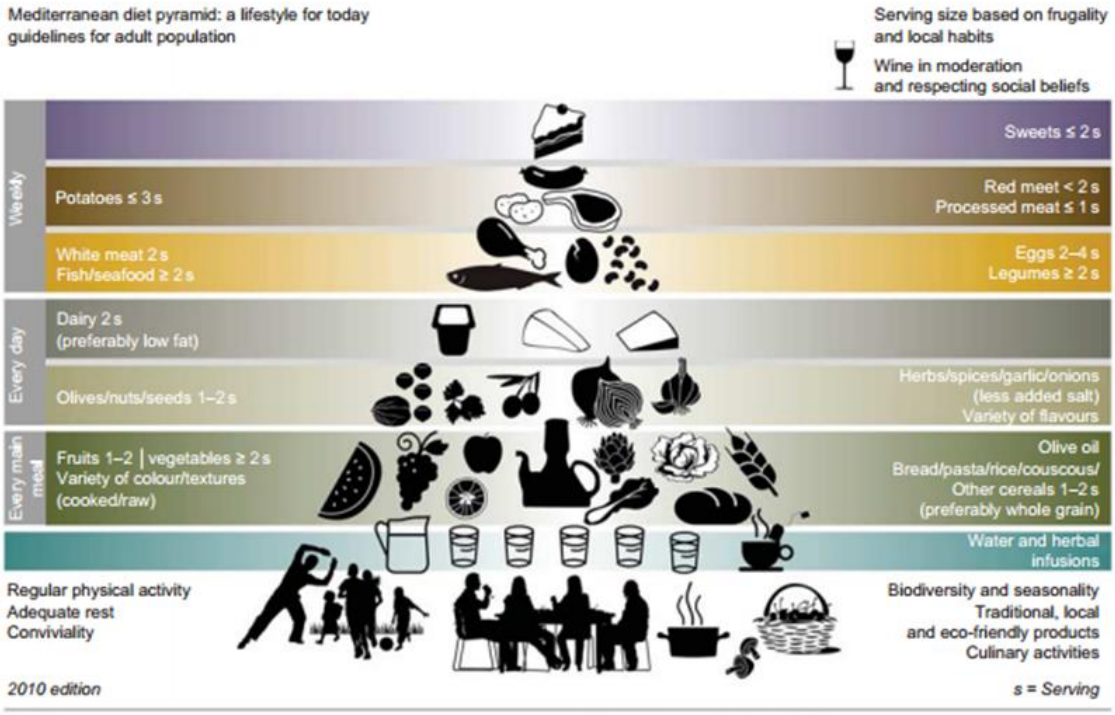


Figure 1.2 The Mediterranean diet pyramid. The composition of the Mediterranean diet as recommended by the Fundación Dieta Mediterránea, from (Bach-Faig et al., 2011) with no modifications.

Additionally, a number of intervention trials have been conducted (PREDIMED, Medi-RIVAGE, GISSI-Prevenzione) or are underway (CORDIOPREV) (Delgado-Lista et al., 2016). From which greater adherence (every 10%) to the MED diet, post MI, significantly reduced the risk of mortality (15%) or coronary events (72%) (Barzi et al., 2003; de Lorgeril et al., 1994), with subjects >60 years demonstrating the greatest benefit (Barzi et al., 2003). While consumption of either a MED diet or low fat diet significantly reduced total and LDL cholesterol, the authors acknowledged that participants were not fully compliant and the MED diet group did not reach the required fibre or MUFA intakes (Vincent-Baudry et al., 2005). Additionally, MED diet intervention in subjects at high cardiovascular risk or meeting ≥3 diagnostic criteria for metabolic syndrome (MetS) significantly reduced BMI, waist circumference (Esposito et al., 2004; Ruiz-Canela et al., 2015), blood pressure, and levels of glucose, insulin, total and LDL cholesterol, with more than half of subjects no longer classified as having MetS after two years (Esposito et al., 2004). Most recently, the comparison of MED diets enriched with nuts or olive oil, with consumption of a low fat diet, in a subgroup from the PREDIMED study, increased cholesterol efflux capacity compared to baseline and the olive oil enriched diet significantly increased the ability of HDL to esterify cholesterol, compared to the low fat diet (Hernáez et al., 2017). In addition, both vegetarian and MED diets significantly reduced weight, fat mass and BMI of subjects after a three month crossover study, with significantly reduced insulin levels, total and LDL cholesterol after the vegetarian diet, while the MED diet significantly reduced triglycerides (Pagliai et al., 2017; Sofi et al., 2016).

However, all of these studies were carried out in the Mediterranean countries of Spain, Italy or France (Marseille), so it is of crucial importance to determine whether the MED diet can demonstrate the observed health effects in regions other than the Mediterranean Basin. This has been investigated by (Kouris-Blazos et al., 1999) where elderly Australian subjects (≥70 years), showing greater adherence to the MED diet, were associated with greater survival, regardless of whether they were from Anglo-Celtic or Greek decent. Which is comparable to a trial conducted in India where CAD patients randomised to the MED diet took significantly fewer cardiac medications after two years, in addition to having significantly reduced risk of non-fatal MI and sudden cardiac death (Singh et al., 2002). With the additional observation that short term consumption (nine weeks) of olive oil by healthy subjects (22–44 years) in Northern, Central and Southern regions of Europe resulted in significantly decreased systolic blood pressure in the non-Mediterranean subjects but not in Mediterranean subjects (Bondia-Pons et al., 2007).

While several of these studies have targeted an elderly population, in various disease states, the effect on immune response has not been extensively investigated, but an anti-inflammatory effect of the diet is alluded to. To date some promising findings have been obtained but only from small scale and short term studies. Twenty free-living elderly subjects (mean 67 years), showed significantly increased expression of MCP-1, postprandially, after three weeks of SFA-rich dietary intake, while the CHO-PUFA-rich diet induced significantly increased expression of TNF-α, compared to MED diet, in cDNA extracted from PBMCs; the increase in MCP-1 was confirmed in plasma samples, in addition to increased IL-6 after consumption of all diets (Camargo et al., 2012). In the ZINCAGE study greater adherence to the MED diet by male elderly Greek subjects was significantly associated with reduced plasma IL-8, with an association made between olive oil consumption and reduced plasma IL-6, IL-8, MCP-1 and TNF-α levels, while red meat consumption was positively associated with these cytokines and chemokines (Dedoussis et al., 2008). Additionally, increased adherence to the MED diet (supplemented with olive oil or nuts) for three months significantly reduced monocyte expression of the cell adhesion molecules, CD49d and CD40, from older subjects (55–80 years), compared to a low fat diet, along with significant reductions in plasma IL-6, in both MED diets, and CRP after the olive oil rich MED diet (Mena et al., 2009). A cross-sectional investigation showed that subjects (51–75 years) consuming a diet of higher quality, assessed by diet quality and healthy eating indexes, which resembled the MED diet with the exception of olive oil and specific changes to meat and fish consumption, displayed a significant trend toward fewer T helper cells and more cytotoxic T cells (Boynton et al., 2007). Additionally, a cross-sectional study showed that elderly subjects, but only non-Hispanic White individuals, with greater adherence to the MED diet (1 point increase) had longer leukocyte telomeres lengths (48 bp increase), while in the whole cohort greater telomere lengths were associated with vegetable intakes above the population median intake (Gu et al., 2015). This is of interest since telomeres provide protection of genetic material located at the chromosomal ends, shortening length of telomeres is considered a marker of ageing (Gu et al., 2015).

Okinawan diets

The Okinawan diet is traditionally consumed in China, South East Asia and Japan, with the highest proportion of energy intake from sweet potatoes, rich in polyphenols (Gavrilova and Gavrilov, 2012), in addition to large quantities of seaweed, and leafy vegetables, and soy as the principle source of protein (Sho, 2001; Willcox et al., 2007). Dietary assessment of elderly (≥70 years) and centenarian subjects in

Okinawa, by two day food record, showed that female centenarians had low energy and vitamin A intakes, and females had inadequate energy, protein, calcium and vitamin B_{12} intakes, while males had intakes close to the Japanese RDAs (only available for up 80 years) (Chan et al., 1997). *In vitro* administration of Okinawan food samples, after chloroform extraction, to human (HL-60) promyelocytic or murine (RAW 264.7) macrophage cell lines induced greater suppression of free radical (O_2^- and NO) generation (≥70%) compared to foods common to Japan (Murakami et al., 2005). Administration of samples from the Gramineae (wild rice and lemongrass) and Zingiberaceae (ginger and turmeric) food groups induced the highest frequency of suppression (Murakami et al., 2005). However, immune parameters have not been investigated in human studies to date.

Nordic diets

The new Nordic diet (NND) recommendations (Åkesson et al., 2013), are similar to the MED diet but the increase in fruit and vegetables emphasises berries, cabbage and root vegetables which are typically grown in Nordic countries, to not only improve health but to be ecologically sustainable (Mithril et al., 2012; Mithril et al., 2013). Elevated IL-1 receptor antagonist (IL-1Ra) was observed in plasma from MetS subjects consuming the control diet compared to those consuming the healthy NND diet, across six centres (Uusitupa et al., 2013); IL-1Ra correlated significantly with SFAs and TG (Lankinen et al., 2016). Significant differences were also observed in plasma levels of the lipid metabolites, plasmalogens (increased) and ceramides (decreased) in subjects with MetS after 12 weeks of NND intake, however, these differences were not sustained and returned to baseline levels by the end of the study (Lankinen et al., 2016). A longitudinal study following Swedish women showed increased NND adherence significantly reduced risk of all-cause mortality, with wholegrains and apples/pears significantly inversely associated (Roswall et al., 2015). However, dietary data was self-reported using FFQ at just one time point, which introduces bias and is not representative of overall dietary intake (Cade et al., 2002). Though similarly, follow-up of a cohort of elderly subjects showed that greater adherence to the NND improved physical performance in women (Perala et al., 2016). While six months intervention with the NND in MetS subjects (18–65 years) had no influence on the faecal *Prevotella: Bacteroides* ratio, when compared to the average Danish diet (Roager et al., 2014). Consumption of an Okinawan-based Nordic diet, including wholegrain, vegetables (including root vegetables), legumes, fruits (including berries), poultry, oily fish and nuts but limiting sugar, white four, red and processed meat and dairy products, caused significant reductions in weight, BMI,

waist circumference and insulin resistance in type II diabetic Scandinavian subjects after a 12 week pilot study (n=30) (Darwiche et al., 2016).

1.1.13 Concluding statement

Numbers of neutrophils (Solana et al., 2012) and cytotoxic ability of NK cells remain relatively unchanged with increased age (Almeida-Oliveira et al., 2011; Sansoni et al., 1993). Changes in numbers of monocyte subsets and their surface expression and cytokine secretion profiles have been observed (Hearps et al., 2012; Pinke et al., 2013; Sadeghi et al., 1999; Seidler et al., 2010) and conflicting observations suggest defects in neutrophil NET formation, respiratory burst and bacterial killing (Butcher et al., 2001; Hazeldine and Lord, 2013; Sauce et al., 2016; Tortorella et al., 1993). Of note the current literature is conflicting with regard to the age-associated changes occurring in the DC population (Agrawal et al., 2007; Della Bella et al., 2007; Jing et al., 2009; Panda et al., 2010; Sridharan et al., 2011; Stout-Delgado et al., 2008), but there appears to be a functional impairment which needs further clarification as the observed defects also demonstrate an influence on $CD4^+$ T cell activation (Liu et al., 2012; You et al., 2013a). An established understanding exists for the influence of ageing on cell-mediated immune functions, with extensive evidence demonstrating the involvement of thymic involution and the subsequent impact on the remaining T cell populations (Briceño et al., 2016; Ferguson et al., 1995; Kang et al., 2004; Saavedra et al., 2017; Wikby et al., 2005). However, the humoral immune response is less clearly understood with conflicting observations of B cell numbers and proliferation rates observed (Colonna-Romano et al., 2009; Lin et al., 2016; Macallan et al., 2005; Paganelli et al., 1992) while functional responses to vaccinations are consistently reduced in the elderly (Ademokun et al., 2011; Frasca et al., 2010; Rubins et al., 1998). The observation that mRNA expression of activation-induced deaminase (AID) protein, which is required for CSR and SHM, is downregulated with age suggests that Igs may be affected by ageing (Frasca et al., 2010; Frasca et al., 2008).

The relatively few whole diet studies that have assessed expression of adhesion markers or cytokine secretion by immune cells have been short in duration (typically three months) and have predominantly been conducted in Mediterranean regions such as Spain or Italy (Camargo et al., 2012; Dedoussis et al., 2008; Mena et al., 2009). A limited number of studies have investigated the Okinawan diet and NND but currently there is no human evidence of immune modulation. Therefore, since there appears to be potential for the MED diet to improve immune parameters, due to the current evidence of reduced cardiovascular risk factors, and reduced expression of

inflammatory mediators, predominantly in an elderly population, there is a timely need to investigate this diet for a longer duration (i.e. one year) in a healthy elderly population residing in non-Mediterranean regions.

Hypothesis

Dietary intervention with a MED diet for one year may increase numbers of DC subsets and increase their ability to secrete cytokines in response to antigenic stimulation in cells from elderly subjects. Additionally, the MED diet may increase the diversity of the Ig repertoire, which is observed to be oligoclonal in the elderly, during early B cell precursor development in the BM.

Aims

To demonstrate the age-associated changes observed in DC numbers and function, and Ig repertoire. To determine whether one-year intervention with the MED diet in elderly subjects (65–79 years) can influence the observed age-associated changes in DCs and the variable region of the Ig heavy chain.

Objectives

To utilise blood samples from elderly subjects on the Nu-AGE dietary intervention study, at baseline, to determine absolute numbers of peripheral mDCs and pDCs and their cytokine secreting ability, in comparison to blood samples collected from young, control subjects, on the Im-AGE study and to subjects' own post-intervention samples. Additionally, to determine whether IgH variable region gene usage and CDR3 length are affected by MED diet intervention, in collaboration with the Babraham Institute.

Chapter 2

Materials and methods

2.1 General materials

Laboratory reagent supplier names have been listed within the text, with product details. The water used within experiments was ultrapure and of 18.2 MΩ cm^{-1} resistance derived from a Milli-Q® Ultrapure water system (Millipore), unless otherwise stated.

2.1.1 Solutions and buffers used

All prepared solutions and buffers used and referred to within this thesis are detailed in Table 2.1.

2.1.2 Dilution of antibodies

All antibodies used throughout this thesis are referred to within the text and the clones, conjugated fluorochromes, dilutions used and suppliers, of which, are detailed in Table 2.2.

2.2 Methodology

2.2.1 Recruitment of young subjects

The Im-AGE project obtained ethical approval by NRES Committee South West-Cornwall, Plymouth and Exeter (15/SW/0038) before the study commenced (Appendix I). Young subjects (18–40 years) were recruited at the Norfolk and Norwich University Hospital phlebotomy department (Appendix II). A total of 45 participants were recruited for immune analysis at the Institute of Food Research. Eligible participants were apparently healthy and free from current or recent (three months) chronic disease, gave informed consent (Appendix III). Volunteers would not be eligible if they had had recent changes to medications, had type I diabetes (T1D), were using steroids or immunomodulatory medication, or taking antibiotics currently or within the previous two months. Additionally, anyone already participating in another study, a regular blood donor or any individual unable to give informed consent would not be eligible to take part. These criteria were the same as for the Nu-AGE participants except for the changed age category and with the additional exclusion of pregnant and breast feeding women, Table 2.1.

Buffer	Reagent/ supplement added	Proportion (conc.)	Supplier details
Flow cytometry buffer:	Phosphate buffered saline (PBS) solution	99.85%	Sigma Aldrich
	Heat-inactivated foetal bovine serum (FBS) (Endotoxin certified <0.6 EU/ml)	0.5%	Biosera
	10% Formalin	1%	Sigma Aldrich
Freeze medium:	Heat-inactivated FBS (Endotoxin certified <0.6 EU/ml)	90%	Biosera
	Dimethyl sulphoxide (DMSO)	10%	Sigma Aldrich
Thaw medium:	Roswell Park Memorial Institute (RPMI) 1640 media	90%	Sigma Aldrich
	Heat-inactivated FBS (Endotoxin certified <0.6 EU/ml)	10%	Biosera
1x Red blood cell lysis solution:	10x Red blood cell lysis solution	10%	Miltenyi-Biotec
	Double distilled water	90%	-
Tissue culture medium:	RPMI 1640	90%	Sigma Aldrich
	Heat-inactivated FBS (Endotoxin certified <0.6 EU/ml)	10%	Biosera
	L-glutamine	2mM	Lonza

	Penicillin/ streptomycin	100 U/ 0.1 mg/ml	Lonza
MACs buffer:	PBS	99.1%	Sigma Aldrich
	Ethylenediaminetetra-acetic acid (EDTA)	0.4% (0.5M)	Lonza
	Heat-inactivated FBS (Endotoxin certified <0.6 EU/ml)	0.5%	Biosera

Table 2.1 Composition of solutions and buffers used within this thesis and source of reagents.

Antibody	Dilution	Supplier
Anti-BDCA mAbs (CD1c, clone: AD5-8E7; CD303, clone: AC144; CD141, clone: AD5-14H12; CD14-Pe-Cy5 and CD19-PE-Cy5)	1:6	Miltenyi-Biotec
Isotype control mAbs (mouse IgG2a-PE, mouse IgG1-FITC, mouse IgG1-APC, mouse IgG2a-PE-Cy5 and mouse IgG1-PECy5)	1:6	Miltenyi-Biotec
CD1c-PE (Clone: AD5-8E7)	1:12.5	Miltenyi-Biotec
CD303-PE (Clone: AC144)	1:12.5	Miltenyi-Biotec
CD304-PE (Clone:AD5-17F6)	1:12.5	Miltenyi-Biotec
CD14-FITC (Clone:TÜK4)	1:40	Biolegend
CD19-FITC (Clone:LT19)	1:20	Biolegend
CD3-FITC (Clone: UCHT1)	1:5.5	Becton Dickinson (BD)
CD16-FITC (Clone: 368)	1:5.5	Becton Dickinson (BD)
HLA-DR-Alexa Fluor 700 (Clone: L243)	1:25	Biolegend
PE rat IgG2a, isotype control (Clone: 543.10)	1:12.5	Miltenyi-Biotec
PE mouse IgG1, isotype control (Clone: IS5-21F5)	1:12.5	Miltenyi-Biotec
FITC mouse IgG1, isotype control (Clone: IS5-21F5)	1:12.5	Miltenyi-Biotec

Alexa Fluor 700 Mouse IgG2a, isotype control (Clone: MOPC-173)	1:50	Biolegend
IL-8-PE-Cy7 (Clone: E8N1)	1:50	Biolegend
IL-1β-Alexa Fluor 647 (Clone: JK1B-1)	1:50	Biolegend
IL-6-Pacifc Blue (Clone: MQ213A5)	1:25	Biolegend
PE-C7 mouse IgG1, isotype control (Clone: RTK2071)	1:200	Biolegend
Alexa Fluor 647 Mouse IgG1, isotype control (Clone: MOPC-21)	1:50	Biolegend
Pacific Blue Rat IgG1, isotype control (RTK2071)	1:500	Biolegend
CD19-VioBlue (Clone: LT19)	1:10	Miltenyi-Biotec

Table 2.2 Details of all monoclonal antibodies (mAbs) used within this thesis.

2.2.2 Recruitment of elderly subjects

The Nu-AGE project obtained ethical approval by NHS NRES Committee East of England - Norfolk, (REC reference 12/EE/0109) before the study commenced (Appendix IV). Elderly subjects (65–79 years) were recruited (Appendix V) and a sub group of the total participants gave additional consent for analysis of immune function at the Institute of Food Research (IFR) (Appendix VI). A total of 120 participants were recruited for this analysis by the study team at the Clinical Research and Trials Unit (CRTU) at the University of East Anglia (UEA). Sixty subjects were enrolled into each arm of the intervention. Eligible participants were apparently healthy and free from current or recent (three months) chronic disease and aged 65–79 years. Volunteers would not be eligible if they had had recent changes to medications, had type I diabetes, were using steroids or taking antibiotics currently or within the previous two months. Additionally, anyone already participating in another study or any individual unable to give informed consent would not be eligible to take part. For full list of exclusion criteria, Table 2.2.

The Im-AGE and Nu-AGE studies were conducted in full compliance with the principles of the declaration of Helsinki (2013 version) and following good clinical practice (GCP). There were no conflicts of interest in relation to these studies.

2.2.3 Subject demographics

For the Nu-AGE study pre-baseline data was collected in the form of a self-reported seven-day food diary (7DD) (Appendix VII), a general questionnaire to provide socio-economic information, followed by anthropometric measurements at baseline at the CRTU. This data collection was repeated one year post-intervention. In order to improve accuracy of the diet diaries, photographs of common portion sizes and servings such as mugs, glasses and spoons were provided, additionally a member of the study team attended a home visit which allowed more accurately assessment of portion sizes and typically consumed brands of foods or foods which may not be clearly identified in the diet diary. For the Im-AGE study a lifestyle questionnaire (Appendix VIII) was completed by the participants.

2.2.4 Dual x-ray bone densitometer (DXA) scans

Bone mineral density (BMD) and bone composition were determined using whole body DXA scans (DXA Discovery Wi dual-energy X-ray absorbtiometer; Hologic Inc.), at pre- and post-intervention study visits, by a trained member of the study team according to a standard protocol. BMD was measured at the lumbar spine and proximal femur.

Inclusion criteria	Exclusion criteria
Male or female aged 18–40 years old	Current manifestation of disease (such as aggressive cancer)
Free from chronic disease for the past two years	Unstable organ failure or organ failure requiring a specific diet
Free and independent living	History of severe heart disease, chronic kidney disease, liver cirrhosis, respiratory problems
Willing to provide a one-off blood sample	Diabetes Mellitus type I
	Chronic use of corticosteroids or any other immunomodulatory medication
	Antibiotic use within the previous two months
	Change in habitual medications within the previous three months (e.g. statins, thyroxin)
	Participation in any intervention study, or sampling donation of blood that may increase volume taken over 500 mL in a four month period.
	Regular blood donor
	Malnutrition (diagnosed as a BMI lower than 18.5 kg/m^2)
	Loss of more than 10% body weight within six months
	Pregnant and breastfeeding women
	Unable to provide informed consent

Table 2.3 Inclusion and exclusion criteria applied to recruiting participants onto the Im-AGE study.

Inclusion criteria	Exclusion criteria
Male or female aged 65–79 years old	Current manifestation of disease (such as aggressive cancer)
Free from chronic disease for the past two years	Unstable organ failure or organ failure requiring a specific diet
Free and independent living	History of severe heart disease, chronic kidney disease, liver cirrhosis, respiratory problems
Willing to participate for one year	Diabetes Mellitus type I
	Chronic use of corticosteroids
	Antibiotic use within the previous two months
	Change in habitual medications within the previous three months (e.g. statins, thyroxin)
	Participation in another dietary intervention study, or sampling donation of blood that may increase volume taken over 500 mL in a four month period
	Malnutrition (diagnosed as a BMI lower than 18.5 kg/m^2)
	Loss of more than 10% body weight within six months
	Presence of frailty according to the criteria of (Fried et al., 2001)
	Unable to provide informed consent

Table 2.4 Inclusion and exclusion criteria applied to recruiting participants onto the Nu-AGE study.

2.2.5 Blood sample collection

For both studies 23 ml blood was taken, after informed consent had been given, by a trained research nurse or phlebotomist by venepuncture; 3 ml with sodium heparin and 20 ml with EDTA (BD; Bunzyl Healthcare). Blood samples were analysed within four hours of collection. In addition to this PhD work, further blood samples (total volume 100 ml) were collected from all Nu-AGE volunteers at pre- and post-intervention visits, these samples included 58mL processed for plasma samples, using EDTA tubes (minimum plasma yield of 26mL). The plasma collected was used for analysis of lipid profiles (total cholesterol, LDL cholesterol, HDL cholesterol and triglycerides).

2.2.6 Isolation of peripheral blood mononuclear cells (PBMCs)

Blood collected via venepuncture into EDTA-vacutainers was used to isolate PBMCs by overlaying the blood on a Ficoll-Hypaque solution (Sigma Aldrich, density 1.077 g/ mL) using leucosep tubes (Greiner Bio-one). PBMCs were counted using a haemocytometer and trypan blue stain to assess viability. Approximately 2 x 10^6 PBMCs were frozen in heat-inactivated FBS (Biosera) containing 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich) in aliquots of 1.8 ml at -80ºC; aliquots were cryopreserved using Nalgene Cryo Freezing containers (Nalgene Nunc International, Rochester, New York) to ensure a freezing rate of -1ºC/ minute. After at least 48 hours the aliquots were removed from the freezing containers and placed into long term storage containers within the -80ºC freezer.

2.2.7 Thawing of PBMCs

Previously frozen PBMCs were thawed in a 37 ºC water bath and washed in an excess of thaw media (90% RPMI 1640 (Sigma Aldrich) and 10% heat-inactivated FBS (Biosera)) to remove DMSO from cells, within 20 minutes of thawing. Cell pellets were resuspended in media containing 90% RPMI 1640, 10% heat-inactivated FBS, and supplemented with 2 mM L-glutamine and 100 U penicillin and 0.1 mg/ ml streptomycin antibiotics. Cells were counted using the viability stain, trypan blue, and resuspended to give a final concentration of 0.5 x 10^6 cells / 200 µl. Cells were aliquoted into 96 well flat bottom tissue culture plates and covered with a plate seal before being left to recover at 37ºC in 5% CO_2 for 18 hours.

2.2.8 Statistical analysis

Statistical analysis carried out for this thesis was conducted using GraphPad Prism Version 7 or 7.02. The specific tests performed for the analysis of data collected are detailed in relevant chapters, at the end of the methods section.

Chapter 3

Impact of age on the distribution and function of plasmacytoid and myeloid dendritic cells

3.1 Introduction

The increased susceptibility to infection with increased ageing, such as that demonstrated between 2003 and 2015 where increased deaths, particularly of the very old, coincided with a peak in hospital admissions and intensive care for influenza (Wells, 2016). Since, antigen presentation is of crucial importance for protection against infection, respiratory or otherwise, and DCs are a major initiator of primary immune responses by presenting unique antigen to naïve T cells (Uyemura et al., 2002), this cell type is therefore of interest with regard to ageing.

3.1.1 Effect of ageing on DC numbers

Whether DCs continue to be produced with increasing age could be questioned since it has been shown that with increasing age there are changes in the numbers of both mDCs and pDCs (Della Bella et al., 2007; Jing et al., 2009; Pérez-Cabezas et al., 2007; Shodell and Siegal, 2002), though the findings thus far are inconsistent, with some observations of no differences between age groups (Agrawal et al., 2007). To date different antibody combinations have been applied to surface stain immune cells (within whole blood or PBMCs), using lineage negative staining in combination with positive staining for HLA-DR, CD11c and CD123 (Della Bella et al., 2007; Jing et al., 2009; Shodell and Siegal, 2002), or the use of specific markers for DCs of CD1c and CD303 (Pérez-Cabezas et al., 2007). In addition, whole blood or isolated PBMCs have been used as a starting material for analysing DCs, with DC counts determined using a combination of flow cytometry and leukocyte cell counts using flow count fluorospheres (Pérez-Cabezas et al., 2007) or haemocytometers (Ciaramella et al., 2011; Della Bella et al., 2007). The lack of consistency and standardisation in methodology may explain the inconclusive observations. However, since DCs are crucial for the generation of adaptive immune responses, it seems plausible that there may be an effect on DCs with increasing host age which would influence downstream T and B cell responses.

3.1.2 Effect of ageing on DC function

The effect of host ageing on the production of efficient T cell responses by DCs has shown that T cells and DCs from aged mice resulted in reduced proliferative

responses in mixed leukocyte reactions (You et al., 2013a) and adoptive transfer experiments where T cells from old transgenic mice (20–24 months) transferred to young mice (2–4 months) (source of APCs) showed significantly greater (3-fold; $p<0.01$) T cell proliferation compared to old mice, though there were still impairments since T cells from young transgenic mice transferred to young mice demonstrated 7.4-fold higher recovery of T cells ($p<0.01$) (Pereira et al., 2011). This suggests that host age is more influential than whether T cells are from a young or old host on APCs, in terms of their ability to induce T cell proliferation. Measurement of T cell proliferation using carboxy fluorescein succimidyl ester (CFSE) and IFN-γ production both showed ~50% reduction, when using an *in vitro* model of HLA-A2+ restricted, influenza matrix protein ($M1_{58-66}$) specific T cell line, generated from PBMCs from a healthy human donor; after co-culture with HLA-matched influenza infected DCs from old subjects (Liu et al., 2012).

Cytokines are produced by and induce the differentiation of DCs from immature into mature cells to allow interaction with antigen-specific T cells (or B cells) (Blanco et al., 2008). Therefore, the ability of DCs to produce cytokine after antigenic exposure, as well as the quantity of the cytokines produced is important for DC function, which may be affected by host ageing. TLR stimulation of mDCs and pDCs in PBMCs derived from both elderly (≥65 years) and young (21–30 years) healthy subjects induced less IL-6, TNF-α and IL-12(p40) (Panda et al., 2010), which was also observed when the experiment was repeated using DCs enriched from PBMCs, along with reduced IFN-α. While this finding does not fit with the "inflamm-aging" theory, the observation that unstimulated PBMC samples showed highly statistically significant increases ($p<0.0001$) in IL-12p40, TNF-α and IL-6 secretion by mDCs, and TNF-α and IFN-α by pDCs from older subjects when compared to younger subjects, suggests an underlying level of inflammation prior to TLR ligand exposure in the elderly (Panda et al., 2010). Similarly the percentage of DCs expressing IL-12 upon LPS stimulation was lower in whole blood samples from elderly than young subjects (Della Bella et al., 2007), in addition to reductions in IFN-α production by pDCs within PBMCs, and IL-6 and TNF-α by isolated pDCs upon stimulation, comparing elderly to young subjects (Jing et al., 2009).

However, enhanced secretion of TNF-α, IL-12p70 and CXC chemokine ligand 10 (CXCL10) has also been observed by MoDCs generated from aged subjects infected with *Chlamydophila pneumoniae* (Cpn) (Prakash et al., 2014), with similar findings when human DNA, isolated from human blood (as a model self-antigen), was delivered intracellularly to MoDCs, from blood of elderly subjects (65–90 years) with

increased secretion of IL-6 and IFN-α in cell culture supernatants (Agrawal et al., 2009). Significant increases in IL-6 and TNF-α ($p<0.01$) secretion were observed after LPS stimulation of MoDCs (Agrawal et al., 2007) and peripheral blood mDCs ($p<0.05$) from elderly subjects (60–80 years) (Janssen et al., 2015), compared to DCs from young subjects (20–35 years), in addition to significant reductions in IL-10 (~1.5 fold), IFN-λ (~1- fold) and IFN-α (~2-fold) secretion by MoDCs after stimulation with Cpn (Prakash et al., 2014). These data imply that DCs from the elderly have an enhanced ability to secrete pro-inflammatory mediators while secretion of anti-inflammatory mediators is inhibited, additionally TLR4 responses of these elderly individuals appears intact (Janssen et al., 2015). However, not all of the studies to date confirm this and many, discussed here, have observed reduced secretion of TNF-α, IL-6 and IL-12 (Della Bella et al., 2007; Jing et al., 2009; Panda et al., 2010) so there are inconsistencies within the current literature of cytokine secretion by DCs from elderly compared to young subjects; with different source of cells used within these experiments providing a potential cause for the differing results.

3.1.3 Effect of age on adipokine production by PBMCs

Since ageing is associated with increased risk of disorders and diseases such as atherosclerosis, CVD and T2D, the possibility that adipokines may play a part in the ageing process, via the response of APCs or T cells to stimulation, is of interest. Thus, other cytokines of interest with regards to ageing are of adipokine origin and include resistin, leptin, adiponectin, adipsin, monocyte chemoattractant protein 1 (MCP-1) and retinoic binding protein 4 (RBP4). Resistin is thought to originate from fat-infiltrating immune cells, and since induction of resistin secretion has been observed as a result of LPS treatment of human macrophages, which was further induced by TNF-α treatment and addition of neutralising antibodies to TNF-α, IL-6 and IL-1β (Lehrke et al., 2004). This suggests that endotoxin induced resistin secretion is mediated by the secretion of inflammatory cytokines and that the low grade inflammatory state, often observed in ageing may enhance resistin secretion further upon exposure to inflammatory stimuli.

3.1.4 Gaps in knowledge; variation in methodology and inconclusive results

Since the studies carried out to date have resulted in inconsistent findings, likely as a result of the variation in the methodologies used, direct comparisons are difficult. The aim of this work was to use the present commonly agreed method by the Nomenclature Committee of the International Union of Immunological Societies (Ziegler-Heitbrock et al., 2010) for identification of DC subsets and to use the most robust method of detecting DC subsets in terms of sample choice, choice of antibody

panel and method of cell counting to provide robust data which can aid in clarifying the effect of age on DC subsets. Since, a decline in DC numbers with age could impact on antigen presentation this chapter also investigated the impact of changes in DC subsets on cytokine production by stimulating DCs, *ex vivo*.

3.1.5 Rationale for choice of cytokines to investigate

IL-8 was found to be the predominant cytokine produced upon TLR stimulation of CD1c-DCs (Piccioli et al., 2007) and IL-1β is secreted as a result of antigen specific DC:$CD8^+$ T cell interactions during antigen presentation (Gardella et al., 2000). Significant increases in IL-6 secretion (up to 2-fold greater) by DCs have been observed in numerous studies with increasing age of subjects (Agrawal et al., 2007; Agrawal et al., 2009; Janssen et al., 2015; Prakash et al., 2014), while others have shown significant reductions (up to 2-fold lower, depending on stimulus) in secretion with increasing age of the host (Panda et al., 2010). Additionally, IL-6 and IL-1β are components of inflammaging (Ostan, 2008; Salvioli et al., 2013). These cytokines (IL-6, IL-8 and IL-1β) were investigated in terms of secretion by DCs, comparing samples derived from young and elderly subjects, within this thesis. Additionally, since IL-8, MCP-1 and resistin have been associated with increased age (Lee et al., 2007) and IL-6, TNF-α and IL-1β secretion have been observed with age-associated conditions such as atherosclerosis and T2D (Pickup, 2004; Spranger et al., 2003), but the immune role is less well understood, secretion of a panel of adipokines was assessed from PBMC samples isolated from young and elderly subjects.

3.1.6 Aims and objectives

Are DCs effected numerically or functionally with increasing age? Also, do PBMCs of elderly subjects produce more adipokines? To answer these questions the following objectives were undertaken:

Objective 1: Determine absolute numbers of mDCs and pDCs within peripheral blood extracted from young (18–40 years) and elderly (65–79 years) subjects using multiparameter flow cytometry

Objective 2: Determine cytokine response of PBMCs of young and elderly donors to TLR stimulation.

3.2 Methodology

3.2.1 Recruitment of subjects

Study subjects were recruited onto either the Im-AGE (18–40 years) or Nu-AGE (65–75 years) studies; details provided in Chapter 2 in sections 2.2.1, and 2.2.2, respectively. The inclusion and exclusion criteria used to select suitable study participants can be found in Tables 2.3, and 2.4, respectively.

3.2.2 Blood dendritic cell enumeration

The protocol was carried out as detailed in the manufacturers' instructions with whole blood (Blood Dendritic Cell Enumeration kit, human; 130-091-086, Miltenyi Biotec) using the mAbs CD1c-PE (Clone: AD5-8E7), CD303-FITC (Clone: AC144), CD14-PE-Cy5, CD19-PE-Cy5 and CD141-APC (Clone: AD5-14H12). As controls, isotype matched mouse IgG2a-PE, IgG1-FITC, IgG1-APC, Ig-G2a-PE-Cy5 and IgG1-PE-Cy5 antibodies were used. Dead cell discriminator was also included. After incubation on ice under a 60 W light bulb for 10 minutes, red blood cells were lysed using 1x red blood cell lysis solution (details in Chapter 2, Table 2.1) for 10 minutes in the dark, at 21ºC. Following two washing steps, by centrifugation at 300 x g for 5 minutes, samples were fixed with 3.7% formaldehyde in PBS and resuspended in 600µL flow cytometry buffer (details in Chapter 2, Table 2.1). 100 µL of Flow-count™ fluorospheres were added to each tube prior to acquisition for an accurate absolute count of leukocytes (Flow-Count™ Fluorospheres, 7547053, Beckman Coulter). Data was acquired on the Beckman Coulter Cytomics FC500 MPL (100 µL sample/ run), and the Sony EC800 (150 µL sample/ run), so that back-up data was available, a minimum of 1000 events were acquired within the flow count fluorosphere gate, and a minimum of 100,000 events were acquired within the cell gate, which excluded debris from analysis.

Single-stained compensation controls, prepared using Ultracomp ebeads (Affymetrix eBioscience), and unstained control samples were run on both cytometers to allow for manual compensation to be applied to all sample data acquired. Acquired data was analysed using FlowJo™ software (TreeStar, San Carlos, CA), Version 10. The gating strategy (Figure 3.1) for Im-AGE samples was consistent with that set out by Miltenyi-Biotec.

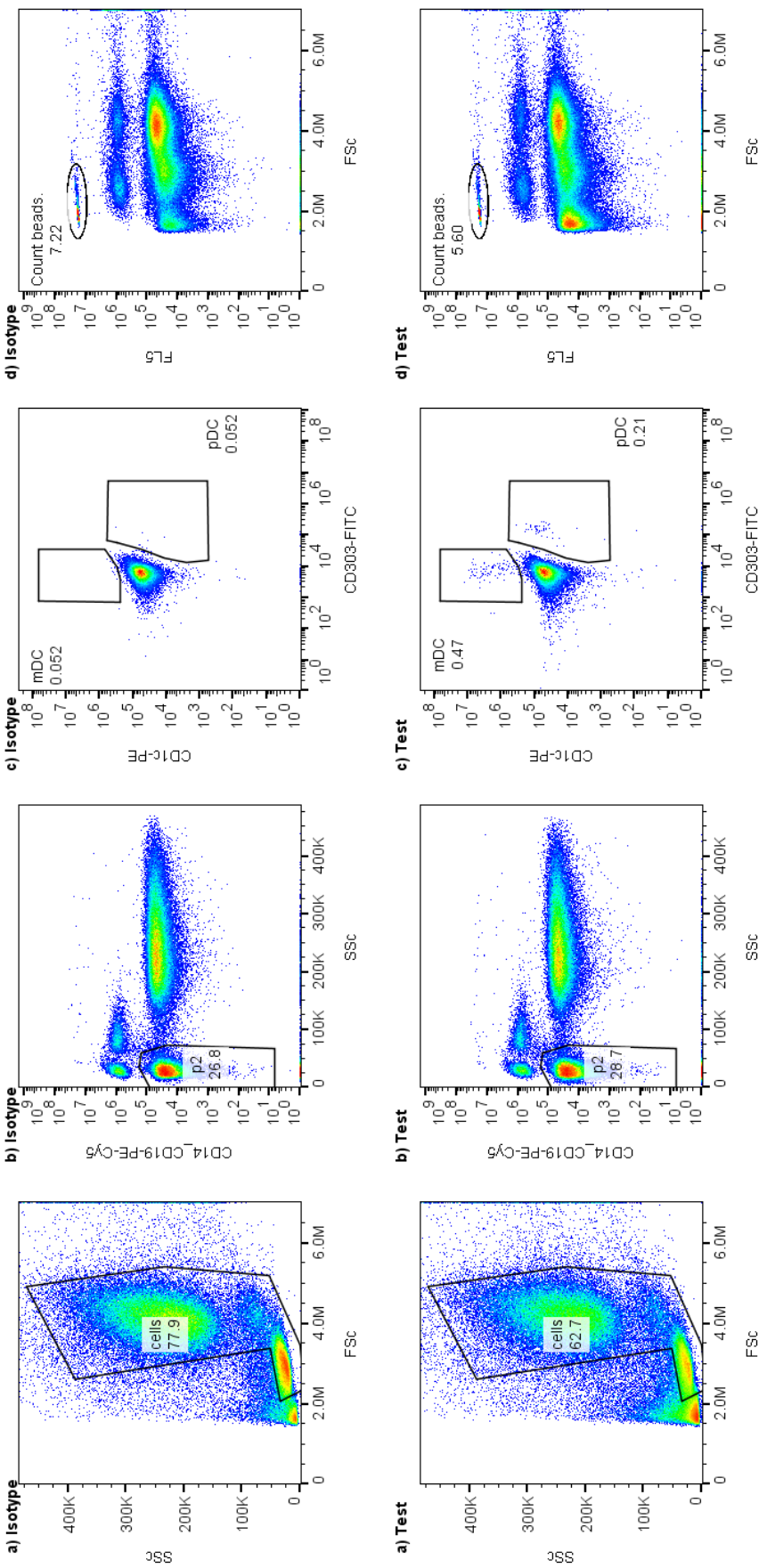


Figure 3.1 Electronic gating strategy applied to all samples using FlowJo™ V.10.

Figure 3.1 Electronic gating strategy applied to all samples using FlowJo™ V.10. Whole blood was stained with the Miltenyi Biotec DC enumeration Test cocktail (CD1c-PE, CD303-FITC, CD14-PE-Cy5, CD19-PE-Cy5 and CD141-APC) and with the control cocktail (Mouse IgG2a-PE, Mouse IgG1-FITC, Mouse IgG1-APC, CD14-PE-Cy5 (isotype: Mouse IgG2a) and CD19-PE-Cy5 (isotype: Mouse IgG1)), followed by dead-cell discriminator (DCD). After activation of the DCD, the red blood cells were lysed and the washed cells are fixed with 3.7% formalin before running on the Sony EC800 iCyt Flow Cytometer. a) Acquired data were gated to exclude debris and any remaining red blood cells; cells gate, b) cells within cells gate were gated to exclude B cells and granulocytes as well as dead cells; gate P2, c) gate P2 allowed detection of cells positive for CD303-FITC, gate pDC, and cells positive for CD1c-PE, gate mDC, d) count beads were gated to ascertain the absolute number of flow count fluorospheres detected, for use in calculating the absolute total counts of mDCs and pDCs.

3.2.3 Calculation of mDC and pDC subset counts

For each individual sample the absolute number per µL of blood of the pDC and mDC subsets was calculated, by subtracting the number of cells in the DC gate for the isotype control, from the count in the DC gate for the test sample. This number, for each subset, was then divided by the count of Flow-count™ fluorospheres and multiplied by the assayed concentration of the Flow-count™ fluorospheres; calculated by the manufacturer and provided with each new lot of beads.

3.2.4 Functional analysis of blood DCs

PBMC stimulation

Frozen PBMCs were thawed in a 37 ºC water bath and washed in an excess of thaw media (90% RPMI 1640 and 10% heat-inactivated FBS) to remove DMSO from cells, within 20 minutes of thawing. Cells pellets were resuspended in tissue culture media supplemented with 2mM L-glutamine, 100 U penicillin and 0.1 mg/ml streptomycin (details in Chapter 2, Table 2.1). Cells were counted using the viability stain, trypan blue (Sigma Aldrich, T8154), and resuspended to give a final concentration of 0.5 x 10^6 cells / 200 µl. Cells were aliquoted into 96 well flat bottom tissue culture plates (Sarstedt) and covered with a plate seal before being left to recover at 37ºC in 5% CO_2 for 18 hours.

PBMCs were then incubated with 1 µg/ml LPS (Ultrapure LPS, E. coli 0111:B4; Invivogen) and 2.5 µg/ml R848 (Imidazoquinoline compound, Invivogen), in the presence or absence of 2 µM monensin (Sigma Aldrich, M5273) for 3 hours. Unstimulated controls were incubated with fresh tissue culture media, with or without 2 µM monensin. Supernatants were stored at -80°C prior to analysis.

Intracellular staining

The mAbs for the surface markers CD1c-PE (Clone: AD5-8E7), CD303-PE (Clone: AC144), CD304-PE (Clone: AD5-17F6), (all from Miltenyi-Biotec), CD3-FITC (Clone: UCHT1), CD16-FITC (Clone: 3G8), (Becton Dickinson), CD14-FITC (Clone: TÜK4), CD19-FITC (Clone: LT19), HLA-DR-Alexa Fluor 700 (Clone: L243) (all from Biolegend) and Fc Receptor block (Miltenyi-Biotec) were added and incubated with the samples for 25 mins, at 4ºC in the dark. The corresponding isotype controls IgG1-FITC (Biolegend), IgG2a-PE, IgG1-PE (Miltenyi-Biotec) and IgG2-AF700 (Biolegend) were also used.

Cell membranes were fixed and cells permeabilised using Leucoperm cell fixation and permeabilisation reagents (AbD Serotec) then stained for intracellular cytokines

with the mAbs IL-1β-PE-Cy7 (Clone: JK1B-1), IL-6-Pacific Blue (Clone: MQ2-13A5) and IL-8-Alexa-Fluor-647 (Clone: E8N1), and the corresponding isotype controls PE-Cy7-Mouse IgG1 and Alexa-Fluor-647-Rat IgG1 were also used (all from Biolegend). Cells were incubated for 30 minutes at 21ºC, in the dark, washed thoroughly with PBS supplemented with 0.5% FBS then resuspended in flow cytometry buffer containing 1% formalin. Data was acquired on the BD LSR Fortessa cytometer. Spectral overlap that occurred between channels was manually compensated in FlowJo™ software Version 10, after measurement of single-stained compensation controls prepared using Ultracomp ebeads (Affymetrix eBioscience) and unstained control samples. Data was analysed using FlowJo™ software Version 10 according to the gating strategy detailed in Figure 3.2.

3.2.5 Multiplex immunoassay of culture supernatants

Supernatants were thawed on ice and analysed by Multiplex immunoassay (LEGENDplex Human Adipokine panel, Biolegend) to assess overall cytokine and chemokine secretion from the PBMCs; targeting IL-8, IL-1β, IL-6, MCP-1, TNF-α, leptin, IL-10, adiponectin, adipsin, IFN-γ, IP-10 (CXCL10), RBP4 and resistin secretion. The assay was performed at 21ºC in a 96-well V-bottom microplate (Greiner-Bio), according to manufacturers' instructions (Figure 3.3), using cell culture supernatant, previously frozen at -80 ºC. The standard provided within each kit was serially diluted (1:4) six times after the top standard (neat standard); in addition to assay buffer (zero standard). To perform the assay, 25 µL of assay buffer was added to all wells, 25 µL of diluted standard was added to standard wells and 25 µL of sample (cell culture supernatant) was added to sample wells, followed by 25 µL pre-mixed beads and 25 µL detection antibodies to all wells. The plate was incubated in the dark for 2 hours on a plate shaker, 1000 rpm. After the incubation 25 µL of Streptavidin-PE conjugated beads were added to all wells, followed by a 30 minute incubation on the plate shaker, 1000 rpm. The plate was centrifuged at 1000 x g for 5 minutes, after removing the supernatant 200 µL wash buffer was added before a further centrifugation step. Each well was resuspended in 300 µL wash buffer before acquiring data on the flow cytometer (BD Fortessa X-20); mean fluorescence intensity (MFI) for the top standard was kept consistent between every experiment.

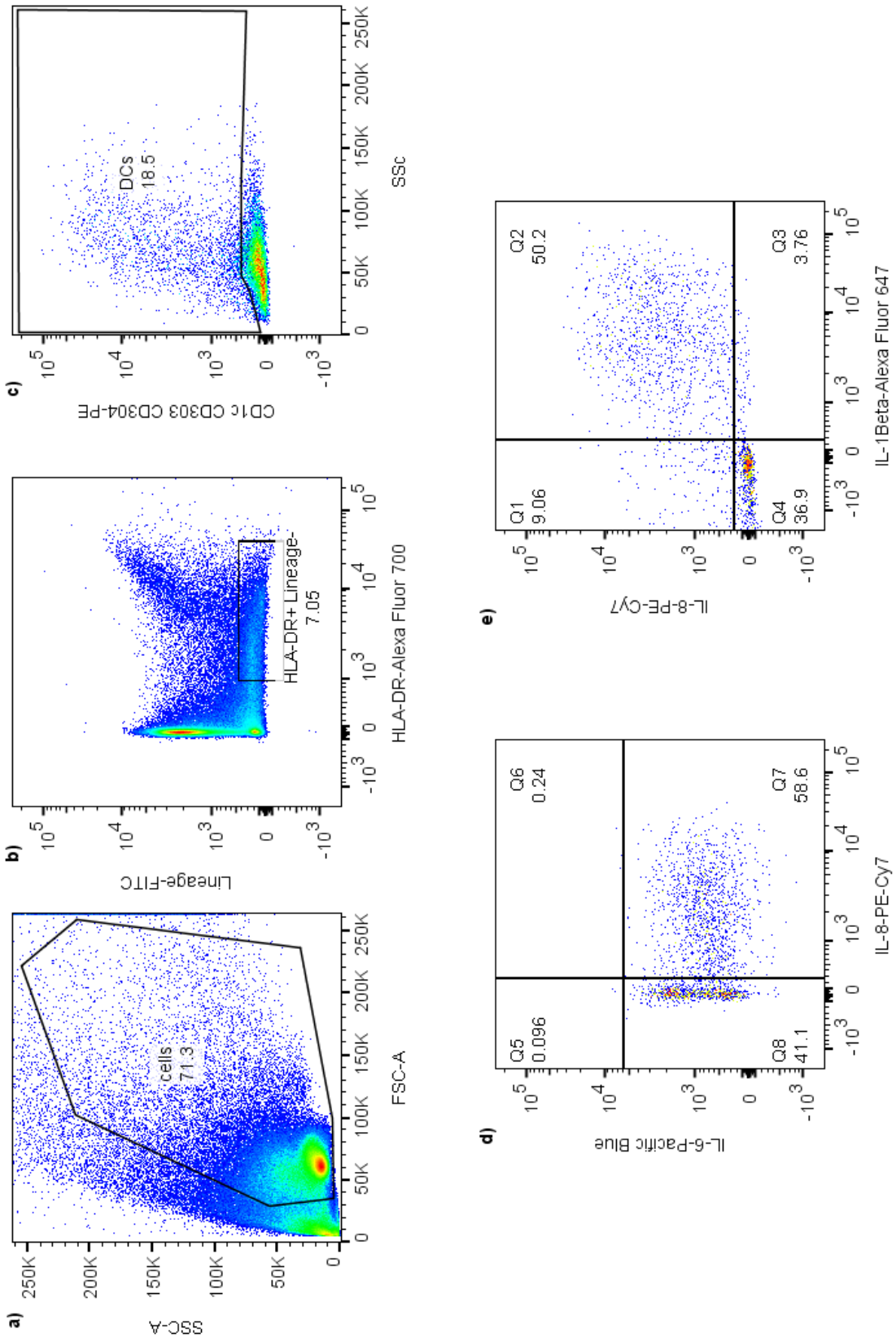


Figure 3.2 Gating strategy applied to PBMC samples to identify DCs producing IL-6, IL-8 and IL-1β.

Figure 3.2 Gating strategy applied to PBMC samples to identify DCs producing IL-6, IL-8 and IL-1β. After 3 hour stimulation with or without LPS and R848, peripheral blood mononuclear cells (PBMCs) were surface stained with the monoclonal antibodies CD1c-PE, CD303-PE, CD304-PE, CD3-FITC, CD16-FITC, CD14-FITC, CD19-FITC, HLA-DR-Alexa Fluor 700 to allow identification of DCs, and Fc Receptor block. Intracellular cytokine staining was performed, after permeabilisation of the cell membranes, with the intracellular monoclonal antibodies IL-1β-PE-Cy7, IL-6-Pacific Blue and IL-8-Alexa-Fluor-647. Fixed samples were run on the BD LSR Fortessa and at least 100,000 events were acquired in the cell gate, data were analysed using FlowJo™ software version 10. a) FSc versus SSc plot allowed cells to be gated and debris to be excluded based on light scattering properties of cells, b) Lineage (FITC) versus HLA-DR (Alexa Fluor 700) plot excluding lineage$^+$ cells (T cells, B cells, monocytes) and gating on HLA-DR$^+$ MHC-II expressing cells, c) CD1c, CD303, CD304 (PE) versus SSc to gate only HLA-DR$^+$ cells that expressed the specific DC markers, d) IL-6 (Pacific Blue) versus IL-8 (PE-Cy7) identified DCs that expressed these cytokines singly or in combination, e) IL-8 (PE-Cy7) versus IL-1β (Alexa Fluor 647) identified DCs that expressed these cytokines singly or in combination.

Acquired data were analysed using the Biolegend LEGENDplex software. Briefly, standard curves are produced for each of the 13 analytes, these standard curves allowed the concentrations of each analyte, within the samples measured, to be determined. This was based on mean fluorescence intensity (MFI) values which related to known concentrations for serial dilution of the standards. The standard curves also provided validation for each experiment. Inter-plate variability was determined for experiments carried out on different days.

3.2.6 Statistical analysis

Subject demographics of young and elderly subjects (at baseline) were compared using Mann Whitney U test, after carrying out D'Agostino & Pearson normality tests, using GraphPad Prism Version 7.

Blood mDC and pDC phenotypes were compared between elderly and young volunteers using a Welch-Satterthwaite t-test on rank transformed data. For mDC, the size of the difference (Hodges Jr and Lehmann, 1963) and the 95% confidence intervals (Moses, 1965) were estimated for a Wilcoxon Mann Whitney U test.

GraphPad Prism Version 7 was used to determine the differences in proportions and cell counts of DCs which were positive for IL-6, IL-8 and IL-1β secretion between young and elderly subjects using Mann-Whitney U tests, after performing a D'Agostino & Pearson normality test to determine non-gaussian distribution. One-way analysis of variance (ANOVA) using the Kruskal-Wallis test with Dunn's multiple comparisons *post-hoc* test was used to identify any differences in proportions of single and double positive cells.

GraphPad Prism Version 7 was used to determine inter-plate variability between multiplex immunoassays using repeated-measures ANOVA. Differences in concentration of each analyte between unstimulated and stimulated samples were calculated using two-tailed paired t-tests, differences in concentration change from unstimulated level between young and elderly subjects was determined using two-tailed Mann-Whitney U tests for each analyte.

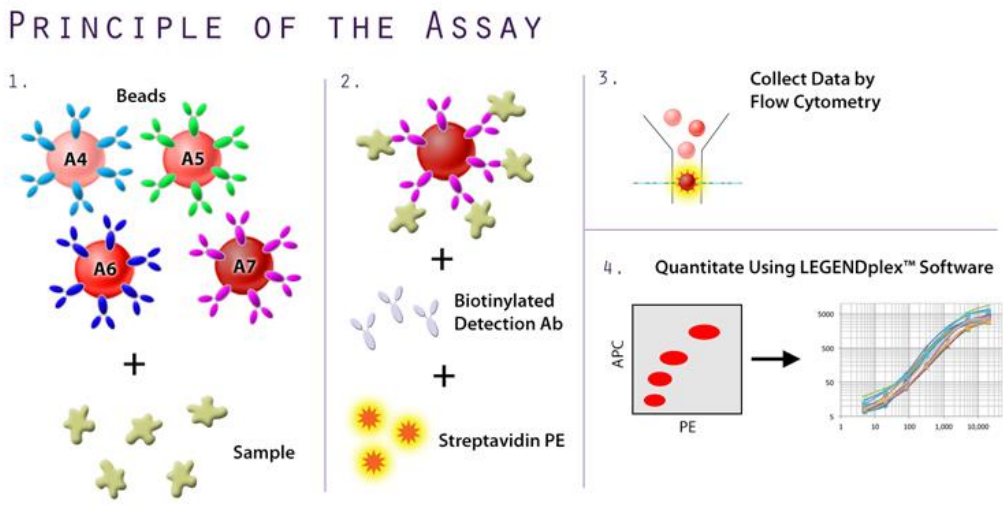


Figure 3.3 Schematic of Biolegend's LEGENDplex multiplex immunoassay; (Biolegend, 2016). The kit comprised two sets of pre-mixed capture beads which were different sizes (set A and B) within which the beads had different levels of APC fluorescence. Each bead within the set bound to a specific analyte. Upon incubating samples with the pre-mixed beads any analyte present would bind to the beads. Biotinylated detection antibody would bind to its specific analyte bound to the capture beads. Addition of streptavidin PE provided the fluorescent signal which was detected by the flow cytometer, the intensity of the signal indicated the proportion of analyte bound. The LEGENDplex software used the mean fluorescence intensity of each analyte detected within each sample, with the standard curves based on the serial dilution of the standard, to calculate concentrations in pg/ml of each analyte within each sample.

3.3 Results

3.3.1 Subject demographics

Mean ages of the two groups were 30.71 years for the young cohort and 70.33 years for the elderly cohort (Table 3.1). In both groups there was a greater number of female than male participants, however, this was not significantly different. The mean weight and BMI of participants between groups was similar though the upper range of weights of the elderly cohort was greater than for the young group. When considering height there was a significant difference between the young and elderly groups, with the elderly having a lower mean value, which is of importance because losing height with age would have implications on BMI.

3.3.2 Numerical analysis of blood DC subsets

To obtain an accurate comparison of DC subset counts between the two age groups, with minimal sample processing whole blood was used and an antibody panel comprising the blood DC specific markers CD1c and CD303, excluding CD14 and CD19 positive monocytes/macrophages and B cells, respectively.

Comparing the young to the elderly cohort the mDC phenotype, using a Welch-Satterthwaite t-test on rank transformed data, there was sufficient evidence to reject the null hypothesis of equal cell counts in each group. The data showed that the young cohort had greater numbers of mDCs compared to the elderly cohort, with the difference estimated to be 0.4831 (0.1622, 0.7932; 95% Confidence interval [CI]) with a corresponding significance level of p=0.0043, Figure 3.4a. When mDC counts were plotted against age, Figure 3.4b, a trend for reduced cell numbers with increasing age was seen.

However, when comparing the pDC phenotype between the young and elderly cohorts there was insufficient evidence to reject the null hypothesis of no difference between the cell counts in each group at the 5% significance level (P=0.3108), Figure 3.5. Additionally, upon comparison of the mDC: pDC ratio between peripheral blood samples taken from the young cohort and the elderly cohort, there was no significant difference between the two groups; indicated by a significance level of P=0.2078, Figure 3.6.

		Im-AGE (n=45)	Nu-AGE (n=122)	p value
Age (years)	Mean (SD)	30.71 (6.36)	70.33 (4.16)	<0.0001
	Range	20–40	65–79	
Gender	% Female	57	61	0.8589 (ns)
Weight (kg)	Mean (SD)	74.46 (6.36)	73.27 (13.85)	0.6676 (ns)
	Range	53.98–108.0	49.50–128.50	
Height (cm)	Mean (SD)	169.30 (0.10)	165.70 (9.16)	0.0311
	Range	147.30–191.00	145.60–188.20	
BMI (kg/m^2)	Mean (SD)	26.13 (3.66)	26.62 (3.96)	0.7237 (ns)
	Range	19.86–34.15	18.50–43.20	

Table 3.1 Baseline anthropometric data for Im-AGE and Nu-AGE participants. SD = Standard deviation, ns= not statistically significant**.**

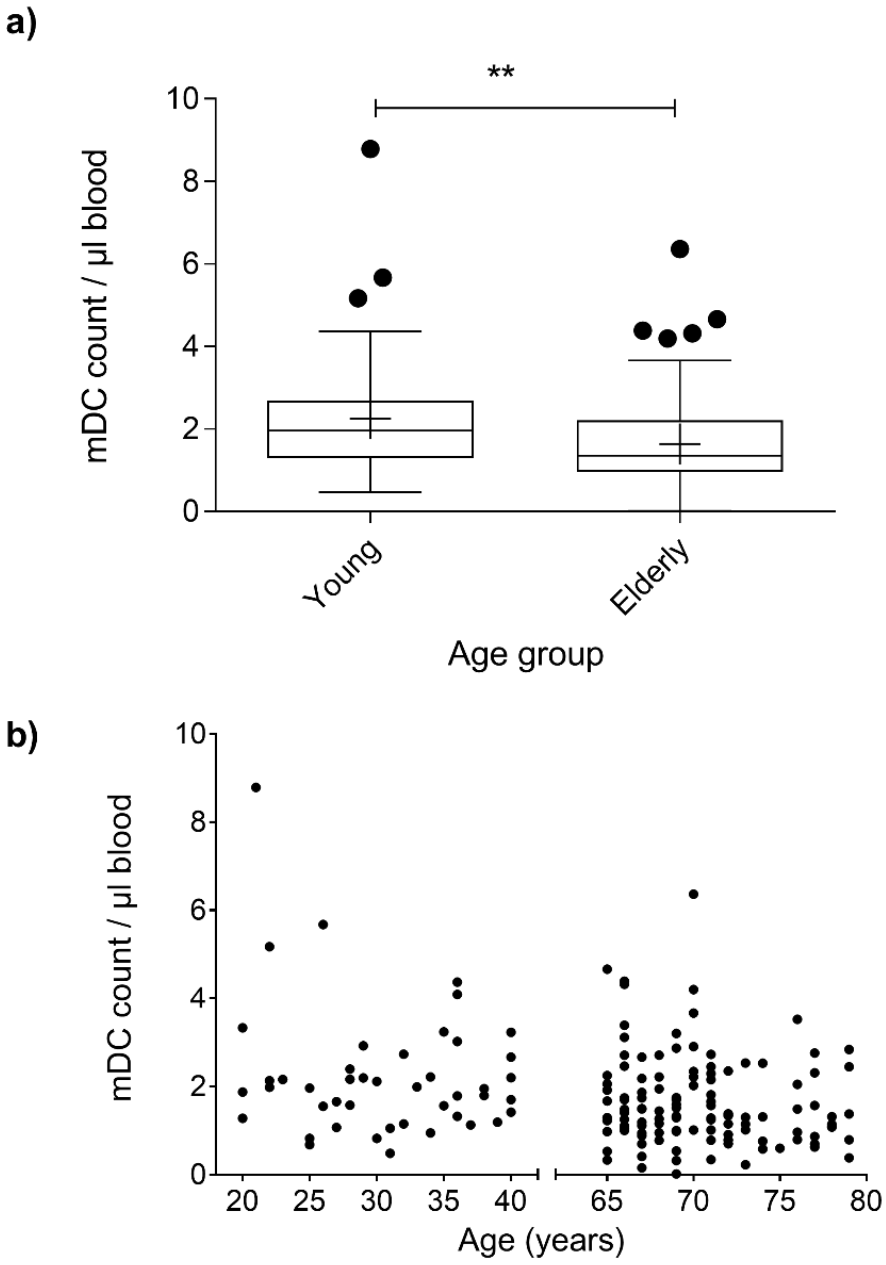


Figure 3.4 mDC counts for the Im-AGE (young) and Pre-intervention Nu-AGE (elderly) cohorts. Whole blood was stained with antibodies reactive with CD1c to identify mDCs and to CD14 and CD19 to exclude CD14+ monocytes and CD19+ B cells, of which a high proportion express CD1c. N=45 in the young cohort and n=120 in the elderly cohort. a) Shows box and whisker plot of mDC counts for young and elderly subjects extending from the 25th to the 75th percentiles with the line through the box representing the median and plus (+) representing the mean value. Whiskers were determined using Tukey's method using the 25th and 75th percentile plus 1.5 times the interquartile range (IQR) as the end of the whiskers. Dots represent individual participants where the values fell above the 25th or 75th quartile plus 1.5 times the IQR. Welch-Satterthwaite t-test on rank transformed data was used to determine the presence of differences between the young and elderly cohorts; significance assumed at $p < 0.05$, ** $p < 0.01$, $p = 0.0043$. b) Scatter plot of the entire dataset (young and elderly subjects) showed mDC counts per µL blood as age increases.

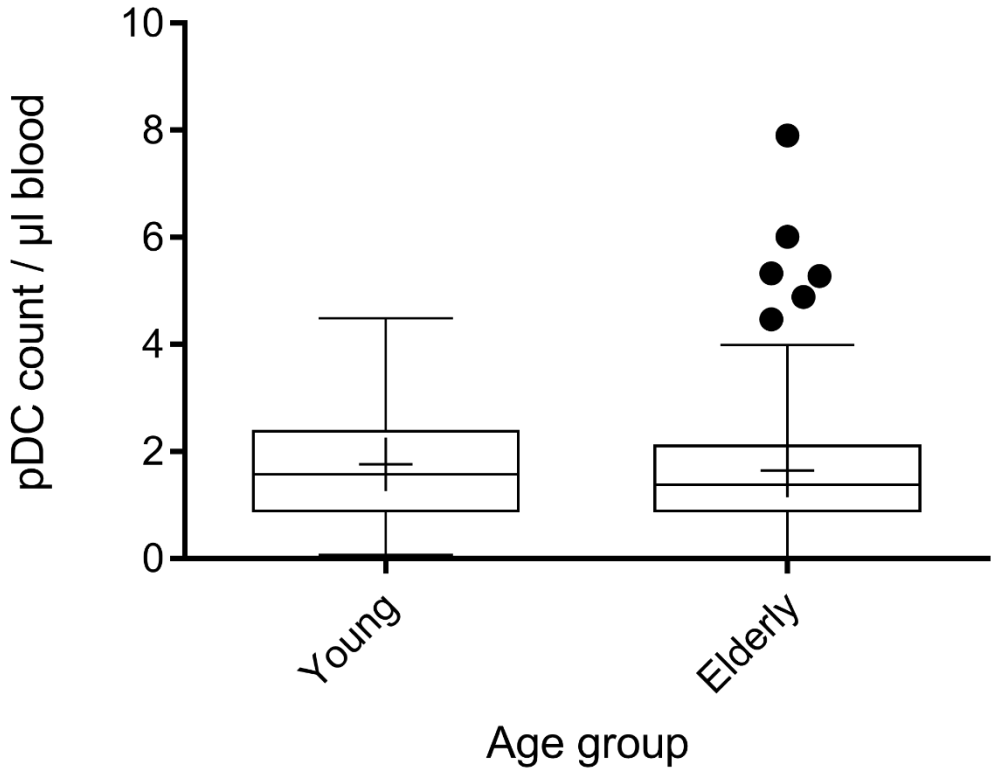


Figure 3.5 pDC counts for the Im-AGE (young) and Pre-intervention Nu-AGE (elderly) cohorts. Box and whisker plot of pDC counts for young and elderly subjects extending from the 25th to the 75th percentiles, the line through the box representing the median and plus (+) representing the mean value. Whiskers were determined using Tukey's method using the 25th and 75th percentile plus 1.5 times the interquartile range (IQR) as the end of the whiskers. Dots represent individual participants where the values fell above the 25th or 75th quartile plus 1.5 times the IQR. Welch-Satterthwaite t-test on rank transformed data was used to determine the presence of differences between the young and elderly cohorts; significance assumed at $p < 0.05$, $p = 0.3108$. N=45 in the young cohort and n=120 in the elderly cohort.

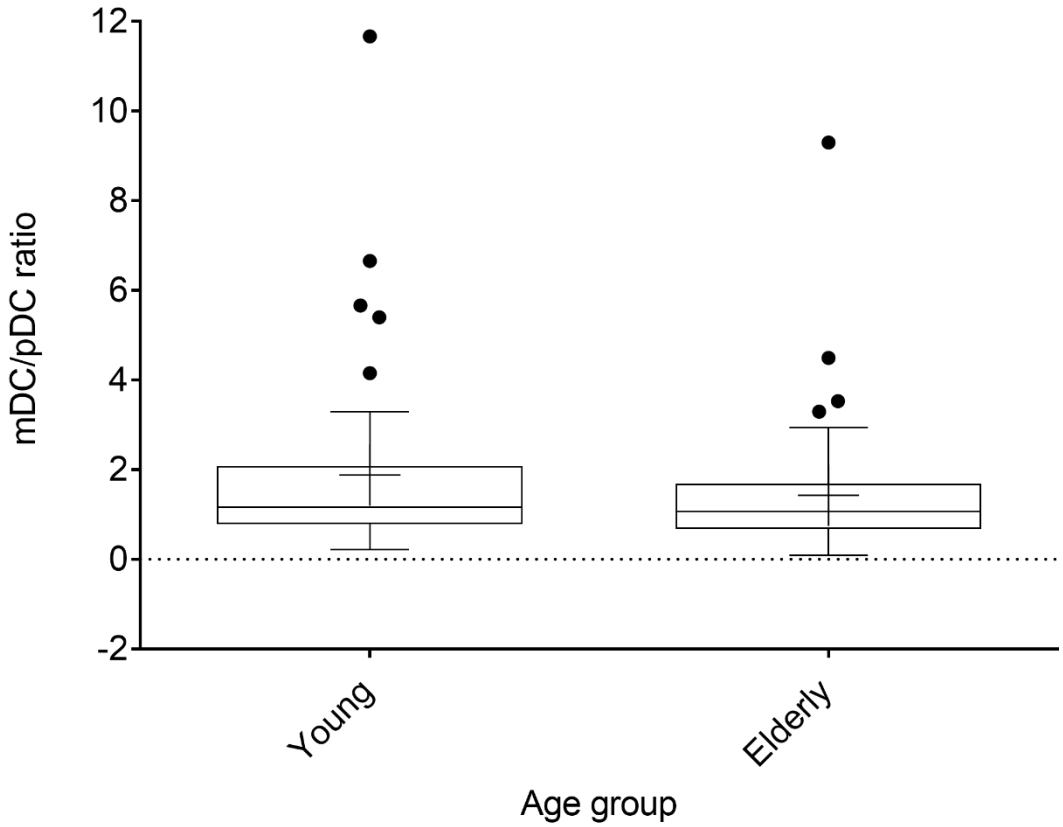


Figure 3.6 mDC: pDC ratio for the Im-AGE (young) and Pre-intervention Nu-AGE (elderly) cohorts. Box and whisker plot of mDC: pDC ratios for young and elderly subjects extending from the 25th to the 75th percentiles, the line through the box representing the median and plus (+) representing the mean value. Whiskers were determined using Tukey's method using the 25th and 75th percentile plus 1.5 times the interquartile range (IQR) as the end of the whiskers. Dots represent individual participants where the values fell above the 25th or 75th quartile plus 1.5 times the IQR. Welch-Satterthwaite t-test on rank transformed data was used to determine the presence of differences between the young and elderly cohorts; significance assumed at $p < 0.05$, $p = 0.2078$**.** N=45 in the young cohort and n=120 in the elderly cohort.

3.3.3 Functional analysis of blood DCs

DC cytokine secretion

Intracellular staining (ICS) is a robust method for observing cytokine production by individual cell populations within a complex multicellular sample that obviates the need for further manipulation for isolation of cells of interest (Freer and Rindi, 2013; Pala et al., 2000). As the study samples were collected over the period of two and a half years it was not possible or appropriate to use fresh PBMCs for these experiments, therefore isolated PBMCs were cryopreserved prior to analysis. The median cell count post-thawing was 6.46 x10^6 cells, and the estimated recovery range was 2–85% of those stored. However all samples were resuspended at a final concentration of 0.5 × 10^6 / 200 µl to ensure that the same number of live cells was used in each culture.

PBMCs were surface stained to distinguish DCs and intracellularly stained with anti-IL-8, IL-6 and IL-1β antibodies. Comparing DCs from young and elderly subjects showed that the proportion of IL-8^+ and IL-$1\beta^+$/IL-8^+ double positive cells were significantly greater in the cells derived from young subjects compared to those from elderly subjects; this was apparent both with and without LPS/R848 stimulation (Figure 3.7a and b). The proportion of DCs secreting IL-1β was not significantly different between samples derived from young and elderly subjects (p=0.324). Additionally, a highly significant difference in secretion of IL-8^+/IL-6^- was observed in DCs from young subjects, compared to DCs from elderly subjects (Figure 3.8). While, the significant difference observed in IL-8^+/IL-6^+ DCs in samples derived from young subjects compared to those from the elderly, was only seen after LPS/R848 stimulation (Figure 3.8a and b). The proportion of single positive cells for IL-8 (IL-6^-/IL-8^+) was significantly greater in samples from young subjects, while IL-6 single positive cells were not significantly different between the two groups; this was apparent for both unstimulated and stimulated samples. Further analysis, by one-way ANOVA, identified that there were significant differences in the proportion of IL-$1\beta^-$/IL-8^+ DCs and IL-$1\beta^+$/IL-8^- cells, as well as between IL-8^- IL-6^+ compared to IL-8^+ IL-6^- cells, and IL-8^+ IL-6^+ compared to IL-8^+ /IL-6^- DCs, in LPS and R848 stimulated samples from young subjects. While in the DCs from elderly subjects there were only significant differences observed between proportions of IL-8^+ /IL-6^+ compared to IL-8^+/IL-6^- DCs in LPS and R848 stimulated samples.

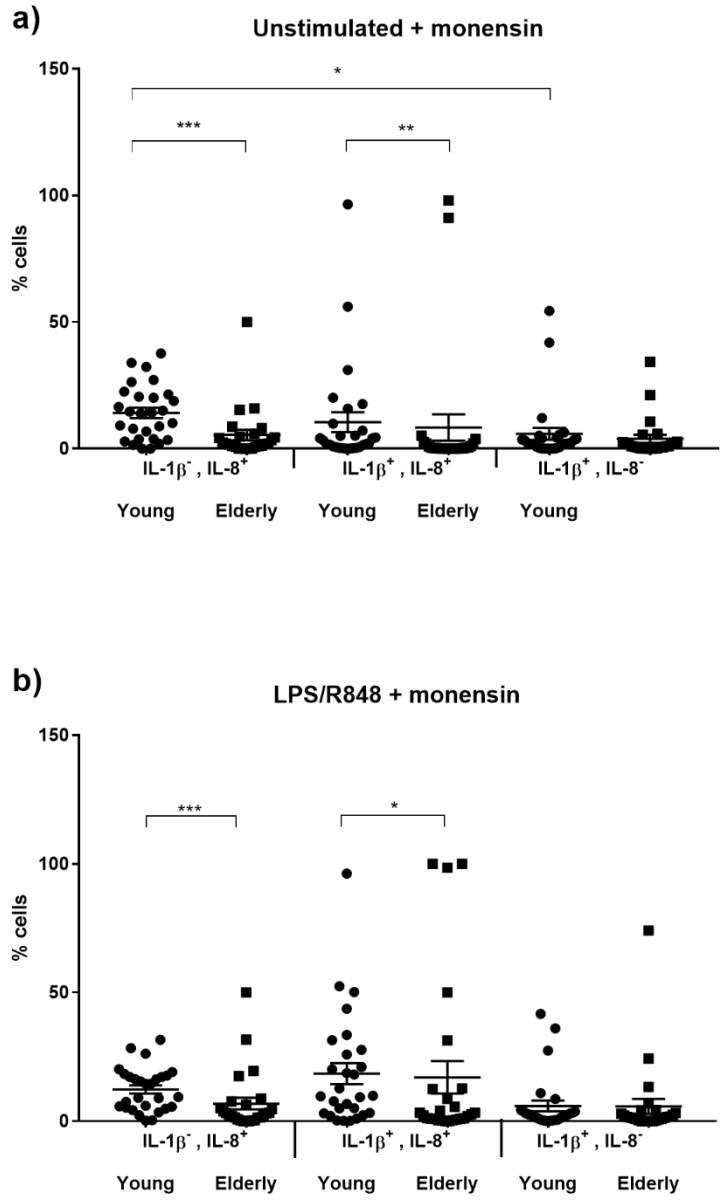


Figure 3.7 Scatter plots showing proportion of cytokine producing DCs from young and elderly subjects. PBMCs were cultured *in vitro* for 3 hours with tissue culture media alone (a) or in media containing LPS and R848 (b) in the presence of 2μM monensin. PBMC samples were subsequently surface stained with monoclonal antibodies against HLA-DR, CD1c, CD303 and CD304, CD14, CD16, CD19 and CD3, permeabilised and stained with anti-IL-1β, IL-6 and IL-8 antibodies. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 indicate significance between age groups as measured by Mann-Whitney U tests, and *post-hoc* analyses, performed by one-way ANOVA using the Kruskal-Wallis test with Dunn's multiple comparisons *post-hoc* test, to identify any differences in proportions between the three cell types by one-way ANOVA.

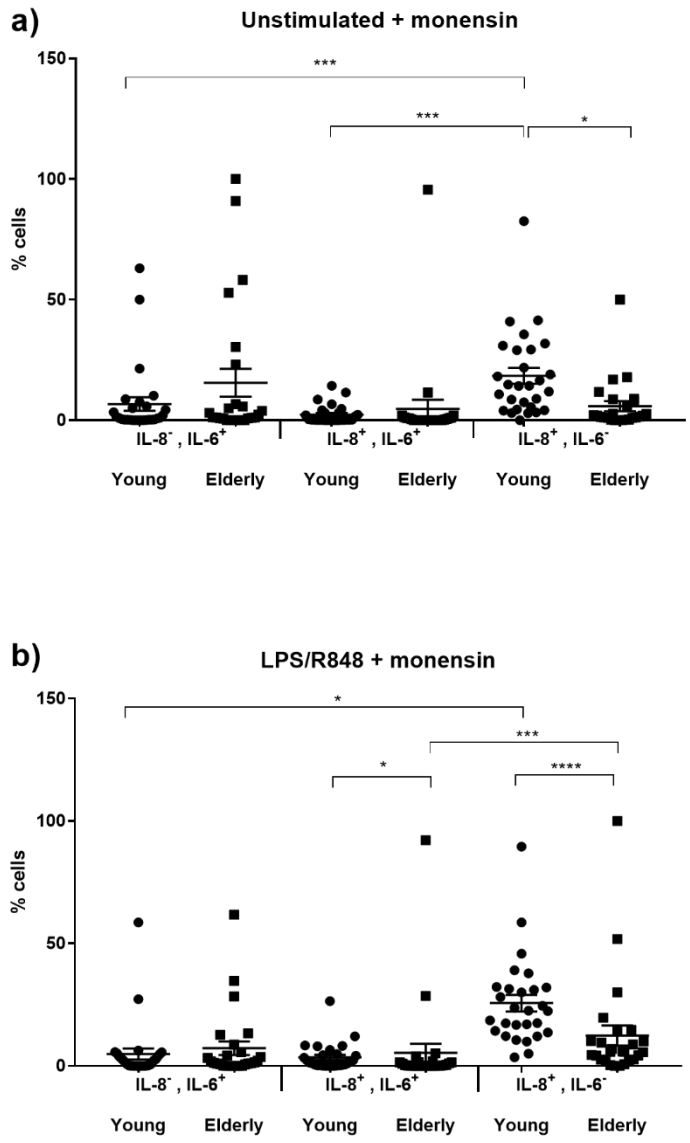


Figure 3.8 Scatter plots showing proportion of cytokine DCs from young or elderly subjects. PBMCs were cultured *in vitro* for 3 hours with tissue culture media alone (a) or in media containing LPS and R848 (b) in the presence of 2µM monensin and stained with anti-HLA-DR, CD1c, CD303 and CD304, CD14, CD16, CD19 and CD3 monoclonal antibodies. After permeabilisation cells were stained with anti-IL-1β, IL-6 and IL-8 monoclonal antibodies. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 indicate significance between age groups as measured by Mann-Whitney U tests, and *post-hoc* analyses, performed by one-way ANOVA using the Kruskal-Wallis test with Dunn's multiple comparisons *post-hoc* test, to identify any differences in proportions between the three cell types by one-way ANOVA.

Total PBMC cytokine secretion

Supernatants were aspirated from pelleted PBMC samples after the three hour incubation and utilised in multiplex bead based immunoassays (LEGENDplex Human Adipokine panel; Biolegend).

Due to the large number of samples analysed and the ability to only process 40 unique samples per plate, it was not possible to avoid a "plate effect" and differences in detection range between plates resulting in different limits of detection for analytes. Therefore, to ensure that any changes observed between groups were independent of the "plate effect", the absolute concentrations for each serial dilution of the standards, which were run on every plate, were compared. Concentrations for all dilutions of the standard were comparable between all plates (Suppl Table 3.1, Appendix IX) and all corresponded to the expected concentration (Figure 3.9 a–m). The co-efficient of variance (CV) for each analyte on each plate (Table 3.2) were all within acceptable limits of less than 5%, highlighting the precision of each dataset. RBP4 was not detectable on plate one due to degradation of the beads as a result of a delay in running the plate due to breakdown of the cytometer. All R^2 values were ≥0.998. Repeated-measures ANOVA was used to determine whether there were differences between plates for each analyte, with all p values greater than 0.05 (range: 0.34–0.9). The plate effect was therefore considered insignificant, accounting for <0.1% of the total variance for all analytes except IL-8, which accounted for 3.91%. Since the detected concentrations for all of the known standards were not significantly different and had CVs within the acceptable range for the assay, there was no need for normalisation of the data and any differences observed within the dataset were real and not due to inter-assay differences.

Differences in secretion between baseline (unstimulated) and post-stimulation with LPS and R848 for the young, as determined by paired t tests (Figure 3.10) was significantly different for MCP-1, IL-1β, IP-10, IL-10, IL-8, IL-6 and TNF-α. However, the difference in secretion by PBMCs from elderly subjects (Figure 3.11) was only significant for IL-1β, IL-8, IL-6 and TNF-α when comparing stimulated to unstimulated samples. Upon comparison of cytokine secretion in samples from young to elderly subjects the levels of secretion were ~10-fold lower in samples from the elderly. To ensure accurate comparisons between samples any samples that resulted in values below the level of detection were recorded as 0.0 pg/ml.

Comparisons of the change from baseline after LPS and R848 stimulation between young and elderly subjects clearly showed that the PBMCs from young subjects secreted higher concentrations of MCP-1, TNF-α, IL-8, IL-1β and IL-6 compared to those from elderly subjects, all of which were significantly greater ($p<0.05$) (Figure 3.12a–e). Resistin secretion decreased after stimulation of the PBMCs from the young subjects (mean of 29 donors), while secretion from PBMCs derived from elderly subjects was statistically higher between these groups (Figure 3.12f). Levels of adiponectin were not significantly different between young and elderly subjects (Figure 3.12g).

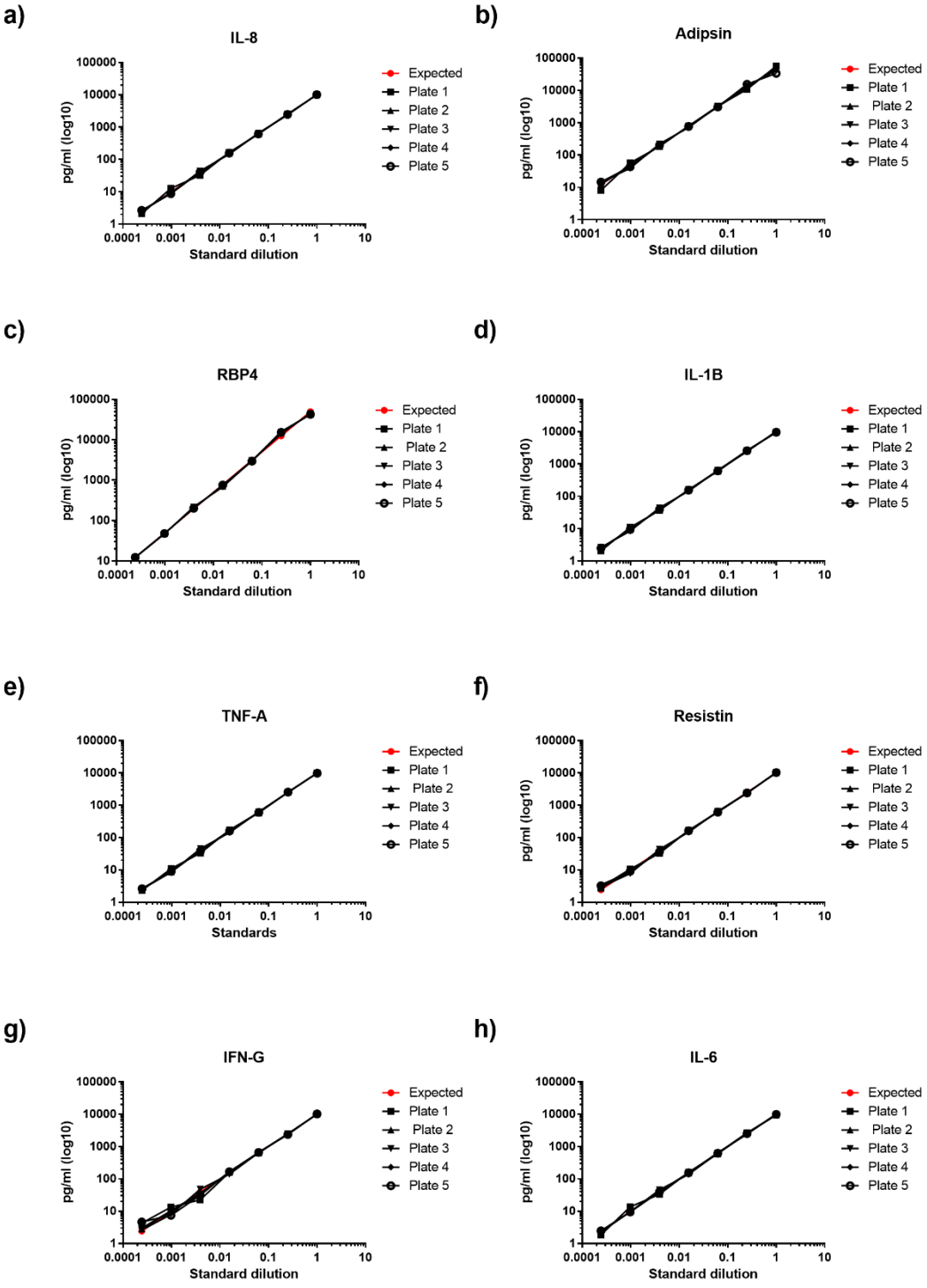


Figure 3.9 Concentrations of standards across the serial dilution from each plate overlaid with the expected concentrations for each of the 13 analytes analysed.

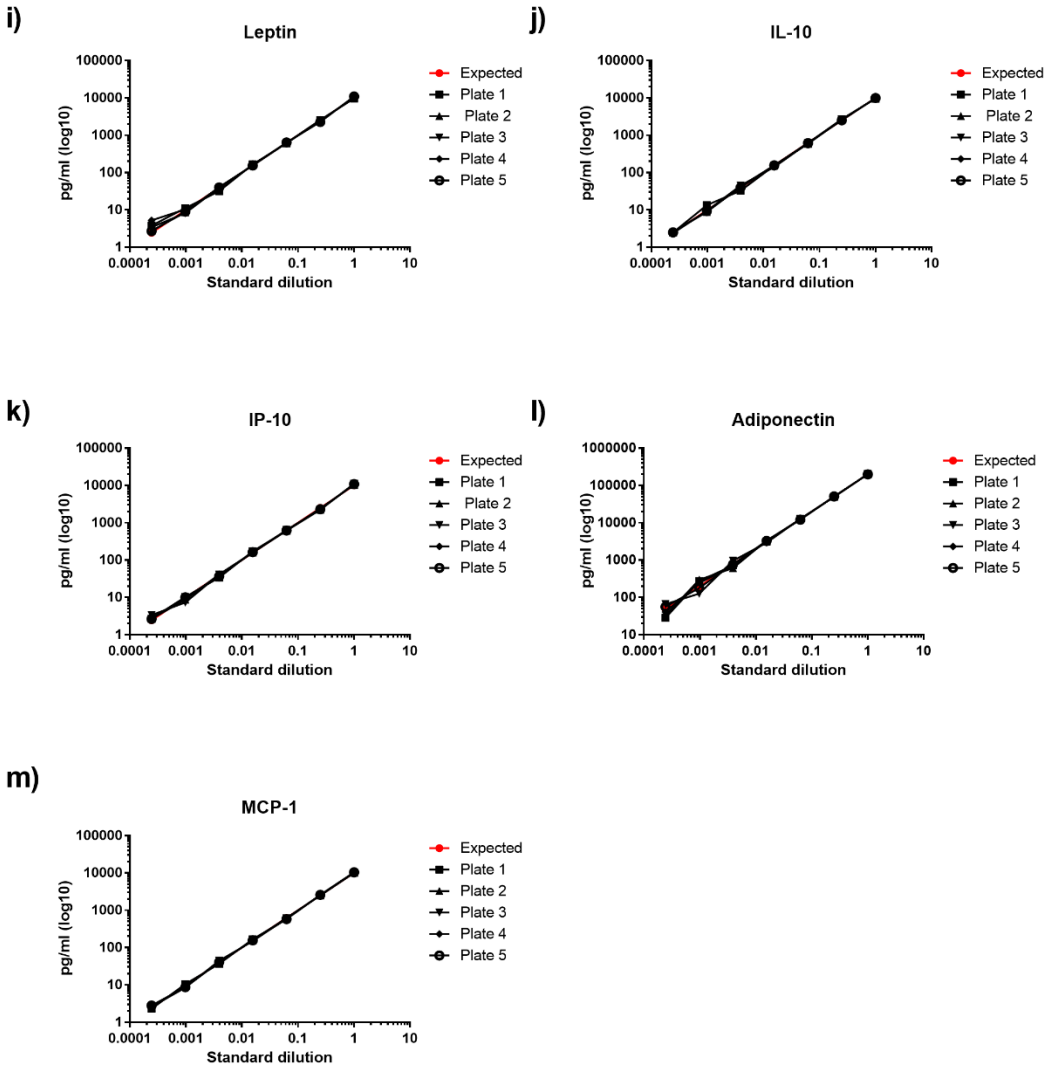


Figure 3.10 Continued. Concentrations of standards across the serial dilution from each plate overlaid with the expected concentrations for each of the 13 analytes analysed. A standard curve was prepared for every assay run on a new plate. For this the standard supplied with each kit, all kits used had the same lot number and batch number, was serially diluted (1:4 dilutions) from the top standard which had expected concentrations of 10,000 pg/ mL for MCP-1, IL-1β, IP-10, IL-10, IL-8, Leptin, IL-6, IFN-γ, Resistin and TNF-α, 50,000 pg/ mL for Adipsin and RBP4, and 200,000 pg/ mL for Adiponectin. Six 1:4 dilutions were performed after the top standard, with a final dilution of 0 pg/mL; which contained only assay buffer. Each serial dilution of standards with the resulting concentrations was overlaid with the expected theoretical values provided by Biolegend, graphs are on a Log10 scale.

3.4 Discussion

3.4.1 Baseline characteristics are comparable between study populations

There are significant differences between the heights of young and elderly subjects, as would be expected since height loss occurs in adults with increasing age (Cline et al., 1989; Sorkin et al., 1999) which has been linked to osteoporosis (Berecki-Gisolf et al., 2010) and changes in hormone secretion in post-menopausal women (Cauley et al., 2001). This is an important consideration when making measurements such as BMI as increases in BMI could be as a result of height loss instead of weight gain (Sorkin et al., 1999). However, the observation that the mean heights for both the young and the elderly subjects were greater than the Reference Man values of 170 cm for men and 160 cm for women (Snyder, 1975) is consistent with a more recent study showing that more than 200 healthy subjects were both heavier and taller as well as having greater fat and muscle masses than the Reference Man (Later et al., 2010); this implies that UK population height and body composition has increased in recent years, compared to 40 years ago. While the mean weight and BMI values are not significantly different, it is of interest that the upper BMI range for the elderly cohort reached 43.2 kg/m^2, while the upper range for the young cohort is 34.15 kg/m^2, highlighting that some individuals in the Nu-AGE study are classified within obesity class III, while the young cohort range from normal weight to obesity class I (WHO, 2009). Increased adiposity has been observed with increasing age in a ten year longitudinal study where 129 elderly subjects significantly increased their total fat mass (1 kg for men, 1.3 kg for women; $p<0.05$) and body fat percentage (1.2–1.3%; $p<0.05$) (Hughes et al., 2004). To obtain a better representation of baseline body composition waist: hip circumference ratios would be advantageous, in addition to the BMI recorded, especially since in addition to increased adiposity, risk of T2D and CVD among many other conditions increase with age, all of which are thought to accompany a pro-inflammatory state (Vasto et al., 2007).

Standards	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5
Adiponectin	0.75	1.72	1.53	0.82	0.64
Adipsin	1.42	0.4	2.26	0.22	0.88
RBP4	N.d	1.06	1.3	0.62	0.69
MCP-1	1.22	1.22	1.73	0.91	1.13
IL-1B	1.19	0.59	1.8	0.32	0.32
IP-10	2.07	0.99	1.73	0.86	1.12
IL-10	2.42	1.04	1.33	0.61	0.33
IL-8	1.81	0.35	1.09	0.57	0.7
Leptin	1.33	0.65	0.42	1.19	0.97
IL-6	2.19	0.75	1.57	0.71	0.25
IFN-G	0.98	0.84	1.13	0.87	1.58
Resistin	1.27	0.66	1.26	0.81	0.83
TNF-A	1.31	0.99	1.23	0.52	0.57

Table 3.2 Table of Coefficient of Variance (CV) values for all 13 analytes for all plates run.

3.4.2 Numbers of mDCs are reduced in the elderly, while pDC numbers remain similar to young subjects

The reduction of mDCs but not pDCs with age is consistent with that by Della Bella et al. (2007) who also analysed whole peripheral blood samples for their immunophenotypic analysis and counting of blood DCs. The authors used lineage negative and HLA-DR positive gating accompanied by positive staining for CD11c for mDCs and CD123 for pDCs, which is different to the staining approach used here. It is possible therefore that processing of blood samples may be a contributory factor for differing results; previous studies of DC subset counts using the same antibodies as Della Bella but using PBMCs isolated from heparinised blood instead of using whole blood (Jing et al., 2009; Panda et al., 2010; Shodell and Siegal, 2002) found reductions in pDCs only. An effect of sample manipulation has been proposed by Gerrits et al. (2007) who compared fresh blood samples to Ficoll-isolated fresh and cryopreserved PBMCs using the anti-CD1c, CD303, CD19 and CD14 antibodies, and found that PBMC isolation resulted in an approximate 3-fold increase in mDCs and pDCs when compared to fresh blood, and cryopreservation produced a 5-fold increase.

However, one previous study using very similar methodology to that used here produced opposing results (Pérez-Cabezas et al., 2007). A possible reason for this may be that the identification of blood DCs by Pérez-Cabezas et al. (2007), using CD1c and CD303, was not accompanied by the exclusion of CD14, CD19 or CD20 so these data may be overstating the numbers of mDCs present. This is important since B cells also express CD1c (Delia et al., 1988) in addition to a recent finding that $CD14^+$ monocytes were also present after CD19 depletion and staining for $CD1c^+$ in PBMCs (Schroder et al., 2016).

With regard to the reduction in mDCs, an interesting finding of increased numbers of $CD14^+$ monocytes and decreased numbers of $CD34^+$ precursors (haematopoietic stem cells) alongside the reduction in mDCs by Della Bella and colleagues (2007) implies that there may be an age-associated impact on the differentiation of these cells, preventing DC differentiation. However, this was not investigated here so these findings cannot be confirmed in the Norfolk cohort.

3.4.3 DCs from elderly subjects produce less IL-8 and have reduced expression of DCs producing IL-6^+ IL-8^+ and IL-$1\beta^+$ IL-8^+

Investigation of *ex* vivo cytokine production by DCs after stimulation with the TLR ligands, LPS and R848, was performed by ICS. To improve performance of ICS after

thawing, the PBMCs were left to “rest” for 18 hours at 37ºC in 5% CO_2 before the addition of stimulants, which has been shown to improve outcomes (Horton et al., 2007). However, the variable recovery rates of PBMCs observed in our samples may have impacted on the results observed, since numbers of DC subsets have been shown to increase 5-fold after cryopreservation (Gerrits et al., 2007). All samples were prepared and stored according to identical methodology in batches so that samples from young and elderly subjects were processed at the same time. In addition all samples were resuspended at a final concentration of 0.5×10^6 / 200 µl to ensure that the same number of viable cells was used in each culture. It is possible that PBMC samples from elderly subjects demonstrated poorer recovery after cryopreservation, compared to samples from younger subjects, since different levels of cellular damage, metabolic states and cell age have been suggested as unavoidable factors which can reduce the success of cryopreservation (Woods et al., 2016). Additionally, while the PBMCs from the Nu-AGE study were frozen for a greater duration of time than the Im-AGE samples, a previous study has shown that storage of PBMCs for 18 months was successful (Valeri and Ragno, 2006).

Adipokine	Age group		Media alone (pg/ml)	Stimulated (pg/ml)	p value
MCP-1	Young	Mean (SEM)	1237 (279.00)	1507 (315.90)	0.004
		Range	1.2–7054	1.24–8080	
	Elderly	Mean (SEM)	11.24 (3.37)	22.05 (8.40)	0.065 (ns)
		Range	0–57.54	0–199.90	
IL-1β	Young	Mean (SEM)	334.70 (191.00)	519.10 (149.40)	0.049
		Range	0–4304	0–3217	
	Elderly	Mean (SEM)	1.12 (0.53)	11.38 (3.36)	0.005
		Range	0–9.49	0–52.37	
IP-10	Young	Mean (SEM)	144.60 (96.79)	302.10 (130.60)	0.001
		Range	0–2830	1.28–3617	
	Elderly	Mean (SEM)	2.37 (0.83)	2.14 (0.89)	0.670 (ns)
		Range	0–17.14	0–21.72	
IL-10	Young	Mean (SEM)	18.89 (7.76)	40.73 (8.84)	0.006
		Range	0–178.90	0–149.90	
	Elderly	Mean (SEM)	0.08 (0.05)	0.13 (0.08)	0.116 (ns)
		Range	0–1.24	0–1.85	
IL-8	Young	Mean (SEM)	2987 (458.10)	4998 (742.30)	0.003
		Range	5.36–8231	5.12–15430	
	Elderly	Mean (SEM)	115.30 (61.45)	346.10 (118.30)	0.007
		Range	0–1610	0–2422	
IL-6	Young	Mean (SEM)	529.70 (229.50)	1392 (240.10)	0.001
		Range	0–4880	0–4470	

	Elderly	Mean (SEM)	2.34 (2.06)	15.43 (7.64)	0.034
		Range	0–53.43	0–187.40	
Resistin	Young	Mean (SEM)	169.40 (42.51)	140.90 (40.04)	0.054
		Range	0–998.40	1.43–991.40	
	Elderly	Mean (SEM)	121.60 (51.93)	136.40 (54.34)	0.264 (ns)
		Range	0–1028	0–869.90	
TNF-α	Young	Mean (SEM)	81.06 (49.42)	2782 (459.60)	<0.0001
		Range	0–1207	0.9–7550	
	Elderly	Mean (SEM)	0.77 (0.59)	48.27 (16.41)	0.008
		Range	0–15.42	0–350.90	

Table 3.3 Mean concentration for each adipokine in cell culture supernatants from PBMCs with or without LPS/R848 stimulation from young or elderly subjects. Footnotes: Adiponectin, Adipsin, RBP4, leptin and IFN-γ were not detected from one, or both conditions so comparisons could not be made. Paired t tests were performed to compared unstimulated to stimulated for each analyte, significance assumed at $p<0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

The proportions of DC secreting IL-8 alone or in combination with IL-1β or IL-6 are significantly reduced with age after stimulation with LPS and R848. The highest proportion of cytokine secretion by DCs derived from both young and elderly donors is seen for IL-8^+ IL-6^- producing DCs. The results from unstimulated and stimulated DCs are similar, except no statistically significant difference is observed between IL-8^+ IL-6^+ DCs from young and elderly subjects. This finding suggests that the freeze-thaw process alone may induce spontaneous stimulation, however a similar study using frozen PBMCs as a source of DCs for ICS (Janssen et al., 2015) and after 6 hours stimulation with 0.5 µg/mL of LPS the DCs from elderly subjects shows significantly greater amounts of IL-6 than the unstimulated cells (and TNF-α); the increase in IL-6 is only slight, but sufficient to be statistically significant. Janssen et al. (2015) recruited cytomegalovirus (CMV) seropositive young subjects, to exclude CMV seropositivity as a confounding factor when comparing results with elderly individuals, this is a difference with the present work and could explain the differing results observed between the studies.

Another study confirms the results found here, as stimulation of PBMCs with the TLR ligands pam3CSK4 (TLR1/2), lipoteichoic acid ((LTA) TLR2/6), poly I:C (TLR 3), flagellin (TLR5), R848 (TLR7/8) CpG-odn2216 (TLR9) for 6 hours resulted in significant reductions in IL-6 (in addition to other cytokines tested) (Panda et al., 2010). Similarly, in peripheral blood samples from elderly subjects incubation with LPS resulted in reduced secretion of IL-12 when compared to samples from young subjects (Della Bella et al., 2007). Also, significantly reduced secretion of IL-1β, IL-6 and IL-8 was observed in supernatants using ELISA after 24 hour stimulation of PBMCs with LPS, from healthy elderly subjects recruited according to the SENIEUR protocol compared to healthy young subjects (Gabriel et al., 2002). IL-8 secretion was significantly greater without stimulation in the PBMCs from the young compared to elderly subjects but levels were comparable after stimulation (Gabriel et al., 2002). Similarly, levels of TNF-α and IL-1β within whole blood supernatant after 24 hour LPS stimulation were significantly lower in samples from elderly compared to from young subjects, again determined by ELISA (Bruunsgaard et al., 1999). These experiments however, do not provide information regarding cytokine secretion specifically from DCs, since PBMCs or whole blood were used as the source of cells, and identification of DCs was not possible, unlike ICS techniques.

There are relatively few studies investigating the effects of ageing on DC function that use peripheral blood as the source of DCs. Other findings are based on the responses of MoDCs. The use of granulocyte macrophage colony stimulating factor (GM-CSF)

to induce differentiation of monocytes to DCs has been suggested to mask age-associated alterations in DC function (Panda et al., 2009), and upon comparison of global gene expression profiling using gene-chip data, GM-CSF DCs cluster with monocytes and macrophages, not lymph node DCs (Robbins et al., 2008), suggesting that conclusions drawn from functional studies performed using *in vitro* derived GM-CSF DCs should be made with caution.

The present findings, in combination with those by Panda and colleagues (2010), suggest that there may be an age-associated defect in the cytokine secretion pathways. A previous study using MoDCs found significant changes in intracellular signalling in the MoDCs from elderly compared to young subjects, whereby AKT phosphorylation, a downstream event of PI3K-Akt activation, is significantly reduced (Agrawal et al., 2007). This regulates mitogen-activated protein kinase (MAPK) and NF-κB activation and is implicated in regulation of TLR activation; Agrawal et al. (2007) postulated that reduced phosphorylation of AKT could cause overactivation of MAPK pathway signalling and thus induce cytokine production. A later study showed that DCs from aged subjects displayed elevated levels of NF-κB activation at baseline (Agrawal et al., 2009). However, the use of $CD1c^+$ mDCs yielded different results when investigating the intracellular signalling pathways and found that the p38 pathway was crucial for inhibition of IL-12, since inhibition of p38 stress-activated protein kinase (p38SAPK) increased IL-12 production (Franks et al., 2014). Nevertheless, investigation of $CD303^+$ pDCs also found that the reduction in IFN-I and -III production with age is linked to impairment in interferon regulatory factor 7 (IRF-7) phosphorylation (Sridharan et al., 2011). These findings imply that MoDCs may be more likened to pDCs than mDCs and highlights the differing responses observed between MoDCs and peripheral blood DCs, suggesting that the functional responses of these two cell types cannot be compared. Research by Franks et al. (2014) also confirmed that IL-6 and IL-1β are not regulated by p38SAPK, so the observed reduction in their secretion with age may be due to a defect in a different signalling pathway, or could be as a result of the reduced number of mDCs with increasing age.

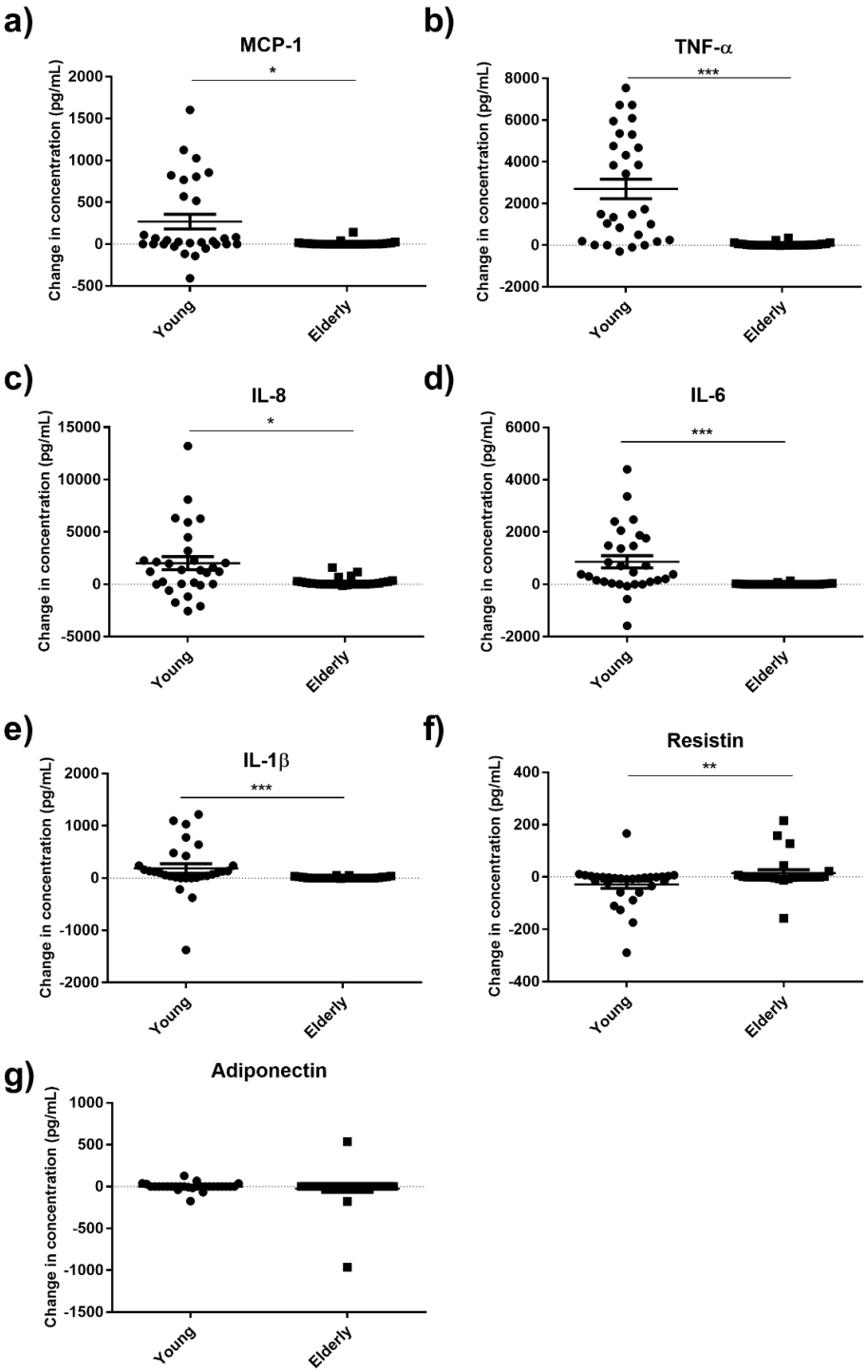


Figure 3.11 Change in concentration with LPS and R848 stimulation of MCP-1, TNF-α, IL-8, IL-6, IL-1β, Resistin and Adiponectin in young compared to elderly subjects; from unstimulated. Concentrations (pg /ml) in unstimulated samples were subtracted from stimulated samples to give a change in concentration as a result of the stimulus. Scatter plots show individual subjects as dots (young) or squares (elderly), horizontal bars represent mean and the error bars indicate SEM. N=29, young; N=26, elderly. Mann-Whitney U tests were performed to compare changes in concentration between young and elderly subjects, significance assumed at $p<0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Another potential reason for the differing results could be the length of time that the PBMCs were stimulated since in addition to use of a different source of DCs, increases in secretion of IL-6 from MoDCs have been observed with LPS stimulation (Agrawal et al., 2007; Ciaramella et al., 2011) for 24 and 48 hours, respectively. The kinetics of DC cytokine secretion has been investigated to show that while DCs "exhaust" their capacity to produce IL-12 after long stimulation periods (such as 24 and 48 hours), capacity to secrete IL-6 remained intact, even at 48 hours (Langenkamp et al., 2000), and could be detected after just 3 to 4 hours. The reduction in mDC numbers observed in the elderly cohort compared to the young cohort, may also be a contributing factor to the reduction in cytokine secretion observed in the present study.

3.4.4 Secretion of adipokines by PBMCs is significantly reduced in the elderly

The concentration of cytokines secreted by LPS/R848 stimulated PBMCs isolated from elderly subjects was 10-fold lower than the concentrations from PBMCs derived from young subjects. As secretion was still observed for most analytes (excluding IL-10, which was below the limit of detection of the assay) the ability to respond to antigenic stimulation was still present, but reduced for specific analytes. The present study found significant reductions in MCP-1, TNF-α, IL-8, IL-6 and IL-1β in PBMCs from elderly subjects. A similar study comparing cell culture supernatants from MoDCs stimulated with LPS (for 20–24 hours) found significantly increased concentrations of IL-6 and TNF-α with single stranded RNA (ssRNA) stimulation also significantly increasing TNF-α secretion in the MoDCs from elderly subjects (Agrawal et al., 2007). However, mDCs and pDCs isolated from PBMCs of young and elderly subjects and stimulated with poly I:C and influenza virus, respectively, found that pDCs secreted less IFN-α, IL-6 and TNF-α compared to young controls, while mDCs secreted comparable levels of cytokines (Jing et al., 2009). Additionally, after 24 hour LPS stimulation of PBMCs, but not whole blood, IL-6, IL-8 and IL-1β secretion decreased (Gabriel et al., 2002), and in whole blood supernatants IL-1β and TNF-α secretion declined in samples from elderly compared to young subjects (Bruunsgaard et al., 1999). Therefore, the source of cell samples may again be a determining factor in whether cytokine secretion increases or decreases upon TLR stimulation.

3.4.5 Resistin secretion by PBMCs increases with age

The present finding of a significant increase in the production of resistin by PBMCs from elderly subjects compared to young subjects after LPS and R848 stimulation is interesting since inflammatory events can induce resistin production (Al Hannan and Culligan, 2015). Resistin, in humans, is expressed predominantly in the BM but is

also present in circulating blood (Filková et al., 2009) and PBMCs have been shown to be an important source (Kaser et al., 2003). Upregulated resistin mRNA expression was observed in PBMCs from type II diabetic women compared to healthy controls (21–49 years), in parallel with plasma resistin levels (Tsiotra et al., 2008); in addition PBMC mRNA expression of IL-1β, TNF-α and IL-6 was significantly elevated in women with T2D. The increased expression of these cytokines may have increased resistin secretion since increased mRNA expression of resistin was observed in the presence of IL-6, IL-1 and TNF-α, along with LPS (Kaser et al., 2003). As the present data shows that LPS and R848 stimulation induces PBMCs from elderly subjects to produce more resistin than those from young subjects, this implies a potential underlying inflammatory state or inflammaging. LPS has been shown to increase resistin gene expression from human PBMCs after a four hour incubation, while this data is only based on samples from three healthy volunteers, the increases in resistin were substantial and consistent between all volunteers (Lu et al., 2002); a more quantitative measure, such as a cytokine bead array, would aid in the explanation. Resistin has also been shown to suppress the ability of MoDCs to secrete IL-6, TNF-α and IL-12 (p40) after incubation with LTA from *Staphylococcus aureus* for 24 hours, but not without these stimulatory conditions (Son et al., 2008). This suggests that the observed decreases in IL-6 and TNF-α, and possibly the other cytokines in the present study could have been due to the elevated secretion of resistin in the elderly subjects.

Contrary to these findings, previous research has shown that plasma concentrations of resistin in a healthy population of over 250 subjects did not differ with donor age (Vilarrasa et al., 2005). This may have been because the recruited cohort were healthy since previous observations of higher serum levels of resistin were also associated with increasing risk of CVD events (Gencer et al., 2016). Resistin is also influenced by insulin resistance and adiposity, which was demonstrated upon comparison of offspring of non-long-lived individuals and centenarians (Ostan et al., 2013). This study showed increased levels of plasma resistin which were positively associated with waist circumference, while centenarian offspring had significantly lower resistin levels that are unaffected by prevalence of metabolic syndrome (MetS). Additionally, in non-long-lived offspring prevalence of MetS increased with increasing levels of resistin (Ostan et al., 2013); suggesting genetic predisposition could also be influential.

When considering the two cohorts of volunteers recruited for this study there were no significant differences in mean BMI or weight of the individuals. After identifying

individuals that exhibited increases in resistin concentration, from unstimulated to stimulated samples, BMI or weight was not consistently high for these individuals. In fact, out of the four individuals identified only one had a high BMI (pre-obese) (27.1 kg/m^2), the rest were within the normal range (18.50–24.99 kg/m^2) (WHO, 2009) and the highest concentration recorded was by an elderly individual with a healthy BMI of 23.0 kg/m^2. This implies that while adiposity is commonly attributed with elevated resistin levels this may not be the cause of the observed increase in resistin in this study. However, BMI may not be the best means to assess body composition and thus increased adiposity in some cases may only be observed by taking measurements such as waist and hip circumferences, in addition to bioelectrical impedance or DXA scan, especially since increases in body fat and reductions in fat free mass occur with age and differences were only seen in resistin concentration when subjects were grouped by body fat content as opposed to BMI (Vilarrasa et al., 2005).

An interesting comparison to this data would have been assessment of baseline plasma or serum levels of adipokines to establish the levels at the time of blood draw, since healthy ageing males and female centenarians have shown higher levels of adiponectin (typically considered to be anti-inflammatory) (Adamczak et al., 2005; Arai et al., 2006). While leptin, TNF-α, IL-6 and MCP-1 are thought to be pro-inflammatory and may increase with normal ageing as they are associated with increased adiposity and insulin resistance, while centenarians remain insulin sensitive (Arai et al., 2011).

3.4.6 Impact of findings

The reduction in mDC numbers with age observed in the present study using the current methodological approach defined by the Nomenclature Committee of the International Union of Immunological Societies (Ziegler-Heitbrock et al., 2010) may lead to improved validity of future studies, if a commonly agreed method could be adopted. Additionally, the source of cells may impact on the response of DCs in *in vitro* stimulation assays, and while MoDCs are a crucial source of DCs, particularly in terms of generating large numbers of cells, they do not appear to have the same functional characteristics as peripheral blood derived DCs identified within PBMCs or isolated from PBMCs.

MCP-1, TNF-α, IL-8, IL-6 and IL-1β were reduced with age in this study upon TLR stimulation, which adds to previously observed reductions in the ability of DCs of elderly subjects to secrete these, and other, cytokines. Further research into the

underlying causes, potentially in terms of cytokine signalling pathways, would be of great importance. Additionally, the increase in resistin secretion may have inhibited the secretion of these cytokines.

An interesting outcome of this research is the observation of increased resistin concentrations upon LPS and R848 stimulation of PBMCs from elderly subjects. The impact of this finding, in addition to the current understanding of resistin in terms of healthy ageing, implies that preservation of insulin sensitivity with increased age may be of importance to preserve health of the elderly. The biological impact of this finding is that since elevated levels of resistin secretion, predominantly observed in plasma, have been associated with negative health outcomes such as MetS, CHD and insulin resistance, resistin levels may be implicated in the prevalence of these age-associated disorders and diseases. Resistin could provide a potential therapeutic target for alleviating the increased economic and social pressures associated with our ageing population.

Chapter 4

Study design, subject characteristics and compliance

4.1 Introduction

The Nu-AGE study, new dietary strategies addressing the specific needs of the elderly population for healthy ageing in Europe, is a multicentre study with sites in five countries; the United Kingdom (UK), the Netherlands, Italy, France and Poland. A total of 1250 participants were recruited throughout the study with the UEA recruiting 250 of the participants. For the research carried out at the IFR for this thesis, 120 of UEA-participants gave additional consent for analysis of immune function using their samples. The participants were recruited onto the study via advertisements in Norwich in locations relevant to the cohort in question, in addition to GP surgeries. The design of the study was a randomised, controlled intervention trial, one year in duration, which comprised of two groups; intervention and control. As a laboratory researcher not interacting with the study participants, all of the work carried out for this thesis was blinded to treatment allocation of samples. This chapter addresses the design of the study in terms of the observed subject characteristics and the dietary consumption of all participants as assessed by diet diary analysis, in addition to compliance data with the overarching aim to address whether study participants in the intervention arm of the study consumed the study provided olive oil, as directed.

4.1.1 Gaps in knowledge

The studies carried out to date have used *in vitro* models, with very few studies using human samples collected after dietary intervention, of which the dietary interventions consisted of consumption of an additional single dietary component such as the prebiotic (B-GOS) (Vulevic et al., 2008; Vulevic et al., 2015) or FA containing supplements, oils and fish (Bechoua et al., 2003; Grieger et al., 2014; Han et al., 2012; Meydani et al., 1991). The study populations for these studies were older subjects (50–79 years), though the inclusion of subjects >50 years is not representative of the elderly population. Also, previous *in vitro* studies are not representative of the ageing population, making the data less transferable to an ageing population. In order to gauge an accurate understanding of the effect of changing dietary intake, the whole diet needs to be monitored to account for any interactions which may occur between food components.

4.1.2 Rationale for studying the Mediterranean diet

The MED diet has been chosen for this work because this diet has been shown to be effective in reducing risk factors and increasing overall survival in patients with CHD, MetS and post-MI (Esposito et al., 2004; Panagiotakos et al., 2009; Trichopoulou et al., 2005), which are diseases associated with ageing. There are indications that this diet may be able to improve immune parameters since increased adherence significantly reduced plasma levels of IL-6, IL-8, and the components olive oil and red meat showed significant negative and positive associations with plasma IL-6, IL-8, MCP-1 and TNF-α, respectively (Dedoussis et al., 2008; Mena et al., 2009).

4.1.3 Aims and objectives

This chapter addresses the specific details of the dietary intervention study carried out and addresses the questions were the two study groups different in terms of demographics and body composition, and were the subjects compliant to their allocated dietary intervention?

Objective 1: Compare subject characteristics collected at baseline for subjects in each group of the dietary intervention study by members of the study team

Objective 2: Calculate Mediterranean diet scores using the diet diary reports to compare compliance in both study groups

Objective 3: Determine hydroxytyrosol sulphate (HTS) concentration within urine samples collected from participants from both study groups.

4.2 Methodology

4.2.1 Composition of the Nu-AGE study diet

The study participants were randomly allocated into two groups, control and intervention; the control group were provided with a standard healthy living advice leaflet from the British Dietetic Association (Appendix X) and asked to maintain their habitual dietary intake. The participants within the intervention group were provided with dietary advice sheets (Appendix XI) and individual dietary advice by members of the study team at the CRTU at the UEA in order to achieve the quantitative requirements for the Nu-AGE dietary intervention (Table 4.1). This advice was based on the information provided within the seven-day food records collected at baseline. Study participants randomised to the dietary intervention arm of the study were given extra virgin olive oil, wholegrain pasta and low fat margarine rich in MUFA and PUFA, freely throughout the study. The study team distributed these products to intervention participants at baseline, four, and eight months, when the participants attended the CRTU, either for their baseline measurements or for their interim interview questionnaires to assess blood pressure, cognitive function and physical function; data not shown.

4.2.2 Randomisation and blinding of study participants

Study participants were recruited onto the Nu-AGE study upon provision of informed written consent and providing they fulfilled the eligibility requirements detailed in Table 2.4; full details in Chapter 2, Section 2.2.2. Computer software developed in-house by the University of Bologna was used by the study team to randomise the participants to the study groups; this was stratified by age, sex and BMI. The software was limited in that postcode was not included in the process of randomisation so that co-habiting participants were randomised together with the aim of increasing compliance. Study participants whom provided additional informed consent for the collection of additional blood samples were included in the immunologic measurements. The same computer software was used to randomise these study participants, which was also stratified by age, sex, and BMI, to ensure that the groups were equal. It was not possible to blind the participants to their allocated study group, however, all samples received and handled were coded with unique sample identification numbers with no indication of grouping, making all analyses anonymous and blind. Only the study co-ordinator and selected members of the study team had access to volunteer names and codes.

4.2.3 Interaction of study team with participants

Study participants attended the CRTU, after an eight hour (overnight) fast, for a baseline visit which lasted for two hours, to provide anthropometric measurements, DXA scans, (further details in Chapter 2, sections 2.2.3 and 2.2.4) blood samples (further details in Chapter 2, Section 2.2.5), and a twenty-four hour urine sample (Appendix XII). After one year of MED diet intervention, or maintenance on their habitual diets, study participants returned to the CRTU for the same measurements. Ethical approval was only granted for the collection of blood and urine samples at the pre- and post-intervention study time points, so further samples could not be collected without the provision of a substantial amendment to the ethical application. Additionally, the large volume (100 ml) of blood collected at each visit, and the difficulty in cannulating the elderly subjects meant it would have been difficult to obtain further samples of blood from these subjects. Dietary intervention

Subjects enrolled onto the intervention arm of the Nu-AGE study were given tailored dietary advice in order to change their own diet to a MED diet, according to set guidelines (Annex VIII). The guidelines stated that participants should aim to consume 4–6 servings of whole-grains per day, at least two servings of fruit per day, at least 300 g of vegetables per day, 500 ml dairy per day, 125 g fish twice a week, meat and poultry four times per week, 20 g nuts twice a week, 150 g potato, pasta or rice per day, 2–4 eggs per week, 20 g extra virgin olive oil (generic brand supplied; 1.5g SFA, 14.7g MUFA, 1.6g PUFA/ 20g serving) per day and 30 g Flora Original margarine per day (supplied; 3g SFA,3.3g MUFA, 6.9g PUFA/ 30g serving). In addition, if participants consumed alcohol then red wine was to be selected with a maximum of one glass per day for women and one to two glasses for men.

Food group	Quantity required in dietary intervention
Whole grains	6 servings per day; 1 serving=25 g bread, 50 g breakfast cereal
Fruits	2 servings per day; 1 serving=1 apple, 1 banana, 8 small plums
Vegetables and legumes	330 g per day, once per week 200 g legumes
Dairy and cheese	500 ml dairy per day (of which 30 g cheese)
Fish and other seafood	2 times per week; 1 portion=125 g
Meat and poultry	4 times per week; 1 portion=125 g
Nuts	2 times per week; 20 g portion
Potatoes, pasta and rice	150 g per day; 80 g (raw weight) whole grain rice or pasta at least twice a week
Eggs	2–4 times per week
Oil or fat	20 g oil per day, 30 g margarine per day; maximum of 50 g fat per day. Should be olive oil and low fat margarine rich in MUFA and PUFA
Alcohol	Maximum of 1–2 glasses per day for men, and 1 glass per day for women. Preferably red wine, if not abstain
Fluid	1.5 litre per day, including milk
Salt	Reduce added salt, and intake of ready meals (soups, gravy, sauce)
Sugar	Limit consumption of sugar and sweetened drinks (replace with fruit or yoghurt, no/reduce sugar in tea or coffee).

Table 4.1 Quantitative dietary guidelines given to study participants whom were allocated to the intervention arm of the study.

4.2.4 Study compliance

Study participants were required to complete seven-day diet diaries (7DD) at pre-intervention and post-intervention, which were used to assess study compliance. In addition, twenty-four hour urine samples were collected from study participants at baseline and one year post-intervention. These samples were aliquoted by the study team and frozen at -80°C until further analysis, for urine collection instruction sheet provided to volunteers see Appendix XII. The samples selected for these analyses were derived from the same subjects for whom PBMC samples were available (n=34), since PBMCs could not collected from the whole subset of Nu-AGE subjects due to unavoidable, unforeseen circumstances.

Seven-day diet diary (7DD) analysis

Self-reported 7DDs were completed by the study participants at the baseline and post-intervention study days. Participants were asked to record all food intake over seven consecutive days using the eight sections for each day; before breakfast, breakfast, during the morning, lunch, during the afternoon, evening meal, evening snack, during the night. Participants were required to record the time and place that foods were consumed and a description which included any brand names and methods of preparation, in addition to the portion size using household measures. The diaries included a section to record any recipes used and a notes section for any other additional information (Appendix VII).

Calculation of the Mediterranean Diet score

Using the output food group data from the 7DDs nine overall food groups were defined, these were total cereals, total fruit which included nuts, total vegetables, total legumes, total fish, total meat and meat products, total dairy, total alcohol and total olive oil. To determine values for these overall food groups the sum of the recorded intakes of all recorded food groups that constituted that category, such as for the cereal food group white bread, brown bread, wholemeal bread, luxury bread, rusk (crispbread), white rusk, non-white rusk, non-wholegrain breakfast cereal, wholegrain breakfast cereal, pastry, refined cereal products (flour), refined cereal products (rice, pasta), wholemeal cereal products (rice, pasta) and stuffed cereal products (rice, pasta) were summed to determine the overall cereal intake. The food groups included for the remaining categories are detailed in Table 4.2.

Overall food group	Food groups included from diet diary output
Cereals	White bread, brown bread, wholemeal bread, luxury bread, rusk (crispbread), white rusk, non-white rusk, non-wholegrain breakfast cereal, wholegrain breakfast cereal, pastry, refined cereal products (flour), refined cereal products (rice, pasta), wholemeal cereal products (rice, pasta) and stuffed cereal products (rice, pasta)
Fruits	Citrus fruits, apple and pear, grape and berries, stone fruits, melon, tropical fruits, mixed fruits (excluding citrus), dried fruit, pure fruit juice, canned or cooked fruit in syrup, canned or cooked fruit in water, salted peanuts, unsalted peanuts, salted nuts, unsalted nuts, unsalted mixed nuts and peanuts, salted mixed nuts and peanuts
Vegetables	Leafy vegetables, fruiting vegetables, root vegetables, cabbages, cruciferous vegetables, stalk/ shoot vegetables, mixed salad, mixed vegetables, mushrooms, grain and pod/ other vegetables, vegetable juice, onion, garlic, pickled vegetables, avocado
Legumes	Legumes
Fish	Oily fish, white fish, fish products (fish in crumbs), seafood/ crustaceans, molluscs
Meat and meat products	Organ meat, red unprocessed meat, lean unprocessed red meat (10g

	fat/100g), fat unprocessed red meat (>10g fat/100g), lean processed meat (10g fat/100g), fat processed meat (>10g fat/100g), lean poultry (10g fat/100g), fat poultry (>10g fat/ 100g), lean cold cuts (10g fat/100g), fat cold cuts (>10g fat/100g), game
Dairy products	Creamy (milk) products, ≥8g fat, full-fat (milk) products ≥2<8g fat, semi-skimmed milk products, >0.5<2g fat, skimmed milk products ≤0.5g fat, powdered milk, cheese ≤30% fat, cheese >30-50% fat, cheese >50% fat, butters, margarines
Alcohol	Alcoholic beverage not stated or mixed, wine, red wine, other wine, fortified beer, spirits/ brandy, liquors, cocktails punches
Olive oil	Olive oil and olives.

Table 4.2 Foods included within each food group from seven-day diet diary entries, used to determine MED diet scores for each participant.

The scoring criteria developed by (Sofi et al., 2014) was used such that the portions of each overall food group were 150 g fruit, 100 g vegetables, 70 g legumes, 130 g cereals, 100 g fish, 80 g meat, 80 g dairy products, 1 alcohol unit=12 g, olive oil consumption was scored as occasional, or regular use, which was interpreted as <5 g/day as occasional, and >20 g/ day as regular use. To calculate the MED diet scores, the sum of each food group was subjected to an IF statement to determine whether the score would be 0, not compliant with the Mediterranean diet, 1, moderately compliant or 2, very compliant. The scores were added together to give an overall score out of 18, Table 4.3

Food group	Score value		
	0	1	2
Fruit	<150 g/ day	150–225 g/ day	>300 g/ day
Vegetables	<100 g/ day	100–250 g/ day	>250 g/day
Legumes	<70 g/ day	70–140 g/ day	>140 g/ day
Cereals	<130 g/ day	130–195 g/ day	>195 g/ day
Fish	<100 g/ day	100–250 g/ day	>250 g/ day
Meat & meat products	>120 g/ day	80–120 g/ day	<80 g/ day
Alcohol	>24 g/ day	<12 g/ day	12–24 g/ day
Olive oil	<5 g/ day	5–20 g/ day	>20 g/ day

Table 4.3 Cut-off values for consumption of each food group to calculate the Mediterranean Diet score, which is the sum of the scores out of a total of eighteen.

High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS/MS) analysis of urine samples

Twenty-four hour urine samples were collected at baseline and one year post-intervention from Nu-AGE participants. In order to determine compliance to the study, concentrations of hydroxytyrsol (HT), a metabolite derived from olive oil, were measured using HPLC coupled to tandem mass spectrometry (MS/MS). The column used was 2.7µm Supelco Ascentis Express C18 (150 mm x 4.6 mm i.d) with a mobile phase of water/ 0.1% formic acid as solvent A, and acetonitrile/ 0.1% formic acid as solvent B, at a temperature of 40°C. The HPLC gradient began with 5% of solvent B, followed by an increase to 55% at 11 minutes for one minute, then increased to 95% for a further four minutes, and was then returned to the initial conditions in 0.1 minutes, with a re-equilibration time of 22 minutes, Figure 4.1. The flow rate was 600 µl/ minute and the injection volume was 10 µl.

The standard used was Hydroxy Tyrosol 3- sulphate sodium salt (Toronto Research Chemical Inc) which has a molecular weight of 232 g. Hydroxytyrosol was detected and quantified according to its ion fragmentation in the tandem MS/MS using multiple reaction monitoring (MRM) mode, and ionisation was performed in the negative mode. Three product ions of hydroxytyrosol sulphate (HTS) were detected at molecular weights of 153, 123 and 80 m/z, Figure 4.2. Taxifolin, a flavanol which is a derivative of quercetin and has a molecular weight of 304 g, was used as the internal standard, the product ion had a molecular weight of 125 m/z. Urine samples (200 µl) were mixed with 10 µl 40 µg/ ml taxifolin in methanol and made up to 600 µl with water. Standards were prepared in water at 2000, 1000, 500, 100, 50 and 0 ng/ ml (ppb); with control samples prepared at the same time in urine, to perform calibration curves in the desired biological matrix.

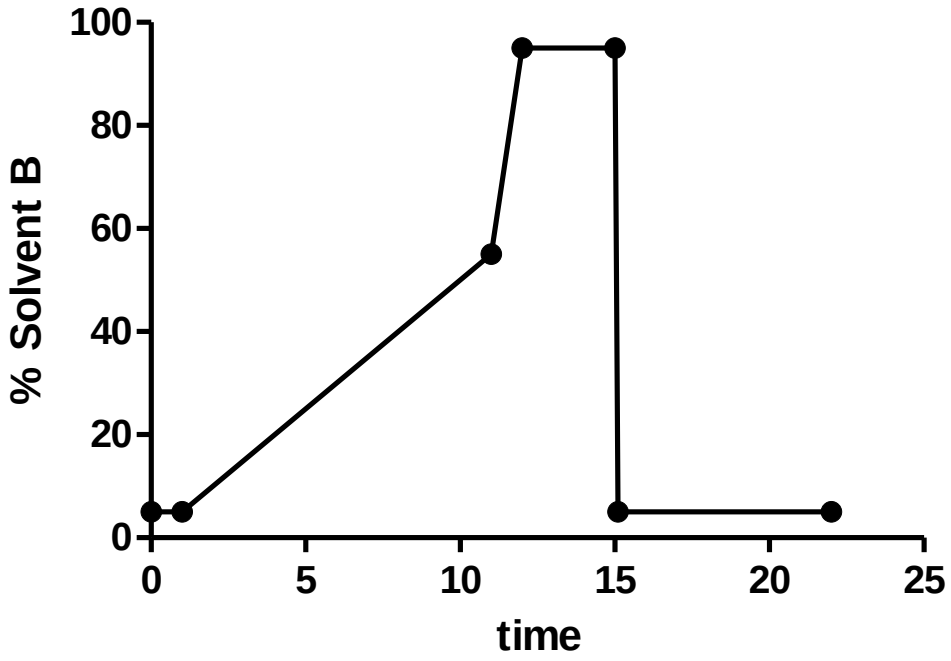


Figure 4.1 HPLC gradient applied to analyse urine samples. The HPLC gradient began initially with 5% of solvent B for 1 minute, then increased to 55% for 11 minutes, before increasing further to 95% for 4 minutes, initial conditions of 5% solvent B were then resumed in 0.1 minutes for the duration of the 22 minute re-equilibration time.

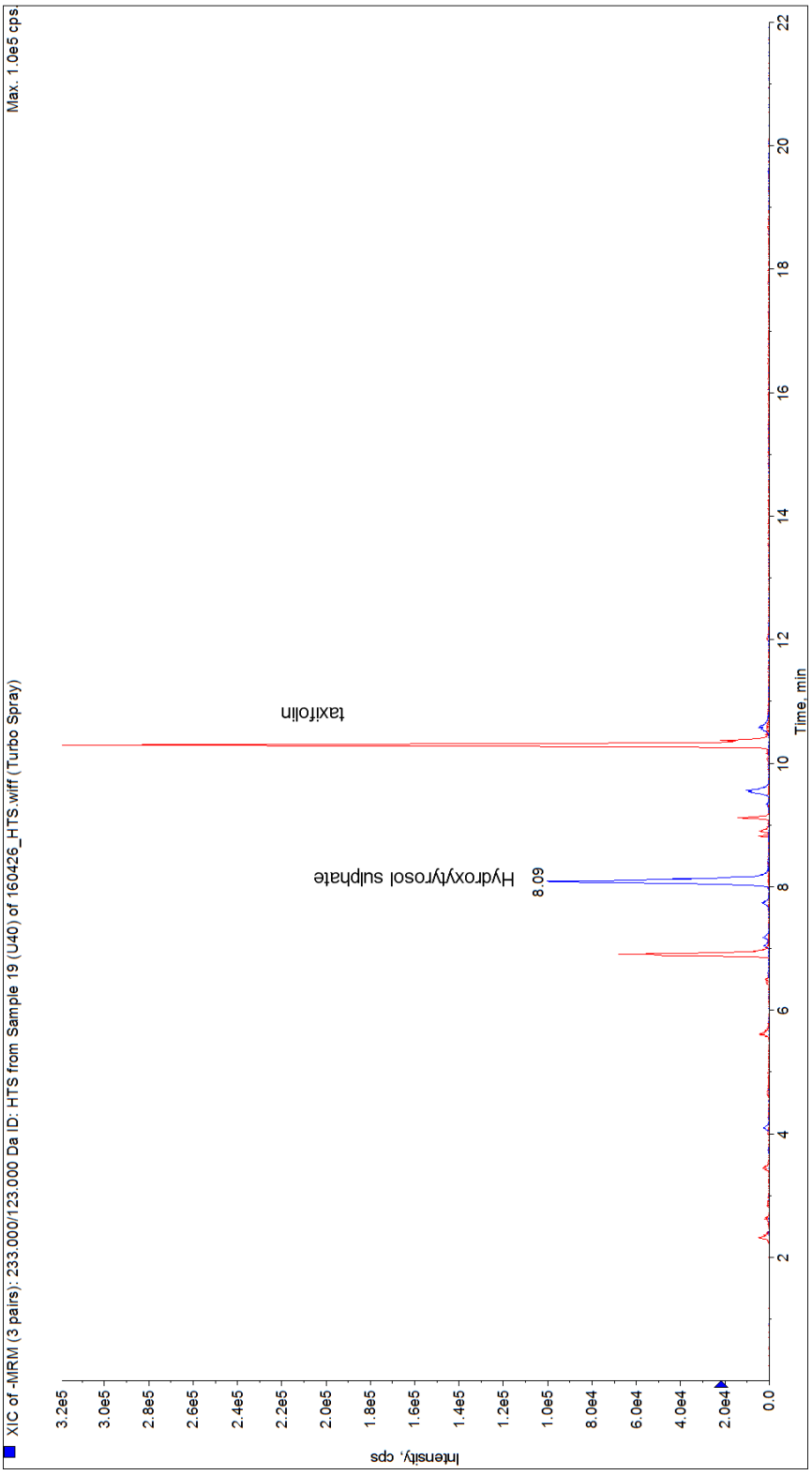


Figure 4.2 Representative chromatogram showing the HTS and taxifolin peaks recorded. The chromatogram shows the intensity against the retention time, with the peaks of interest, HTS and taxifolin, identified. HPLC-MS/MS completed by Mark Philo, Metabolomics Department at the Institute of Food Research.

4.2.4 Statistics

Anthropometric data from Nu-AGE participants at baseline was separated by allocated study group and a two-tailed t-test for unmatched samples comparing the two groups was carried out, with $p<0.05$ showing significance.

Change in MED diet scores from baseline were calculated for subjects in the control and MED diet groups, an unpaired t test was applied to determine whether the changes in MED diet differed between the two groups. Ten subjects dropped out of the study and thus did not provide post-intervention data, to account for this intention to treat (ITT) was applied and missing values were imputed using last observation carried forward (LOCF). Multivariate analysis of the nine food categories, used to calculate the MED diet scores, was carried out using variance scaled single cross-validated partial least squares-linear discriminant analysis (PLA-LDA). The associations of the individual food categories with the overall MED diet scores were assessed using Wilcoxon Mann-Whitney U test, after controlling for the false discovery rate ($q= 0.05$). Change in HTS concentration from baseline was calculated for each subject, and an unpaired t test was applied to determine whether the changes in HTS concentration differed between the two groups.

4.3 Results

4.3.1 Observed subject characteristics

The overall characteristics of the study participants, reported in Table 4.4 show that the age of the participants ranged from 65 to 79 years, with a mean age of 70.33 ±4.16 years. When the two study groups were compared the mean age was similar with the same age range. The gender spread was more favoured towards females although it was not significantly different when the two study groups, control and intervention, were compared. Body mass indices (BMIs) of study participants ranged from normal (18.5–24.0 kg/m^2), overweight (25–29.9 kg/m^2), obese (≥30 kg/m^2) to extreme obesity (≥40 kg/m^2) (WHO, 2009), with the mean BMI of 26.62 ± 3.96 kg/m^2, which classified as overweight. When comparing the two groups, the BMI was similar, however the range of values was greater in the MED diet group with the greatest value being 43.20 kg/m^2. The weight of participants between the two groups was again comparable with similar mean values; however the range of values was larger in the MED diet group, reaching up to 128.50 kg. The height of the participants was similar between the study groups and the ranges were also comparable. In terms of waist circumference the study population was well distributed with relatively equal groups at normal, at risk or high waist circumference (Dobbelsteyn et al., 2001; Qiao and Nyamdorj, 2010), the mean values were similar while the range of values in each group was greater in the MED diet group than the control group. When assessing frailty status, a large proportion of the participants were pre-frail, with two participants recorded as being frail. Table 4.5 shows the results of DXA scans at baseline and one year post-intervention and shows that there were minimal changes to the mean body weight, fat mass, lean mass, bone mineral content (BMC), soft tissue and bone mineral density (BMD). The fat mass and regional fat mass percentages were reduced at post-intervention but minimally. The mean T scores were similar but the upper value of the range was much smaller, indicating deterioration in some subjects.

Of these participants ten dropped out of the study and thus a post-intervention sample was not collected, therefore overall 112 participants completed the study.

		All subjects	Control group (n=57)	MED diet group (n=65)	p value
Age (years)	Mean (SD)	70.33 (4.16)	70.95 (4.07)	69.78 (4.18)	0.32 (ns)
	Range	65–79	65–79	65–79	
Gender (n=)	Male	48	22	26	0.88 (ns)
	Female	74	35	39	
BMI (kg/m^2)	Mean (SD)	26.62 (3.96)	26.56 (3.25)	26.67 (4.51)	0.73 (ns)
	Range	18.50–43.20	20.00–37.40	18.50–43.20	
Weight (kg)	Mean (SD)	73.27 (13.85)	73.18 (12.46)	73.36 (15.05)	0.94 (ns)
	Range	49.50–128.50	52.50–108.90	49.50–128.50	
Height (cm)	Mean (SD)	165.70 (9.16)	165.70 (9.29)	165.60 (9.11)	0.73 (ns)
	Range	145.60–188.20	148.60–187.40	145.60–188.20	
Waist circumference (cm)	Mean (SD)	91.40 (11.80)	91.49 (11.11)	91.31 (12.46)	0.92 (ns)
	Range	63.50–134.80	72.00–122.00	63.50–134.80	
Frailty status (n=)	Non frail	26	12	14	0.74 (ns)
	Pre frail	94	44	50	
	Frail	1	1	0	

Table 4.4 Baseline anthropometric data for Nu-AGE participants. *P;* probability value, ns; not statistically significant, SD; standard deviation.

		Nu-Age cohort pre-intervention	Nu-AGE cohort post-intervention
Body weight (kg)	Mean (SD)	74.8 (13.7)	74.2 (13.5)
	Range	47.7–12.9	48.0–119.5
Fat mass (kg)	Mean (SD)	23.7 (8.1)	23.1 (8.2)
	Range	6.7–50.4	5.6–55.9
Lean mass (kg)	Mean (SD)	48.8 (10.3)	48.8 (10.1)
	Range	31.9–81.1	32.5–78.4
Bone mineral content (BMC) (kg)	Mean (SD)	2.2 (0.5)	2.2 (0.5)
	Range	1.4–3.8	1.3–3.9
Soft tissue (kg)	Mean (SD)	72.5 (13.4)	72.0 (13.2)
	Range	46.1–125.3	46.4–116.1
Fat mass (%)	Mean (SD)	32.5 (8.4)	31.9 (8.6)
	Range	11.7–49.9	9.9–51.3
Regional fat mass (%)	Mean (SD)	31.6 (8.2)	31.0 (8.4)
	Range	11.3–48.8	9.5–50.2
Bone mineral density (BMD) (g/cm^2)	Mean (SD)	1.1 (0.1)	1.1 (0.1)
	Range	0.8–1.5	0.8–1.5
T score	Mean (SD)	-0.9 (1.2)	-0.9 (1.2)
	Range	-3.3–4.1	-3.3–3.0
Fat mass/ lean mass	Mean (SD)	0.5 (0.2)	0.5 (0.2)
	Range	0.1–1.0	0.1–1.1

Table 4.5 Dual x-ray bone densitometry (DXA) results for all Nu-AGE subjects at baseline (n=272), showing mean, standard deviation (SD) and range for each variable.

4.3.2 Diet diary analysis

To assess compliance self-reported 7DDs were kept at baseline and post-intervention by the study participants, which allowed MED diet scores to be calculated to determine how well participants complied as a whole. The MED diet scores were calculated for each participant, pre- and post-intervention, according to the method described by Sofi et al. (2014). The mean MED diet score for the control group was 5.8, and for the MED diet group was 5.5, at baseline (Table 4.6). In both groups, the mean scores decreased at post-intervention, to 4.7 and 5.1, respectively. When comparing mean change in MED diet scores from baseline for the two groups, the control group decreased, on average, by one point, while there was little change in mean score of the MED diet group. The differences between the mean change from baseline between the two groups was significantly different (P=0.03), however, as the mean MED diet score for the MED diet group did not increase this does not show evidence of an association between score and group allocation, control or intervention, despite the significant difference between the two groups (Figure 4.3). This analysis used ITT as a less biased estimate, and it was evident that completion rate was not related to the intervention (ITT), as the distribution of drop outs between the two groups was equal. This was achieved by including all participants who were randomised into the study, regardless of whether or not they completed it, in order to avoid over-confident estimates of the intervention effect which could be achieved as a result of removing non-compliant participants (Gupta, 2011).

	Control (n=57)	MED Diet (n=65)
Pre-intervention		
Mean (SD)	5.8 (1.6)	5.5 (1.7)
Median	6.0	6.0
Range	3–10	2–10
Post-intervention		
Mean (SD)	4.7 (1.4)	5.1 (1.7)
Median	5.0	5.0
Range	1–9	2–7

Table 4.6 Mean and median values for MED diet scores for the control and MED diet groups, at pre- and post-intervention.

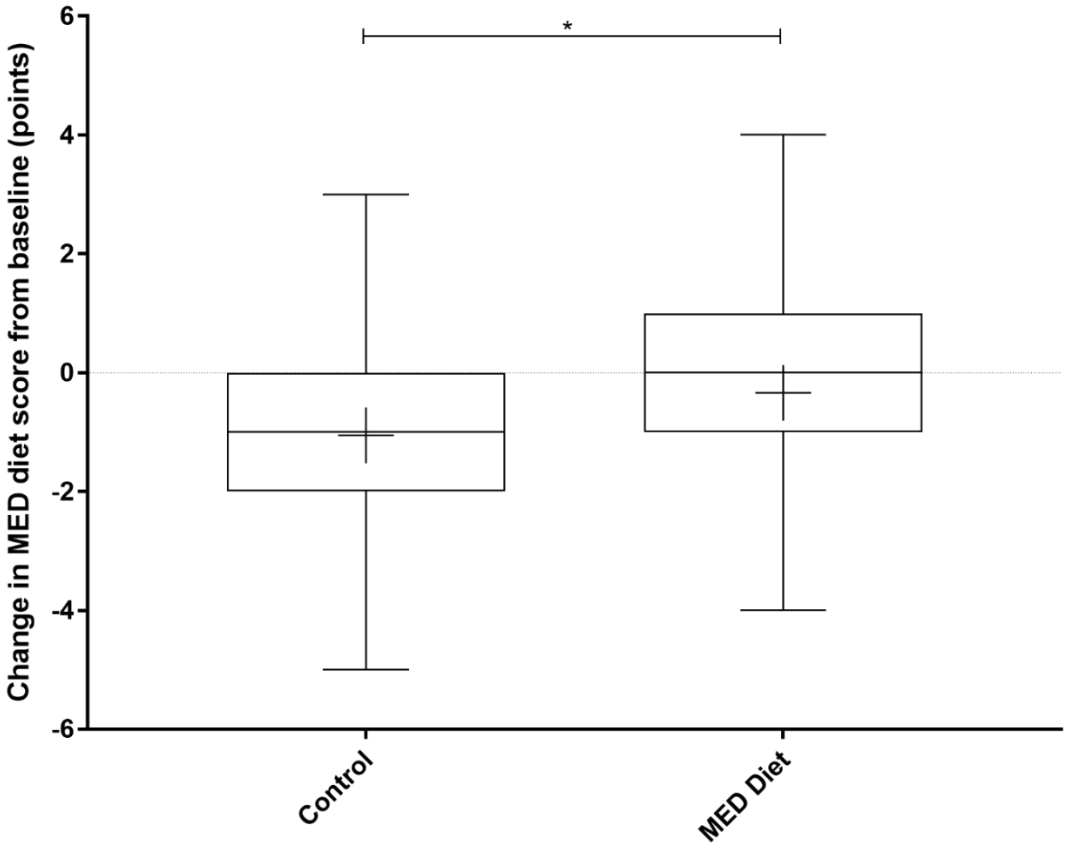


Figure 4.3 Change in Mediterranean diet scores from baseline in the control and MED diet groups. Shown as box and whisker plots which extend to the 25th and 75th percentiles, with the mean change from baseline represented by the plus (+) and median change by the horizontal line through the box plots. Whiskers are determined using Tukey's method, which uses the 25th and 75th percentile, plus 1.5 times the interquartile range (IQR). An unpaired t-test was used to determine whether the changes in the diet score differed between the two groups. *p<0.05.

The MED diet scores were created using the following nine categories; cereals, fruits, vegetables, legumes, fish, meat, dairy, alcohol and olive oil. Multivariate analysis was also carried out in the form of principle component analysis (PCA), (Figure 4.4), showing the first two principle components. The results of the Welch t-test suggest an association between the score and intervention ($p=0.0028$) and whether there was a clear difference between the groups according to the nine food groups. The PCA plot showed no separation in terms of clustering as control group (open circles) and the MED diet group (closed circles) did not separate into clusters within the plot; a lack of variability in the diets between the two groups within the study was shown.

To investigate the predictive ability of correctly classifying the group based on the nine dietary categories, partial least squares-linear discriminant analysis (PLS-LDA) was used, using three PLS factors, as this was the highest number to correctly classify the group (164/253) but the fewest PLS factors in which to achieve this. The null hypothesis of making successful predictions was 0.5 and the model was based on a two-sided comparison of the binomial distribution. Figure 4.5 shows that 11% of the variance in the food intakes can be explained by the predictive component of the model, while 19% of the variance can be explained by the orthogonal scores, which were unrelated to dietary intervention. The figure (4.5) shows that olive oil was the best variable to predict compliance to the MED diet as there was a very low value of unrelated scores (orthogonal scores), while there was between 50–90% correlation with the prediction; the next best predictor was alcohol consumption. There was strong evidence that the prediction of total olive oil intake was associated with the MED diet ($p=1.82 \times 10^{-9}$), as defined by a non-parametric two sample t-test, after controlling false discovery rate, in order to control the number of type one errors of falsely classifying results as significant (Verhoeven et al., 2005). Again, it was observed that total olive oil intake was significantly different between the two groups.

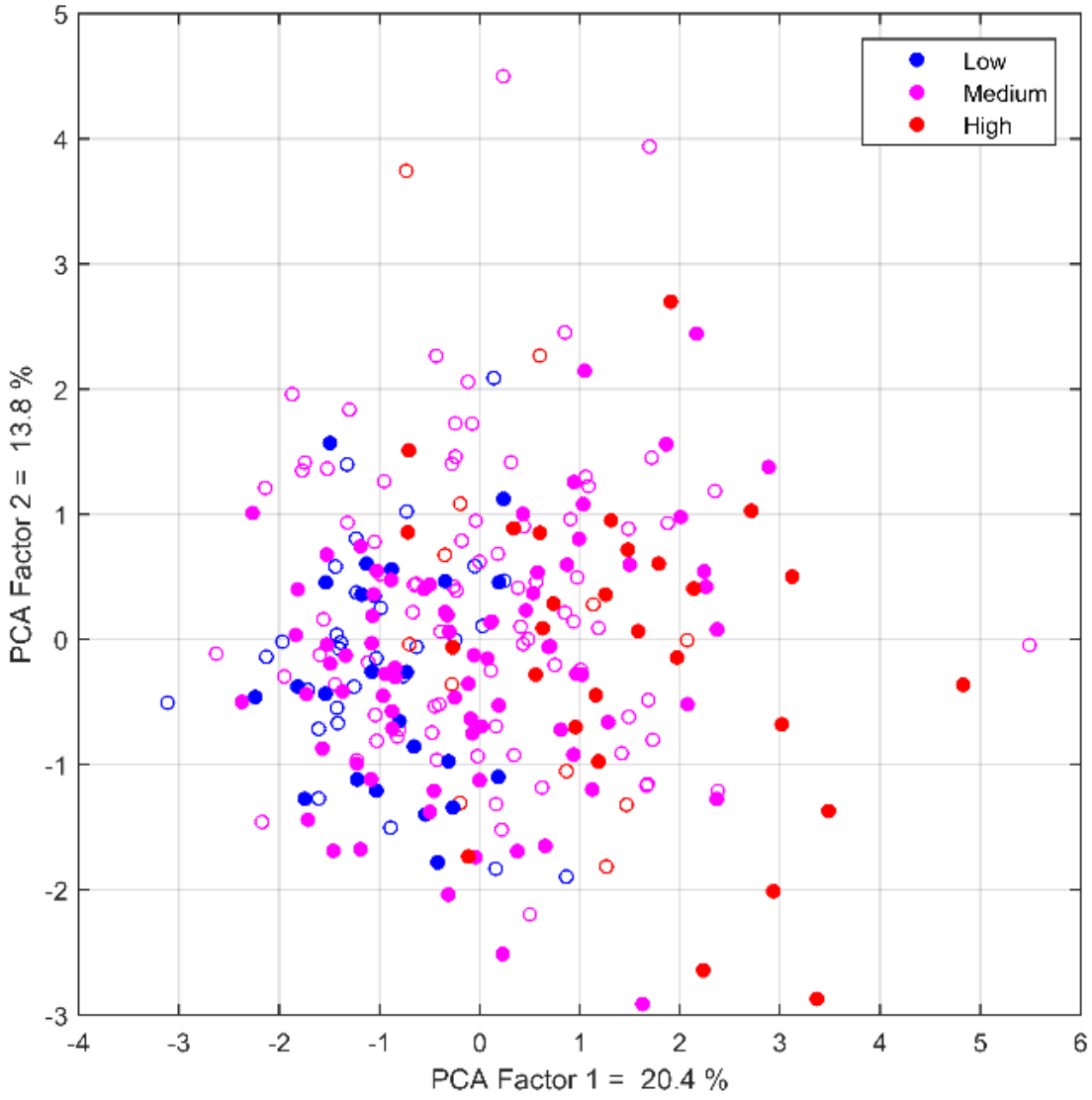


Figure 4.4 Principle component analysis score plot for variance of intake of the nine categories of food intake utilised to compile the Mediterranean diet score. Control group, open circles; MED diet group, filled circles. PCA plot produced in R by Dr Henri Tapp.

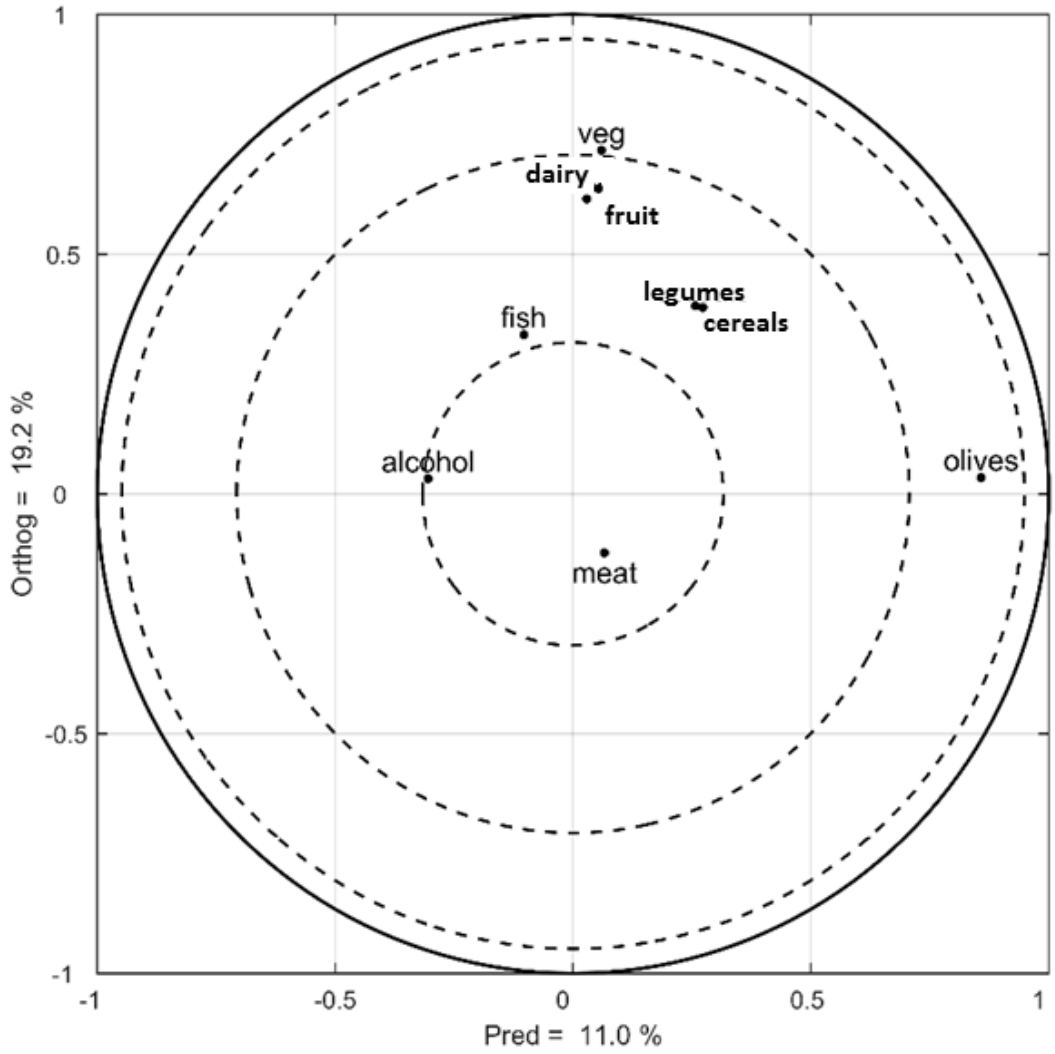


Figure 4.5 Target Plot (Tapp et al., 2011) representing the correlation between predictive and orthogonal scores for a three-component Partial Least Squares regression model. Each dot represents one of the nine food groups, as labelled, the dashed lines represent the 10%, 50% and 90% of the variance, respectively from the inner to the outer circle, in each food group that is explained by these two scores; produced using R by Dr Henri Tapp.

4.3.3 Compliance

To ascertain a more valid and biologically determined indication of study participants' compliance, it was decided that since olive oil was given out as part of the study it would be appropriate to determine levels of one of the main phenolic constituents, HT, in the urine of participants as a biological marker of consumption. Since real life doses (25 ml) of olive oil, containing varying levels of phenolics, have demonstrated dose-dependent increases in urinary HT levels, which are typically in the form of glucuronide conjugates (Khymenets et al., 2016; Khymenets et al., 2011). The metabolites HTS and HT acetate sulphate are suitable compliance markers for olive oil consumption (Rubió et al., 2014), since olive oil undergoes extensive metabolism and biotransformation in the gastrointestinal tract (Corona et al., 2009). Therefore, to assess study compliance HTS was used as a compliance marker for assessing the urine samples.

Briefly, HPLC coupled with tandem MS was used to quantitatively determine the levels of HTS according to ion fragmentation in 24-hour urine samples collected from a subset of the Nu-AGE participants; the same participants whose PBMC samples were utilised for functionality of dendritic cells (Chapter 5) and VDJ-Seq analysis (Chapter 6).

The data show, surprisingly, that HTS was present in all samples at baseline, with a minimum concentration of 90 ng/ml, with one participant having a particularly high baseline concentration of 5154 ng/ml urinary HTS, which fell to 215 ng/ml after one year intervention and was much greater than the highest observed level at post-intervention of all participants studied. This participant was therefore classified as an outlier and was excluded from all further analysis, leaving 17 participants in each group (Table 4.7). Upon grouping the samples according to study group allocation, control or intervention, the MED diet group showed a greater increase from baseline in concentration of HTS, with 11 out of the 17 participants showing an increase as opposed to a decrease from baseline levels (Figure 4.6). When change in HTS concentration from baseline was compared between the control and MED diet groups, using an unpaired t-test, (Figure 4.7) there was strong evidence that HTS concentrations differed between the two groups ($p=0.0063$). Additionally, the group with the higher levels of HTS was also the group which were found to consume significantly greater quantities of olive oil, determined when analysing data from their 7DDs (Figure 4.7).

	HTS concentration (ng/ ml)	HTS concentration (ng/ ml)	
	Baseline	Post-intervention	p value
Control group (n=17)	**Median (IQR)** 268 (48, 1513)	233 (91, 839)	
MED diet group (n=17)	**Median (IQR)** 263 (90, 1201)	442 (90, 1485)	0.0063

Table 4.7 Hydroxytyrosol sulphate (HTS) concentrations (ng/ ml) at baseline and post-intervention for study participants in each group. p value: probability value.

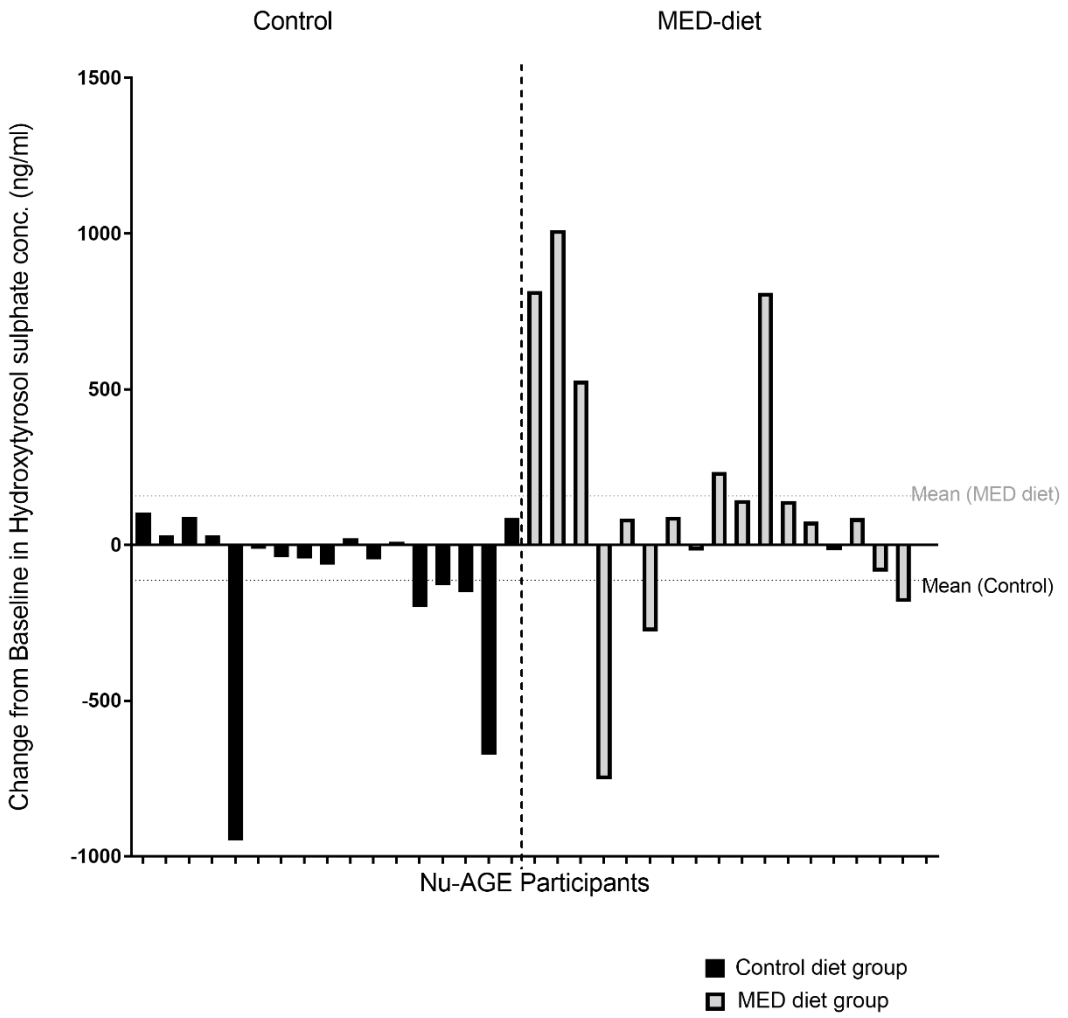


Figure 4.6 Change in hydroxytyrosol sulphate (HTS) concentrations (ng/ml) from baseline in the control and MED diet groups. Each bar represents one Nu-AGE participant, with the height of bars showing increased or decreased concentrations from baseline. N=34; 17 participants in each group. Dotted horizontal lines represent the mean change from baseline for the control group (black), and the MED diet group (grey)**.**

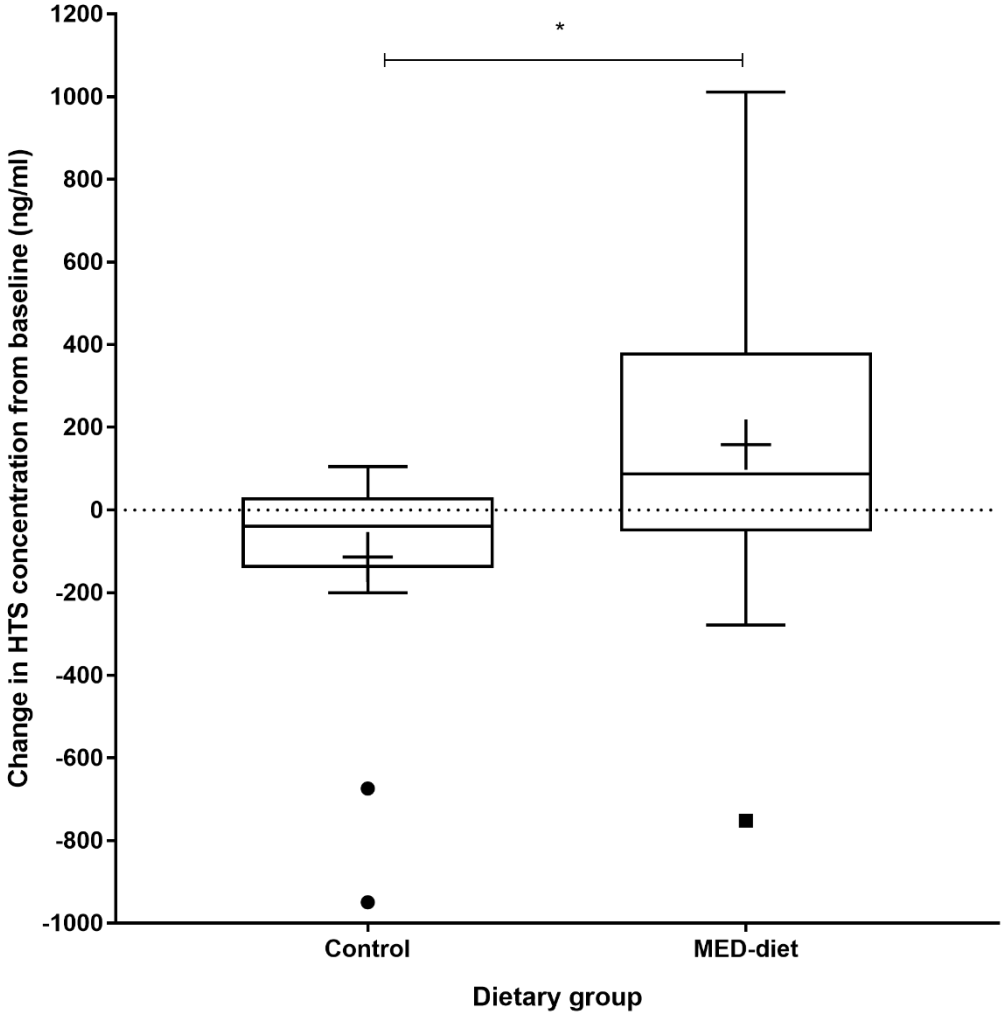


Figure 4.7 Change in hydroxytyrosol sulphate (HTS) concentration (ng/ml) from baseline, for the control and MED diet groups. Shown as box and whisker plots which extend to the 25th and 75th percentiles, with the mean change from baseline represented by the plus (+) and median change by the horizontal line through the box plots. Whiskers are determined using Tukey's method, which uses the 25th and 75th percentile, plus 1.5 times the interquartile range (IQR). An unpaired t-test was used to determine whether the changes in the HTS concentration differed between the two groups.*$p<0.05$.

4.4 Discussion

4.4.1 Subject characteristics are comparable between study groups

Comparison of the control and intervention groups showed that participants are evenly matched in terms of age, gender, weight, BMI, height and waist circumference in each group. While the MED diet group appear to have a greater range of weight, BMI and waist circumference values, the mean values for these characteristics are similar between groups and variability between people is to be expected. When comparing these values to previously published data these data correspond with the EPIC-Norfolk figures as the overall mean values for all of the Nu-AGE participants fall within the range from the men and women in the EPIC study. When looking at gender specific means (data not shown) the values between the two studies are very similar (Park et al., 2011). The DXA results indicated that at one year post-intervention body composition is generally maintained, however, mean T scores, which are used as a measure for diagnosing osteopenia and osteoporosis, are considered low (< –1.0 but >–2.5), and those subjects at the lower end of the range have very low scores (≤-2.5) (NHANES, 2007), characteristic of osteoporosis (Ramos et al., 2012). Additionally, the upper range of T scores is reduced from 4.1 to 3.0, showing a reduction in bone density after one year. Similarly, another MED diet intervention, one year in duration, showed no effect on BMD values in elderly subjects (Bullo et al., 2009).

4.4.2 MED diet scores are higher in the MED diet intervention group than the control group

A recent meta-analysis (Sofi et al., 2014) developed a literature-based MED diet score (MEDI-LITE) utilising median or mean values for each food group from the studies evaluated, and included weighting for the number of subjects enrolled in the study, gender and age. This method includes fruits, vegetables, legumes, cereals, fish, meat and meat products, dairy products, alcohol and olive oil, all of which have a determined portion size calculated from the mean, of the median or mean values in the current literature, to give a final score out of 18 (Sofi et al., 2014). Other methods of measuring adherence to the Mediterranean diet include the absolute-normative Mediterranean score (MEDAS) (Dominguez et al., 2013), which utilises the Food Frequency Questionnaire from the PREDIMED study and comprises 136 items but similarly is based on scoring of either 0 or 1 depending on cut-off values, as well as the Mediterranean diet score (MDS) which was the first scoring method, proposed in 1995 (Pérez-Tasigchana et al., 2016; Trichopoulou et al., 2003; Trichopoulou et al., 1995). The MEDI-LITE score has recently been validated against the MDS in a cohort of 204 healthy subjects (23–78 years) and is able to differentiate adherent from non-

adherent subjects, and demonstrates significant positive correlations between the two scoring methods for all nine food groups (Dinu et al., 2017). Therefore, although different methods of analysis could have been used to calculate these scores, as the methods are similar the overall outcome would likely be the same. The present method was chosen since it utilises absolute values for determining scores for each food group, allowing consistency when comparing its use between studies. The results observed show an insufficient alteration in dietary intake towards a MED diet in the intervention group at post-intervention, and the dietary patterns are similar between the groups at both pre- and post-intervention, implying that the study participants may not have followed the dietary guidelines set out for them by the study team. Self-report diet diaries in nutritional intervention studies are less sensitive when detecting minor changes to the diet and it should also be noted that the methods of self-reporting dietary intakes are limited in their validity. However, comparisons between FFQ and 7DDs have been carried out and although the FFQ is less prone to the introduction of human error since they are machine-readable, while the 7DD requires manual coding (Brunner et al., 2001), the FFQ is associated with participants recording the dietary patterns they believe to be the desired response in an intervention study (Cade et al., 2002). Comparison of 7DD and FFQ with urinary measures of sodium, potassium and nitrogen show the 7DD to be the better method for estimating average intake (Day et al., 2001; McKeown et al., 2001), however, these studies only look at three individual nutrients so cannot predict the ability of the self-report methods for other food groups.

The food group that shows the best prediction of compliance to the MED diet is olive oil, which was expected since olive oil was provided to study participants as part of the intervention study. However, cereals are more influenced by orthogonal scores than predictive scores which is surprising since wholegrain pasta was also provided to study participants. This could be because this food group included all types of cereals, both refined and wholegrain, so intake of large quantities of cereals did not necessarily correlate with high wholegrain intake.

4.4.3 Subjects allocated to the MED diet group have higher urinary HTS concentrations at post-intervention

Olive oil is the predominant source of fat in the MED diet and is high in MUFA but also contains phenolic components, such as HT (Perez-Jimenez et al., 2005). The concentration of HT in olive oil varies dependent on the amount of processing and

the olive variety, in addition to the source of olives (Owen et al., 2000; Ramos-Escudero et al., 2015; Romero and Brenes, 2012). Upon consumption of olive oil the majority of HT is absorbed in the small intestine, since ileostomy effluent post consumption of olive oil phenol rich supplements contained low concentrations of HT (Vissers et al., 2002). While it has been demonstrated, *in vitro,* that HT remains intact after passing through the acidic conditions similar to that of the stomach, with elevated levels observed in the small intestine (Corona et al., 2009), which together suggest that HT is mostly absorbed once the ileum is reached. It is thought that the sulphate conjugate metabolites are produced in the liver after HT has crossed the small intestinal epithelium, and these products of methylation, sulphation and glucuronidation are then excreted in the urine (D'Archivio et al., 2010).

These data show, surprisingly, that HTS is present in all samples at baseline, with the minimum concentration being 90 ng/ml (or 0.39 μM), though similar observations are made in previous studies (Khymenets et al., 2011; Rubió et al., 2014). Additionally, it has been noted that even after strict dietary control and fasting prior to blood sampling it is not possible to clear HT from biological fluids (Miro-Casas et al., 2003), suggesting that detection of HT in all samples is to be expected.

A comparative study looking at the administration of olive oil to rats or humans showed much greater basal urinary levels of HT of 180 μg/ 24 hours from the human samples with concentrations reaching 1118.8 μg/ 24 hours after administration of olive oil (Visioli et al., 2003). This provides further evidence of the difficulty to clear HT from biological fluids and implies that there is variability between participants, explaining the inter-subject variability. Additionally, these comparable studies were investigating solely HT or olive oil, and their designs included control groups provided with a placebo, with baseline measurements taken after a run-in period of a low-phenolic diet (Khymenets et al., 2016; Khymenets et al., 2011; Suárez et al., 2011). The Nu-AGE study differs from this design, making comparisons between these studies difficult. As participants in the present study were not asked to consume a low-phenolic diet prior to the provision of their baseline urine samples, it is possible that they were habitually consuming olive oil or other phenolic compounds, resulting in the presence of HTS in their urine samples.

However, using this as a baseline was still valid as each participant serves as their own control, so if their dietary pattern changed to include greater amounts of olive oil then this would have been observed as an increase from baseline. Upon comparison of the MED diet scores with the concentrations of HTS, it is apparent that the two sets of data do not align, showing that the participants in the MED diet intervention may not have adopted a Mediterranean style diet, but instead only consumed more olive oil, or phenolic rich foods. However, while the data from the MED diet scores demonstrate that this type of data was insufficient to record compliance, it demonstrates that it is important to include a measure of biological compliance, especially if only small changes to the diet were made, since they may not be easily detected from a self-report diary.

One participant has a very high urinary concentration of HTS at baseline of 5154 ng/ml, which decreases to 215 ng/ ml after one year intervention. Upon investigation of the participant's diet diary it is apparent that while their consumption of olive oil is relatively low, at 1.57 g per day, their consumption of red wine is substantially higher at 203 g per day; which may be contributory to the observed elevated HTS concentration. This participant's data are therefore excluded from the overall analysis. Recent investigations into the consumption of alcohol, in particular red wine, have demonstrated that while the concentration of HT in red wine is low, red wine appears to interact with dopamine oxidative metabolism resulting in the formation of HT (de la Torre, 2008; Pérez-Mañá et al., 2015; Pérez-Mañá et al., 2015). The levels of HTS recovered in urine samples may therefore be representative of not just olive oil consumption but a sum of the consumption of olive oil, directly, and red wine consumption. This may add value to the data of this study as red wine is part of the MED diet, so elevated HTS caused by either of these pathways is a sign of compliance, but it does not discriminate between these two dietary components.

Overall, the study population used for this study is suitable as the participants fit the criteria for inclusion and exclusion, as well as being distributed between the two groups effectively, such that neither group is biased in any way. The diet diary data shows that the participants may not have followed the instructions given to them regarding taking part in the study. However, the urinary compliance data gives some validity to the data that this study produces, since it can be seen that participants in the MED diet group display significantly greater change in HTS concentrations compared to the control, suggesting that they consumed some components of the study diet, possibly olive oil or other phenolic rich foods. This shows that theoretically the dietary intake may have caused the changes in immune parameters found. The

choice of HTS as a sole compliance marker is a limitation to this study, and more biomarkers would have produced more conclusive results. Additionally, the Nu-AGE study design was limited because the subjects only attended two visit days, and thus only provided two sets of samples for analysis. While considering the ethical constraints of this study, it would have been easy to obtain further urine samples, so this should have been carried out to give more data points for this analysis, upon successful approval of an ethical amendment.

Chapter 5

Impact of diet on the distribution and function of plasmacytoid and myeloid dendritic cells in aged individuals

5.1 Introduction

With increased age a significant reduction in numbers of mDCs was shown in Chapter 3, in addition to significantly reduced secretion of pro-inflammatory cytokines (IL-1β, IL-8, IL-6, TNF-α and MCP-1) upon TLR stimulation, and elevated secretion of resistin by DCs and PBMCs, respectively. The presence of FAs has been shown to both increase and decrease pro-inflammatory cytokine secretion by DCs (Fogarty et al., 2015; Zapata-Gonzalez et al., 2008; Zeyda et al., 2005), of which the type of FA is of principal importance. In addition, SCFAs and polyphenols have been demonstrated to reduce secretion of IL-6 and IL-12 by LPS-stimulated MoDCs and murine derived DCs, respectively (Delvecchio et al., 2015; Geisel et al., 2014; Nastasi et al., 2015). While there is some evidence that dietary intake can influence cytokine secretion by PBMCs (Vulevic et al., 2008; Vulevic et al., 2015; Zhao et al., 2007), this was not reproduced in other studies (Grieger et al., 2014; Thies et al., 2001; Wu et al., 1999). These findings suggest there is potential for a MED diet to impact on the age-associated changes to DC numbers and function as this traditional dietary pattern includes unsaturated FAs in the form of olive oil and oily fish, and high intakes of wholegrains, fruits and vegetables providing a source of SCFAs and polyphenols.

5.1.1 Evidence of a dietary effect on DCs

Lipids

To date only one study (Rehman et al., 2013) has shown that blockade of FA synthesis, using acetyl CoA carboxylase inhibitor (Tall oil fatty acid, TOFA), inhibited DC production and significantly increased production of pro-inflammatory cytokines (IL-1α, IL-1β, IL-6, IL-10, IL-12, IFN-γ, IP-10 and MCP-1), using MoDCs and murine derived BMDCs. These results imply that FAs have a role in DC production and function. However, no studies, to date, have directly assessed the effect of diet on peripheral blood DC composition and function in elderly populations. While the effect of FA intake on the production of cytokines by DCs has been investigated in both human studies and *in vitro* models. The type of FA has been shown to be important in the DC response, since LPS-stimulated MoDCs released IL-1β and IL-6 and

strongly induced IFN-γ production by T cells with palmitic acid treatment (Stelzner et al., 2016). Additionally, provision of a high fat diet (breakfast, 965 kcal, 65 g fat; lunch, 870 kcal, 42 g fat) induced significantly elevated release of TNF-α, IL-1β and IL-6 by LPS-stimulated MoDCs and $CD1c^+$ human mDCs from T1D patients (18–65 years), but only in the fasted state (Fogarty et al., 2015). These findings suggest that SFA has pro-inflammatory effects on DC function but they were not as a consequence of systemic LPS and that therefore, diets which reduce SFAs in favour of increasing unsaturated FAs may be favourable. Especially since oleic acid (MUFA) (250-500 μM) blocked the palmitic acid induced effects of reduced MHC I expression, inhibited T cell stimulation (Shaikh et al., 2008) and induced secretion of IL-17A by T cells (Stelzner et al., 2016). Whereas, PUFA administration to human derived MoDCs significantly reduced TNF-α, IL-12, IL-10 and IL-6 release (Zapata-Gonzalez et al., 2008; Zeyda et al., 2005). This implies that SFAs act as activators of TLR4, mimicking the action of LPS, and that unsaturated FAs can inhibit this interaction resulting in reduced secretion of pro-inflammatory cytokines and T cell proliferation.

Dietary fibre

In comparison to the present knowledge of FA influence on DCs, much less is known regarding the effect of dietary fibre on DCs. SCFAs, the fermentation end products of dietary fibre, are readily absorbed by colonocytes and released into the bloodstream (Cummings et al., 1987), where they can interact with peripheral DCs. *In vitro* models using human derived MoDCs have shown that addition of SCFAs (butyrate and propionate) or intact fibres results in significant reductions in the pro-inflammatory cytokines IL-12 and TNF-α (Bermudez-Brito et al., 2015b), in addition to reduced gene expression of IL-6, IL-12, surface expression of maturation marker CD83 and a number of chemokines, in the presence of LPS (Nastasi et al., 2015). However, exposure of MoDCs, co-cultured with autologous T cells, to RS (High-maize®) alone was shown to significantly increase secretion of IL-6, IL-12, IL-8, TNF-α, MCP-1, MIP-1α and RANTES, while exposure of RS to IECs (in a transwell, co-culture system) lessened the RS induced pro-inflammatory response with only MIP-1α and TNF-α secretion by DCs showing small, but statistically significant increases (Bermudez-Brito et al., 2015a). The latter approach was more biologically relevant to the *in vivo* situation, however, since DCs would encounter dietary fibres after they have been exposed to intestinal IECs and highlights that dietary fibre may impact on DC function directly, as well as indirectly via the production of SCFAs. These observations are not, however, representative of an ageing population, so it is difficult to extrapolate these findings to the elderly.

Polyphenols

Plant-derived polyphenols have been shown to exhibit protective effects in murine and human derived DCs; by reducing secretion of IL-6, IL-8, IL-12 and IL-23 from LPS-stimulated DCs co-cultured with protocatechuic acid and isothiocyanate sulforophane (Del Cornò et al., 2014; Geisel et al., 2014). Additionally, a recent study found that treatment of LPS-stimulated BMDCs with the polyphenols quercetin and piperine reduced TNF-α mRNA expression (Delvecchio et al., 2015). The lack of studies in elderly subjects, or ageing models, makes it difficult to know whether these effects are relevant to aged individuals.

5.1.2 Effect of diet on adipokine production by PBMCs

Confounding evidence exists for the effects of dietary FAs on immune function in the elderly. Whilst studies have failed to detect an effect on serum or PBMC cytokine concentrations after consumption of large quantities of fish per week (4 portions) (Grieger et al., 2014), fish oil capsules or blackcurrant seed oil, rich in GLA and ALA (Thies et al., 2001; Wu et al., 1999). Other studies using LPS-stimulated PBMCs from hypercholesterolemic subjects (36–69 years) demonstrated reduced secretion of IL-6, TNF-α and IL-1β after consumption of an ALA rich diet (Zhao et al., 2007). Additionally, reduced T cell proliferation has been observed after fish oil consumption (Bechoua et al., 2003; Meydani et al., 1991). The variation in formulation and doses of *n*-3 PUFA administered in the aforementioned studies, makes direct comparison of studies difficult and may account for the conflicting findings in the elderly.

Investigation of dietary fibre consumption in elderly populations is sparse, although provision of a prebiotic mixture (B-GOS) to elderly subjects (64–79 years) significantly increased IL-10 and IL-8 production and reduced IL-6, IL-1β and TNF-α production by LPS-stimulated PBMCs (Vulevic et al., 2008; Vulevic et al., 2015). In addition, reduced TNF-α and IL-6 mRNA expression in PBMCs from the elderly (77–91 years) was seen after 12 week supplementation with 1.3 g/ 250 ml FOS (1.5–3 times per day) (Schiffrin et al., 2007).

Provision of nutritional supplements containing 20% PUFA, 6 g oligosaccharides, 10^9 cfu of *Lactobacillus paracasei* and 62 g carbohydrate, in addition to vitamins and minerals, to Chilean elderly subjects (≥70 years) failed to demonstrate any diet induced effect on LPS-elicited secretion of IL-1 or TNF-α (Bunout et al., 2004) by PBMCs. Of the few whole diet studies investigating the effects of a MED diet on cytokine secretion, investigators have shown reductions in plasma levels of IL-6

(Dedoussis et al., 2008; Mena et al., 2009; Panagiotakos et al., 2007) and IFN-γ (Konstantinidou et al., 2010).

5.1.3 Gaps in knowledge

In Chapter 3 both numbers and function of DCs in the elderly were shown to be reduced with age. Studies investigating the impact of dietary intervention of DC function have shown potential for ameliorating the effects of chronic age-related inflammation, as dietary MUFAs and PUFAs can inhibit cytokine secretion induced by prolonged activation of TLRs by SFAs or bacterial LPS (Rehman et al., 2013; Zapata-Gonzalez et al., 2008; Zeyda et al., 2005). In addition SCFAs, have been shown to interact with DCs in a similar manner by inhibiting LPS-induced pro-inflammatory cytokine secretion (Nastasi et al., 2015). However, the studies carried out have relied on cell lines and animal models or, have been conducted by directly adding FAs, SCFAs or polyphenols to human derived cells. There is therefore a need for human intervention studies to test these hypotheses *in vivo* in the context of consumption of food, as opposed to supplementation with single compounds to account for any interactions which may occur between food components in the appropriate target population. Furthermore, the study populations used within previous interventions were described as “older” subjects, but included subjects as young as 55 years in one study (Zhao et al., 2007), while the previous *in vitro* studies are not representative of ageing individuals, making the data less relevant to this population.

Additionally, since reduced secretion of cytokines (IL-1β, IL-8, IL-6 TNF-α, and MCP-1) and increased secretion of resistin by LPS/R848 stimulated PBMCs was observed in Chapter 3, the potential for dietary intervention to ameliorate this is worth investigating, particularly as few studies to date have looked at the effect of diet on resistin secretion. Although associations have been made with “healthy diets” containing lower SFA and elevated MUFA (Cabrera de León et al., 2014; Fargnoli et al., 2008). Resistin may have negative health effects, such as the suggested association with insulin resistance and elevated resistin levels (Koerner et al., 2005), so exploration of dietary sources to reduce the increases associated with age is of interest, however the health effect of elevated resistin is not certain at present.

5.1.4 Rationale for investigating the Mediterranean diet

Combining the aforementioned dietary components into a whole diet is beneficial in determining whether these effects are only apparent *in vitro* or when consumed in isolation, or whether they are still apparent when the components are consumed

naturally, as part of the whole diet. The MED diet is an appropriate diet to investigate since it encompasses increased quantities of dietary fibre through wholegrains, fruits and vegetables, and MUFA is the predominant source of fat, from olive oil, with increases in PUFA from fish intake but reduced SFA intakes (Willett et al., 1995). The increased fruit and vegetable intakes are also associated with increased polyphenol consumption. Therefore this diet includes all of the components with potential to influence DC production and function. As the MED diet has been shown to have a positive effect in non-MED populations in Australia and Sweden on longevity (Kouris-Blazos et al., 1999; Tognon et al., 2011), it is feasible that adoption of this dietary pattern in the Norfolk Nu-AGE cohort would be beneficial.

5.1.5 Aims and objectives

Does consumption of a MED-style diet by elderly subjects restore the reduction in mDCs observed with age? Also, can this dietary intervention increase the secretion of MCP-1, TNF-α, IL-8, IL-6 and IL-1β in DCs and PBMCs which were observed to decrease with age, or, reduce elevated levels of resistin secretion by PBMCs observed with increasing age?

Objective 1: Determine absolute numbers of mDCs and pDCs within peripheral blood extracted from elderly Nu-AGE subjects at pre- and post-intervention after consumption of a MED-style or control diet, using multiparameter flow cytometry

Objective 2: Determine cytokine response of DCs and PBMCs from elderly Nu-AGE subjects, pre- and post-dietary intervention, to TLR stimulation, using intracellular staining and multiparameter flow cytometry, and multiplex bead based immunoassays.

5.2 Methodology

5.2.1 Recruitment of Nu-AGE participants, and sample collection

Study participants were recruited onto the Nu-AGE study upon provision of informed written consent and providing they fulfilled the eligibility requirements detailed in Table 2.4; full details in Chapter 2, Section 2.2.2. Blood samples were collected from participants at the baseline and post-intervention study visit days, as described in Section 2.2.5.

5.2.2 Blood DC enumeration

The protocol was carried out following the manufacturers' instructions using whole blood (Blood Dendritic Cell Enumeration Kit, Human; 130-091-086, Miltenyi Biotec). (See Chapter 3, Section 3.2.2).

Single-stained compensation controls, prepared using Ultracomp ebeads (Affymetrix eBioscience), and unstained control samples were run on both cytometers to enable manual compensation to be applied to all sample data acquired. Acquired data was analysed using FlowJo™ software (TreeStar, San Carlos, CA), Version 10. The gating strategy (Figure 3.1, Chapter 3) for Nu-AGE samples was consistent with that described by Miltenyi-Biotec.

5.2.3 Functional analysis of blood DCs

PBMC stimulation

Frozen PBMCs were thawed in a 37ºC water bath and washed within 20 minutes in an excess of thaw media (90% RPMI 1640, 10% heat-inactivated FBS) to remove DMSO (Sigma Aldrich). Cell pellets were resuspended in tissue culture media including the same supplements as used in earlier experiments. Cell viability was determined using trypan blue viability dye exclusion (0.4%; Sigma Aldrich) and resuspended in a final concentration of 0.5×10^6 cells / 200 µl. Cells were aliquoted into 96 well flat bottom tissue culture plates (Sarstedt) and covered with a plate seal and left to recover at 37ºC in 5% CO_2 for 18 hours.

Cells were incubated in the presence or absence of LPS and R848 ± monensin, as previously described (Chapter 3, Section 3.2.4). PBMC culture supernatants were stored at -80°C prior to analysis.

Intracellular staining

Staining for surface markers, fixation of cell membranes, cell permeabilisation and intracellular cytokine staining was performed as previously described (Chapter 3, Section 3.2.4).

Data was acquired on the BD LSR Fortessa cytometer. Spectral overlap between channels was manually compensated in FlowJo™ software version 10, after measurement of single-stained compensation controls prepared using Ultracomp ebeads (Affymetrix eBioscience) and unstained control samples. Analysis of data was performed using FlowJo™ software Version 10 according to the gating strategy detailed in Figure 3.2 (Chapter 3).

Multiplex immunoassay of PBMC culture supernatants

Supernatants were thawed on ice and analysed by Multiplex immunoassay (LEGENDplex Human Adipokine panel, Biolegend) using the human adipokine panel (IL-8, IL-1β, IL-6, MCP-1, TNF-α, leptin, IL-10, adiponectin, adipsin, IFN-γ, IP-10 (CXCL10), retinoic binding protein 4 (RBP4) and resistin). The assay was performed at 21ºC in a 96-well V-bottom microplate (Greiner-Bio), according to manufacturers' instructions. Sample preparation and data acquisition were performed as detailed in Chapter 3 (Section 3.2.5).

5.2.4 Statistical analysis

Demographic information for study participants within each dietary group (control or MED diet intervention) was recorded at baseline and at the one-year post-intervention time point and the change from baseline to post-intervention was compared between groups by unpaired t tests using GraphPad Prism 7.02.

Blood mDC and pDC phenotypes were compared between groups by change from baseline to post-intervention using Mann Whitney U tests.

GraphPad Prism 7.02 was used to determine differences in proportions and cell counts of DCs positive for IL-6, IL-8 and IL-1β secretion at pre- to post-intervention for each intervention group using Wilcoxon matched pairs signed rank tests, after performing a D'Agostino & Pearson normality test to determine non-gaussian distribution. *Post-hoc* analyses were performed by one-way ANOVA using the Kruskal-Wallis test with Dunn's multiple comparisons *post-hoc* test to identify any differences in proportions of single and double positive cells.

GraphPad Prism 7.02 was used to determine differences in concentration of each analyte between unstimulated and stimulated samples using two-tailed paired t tests for each group at pre-intervention and post-intervention. Differences in concentration from unstimulated were compared per group, pre- versus post-intervention using paired t-tests. Comparison of post-intervention values to young subject values was determined by Mann Whitney U test for each analyte.

5.3 Results

5.3.1 Subject demographics

The intervention was a year in duration and thus the significant differences observed in age between pre- and post-intervention were expected (Table 5.1). Both groups had lower mean weight (-0.5 kg) and BMI (-0.3 kg/m^2) after the year intervention, but the change from baseline between the two groups was not significantly different for either variable. Similarly, change from baseline in height, waist circumference and frailty status were comparable with no significant difference observed between the groups at post-intervention. Ten participants dropped out of the study, however the gender ratios of the two groups remained similar at post-intervention, with no significant difference observed between the groups at post-intervention. Plasma lipid analysis shows that while all subjects had high total and LDL cholesterol, and normal HDL and triglyceride concentrations, these concentrations were unaffected in both dietary groups, Figure 5.1.

5.3.2 Numerical analysis of blood DC subsets

No effect was observed in numbers of either mDC (Figure 5.2) or pDC (Figure 5.3) subset, which remained consistent over the dietary intervention for both groups. There was therefore insufficient evidence to reject the null hypothesis of no difference between the cell counts in each group at the 5% significance level. Additionally, the ratio of mDC: pDC was also unaffected by dietary intervention (Figure 5.4).

5.3.3 Functional analysis of blood DCs

DC cytokine secretion in control diet samples

PBMCs were surface stained to distinguish DCs and intracellularly stained with anti-IL-8, IL-6 and IL-1β antibodies. Significant reductions in IL-1β^+ /IL-8^+ DCs were observed between pre- and post-intervention in the control group with LPS/R848 stimulation (Figure 5.5a, b; p=0.0264). A significant reduction was also observed in IL-8^- /IL-6^+ DCs in unstimulated samples from the control group (Figure 5.6a and b; p=0.0269).

Post-hoc analyses (by one-way ANOVA) showed significant differences in the proportion of IL-8^+ /IL-6^+ DCs and IL-8^+ /IL-6^- DCs in the control group after LPS/R848 stimulation (p=0.0386). Additionally, numbers of IL-8^+ /IL-6^+ DCs at pre-intervention were significantly reduced compared to IL-8^+ /IL-6^- DCs at post-intervention (p=0.0380), after LPS/R848 stimulation.

		Pre-intervention		Post-intervention				
		Control Group (n=57)	**MED Diet Group (n=65)**	**Control Group (n=54)**	**MED Diet Group (n=61)**	**Mean CFB in Control Group**	**Mean CFB in MED Group**	**p values (Control v MED)**
Age (years)	Mean (SD)	71.0 (4.1)	69.8 (4.2)	71.6 (3.8)	70.8 (4.2)	0.6	1.0	0.55
	Range	65–79	65–79	66–80	66–80			
Gender (%)	Female	61	60	61	57	0	-3	>0.99
BMI (kg/m^2)	Mean (SD)	26.6 (3.3)	26.7 (4.5)	26.3 (3.6)	26.4 (4.6)	-0.3	-0.3	0.99
	Range	20.0–37.4	18.5–43.2	18.9–37.9	18.2–45.8			
Weight (kg)	Mean (SD)	73.2 (12.5)	73.4 (15.1)	72.7 (12.6)	72.9 (14.7)	-0.5	-0.5	0.40
	Range	52.5–108.9	49.5–128.5	52.0–101.0	49.7–118.7			
Height (cm)	Mean (SD)	165.7 (9.3)	165.6 (9.1)	166.1 (9.3)	165.8 (9.0)	0.4	0.2	0.26
	Range	148.6–187.4	145.6–188.2	149.6–186.5	146.6–187.1			
Waist circumference (cm)	Mean (SD)	91.5 (11.1)	91.3 (12.5)	91.2 (11.7)	91.5 (12.6)	-0.3	0.2	0.96
	Range	72.0–122.0	63.5–134.8	69.1–127.9	65.0–126.7			
Frailty status (%)	Non frail	77	77	78	89	1	12	0.62
	Pre frail	21	22	17	15	-4	-7	
	Frail	0.04	0	0	0	-0.04	0	

Table 5.1 Characteristics of study participants at pre- to post-intervention. p values determined by paired t tests. CFB: Change from baseline, ns: not statistically significant, SD: Standard deviation.

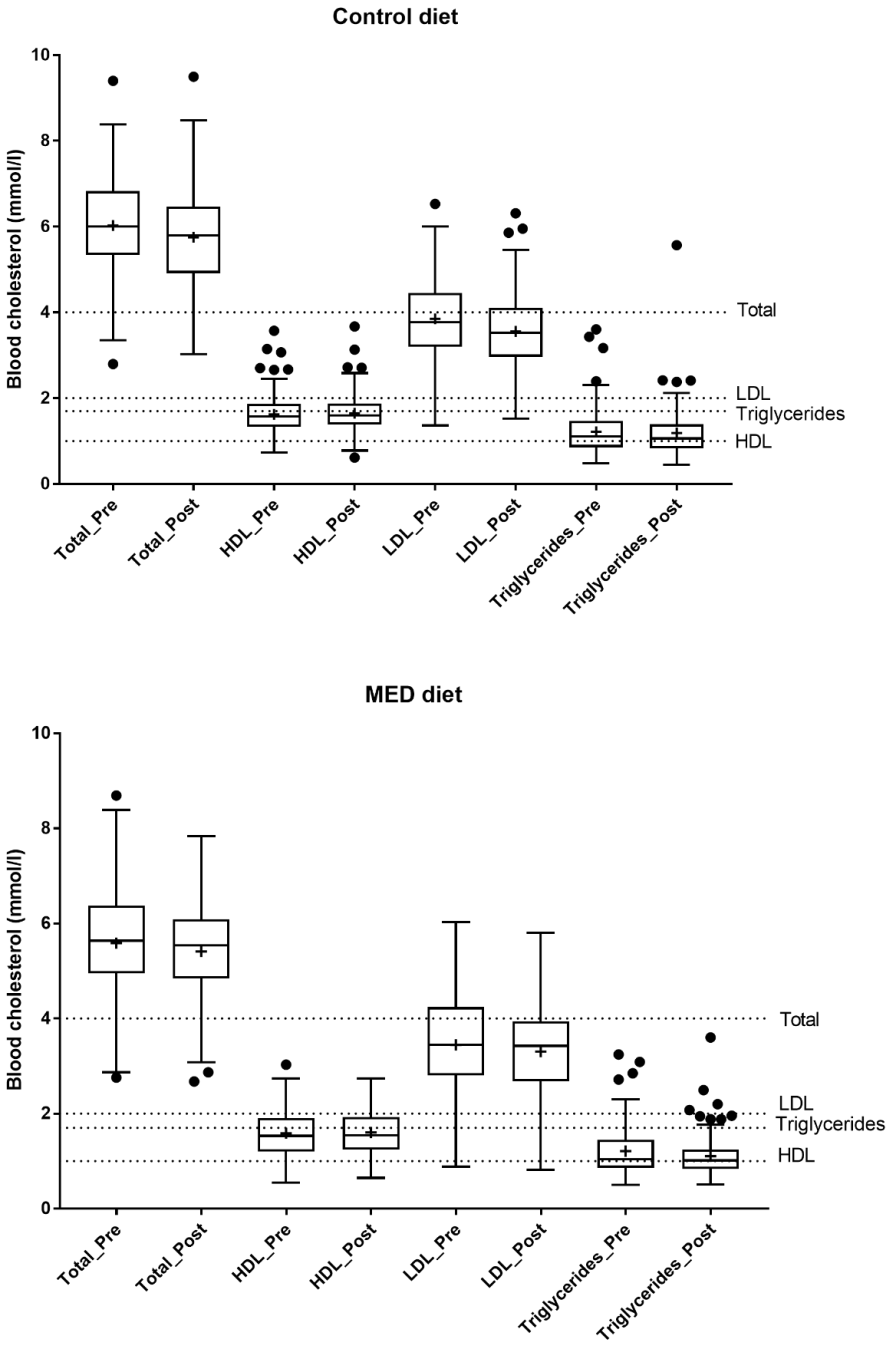


Figure 5.1 Plasma lipid concentrations at pre- and post-intervention for all subjects, separated by allocated diet group. Plasma concentrations for all Nu-AGE subjects in the control (top graph) and MED-diet group (lower graph) are shown as box and whisker plots which extend from the 25th to the 75th percentiles, with the horizontal line representing the median and the plus (+) representing the mean. Whiskers are determined using Tukey's method, which uses the 25th and 75th percentile plus 1.5 times the interquartile range (IQR). The dotted lines indicate the British Heart Foundation's recommended levels of blood cholesterol and triglycerides; total cholesterol <4 mmol/l, LDL cholesterol <2 mmol/l, triglycerides <1.7 mmol/l and HDL cholesterol >1 mmol/l (BHF, 2017). n=134, control group; n=138, MED-diet group.

DC cytokine secretion in MED diet samples

In the MED diet group no significant differences were observed on comparison of the same cell type from pre- to post-intervention in LPS/R848 stimulated or unstimulated samples ($p>0.05$).

Post-hoc analyses were performed and showed significant differences between IL-1β$^-$ /IL-8$^+$ DCs at pre-intervention and IL-1β$^+$ /IL-8$^+$ DCs at post- intervention ($p=0.004$), and IL-1β$^-$ /IL-8$^+$ DCs post-intervention compared to IL-1β$^+$ /IL-8$^+$ DCs at pre-intervention ($p=0.0146$) in unstimulated PBMCs. While, LPS/R848 stimulated IL-8$^+$ /IL-6$^+$ DCs at pre-intervention were significantly reduced compared with IL-8$^+$/ IL-6$^-$ DCs at post-intervention ($p=0.0051$), IL-1β$^-$ /IL-8$^+$ DCs at pre-intervention were significantly greater than IL-1β$^+$ /IL-8$^-$ DCs at post-intervention ($p=0.0105$) and IL-1β$^-$ /IL-8$^+$ DCs at post-intervention were significantly greater than IL-1β$^+$ /IL-8$^-$ DCs at pre-intervention ($p=0.0087$); Figure 5.5 a and b.

Significant differences were observed between IL-8$^+$ /IL-6$^+$ and IL-8$^+$ /IL-6$^-$ DCs both at pre- ($p=0.0051$) and post-intervention ($p=0.0008$) with LPS/R848 stimulation. The same was also observed for unstimulated samples ($p=0.0118$, pre; $p=0.0154$, post). While, proportions of IL-1β$^-$ /IL-8$^+$ DCs and IL-1β$^+$ /IL-8$^-$ DCs were significantly different ($p<0.0001$) at pre-intervention with LPS/R848 stimulation (Figure 5.6 a, b).

PBMC cytokine secretion

There were no significant differences in secretion of any of the cytokines analysed in the control group by unstimulated or LPS/R848 stimulated PBMCs at baseline (Table 5.2); determined by paired t-tests. While in the MED diet group LPS/R848 stimulation induced significant increases in IL-1β, IL-8 and TNF-α secretion (Table 5.3). The absolute concentrations recorded for the two groups at baseline were similarly low for Adipsin, RBP4, MCP-1, IP-10, Leptin, IL-6 and IFN-γ. However, adiponectin was detected to mean levels >100 pg/ ml in the control group, but this adipokine was not detected in the MED-diet group, at baseline. As in Chapter 3, any samples that resulted in values below the level of detection were recorded as 0.0 pg/ ml.

At post-intervention, in the control group, the secretion of IL-1β and TNF-α were significantly increased after LPS/R848 stimulation (Table 5.2). The concentration of resistin was significantly reduced post-dietary intervention (Table 5.2). All other analytes remained the same, with no significant differences observed between unstimulated and LPS/R848 stimulated PBMC samples.

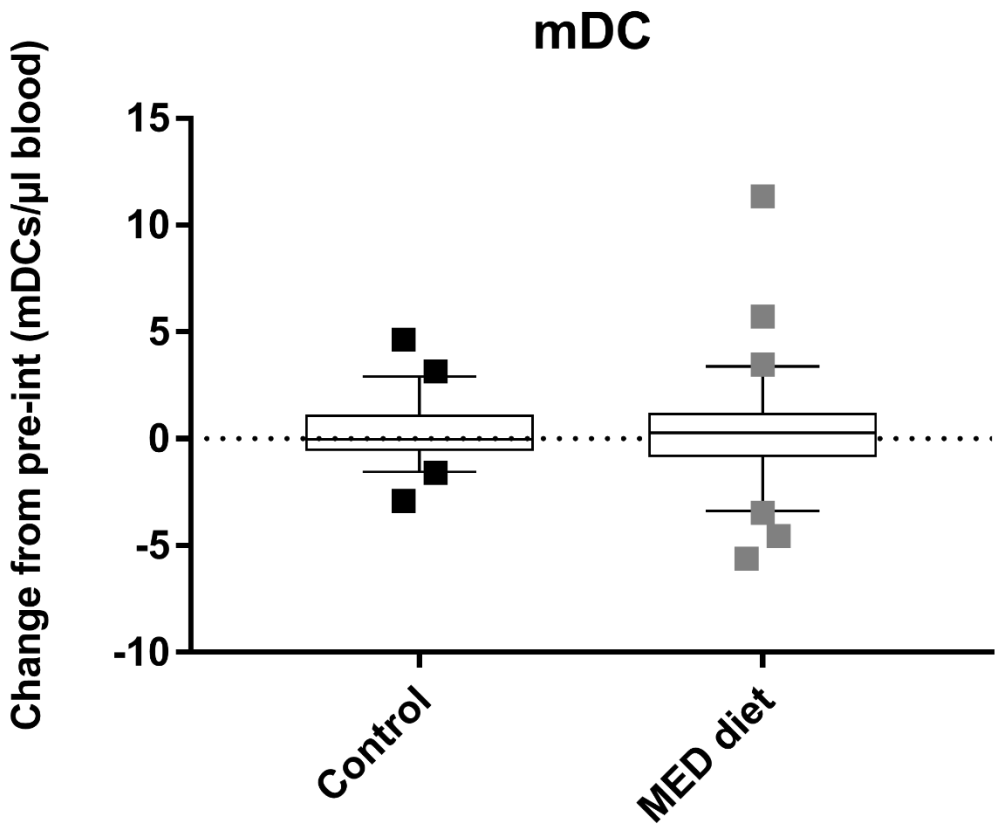


Figure 5.2 mDC counts after dietary-intervention for control and MED-diet study groups. Whole blood from control and dietary intervention groups was stained with anti-CD1c to identify mDCs, and anti-CD14 and CD19 to exclude $CD14^+$ monocytes and $CD19^+$ B cells, of which a high proportion express CD1c. The data shows box and whisker plots of change in mDC counts from pre to post intervention in elderly subjects extending from the 25^{th} to the 75^{th} percentiles, with the horizontal line representing the median. Whiskers were determined using Tukey's method using the 25^{th} and 75^{th} percentile plus 1.5 times the interquartile range (IQR) as the end of the whiskers. Squares represent individual participants where the values fell above the 25^{th} or 75^{th} quartile plus 1.5 times the interquartile range (IQR). A Mann Whitney test was used to determine the presence of differences between the control and dietary intervention cohorts. N= 58, Control group, n= 62, MED diet group, significance assumed at *p<0.05, **p<0.01; p=0.9104.

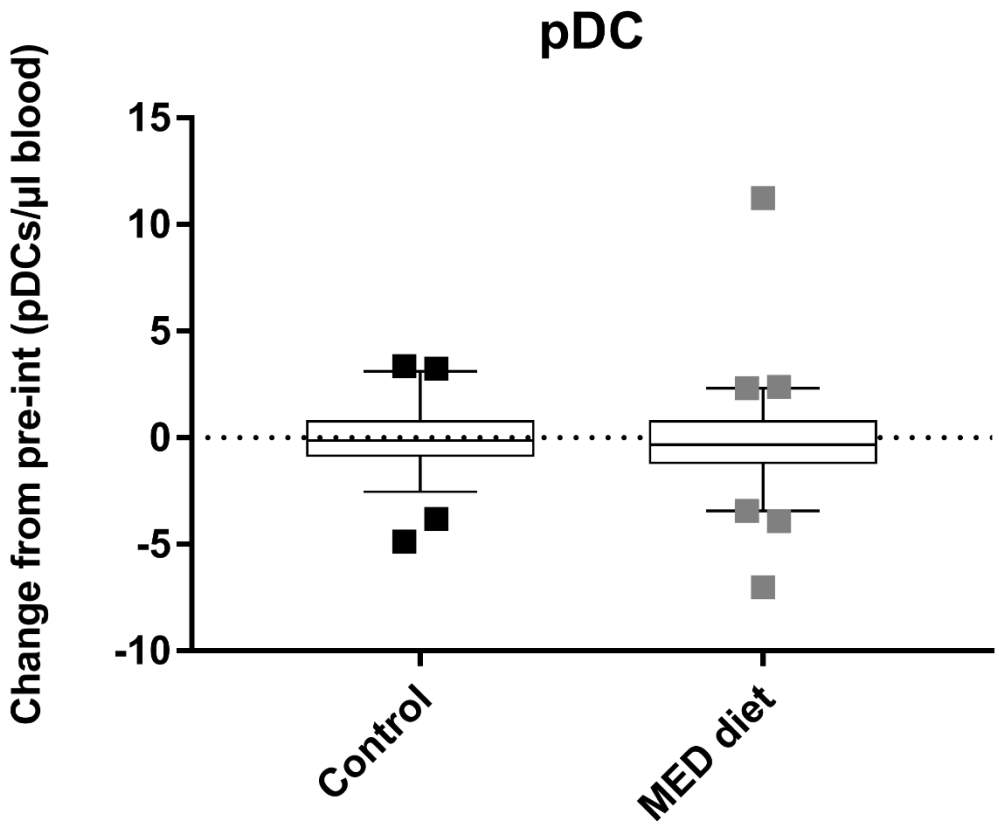


Figure 5.3 pDC counts after dietary-intervention for control and MED diet intervention study groups. Whole blood from control and dietary intervention groups was stained with anti-CD303 to identify pDCs. The data shows box and whisker plots of change in pDC counts from pre to post intervention in elderly subjects extending from the 25th to the 75th percentiles, with the horizontal line representing the median. Whiskers were determined using Tukey's method using the 25th and 75th percentile plus 1.5 times the interquartile range (IQR) as the end of the whiskers. Squares represent individual participants where the values fell above the 25th or 75th quartile plus 1.5 times the interquartile range (IQR). A Mann Whitney test was used to determine the presence of differences between the control and dietary intervention cohorts. N= 58, Control group, n= 62, MED diet Group, significance assumed at *p<0.05, **p<0.01; p=0.3553.

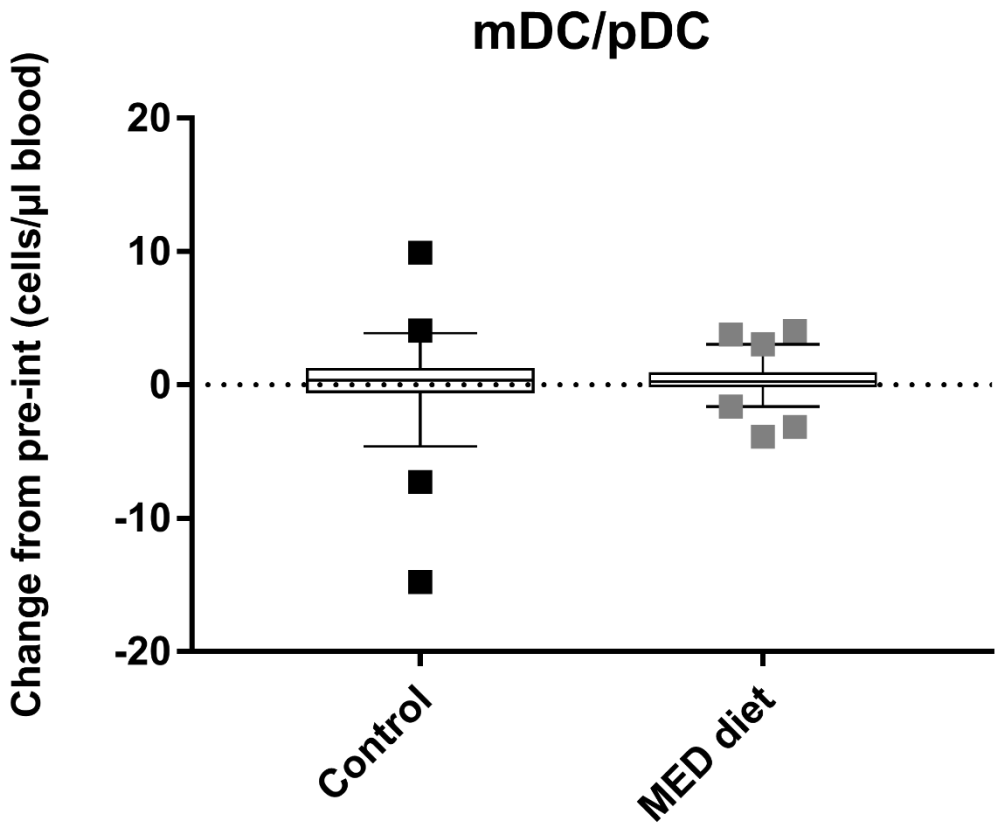


Figure 5.4 mDC/pDC ratios at pre dietary-intervention for control and MED diet intervention study groups. Whole blood from control and dietary intervention groups was stained with anti-CD1c to identify mDCs, anti-CD303 to identify pDCs , and anti-CD14 and CD19 to exclude $CD14^+$ monocytes and $CD19^+$ B cells, of which a high proportion express CD1c. The data shows box and whisker plots of change in mDC: pDC ratio from pre to post intervention in elderly subjects extending from the 25^{th} to the 75^{th} percentiles, with the horizontal line representing the median. Whiskers were determined using Tukey's method using the 25^{th} and 75^{th} percentile plus 1.5 times the interquartile range (IQR) as the end of the whiskers. Squares represent individual participants where the values fell above the 25^{th} or 75^{th} quartile plus 1.5 times the interquartile range (IQR). A Mann Whitney test was used to determine the presence of differences between the control and dietary intervention cohorts. N= 58, Control group, n= 62, MED diet group, significance assumed at *$p<0.05$, **$p<0.01$; $p=0.8404$.

Comparison of PBMC cytokine secretion from pre- to post-intervention showed that all analytes investigated, except for resistin, were not significantly affected by control or dietary intervention. Comparing changes in resistin concentration, between unstimulated pre- and post-intervention for each group revealed that while the resistin levels were not significantly different between the pre- and post-intervention sample in the control group (Figure 5.7c), they were significantly reduced at post-intervention in the MED diet group (Figure 5.7c). However, upon comparison of the two dietary intervention groups, it was apparent that while resistin secretion was significantly different from pre- to post-intervention in the MED diet group, the change in resistin secretion from baseline was not significantly different between the control and the MED diet groups (Figure 5.8c). While, the change from baseline in secretion of TNF-α and MCP-1 were significantly different between the control and MED diet groups (Figure 5.8 a, d).

5.4 Discussion

5.4.1 MED diet intervention results in weight loss

BMI values are significantly reduced in both study groups at post-intervention implying that participants were more aware or cautious of their dietary intake as a result of being on the study. Dietary advice was given to all study participants, as the control group were provided with a healthy eating fact sheet from the British Dietetic Association (Appendix X), therefore, as a result all participants may have altered their dietary habits post-intervention. Additionally, having to complete 7DDs could introduce error since this data is self-reported, reducing its reliability. However, when compared with food frequency questionnaires (FFQ), 7DD have been shown to be better able to predict potassium and nitrogen intakes, which were quantified by urinary biomarker analysis (Day et al., 2001; McKeown et al., 2001). Although, as mentioned in Chapter 4, 7DD are more prone to human error as data is manually coded (Brunner et al., 2001). Only participants on the MED diet intervention group lost a significant amount of weight at post-intervention, though the control group are close to significance at p=0.0567. The MED diet consists of fewer calorie dense sweet products such as cakes and biscuits, as well as less red and processed meat. Therefore the observed weight loss is expected, as seen in a MED diet weight loss study that resulted in greater weight loss than adherence to a low fat diet (Shai et al., 2008).

5.4.2 Diet has no effect on numbers of mDCs or pDCs distribution in elderly subjects

Although, production of DCs may be dependent on FA synthesis (Rehman et al., 2013), no studies have investigated the effect of diet on peripheral blood production of DCs. Our observation that the MED diet has no effect on mDC or pDC numbers is inconsistent with the previous findings of Rehman et al (2013). However the consistency in DC numbers from both subsets at one year post-intervention may be a positive finding as the numbers of mDCs or pDCs do not decrease further with an additional year of age. This has not been explored in previous papers, since resulting numbers, determined from one-off blood or PBMC samples from young and elderly subjects, have been compared, but the elderly have not been followed up over time.

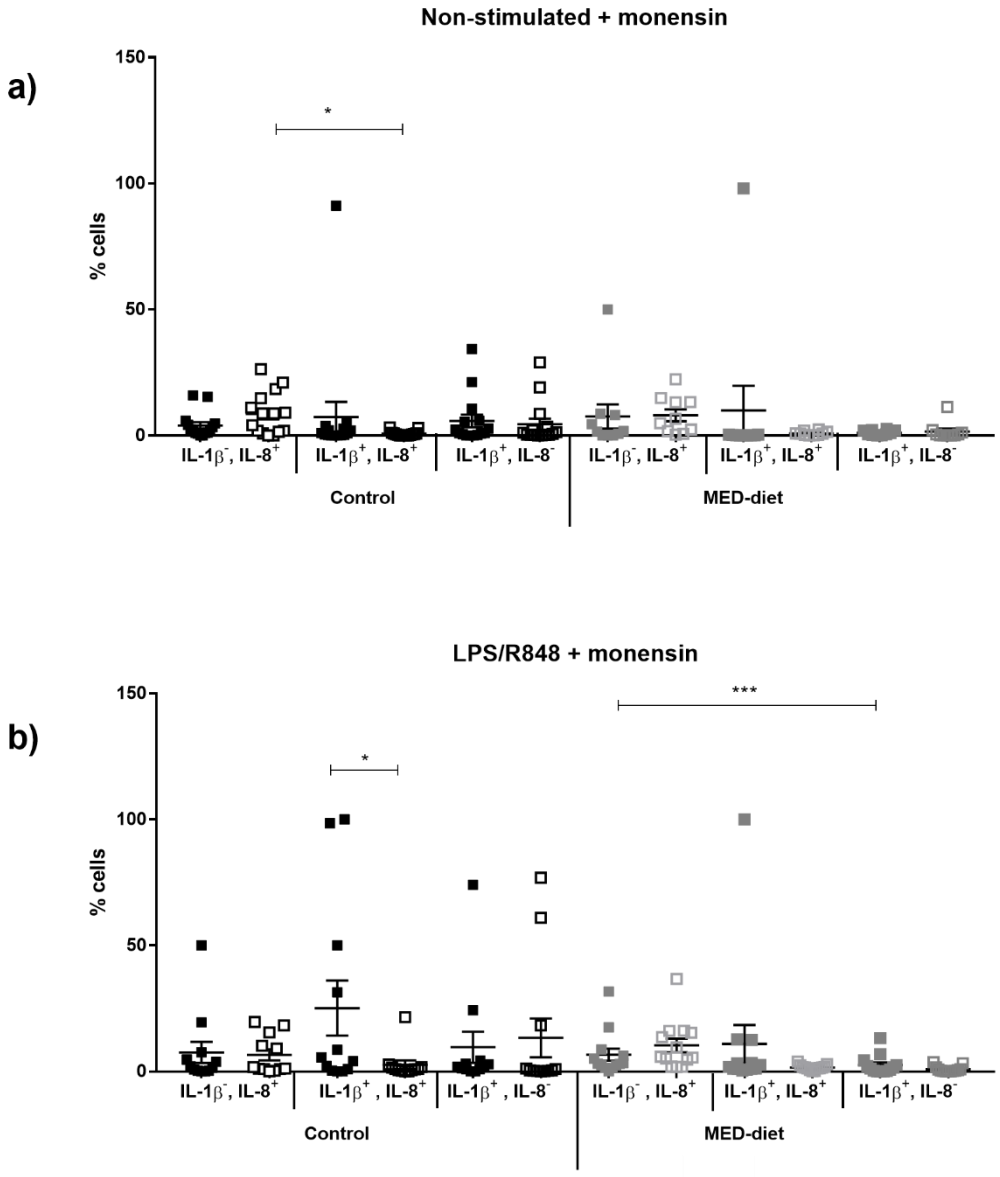


Figure 5.5 Cytokine producing DCs from elderly subjects at pre- and post-dietary intervention. PBMCs from control subjects and MED diet intervention subjects were cultured for 3 hours in complete tissue culture media alone (a), or in media containing LPS and R848 (b) in the presence of 2μM monensin. Samples were subsequently surface stained with anti-HLA-DR, CD1c, CD303 and CD304, CD14, CD16, CD19 and CD3 antibodies, permeabilised and stained with anti-IL-1β and IL-8 antibodies. Squares represent values for each individual subject with filled squares (■) representing pre-intervention values and open squares (□) representing post-intervention values. Significance assumed at $p<0.05$; *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ indicate significance between pre- and post-dietary intervention as measured by Wilcoxon matched pairs signed rank test, and between the three cell types by one-way ANOVA.

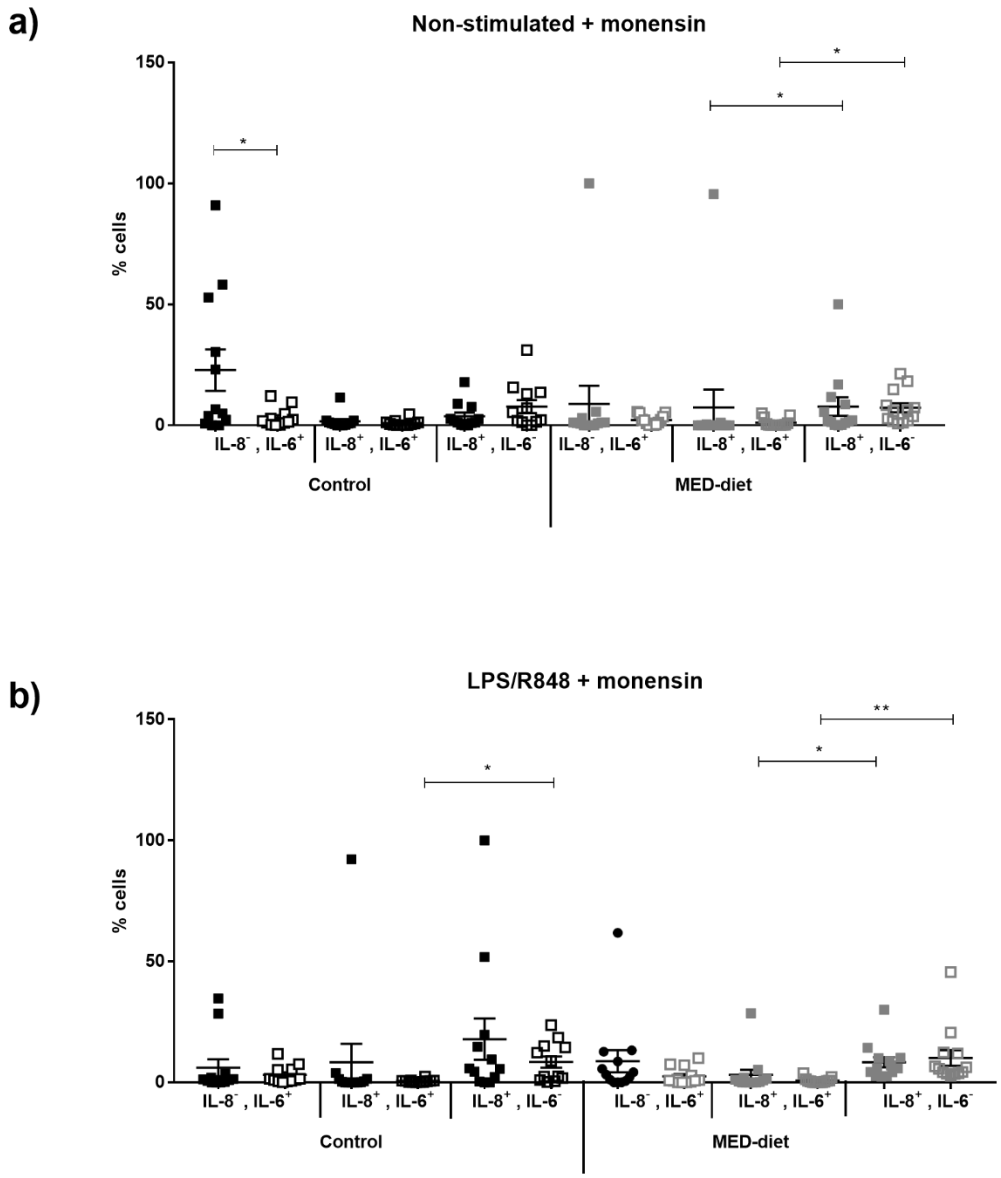


Figure 5.6 Cytokine producing DCs from elderly subjects at pre- and post-dietary intervention. PBMCs from control subjects and MED diet intervention subjects were cultured for 3 hours in complete tissue culture media alone (a), or in media containing LPS and R848 (b) in the presence of 2μM monensin. Samples were subsequently surface stained with anti-HLA-DR, CD1c, CD303 and CD304, CD14, CD16, CD19 and CD3 antibodies, permeabilised and stained with anti-IL-8 and IL-6 antibodies. Squares represent values for each individual subject with filled squares (■) representing pre-intervention values and open squares (□) representing post-intervention values. Significance assumed at $p < 0.05$; *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ indicate significance between pre- and post-dietary intervention as measured by Wilcoxon matched pairs signed rank test, and between the three cell types by one-way ANOVA.

5.4.3 The MED diet changes DC cytokine secretion

LPS/R848 stimulation of PBMCs does not induce any changes in the same cell type at pre- and post-intervention, indicating that the MED diet did not change DC cytokine secretion after one year intervention. However, significant differences were observed between pre- and post- MED diet intervention between IL-8^+ /IL-6^+ and IL-8^+ /IL-6^- DCs, IL-$1\beta^-$ /IL-8^+ and IL-$1\beta^+$ /IL-8^- DCs and IL-$1\beta^+$ / IL-8^- and IL-$1\beta^-$ /IL-8^+ DCs, after LPS/R848 stimulation. The stimuli applied to these samples was a combination of LPS, derived from *E. coli 0111:B4* which activates the TLR4 pathway, and the synthetic compound R848, which has been shown to activate cells via TLR7 and 8 (Jurk et al., 2002). LPS induced responses may therefore be more reliable, since LPS would reflect the true *in vivo* response, while the synthetic R848 may not. However, the combination of LPS and R848 demonstrated strong induction of IL-10, IFN-γ and IL-17A in $CD4^+$ T cells co-cultured with MoDCs, an effect which was not observed with each stimuli alone (Lombardi et al., 2009).

In comparison to the age associated effects observed in Chapter 3 of reduced IL-6^+ /IL-8^+ and IL-$1\beta^+$ /IL-8^+ expressing DCs, post MED diet there are fewer of these DCs although differences did not reach statistical significance. While secretion of IL-8 is seen to decrease with age, post MED diet IL-8 secretion increases, with significantly greater proportions of IL-8^+ /IL-6^- DCs compared to IL-8^+ /IL-6^+ DCs, as well as greater proportions of IL-$1\beta^-$ /IL-8^+ DCs compared to IL-$1\beta^+$ /IL-8^- DCs. These differences may be indicative of EPA and DHA (from consumption of oily fish) since these FAs diminish IL-6 secretion by LPS stimulated MoDCs, with DHA treatment providing the most prominent effects (Zapata-Gonzalez et al., 2008). Additionally, DHA decreases IL-1β secretion after palmitic acid induction (Snodgrass et al., 2016), though these results were derived from a monocyte cell line, so cannot be directly compared to data from human blood derived DCs. The recent findings of Stelzner et al. (2016), which used LPS-stimulated MoDCs, yielded similar findings of palmitic acid induced increases in IL-1β and IL-6, although oleic acid produced no effect. Additionally, no effect of human consumption of DHA and EPA rich capsules was observed on cytokine secretion by PBMCs isolated from these subjects (Kew et al., 2004). Therefore, DHA inhibition of DC activation via PPARγ and retinoic X receptors (RXR) (Zapata-Gonzalez et al., 2008) could explain the present findings of reductions in secretion of IL-6, although current evidence is limited due to very few studies being carried out to date.

Adipokine	Study time point		Media alone	Stimulated	p values
Adiponectin	Pre-int	Mean (SEM)	217.80 (135.20)	162.80 (162.80)	0.612 (ns)
		Range	0–1254	0–1791	
	Post-int	Mean (SEM)	n.d	n.d	-
		Range	n.d	n.d	
Adipsin	Pre-int	Mean (SEM)	21.28 (9.66)	25.13 (10.80)	0.551 (ns)
		Range	0–98.29	0–91.65	
	Post-int	Mean (SEM)	96.99 (62.22)	89.06 (58.75)	0.076 (ns)
		Range	0–710.70	0–669.50	
RBP4	Pre-int	Mean (SEM)	1.85 (1.85)	1.60 (1.60)	0.341 (ns)
		Range	0–20.29	0–17.58	
	Post-int	Mean (SEM)	40.07 (16.22)	36.87 (16.78)	0.115 (ns)
		Range	0–183.30	0–192	
MCP-1	Pre-int	Mean (SEM)	4.50 (3.02)	6.81 (4.69)	0.205 (ns)
		Range	0–34.13	0–52.88	
	Post-int	Mean (SEM)	16.48 (6.70)	29.77 (12.53)	0.177 (ns)
		Range	0–71.79	1.11–121	
IL-1β	Pre-int	Mean (SEM)	1.83 (1.01)	4.58 (2.49)	0.275 (ns)
		Range	0–9.49	0–25.03	
	Post-int	Mean (SEM)	25.69 (17.19)	29.22 (17.06)	0.026
		Range	0–153.70	0–158.90	
IP-10	Pre-int	Mean (SEM)	4.59 (1.75)	3.29 (1.93)	0.227 (ns)
		Range	0–17.14	0–21.72	
	Post-int	Mean (SEM)	1.35 (0.59)	1.32 (0.60)	0.963 (ns)
		Range	3.48–1751	4.87–1668	
IL-10	Pre-int	Mean (SEM)	n.d	n.d	-
		Range	n.d	n.d	

	Post-int	Mean (SEM)	n.d	n.d	-
		Range	n.d	n.d	
IL-8	Pre-int	Mean (SEM)	32.84 (19.74)	104.4 (52.48)	0.065 (ns)
		Range	0–220.70	0–580.50	
	Post-int	Mean (SEM)	318.5 (185.80)	552.10 (169.80)	0.070
		Range	3.48–1751	4.87–1668	
Leptin	Pre-int	Mean (SEM)	6.35 (1.48)	3.08 (3.08)	0.198 (ns)
		Range	0–45.20	0–33.90	
	Post-int	Mean (SEM)	n.d	0.70 (0.70)	0.341 (ns)
		Range	n.d	0–7.72	
IL-6	Pre-int	Mean (SEM)	0.50 (0.50)	3.90 (2.83)	0.185 (ns)
		Range	0–5.48	0–29.38	
	Post-int	Mean (SEM)	32.38 (22.26)	33.60 (19.39)	0.761 (ns)
		Range	0–214.30	0–181.10	
IFN-γ	Pre-int	Mean (SEM)	12.69 (10.14)	6.11 (6.11)	0.176 (ns)
		Range	0–110.7	0–67.21	
	Post-int	Mean (SEM)	0.25 (0.25)	0.24 (0.24)	0.341 (ns)
		Range	0–2.72	0–2.62	
Resistin	Pre-int	Mean (SEM)	164.90 (100.30)	183.70 (100.50)	0.531 (ns)
		Range	0–1028	0–869.90	
	Post-int	Mean (SEM)	309.10 (124.00)	285.10 (120.70)	0.124 (ns)
		Range	0–1245	0–1246	
TNF-α	Pre-int	Mean (SEM)	0.09 (0.09)	16.68 (7.67)	0.054 (ns)
		Range	0–0.96	0–75.91	
	Post-int	Mean (SEM)	2.95 (2.01)	53.26 (16.63)	0.016
		Range	0–18.85	0–151	

Table 5.2 Absolute concentrations of cytokines in PBMC culture supernatant, from elderly subjects in the control dietary intervention group, at pre- and post-intervention. SEM = standard error of the mean, ns = not statistically significant.

Adipokine	Age group		Media alone	Stimulated	p values
Adiponectin	Pre-int	Mean (SEM)	n.d	n.d	-
		Range	n.d	n.d	
	Post-int	Mean (SEM)	27.39 (20.24)	20.06 (20.06)	0.220 (ns)
		Range	0–260.70	0–260.70	
Adipsin	Pre-int	Mean (SEM)	3.83 (2.12)	4.74 (3.89)	0.743 (ns)
		Range	0–23.14	0–57.72	
	Post-int	Mean (SEM)	103.40 (48.23)	92.73 (48.59)	0.132 (ns)
		Range	0–661.30	0–651.00	
RBP4	Pre-int	Mean (SEM)	n.d	n.d	-
		Range	n.d	n.d	
	Post-int	Mean (SEM)	41.87 (11.20)	28.42 (7.81)	0.089 (ns)
		Range	0–141.70	0–79.50	
MCP-1	Pre-int	Mean (SEM)	16.19 (5.12)	33.22 (13.63)	0.093 (ns)
		Range	0–57.54	0–199.90	
	Post-int	Mean (SEM)	199.80 (183.30)	179.20 (166.00)	0.264 (ns)
		Range	0–2398	0–2170	
IL-1β	Pre-int	Mean (SEM)	0.59 (0.54)	16.37 (5.25)	0.009
		Range	0–8.17	0–52.37	
	Post-int	Mean (SEM)	41.26 (30.94)	16.57 (11.54)	0.447 (ns)
		Range	0–401.60	0–152.20	
IP-10	Pre-int	Mean (SEM)	0.73 (0.33)	1.30 (0.61)	0.225 (ns)
		Range	0–4.28	0–7.67	
	Post-int	Mean (SEM)	30.97 (28.74)	9.89 (7.62)	0.338 (ns)
		Range	0–375.70	0–101.00	
IL-10	Pre-int	Mean (SEM)	0.13 (0.09)	0.22 (0.13)	0.169 (ns)
		Range	0–1.24	0–1.85	
	Post-int	Mean (SEM)	3.27 (2.98)	0.82 (0.52)	0.348 (ns)

		Range	0–38.87	0–6.39	
IL-8	Pre-int	Mean (SEM)	175.80 (104.20)	523.40 (191.30)	0.018
		Range	0–1610	7.07–2422	
	Post-int	Mean (SEM)	810.60 (395.90)	961.10 (480.10)	0.226 (ns)
		Range	0–4738	17.27–6130	
Leptin	Pre-int	Mean (SEM)	n.d	n.d	-
		Range	n.d	n.d	
	Post-int	Mean (SEM)	0.94 (0.94)	1.19 (0.80)	0.716 (ns)
		Range	0–12.16	0–8.02	
IL-6	Pre-int	Mean (SEM)	3.69 (3.56)	23.89 (12.82)	0.056 (ns)
		Range	0–53.43	0–187.40	
	Post-int	Mean (SEM)	218.60 (208.80)	15.71 (6.24)	0.342 (ns)
		Range	0–2723	0–64.81	
IFN- γ	Pre-int	Mean (SEM)	n.d	0.22 (0.22)	0.334 (ns)
		Range	n.d	0–3.30	
	Post-int	Mean (SEM)	0.83 (0.63)	n.d	0.217 (ns)
		Range	0–8.01	n.d	
Resistin	Pre-int	Mean (SEM)	89.80 (53.89)	101.70 (60.39)	0.204 (ns)
		Range	0–747.20	0–790.70	
	Post-int	Mean (SEM)	304.60 (110.40)	251.10 (101.80)	0.049
		Range	14.03–1320	12.98–1262	
TNF-α	Pre-int	Mean (SEM)	1.28 (1.02)	71.44 (26.68)	0.021
		Range	0–15.42	0–350.90	
	Post-int	Mean (SEM)	394.20 (393.10)	18.35 (6.57)	0.360 (ns)
		Range	0–5111	0–64.89	

Table 5.3. Absolute concentrations of cytokines in PBMC culture supernatant, from elderly subjects in the MED diet intervention group, at pre- and post-intervention. SEM = standard error of the mean, n.d. = not detected, ns = not statistically significant.

Another consideration, with regard to the reduction in IL-6^{+} DCs at post-intervention could be the increase in dietary fibre, since a pilot study showed significant reductions in blood levels of IL-6 after 30 participants supplemented their diets for 30 days with pasta enriched with β-glucans (6%) (Barera et al., 2016). This finding, along with downregulation of IL-6 mRNA expression and IL-6 secretion upon butyrate and propionate treatment of LPS-stimulated MoDCs (derived from healthy donors) (Nastasi et al., 2015) or PBMCs derived from elderly subjects given the prebiotics B-GOS (Vulevic et al., 2008) suggests a role for dietary fibre and the microbiota generated SCFAs in controlling DC secretion of IL-6. However, since the present data also demonstrates some changes in cytokine secretion by DCs sampled from participants in the control group the impact of these findings is reduced.

5.4.4 The MED diet reduces resistin production

Resistin concentration, after stimulation with LPS/R848 is significantly reduced from pre-intervention ($p=0.0367$) after a one-year intervention of a MED diet. This finding could be related to the weight loss of the participants in the intervention group, as a recent study found resistin levels positively correlated with BMI as well as abdominal visceral and subcutaneous fat volume and mass, determined by computer tomography (CT) (Gencer et al., 2016). However, a weight loss study found no effect on plasma resistin levels after a short term, four week reduced calorie diet, resulting in a mean weight loss of 3.4 kg (Wolfe et al., 2004). Additionally, no association was observed between plasma resistin levels and intra-abdominal fat levels, insulin sensitivity nor MetS, and only a weak association was observed with BMI (Utzschneider et al., 2005). The relationship between the adiposity and resistin levels is likely therefore to be complicated and the weight loss achieved by the participants in the present study may not be causal in reducing resistin levels. This is especially apparent as the control group also lost weight, but there was no significant difference in weight loss between the two groups at post-intervention, and the control group showed no significant difference in resistin levels at post-intervention ($p=0.2052$). However, it should be noted that upon comparison of the change in resistin secretion from baseline, between the control and MED diet group, there was no significant difference (Figure 5.8c).

As the association of resistin levels with adiposity is uncertain, the dietary composition may account for the observed changes which has been demonstrated in a similar study, where adherence to a “healthy diet” resulted in significantly lower resistin concentrations (Fargnoli et al., 2008). In the study by Fargnoli et al. (2008) participants’ diets were assessed according to the Alternate Healthy Eating Index

(AHEI) to score intakes of fruits, vegetables, ratio of white to red meat, *trans* fats, ratio of PUFA to SFA, cereal fibre, nuts, soy and alcohol. Additionally, a cross-sectional study investigating over six thousand participants found a positive association with SFA intake and serum resistin levels, while greater intake or MUFA and adherence to a MED diet was inversely associated with serum resistin levels (Cabrera de León et al., 2014). These studies were observational and correlative. However, our findings validate these observations since the reduction in resistin may be attributed to the dietary intervention. Another key difference between these studies and the present study is the use of serum for cytokine and adipokine analysis compared to the analysis of supernatant samples derived from LPS-stimulated PBMCs. Therefore, the present findings represent the functional response of PBMCs derived from elderly participants on the intervention study, as compared to the pre-existing inflammatory state of participants, without any experimental challenge. Further studies similar to the present study are required to confirm our findings. Particularly, as although a significant reduction in resistin was observed after the MED diet intervention, the change in secretion from baseline was not significantly different to that observed for the control group. This is likely due to the observations that the two groups did not report differences in their dietary intakes in the self-reported diet diaries, which suggest that either one, or both of the groups did not follow the study guidelines provided to them. Therefore, future studies, with more stringent control over dietary intake and more frequent contact with the study participants would be beneficial. Additionally, as the intervention was not blinded to the subjects, it is possible that both groups improved their diets, as a reduction in resistin has been correlated with more nutritious diets (Cabrera de León et al., 2014; Fargnoli et al., 2008).

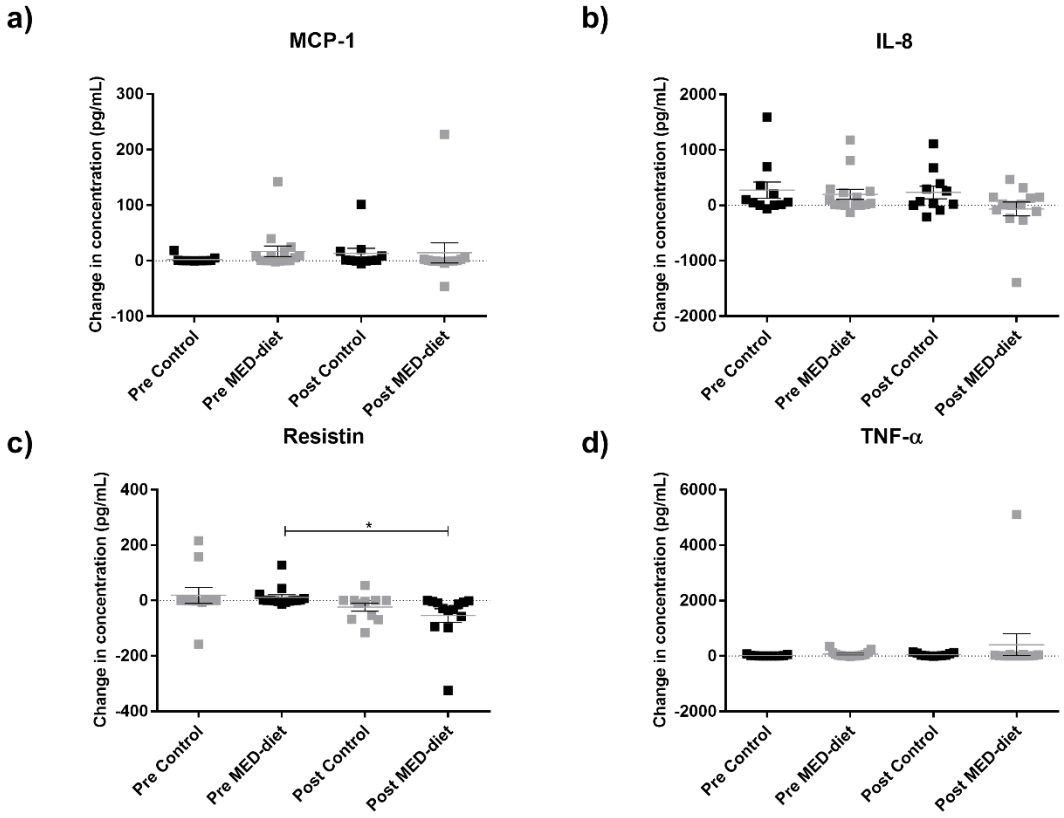


Figure 5.7 Change in cytokine production by PBMCs in response to LPS and R848 stimulation in pre- versus post-dietary intervention. PBMCs from control subjects and MED diet intervention subjects were cultured for 3 hours in complete tissue culture media alone, or in media containing LPS and R848 in the presence of 2μM monensin. PBMC culture supernatants were analysed by multiplex bead based immunoassay to determine absolute concentrations of each analyte in the samples. Concentrations (pg /mL in unstimulated samples were subtracted from stimulated samples to give a change in concentration as a result of the stimulus. Squares and dots represent individual values for change in secretion of each cytokine by PBMCs, error bars represent the SEM. Determination of significant differences between pre- and post-intervention by paired t-tests, comparison of post values to young values (●) determined by Mann Whitney U test, significance assumed at *$p<0.05$, **$p<0.01$, ***$p<0.001$.

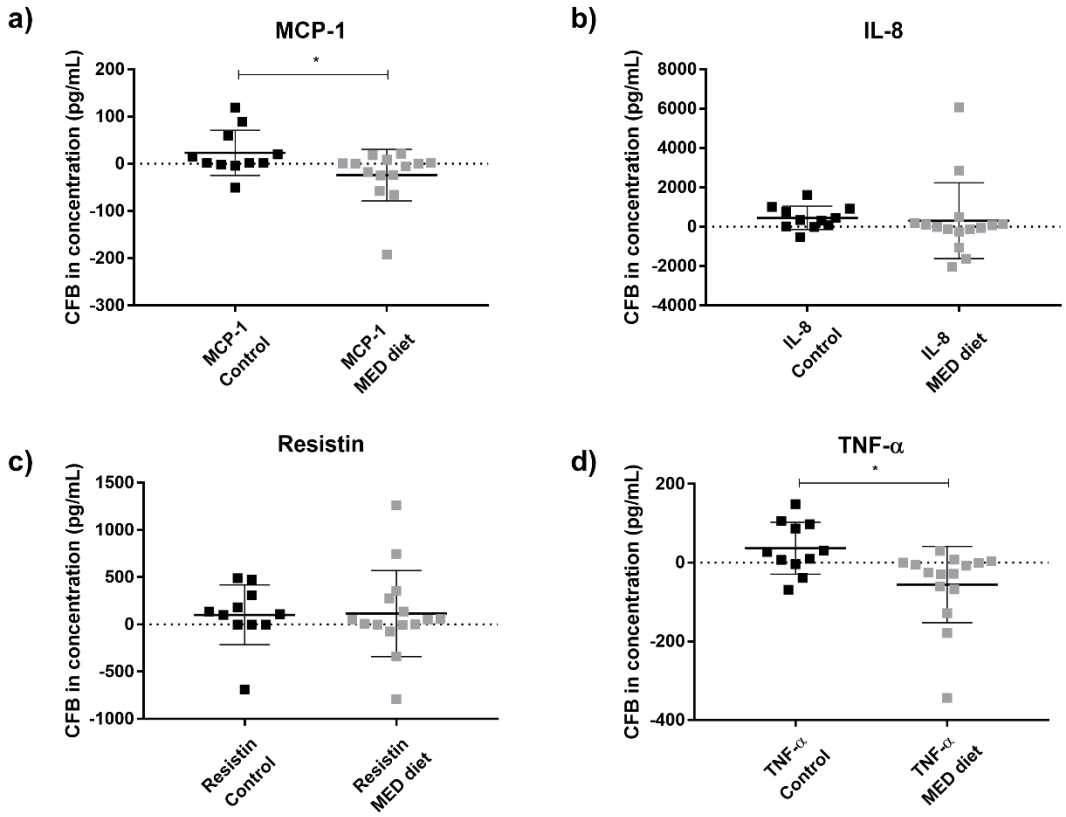


Figure 5.8 Change from baseline in cytokine production by PBMCs in response to LPS and R848 stimulation after one-year of control and MED-diet intervention. PBMCs from control subjects and MED diet intervention subjects were cultured for 3 hours in complete tissue culture media containing LPS and R848 in the presence of 2μM monensin. PBMC culture supernatants were analysed by multiplex bead based immunoassay to determine absolute concentrations of each analyte in the samples. Concentrations (pg/mL) of each cytokine at pre-intervention were subtracted from the post-intervention values to give a change from baseline value. Change from baseline in the control and MED diet groups were compared using a Mann Whitney U test, with significance assumed at *$p<0.05$. CFB: change from baseline, IL-8: interleukin-8, MCP-1: monocyte chemoattractant protein-1, MED: Mediterranean, PBMC: peripheral blood mononuclear cell, pg: picogram, TNF-α: tumour necrosis factor alpha.

We show, however, that the MED diet consuming study participants consume an increased amount of phenolic compounds, most likely from olive oil, consistent with elevated urinary HTS concentrations in this group. Since it is apparent that the subjects were compliant the effects observed may be a consequence of altered dietary intake. In considering FA intake calculated form the self-report 7DD it is apparent that the MED diet group consumed significantly less saturated fat ($p<0.0001$) compared to the control group while the MUFA and PUFA intakes were not significantly different between the groups ($p=0.063$ and 0.902, respectively). As shown in Figure 5.9 the MUFA intake is decreased compared to baseline for both groups in the present study, suggesting the effects observed may be attributed to changes in the SFA: MUFA or SFA: PUFA ratio, or simply the reduced intake of SFA. However, this cannot be confirmed since we assume that the control group were not compliant, as the MED diet scores were not different between the two groups and therefore these analyses lack an effective control group to confirm our findings.

A consideration for the present findings is the evidence that resistin expression is regulated by PPARγ activators (Patel et al., 2003) and as such it is possible that the dietary intervention may have impacted on PPARγ initiated signal transduction. Increased MUFA intake (via the consumption of olive oil) or PUFA (via the consumption of oily fish) by study participants decreases PPARγ signal transduction and thus inhibits cytokine secretion, as seen in previous studies (Zapata-Gonzalez et al., 2008; Zeyda et al., 2005), giving potential for effects on adipokine secretion. Provision of 0.4 g or 1.8 g EPA and DHA to elderly subjects resulted in altered gene expression profiles of PBMCs after a period of only 26 weeks consumption, with downregulation of PPARα observed (Bouwens et al., 2009). This finding corresponds with the data from previous studies showing that inhibition of cytokine secretion upon treatment with EPA and DHA involved PPARγ (Marion-Letellier et al., 2008; Zapata-Gonzalez et al., 2008). An interesting confounding observation is that MUFA consumption had the opposite effect and resulted in upregulation of PPAR signalling (Bechoua et al., 2003). It would be interesting to investigate this in future work aimed at determining whether PPAR signalling is involved and whether the effect is induced by the reduced SFA: MUFA ratio or the SFA: PUFA ratio.

Previous studies show that IL-6, TNF-α, and IL-1β can induce secretion of resistin and that the insulin sensitizer, rosiglitazone, can neutralise these cytokines by activating PPARγ (Lehrke et al., 2004), suggesting that the presence of low grade chronic inflammation may enhance secretion of resistin in the elderly subjects in the present study. Since in the present study levels of IL-6, TNF-α and IL-1β are already low in the elderly subjects (Tables 5.2 and 5.3) and a significant reduction in TNF-α was observed after one year MED diet intervention (Figure 5.8d), the diet could potentially induce a reduction in resistin secretion via activation of PPARγ. Therefore, *n*-3 PUFA or MUFA consumption could impact on cytokine signalling pathways and thus inhibit the secretion of resistin, as a consequence of the observed reduction in consumption of SFAs by the MED diet intervention group and the confirmed intake of olive oil by the urinary HTS concentrations. However, this study is limited by the poor control group and therefore these data need validating in future studies with more stringent control over dietary intake and more frequent contact with the study participants. (Cabrera de León et al., 2014; Fargnoli et al., 2008).

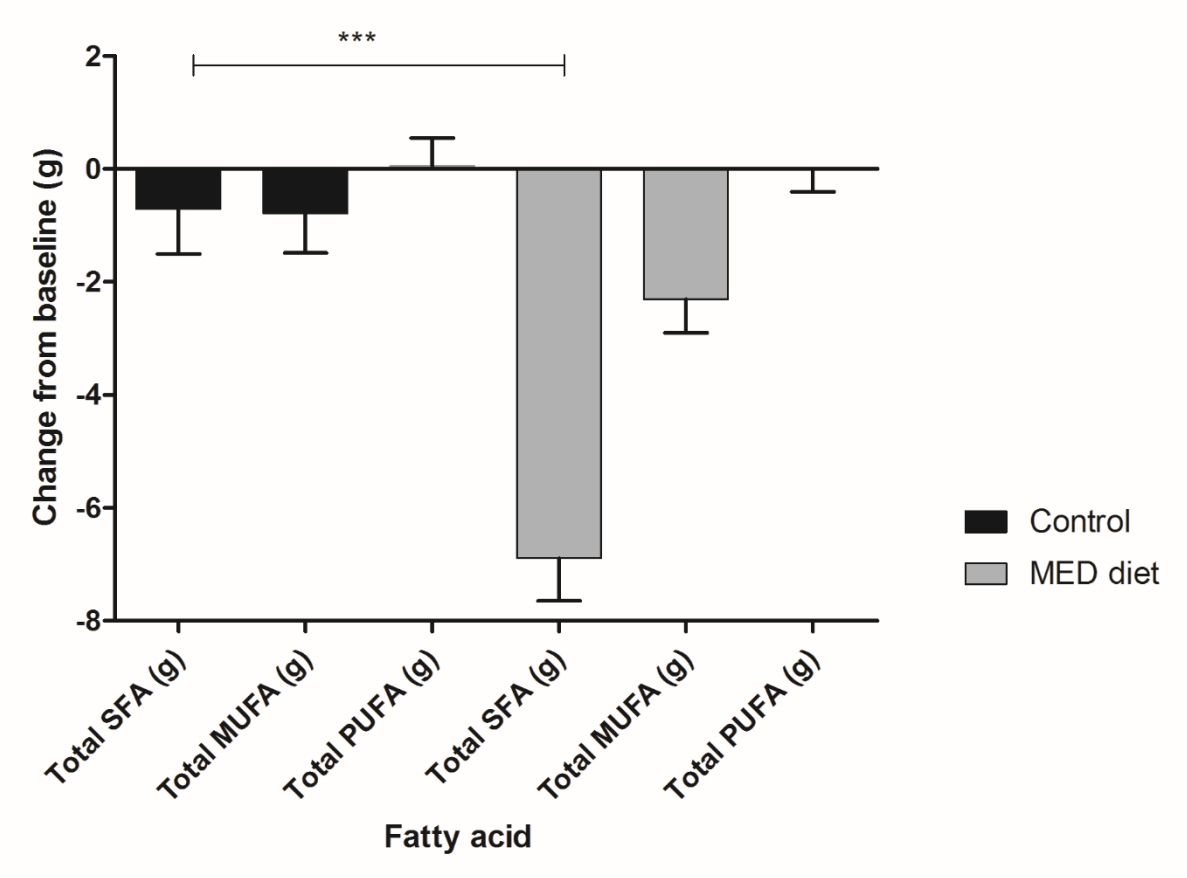


Figure 5.9 Change in fatty acid intake, from baseline, in study participants from the control and intervention study groups. Nutrient intakes were determined, by members of the study team, using dietary analysis software (WISP, Tinuviel Software) to input the 7-day diet diary information self-reported by the study participants. Fatty acid intakes in terms of saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) were calculated in grams (g) and the change in intake from baseline calculated by subtracting the baseline intake from the post-intervention intake. Bars represent mean with the SEM (indicated by error bars). Determination of significant differences between groups for each type of FA by Mann-Whitney U test, significance assumed at *p<0.05, **p<0.01, ***p<0.001.

5.4.5 Impact of findings

The study participants in the MED diet intervention group show a significant reduction in body weight at post-intervention, which is of importance since increased adiposity is often attributed with ageing and the associated negative health consequences. Maintaining a healthy weight with increasing age is important and thus this dietary pattern may prove to be an appropriate long term method of reducing weight in these individuals.

The significant reduction in resistin production after the MED-diet intervention is of biological relevance since elevated resistin is associated with insulin resistance, CVD risk and obesity (Koerner et al., 2005). Therefore, if only one year of dietary change to increase consumption of phenolic-rich food can reduce resistin levels, this could have beneficial implications as a potential target for reducing the burden of these age-associated conditions. Other studies have also shown that dietary modification can reduce resistin levels in serum (Cabrera de León et al., 2014; Fargnoli et al., 2008). This data is also of biological significance for the study participants since four were taking medications for T2D, including simvastatin and metformin, and 55 were taking medications for high cholesterol, therefore, these participants are an at risk group for developing MetS, especially since the mean BMI classifies them as overweight in both intervention groups, even after weight loss. These data are limited by the assumed lack of compliance by the control group and therefore further dietary interventions are necessary in order to replicate these findings using a more stringently monitored control group.

Further investigation into the mechanistic action responsible for this reduction in resistin secretion would be of interest since while there is speculation in the field as to the mechanism, little is currently known due to very few human studies. Especially since this finding could be relevant for the future investigation of drug targets to treat these conditions, such as the current pharmaceutical use of the PPARγ agonist (thiazolidinedione; TZD) to treat insulin resistance in patients with T2D, which is associated with numerous adverse side effects such as body weight gain, fluid retention, heart failure, bone fractures and increased risk of bladder cancer (Cariou et al., 2012). Patel et al. (2003) have shown that expression of resistin by human macrophages can be reduced with extensive (96 hour), but not short-term (24 hour), exposure to rosiglitazone, which is a TZD. Therefore, the observation that dietary modification to a MED diet shows similar reductions in resistin secretion is of great interest, since if the public could be persuaded to adopt this dietary pattern, there may be potential for the age-associated increase in resistin, as seen in Chapter 3, to be

prevented, and thus the need for such drugs to improve insulin sensitivity might be reduced, in addition, potentially, to those required for other inflammatory conditions.

Chapter 6

Impact of a Mediterranean diet on the diversity of the aged immunoglobulin repertoire

6.1 Introduction

With increased age there is an increased susceptibility to infection and despite high uptake (72.7%) of the influenza vaccination in the over 65 age group, the elderly are typically the predominant group infected by influenza virus within the population (Public Health England, 2015a). Vaccination responses rely on the production of antigen-specific cell-mediated and humoral immune responses and the subsequent production of immunological memory, which includes the production of plasma cells which secrete specific antibodies to destroy pathogens or inhibit their ability to interact with host cells (Sallusto et al., 2010). Plasma cells, which reside in the BM, secrete specific antibodies, in an antigen-independent manner to maintain effective concentrations within the serum, and also to respond to re-encounter with the same pathogen, after rapid proliferation and differentiation of memory B cells (Sallusto et al., 2010). In parallel to the quantitative measures of age-associated reduction in serum IgM and hemagglutination inhibition (HI) responses post vaccination (Ademokun et al., 2011; Frasca et al., 2010), and delayed serum IgA production post pneumonia vaccination (Ademokun et al., 2011), qualitative alterations to the Ig repertoire have also been observed. Early observations in aged mice showed loss of diversity and increased oligoclonality of the antibodies produced (Nicoletti et al., 1991; Nicoletti et al., 1993). This failure to produce sufficiently diverse antibody repertoires has been observed in the elderly, whom demonstrate poor immune function (Bolland et al., 2016), which could explain why the elderly are predominantly infected with influenza despite high vaccination uptake (Public Health England, 2015a). This poses the question of whether it is possible to intervene at the Ig level, to improve humoral immune responses in the elderly. Therefore, the potential for dietary intervention to influence the Ig repertoire will be investigated within this chapter. The gene names and definitions of Igs approved by the HUGO Nomenclature Committee (HGNC) (Gray et al., 2016) will be used throughout this chapter to identify Ig heavy chain variable (IGHV) regions, which classifies by group (IGHV), of which there are subgroups (IGHV3), which comprise genes (IGHV3-15), these genes are ordered within the locus (IGH (14q32,33)) and alleles (IGHV3-15*01) are a variant of the gene (Lefranc et al., 2003). Functionality refers to germline V, D, J and C sequences where

coding regions have an open reading frame (ORF) with no stop codon and no effect within splicing sites, recombination signals or regulatory elements (Lefranc et al., 2003).

6.1.1 Evidence of collapse of immunoglobulin repertoire with age

Assessment of CDR3 length and diversity are useful determinants of Ig repertoire diversity (Pickman et al., 2013), since the CDR3 is the most hypervariable region of the Ig encoding antigen specificity and reactivity. The length of CDR3 of IgM and IgG heavy chains has shown no differences with age, when comparing young (24–31 years) to aged subjects (>65 years) (Wang and Stollar, 1999; Xue et al., 1997). These studies were carried out using PBMC or single cell PCR and Sanger sequencing prior to the advent of more sensitive and accurate methodologies utilising high-throughput and deep sequencing (van Dijk et al., 2014). More recent work has shown shorter CDR3 lengths in IgM naïve B cells from old subjects (65–92 years and 86–94 years, respectively) (Chong et al., 2003; Pickman et al., 2013), but similar lengths for IgM memory and IgG CDR3 regions with age (Chong et al., 2003).

Spectratype size analyses, examine CDR3 length distribution of the Ig heavy chain (IgH). Non-normally distributed spectratypes indicate multiple identical or near identical sequences, implying increased clonality, and thus reduced Ig repertoire diversity of B cells derived from elderly, frail subjects (Gibson et al., 2009). This finding, accompanied with significantly fewer samples matching Gaussian distribution in spectratypes of elderly subjects, prior to vaccine challenge, when compared to young subjects (Ademokun et al., 2011) suggests that there are age-associated reductions in the level of diversity in the IgH repertoire. While CDR3 length was significantly longer for IgH from naïve B cells from elderly subjects at baseline and both time points post vaccination (7 and 28 days) (Ademokun et al., 2011). However, recently, computational models have been applied to spectratype analyses to obtain more information, resulting in the identification that, when excluding frail individuals, CDR3 lengths of IgH from elderly subjects were positively skewed towards shorter lengths with more clonal expansions present (Pickman et al., 2013), after re-analysis of the CDR3 data from Gibson et al. (2009). Two-fold increases in the number of clones of the IgA isotype in B cells from the older subjects, compared to young, was also observed (Wu et al., 2012a). Older subjects had larger CDR3 regions of the heavy chain and lower mutational frequency compared to young subjects (Wu et al., 2012a), in addition to persistence of B cell clonality 28 days post-vaccination (Ademokun et al., 2011; Wu et al., 2012a). This implies a delayed immune response

in the elderly; an observation also made in a B cell kinetics study (Macallan et al., 2005).

High-throughput sequencing allows more in-depth and detailed investigation of the IgH variable region (IGHV), including the usage of V, D and J gene subgroups and genes within the antigen binding region (Dunn-Walters, 2016). Earlier studies, utilising single-cell PCR, detected significantly greater use of the IGHV4 gene subgroup, while IGHV3 usage was diminished in the elderly (Wang and Stollar, 1999); with more recent observations supporting this reduction in IGHV3 gene subgroup usage (Martin et al., 2015; Wu et al., 2011). By contrast, reduced usage of the IGHV6 gene subgroup has been observed in B cells from young subjects (Wu et al., 2011). Conversely, no age-associated differences were observed post-influenza vaccination in IGHV gene usage in one study (Wu et al., 2012b), however IGHV3-30 gene usage was significantly increased after pneumonia vaccination (PPS4 and PPS14) in B cell samples from elderly subjects (Kolibab et al., 2005). While, Ig from peripheral B cells from young subjects displayed gene usage dominated by IGHV3-74 and IGHV3-01 for PPS4 and IGHV3-48 for PPS14, along with increased frequency of somatic mutations in samples from young compared to old subjects (Kolibab et al., 2005). It is clear from the current published data that there is a lack of consistency between studies in terms of the age-associated changes to both IgH CDR3 length and V gene subgroup and gene usage within the IGHV when comparing young to aged subjects.

6.1.2 Influence of dietary intervention on the B cell production, function and immunoglobulin repertoire of elderly subjects

Ageing is accompanied by increased adiposity of the BM (Justesen et al., 2001). Since adipocytes and osteoblasts arise from the differentiation of MSCs, which are also involved in the development and differentiation of B cells from HSCs within the BM (Tabera et al., 2008), BM adiposity may impair earlier precursor stages of B cell development; including Ig production. Additionally, the BM is the site of storage for antibody-producing plasma cells (Caraux et al., 2010), and since increases in BM adiposity results in tightly packed adipocytes the reduced space has been suggested to inhibit haematopoiesis (Bonomo et al., 2016), it could also inhibit plasma cell storage; an important part of the memory response (Janeway Jr et al., 2012). The presence of adipocytes within other tissues can influence surrounding cells and tissues via the secretion of adipokines with implications for insulin resistance, fatty liver and hyperlipidaemia (Vázquez-Vela et al., 2008). It is feasible therefore to hypothesise that this could also occur in bone cavities (Yokota et al., 2003), since greater adiponectin secretion was observed from BM adipose tissue than WAT

(Cawthorn et al., 2014). Additionally, osteoblasts have been shown to support the differentiation of HSCs to lymphoid precursors and the subsequent differentiation of B cell precursors and mature B cells, since depletion of osteoblasts severely depleted pre-pro-B and pro B cells from murine BM (Zhu et al., 2007).

High fat diets and calorie restriction (30%) have been shown to increase BM adiposity (Adler et al., 2014; Devlin et al., 2010; Doucette et al., 2015; Halade et al., 2010). Similarly, alcohol increased BM adiposity (Maddalozzo et al., 2009), though this evidence is all derived, to date, from animal models. Six weeks of high fat diet significantly increased BM adiposity (+363%) in C57BL/6 mice compared to regular diet, with an accompanying reduction in BM B cells (-25%) compared to the regular diet (Adler et al., 2014). A significant deficit in total B cells was also observed, after extrapolation from flow cytometry determined total cell counts (Adler et al., 2014). The accumulation of adipocytes after high fat diet was, however, suppressed by increased exercise in C57BL/6 mice (Styner et al., 2014). Feeding of CLA isomers to 12 month old C57BL/6 mice for six months significantly reduced BM adiposity after consumption of the *trans*-10, *cis*-12 CLA (Rahman et al., 2011). Also, addition of oleuropein, a major polyphenol in olive oil, to cell culture media significantly reduced adipocyte differentiation and expression of genes involved in adipogenesis using MSCs from human BM (Santiago-Mora et al., 2011). This suggests the potential for altered FA intake, predominantly in the form of elevated MUFA from olive oil intake, may reduce the age-associated accumulation of adipose tissue in the BM. Since, adiposity of the BM can dysregulate B cell populations in mice (Adler et al., 2014), reducing adipogenesis may influence B cell populations and functions in elderly subjects.

6.1.3 Gaps in knowledge

At present experimental evidence for dietary interventions with outcomes relating to B cell function have been restricted to animal and *in vitro* models (Gurzell et al., 2015; Rockett et al., 2013; Rockett et al., 2012). While, interventions of dietary fat (particularly *n-3* PUFA), dietary fibre, protein and phytochemical intakes have been conducted in human studies, immune parameters are often not investigated.

The Ig repertoire studies of young and elderly subjects have not included complete analysis of VDJ recombination at the DNA level due to PCR-based methodological issues (Bolland et al., 2016), due to the lack of a primer that can detect all genes (95), within the seven V gene subgroups in the human IgH, which altered primer design could resolve (Wood et al., 2013). Similarly, while age-associated effects on B cell

subset numbers and intrinsic genetic alterations in the Ig repertoire have been reported no observations have been made in relation to dietary impact in humans.

6.1.4 Rationale

This study utilised blood samples from human subjects to provide initial and novel data regarding the effects of dietary intervention on immune function, in relation to VDJ recombination within the IGHV region. A VDJ sequencing assay (VDJ-seq) has been developed at the Babraham Institute utilising high-throughput next generation sequencing which captures primer extension products of genomic DNA from J_H gene oligonucleotides without the need for multiple V gene primers, allowing detection of unbiased DJ_H and VDJ_H recombination products (Bolland et al., 2016). Since there are only six human J_H genes (Wood et al., 2013), every DJ_H and VDJ_H recombination event will end with one of these six genes.

6.1.5 Aims and objectives

Does the VDJ-seq assay provide equivalent data to the current literature comparing young and elderly Ig repertoires and does dietary intervention with a 12 month MED diet effect the Ig repertoire? This work is in collaboration with the Babraham Institute and is acknowledged within the text.

Objective 1: Compare the frequency of usage of IGHV gene subgroups and genes between elderly (65–79 years) and younger subjects, using the VDJ-seq assay utilising B cell DNA extracted from PBMC samples derived from Nu-AGE participants; re-analysing sequencing data previously collected from young subjects at the Babraham Institute.

Objective 2: Determine whether dietary intervention influences the frequency of usage of IGHV genes, the CDR3 length of the IGHV and the overall diversity of the Ig by allocation of clonotypes.

6.2 Methodology

6.2.1 Thawing of PBMCs

Frozen PBMCs were thawed as described in Chapter 2. Cells were counted using the viability stain, trypan blue (Sigma Aldrich, T8154), and resuspended to give a final concentration of 1 x 10^6 cells / ml. Cells were “rested” for 2 hours at 37 ºC, before B cells were isolated.

6.2.2 B cell isolation

A subset of Nu-AGE PBMC samples were selected based on those that had a corresponding post-intervention sample and those that had been analysed for DC functionality in Chapter 5. Of which ten samples were selected blinded and at random, due to the cost implications of this analysis, with verification from a colleague not involved in the research that allocation from the two dietary groups had been achieved. PBMC-B cells were purified, from this subset of samples, by negative immunomagnetic selection. Briefly, cells were incubated with 10µl/ 10^7 cells of antibodies specific for T cells, NK cells, monocytes, DCs, granulocytes and erythrocytes (MACS pan B cell isolation kit, Miltenyi-Biotec). Anti-biotin magnetic microbeads (20µl/ 10^7 cells) were added and antibody-labelled cells were separated by passing the labelled samples through MS columns (Miltenyi-Biotec) on a mini-MACS magnet (Miltenyi-Biotec), by three washes with MACS buffer (see Chapter 2 for details). The remaining, untouched, B cell fraction was flushed through the MS column with MACS buffer after removing the MS column from the magnet. Isolated B cells were analysed for purity by flow cytometry by staining samples with a CD19-VioBlue antibody (Miltenyi-Biotec).

6.2.3 DNA Extraction

DNA was extracted from isolated B cells using the DNeasy kit (Qiagen), following the manufacturer's instructions. DNA was quantified, and purity (260/280) and integrity (260/230) ascertained via UV/VIS spectrophotometry using the Thermo Scientific Nanodrop™ 1000; DNA samples were stored at -20°C until the VDJ-seq assay was carried out.

6.2.4 Generation of IGHV gene libraries for high throughput sequencing; VDJ-seq assay

The VDJ-Seq technique was performed by researchers at The Babraham Institute according to the protocol described by (Bolland et al., 2016), with some adjustments including the omission of the step to deplete unrecombined DNA; a brief description is given below.

Fragmentation and repair of DNA samples

DNA was fragmented by sonication, to generate ~500bp, using Covaris E220 system. End repair of sonicated DNA was carried out followed by a purification step using QiaQuick columns (Qiagen).

PE1 adapter ligation

A-tailing of end-repaired DNA and incorporation of two adaptor mixes, which each incorporate six nucleotides at the start of read 1 followed by a known “anchor” sequence of 7 or 8 bp; unique molecular identifier (UMI) adapters; details in Appendix XIII. Fragmented DNA samples were ligated with one of these mixes (mix 1 or 2) to fulfil the criteria for Illumina red/ green laser registration of clusters; sequences of adapter mixes in Appendix XIII.

Enrichment of VDJ recombined fragments

Biotinylated primer extension was carried out using oligonucleotides annealing within each J segment, with five rounds of PCR; details of thermal cycler conditions in Appendix XIV. Primer-extended DNA were enriched for J-containing fragments using J_H specific oligonucleotides according to method detailed by (Bolland et al., 2016).

Incorporation of PE2 adapter by PCR

PCRs were carried out using a mixture of 7 reverse primers 15 bp downstream of the recombination junction with paired-end 2 (PE2) sequence at the 5′ end, combined with a single forward primer annealed to PE1 sequence in the ligated adapter. Up to 1 µg starting DNA was used for each primer extension reaction, 5x primer extension reactions; thermal cycler conditions in Appendix XIV.

Flowcell binding and barcoding sequencing

PCR was carried out using universal Flowcell PE1 primer (forward primer) in combination with one of 12 index PE2 primers (reverse primer) to each sample. Each primer extension reaction used up to 1 µg DNA, thermal cycler conditions and primer sequences detailed in Appendix XIV.

6.2.5 DNA-library quality control

DNA-libraries ready for sequencing underwent analysis using Agilent 2100 Bioanalyser and Kapa qPCR for quantification, and qPCR for quality control of libraries at the Babraham Institute; to ensure that libraries were of sufficient quality and quantity to be sequenced. Bioanalyser results in Appendix XV. DNA-libraries that passed QC tests were sequenced by Illumina MiSeq 2 x 300bp paired end sequencing.

6.2.6 Bioinformatics analysis

Fastq files from Illumina 2x300bp MiSeq paired end sequencing entered Babraham LinkON VDJ-Seq pipeline. The initial pre-clean stage identified paired-end assembled reads (PEAR), removing any unassembled reads. The assembled reads were pre-

filtered to remove germline reads, sequences containing bases of low quality (Q2 sequences) and sequences with UMI bases lower than Q30. The sequences were aligned to J primers, any sequences that fitted more than one J primer sequence were ambiguous and were discarded. To account for any mispriming, five base pairs beyond the primer were used to identify the original J gene. If the five base pairs matched more than one J gene, the sequences were discarded and if they didn't perfectly match the reference they were classed as unclear. The sequences were then demultiplexed by assigning to one of the two anchors; Appendix XVI. The UMI was extracted along with the anchor sequence and placed in the read name. These are used in the deduplication step. Sequences were discarded if they could not be assigned to either of the anchors.

The fastQ files then underwent deduplication in order to remove sequencing errors. Sequences with less than five reads per UMI group were discarded. If the reads within a UMI group were more than 5bp different from the consensus the entire group was discarded to remove early PCR errors and incorrect grouping. The output fasta files containing the consensus sequence were then inputted into the IgBLAST analysis tool (NCBI) (Ye et al., 2013) with the international ImMunoGeneTics information system (IMGT) database to annotate the Ig domain. Clonotypes were assembled and assigned to each read. Clonotypes are clones (reads) with one mismatch in the CDR3, and the same V gene.

Young samples (<40 years) previously analysed using VDJ-seq at the Babraham Institute were re-analysed in the same way as that carried out for the Nu-AGE samples, to enable comparison between the two groups.

6.2.7 Statistical analysis

Frequency of V gene usage within each gene subgroup (IGHV1–7) was compared between young (n=12) and elderly subjects (n=10) using two-way ANOVA with *a priori* Sidak's multiple comparisons tests to compare means for each gene subgroup. Significance assumed at alpha=0.05, using GraphPad Prism V7.02. The same analysis was carried out to compare, pre- to post-intervention, V gene subgroup frequency of usage values for control diet (n=6) allocated subjects, and MED diet (n=4) allocated subjects.

Comparison of V gene usage by young and elderly subjects was carried out after aligning all genes represented within both groups of subjects by multiple unpaired t-tests to compare means of the usage of each gene for the elderly and the young subjects. Statistical significance was determined using the Holm-Sidak method, which

corrects for multiple comparisons, with alpha=0.05, no assumption of consistent standard deviations was made, as each gene was analysed individually; this analysis was performed using GraphPad Prism V7.02.

For V allele analysis pre- and post-intervention frequencies for all V alleles, represented across all Nu-AGE subjects, were compared for each subject by two-way ANOVA with Sidak's multiple comparisons test, using GraphPad Prism V7.02. This allowed comparison of each of the 246 alleles from pre- to post-intervention, in all subjects allocated to the MED diet or control diet intervention, for functional or non-functional alleles.

The frequency distributions of the CDR3 lengths for functional and non-functional genes for all samples at pre- and post-intervention were determined using GraphPad Prism V7.02 and compared using the Kolmogorov-Smirnov (K-S) test for equality of distributions when comparing non-parametric, unpaired data. Samples were grouped according to study group allocation, MED diet or control diet. D'Agustino & Pearson normality tests were performed on the frequency distribution data to determine whether data were normally distributed for functional and non-functional genes in both dietary groups and pre- and post-intervention.

Tree maps were produced using RStudio to show the hierarchical structure of the clonotype data using a space-filling visualisation method, this method efficiently represents the size of clonotypes which can easily be displayed with nesting to subdivide each of the subjects, while colour is used to determine study time point; pre- or post-intervention (van Wijk and van de Wetering).

6.3 Results

6.3.1 V gene subgroup usage frequency

Effect of age

The clone frequencies for all of the V genes within each of the IGHV subgroups IGHV1, IGHV2, IGHV3, IGHV4, IGHV5, IGHV6 and IGHV7 were grouped to determine gene usage within gene subgroups. Comparable profiles of IGHV gene subgroups within the Ig repertoire of naïve B cells were evident for both young and elderly subjects. IGHV3 was the predominant subgroup, followed by IGHV1, while IGHV6 and IGHV7 were represented the least for both young and elderly subjects. The frequency of gene usage within the IGHV3 subgroup was however significantly reduced in the elderly compared to the young samples; $p=0.0037$, Figure 6.1. Conversely, the frequency of gene usage within the IGHV1 subgroup was significantly increased in the elderly compared to the young subjects; $p<0.0001$. All other gene subgroups had similar gene usage between young and elderly subjects; $p>0.05$.

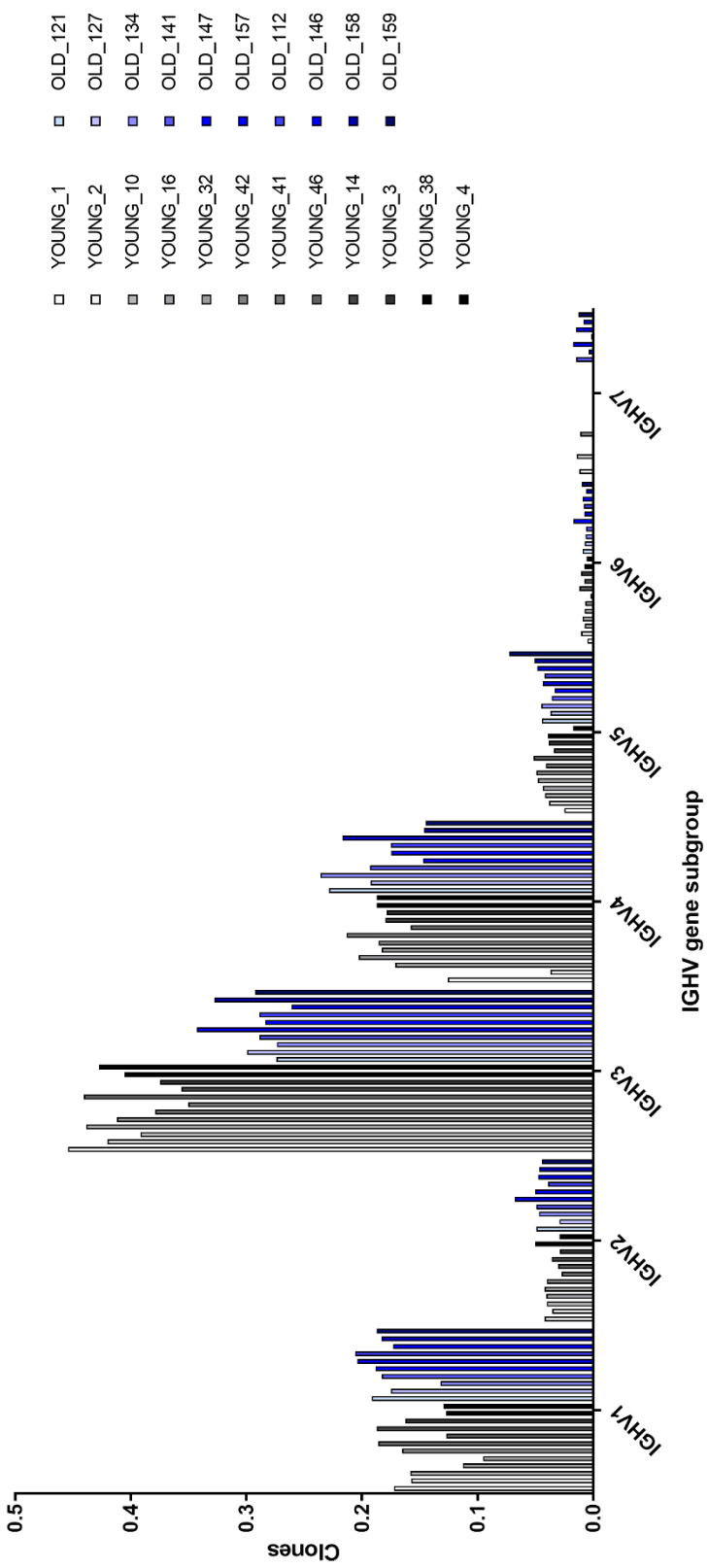


Figure 6.1 Comparison of immunoglobulin heavy chain variable (IGHV) gene usage by gene subgroup for functional genes between young and elderly subjects.

Figure 6.1 Comparison of immunoglobulin heavy chain variable (IGHV) gene usage by gene subgroup for functional genes between young and elderly subjects. 2x 300bp paired end sequencing was carried out using the Illumina MiSeq platform for DNA libraries by Dr Daniel Bolland (Babraham Institute) prepared from DNA extracted from human peripheral B cells. After analysis of the Illumina acquired Fastq files through the Babraham LinkON analysis pipeline to remove sequencing errors and de-duplicate the data, the consensus Fasta files were run through the NCBI IgBLAST tool using the IMGT database and subsequently the clones were assembled and assigned clonotypes; all by Peter Chovanec (Babraham Institute). Frequencies of usage of genes determined for all subjects were grouped by gene subgroup to give an overall frequency of gene variant use within the seven IGHV gene subgroups. Frequencies of variable gene usage within gene subgroups is shown for each subject, greyscale bars refer to young subjects, blue scale bars refer to elderly subjects (65–79 years) from the Nu-AGE study at pre-intervention. Statistical analysis was carried out using GraphPad Prism V7.02 to compare IGHV gene subgroup usage between young and elderly subjects by two-way ANOVA with *a priori* Sidak's multiple comparisons comparing row means.

Effect of dietary intervention

The frequency of V gene usage was determined for all of the V genes and alleles represented within the Nu-AGE subject cohort. Initially, the clone frequencies for all of the genes within the IGHV subgroups IGHV1, IGHV2, IGHV3, IGHV4, IGHV5, IGHV6 and IGHV7 were grouped to determine gene usage within gene subgroups. This showed that the subgroup expressed predominantly in the naïve B cell repertoire from the elderly subjects was IGHV3, in both functional and non-functional subgroups; comparison of all gene subgroups against IGHV3 were significantly different ($p<0.00001$). The functional IGHV4 subgroup was expressed with the second greatest frequency, while IGHV6 and IGHV7 were expressed at the lowest frequencies. Comparing gene subgroup usage across all subjects showed significant differences between all combinations ($p<0.00001$; VH7 versus VH5; VH6 versus VH5; VH6 versus VH2 $p<0.001$) except VH4 versus VH1; VH5 versus VH2; VH6 versus VH2 for functional genes at pre-intervention which did not show statistically significant differences. All combinations were significantly different ($p<0.00001$) with the exception of VH4 versus VH1; VH5 versus VH2; VH6 versus VH2; VH7 versus VH2; VH6 versus VH5; VH7 versus VH5; and VH7 versus VH6 for functional genes at post-intervention (Figure 6.2), and non-functional genes at both pre- and post-intervention (Figure 6.3). Comparison of pre- to post-intervention for the control and MED diet groups showed similar usage of gene subgroups at both time points.

However, a large increase was observed in one MED diet subject in the frequency of IGHV4 clone usage, while a decrease was observed in IGHV1 gene usage (MED146). In terms of the non-functional IGHV frequencies this subject displayed an increase in IGHV4 clone usage, and a decrease in IGHV3 clone usage, while a decrease in IGHV1 gene usage was apparent in MED159. These differences were not, however statistically significantly upon comparison of the pre- and post-intervention frequencies for any of the IGHV subgroups in any of the subjects, regardless of whether they were on the control or MED diet; $p>0.05$.

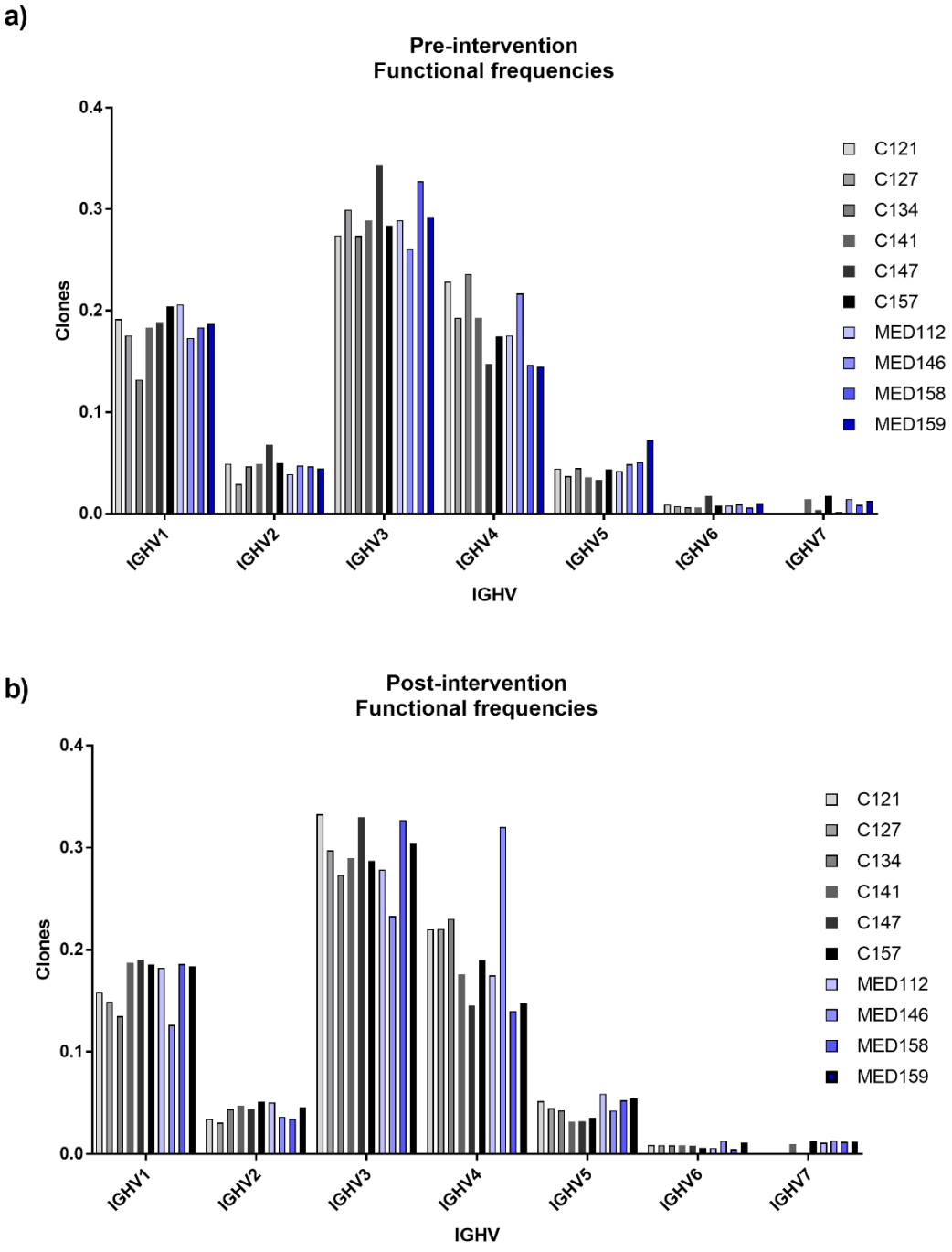


Figure 6.2 Immunoglobulin heavy chain variable (IGHV) gene usage by gene subgroup for functional genes. 2x 300bp paired end sequencing was carried out using the Illumina MiSeq platform for DNA libraries prepared by Dr Daniel Bolland (Babraham Institute) from DNA extracted from human peripheral B cells. After analysis of the Illumina acquired Fastq files through the Babraham LinkON analysis pipeline to remove sequencing errors and de-duplicate the data, the consensus Fasta files were run through the NCBI IgBLAST tool using the IMGT database and subsequently the clones were assembled and assigned clonotypes; all by Peter Chovanec (Babraham Institute). Frequencies of usage of genes determined for all subjects were grouped by gene subgroup to give an overall frequency of gene use within the seven IGHV gene subgroups.

Figure 6.2 (Continued) Immunoglobulin heavy chain variable (IGHV) gene usage by gene subgroup for functional genes. a) Variable gene usage within gene subgroups for all Nu-AGE subjects at pre-intervention. b) Variable gene usage within gene subgroups for all Nu-AGE subjects at post-intervention. Statistical analysis was carried out, using GraphPad Prism V7.02, to compare frequency of usage of each IGHV gene subgroup for control and MED diet allocated subjects from pre- to post-intervention, for functional genes by two-way ANOVA with Sidak's multiple comparisons comparing row means. Each subject's pre- to post-intervention values for each of the gene subgroups were compared by two-way ANOVA, with Sidak's multiple comparison test.

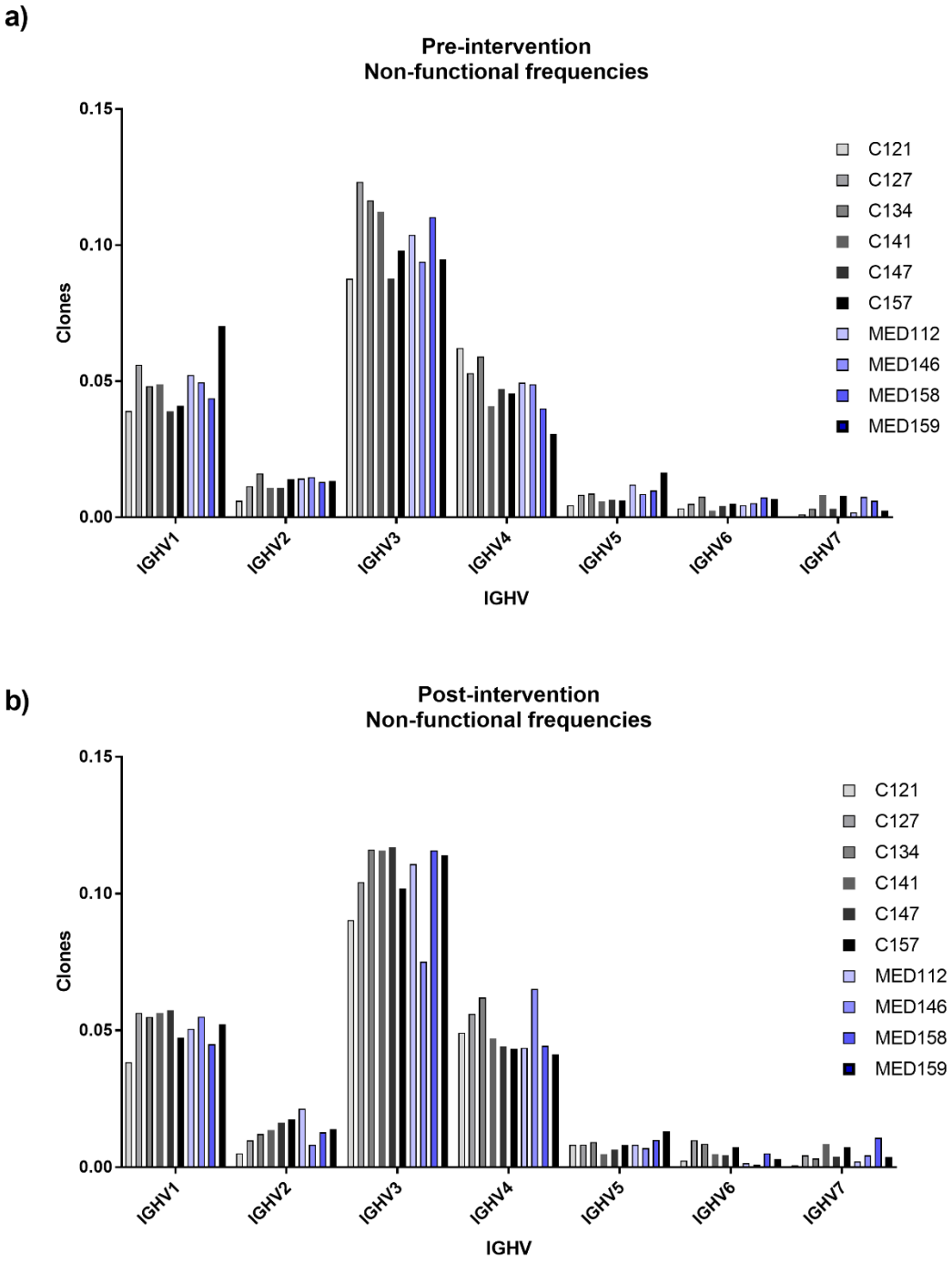


Figure 6.3 Immunoglobulin heavy chain variable (IGHV) gene usage by gene subgroup for non-functional genes. 2x 300bp paired end sequencing was carried out using the Illumina MiSeq platform for DNA libraries prepared by Dr Daniel Bolland from DNA extracted from human peripheral B cells. After analysis of the Illumina acquired Fastq files through the Babraham LinkON analysis pipeline to remove sequencing errors and de-duplicate the data, the consensus Fasta files were run through the NCBI IgBLAST tool which utilised the IMGT database to assemble the clones and assign clonotypes; all by Peter Chovanec (Babraham Institute). Frequencies of usage of genes determined for all subjects were grouped by gene subgroup to give an overall frequency of gene use within the seven IGHV gene subgroups.

Figure 6.3 (Continued) Immunoglobulin heavy chain variable (IGHV) gene usage by gene subgroup for non-functional genes. a) Variable gene usage within gene subgroups for all Nu-AGE subjects at pre-intervention. b) Variable gene usage within gene subgroups for all Nu-AGE subjects at post-intervention. Statistical analysis was carried out, using GraphPad Prism V7.02, to compare frequency of usage of each IGHV gene subgroup for control and MED diet allocated subjects from pre- to post-intervention, for non-functional genes by two-way ANOVA with Sidak's multiple comparisons comparing row means. Each subject's pre- to post-intervention values for each of the gene subgroups were compared by two-way ANOVA, with Sidak's multiple comparison test.

6.3.2 V gene usage frequency

Effect of age on V gene usage frequency

Comparison of IGHV gene usage between young and elderly subjects revealed that the frequency of usage of V genes, IGHV4-28, IGHV3-23, IGHV4-30-2, IGHV3-30 and IGHV3-13 were significantly decreased in the Ig heavy chains of naïve B cells of elderly subjects, compared to young, Figure 6.4. While, usage of IGHV3-15 was significantly increased in the Ig heavy chains of naïve B cells of elderly compared to young subjects. Frequency of usage of all other genes were not significantly different between young and elderly subjects; all $p>0.05$.

Effect of age on regional location of V genes

V gene usage was ordered to determine the regional position of genes on the chromosome, so as to determine whether proximity of the genes to either the 3′ or 5′ end of the chromosome had any influence on gene and allele usage. The sequencing data from 12 control subjects were re-analysed according to a similar pipeline analysis workflow to allow comparison of the baseline elderly sequences to a younger control group (Supplementary Figure 6.1; Appendix XVII). After aligning all genes represented within both subject cohorts in regional order of location on the chromosome, comparisons of IGHV gene and allele usage was compared between young and elderly subjects were made, with a representative example of young compared to elderly subjects shown in Supplementary Figure 6.2; Appendix XVIII. As shown in Figure 6.4 IGHV3-23, IGHV3-30, IGHV4-30-2 and IGHV3-13 were used significantly more frequently in the young than elderly subjects. Conversely, IGHV3-15 was used significantly more frequently in the elderly subjects compared to the young. IGHV4-28 was only expressed in 4/10 elderly subjects, while it was expressed in all 12 young subjects' B cell repertoires. Figure 6.5 shows the frequency of functional IGHV gene usage between young (blue) and elderly (at baseline; green), it is clear that some genes are affected by ageing. IGHV3-30, IGHV3-23, IGHV3-21 and IGHV3-11 were reduced in elderly compared to young subjects with smaller decreases observed in IGHV4-1 and IGHV3-7 in the elderly compared to young subjects. These genes are displayed on the left side of the x axis (Figure 6.5), which indicates that they are closer to the 3′ end of the chromosome. Additionally, IGHV3-15, IGHV4-31, IGHV4-34 and IGHV4-39 were all increased in the elderly compared to young subjects which are located in region I or II of the chromosome, while IGHV1-3 is increased in the elderly compared to the young and this is located very close the 3′ prime end of the chromosome.

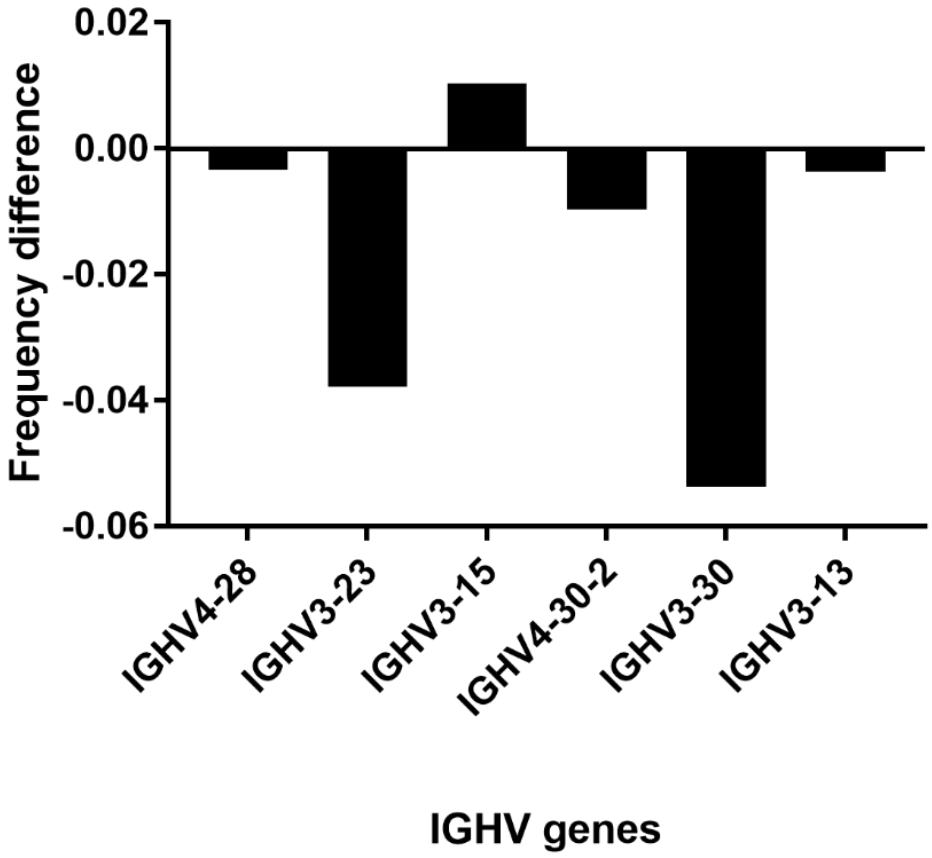


Figure 6.4 Change in frequency of usage for significantly different V genes in IGHV of elderly subjects in comparison with young subjects. Mean frequency of V gene usage was compared between elderly and young subjects for each V gene represented within the study cohort using multiple t tests, one per V gene (row), and the Holm-Sidak multiple comparisons test. Significance was assumed at alpha=0.05. Differences in V gene usage between young and elderly mean values were plotted for individual IGHV genes with probability values < 0.05. IGHV4-28 ($p<0.00001$), IGHV3-23 ($p<0.001$), IGHV3-15 ($p<0.01$), IGHV4-30-2 ($p<0.01$), IGHV3-30 ($p<0.01$) and IGHV3-13 ($p<0.01$).

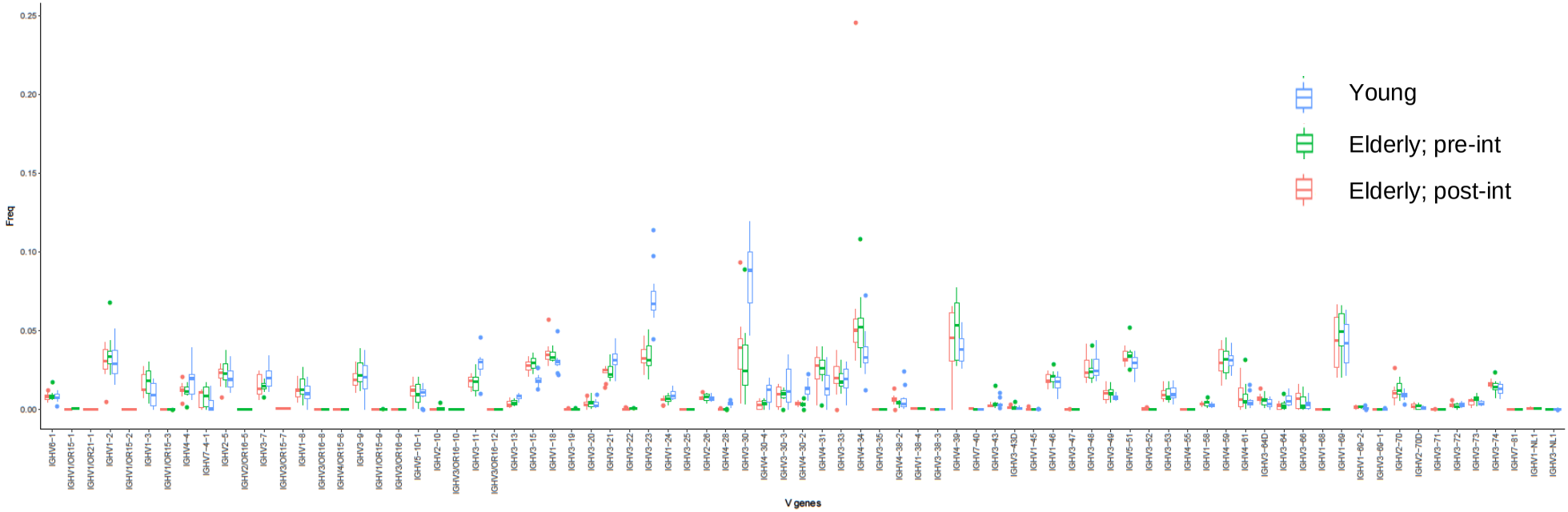


Figure 6.5 Frequency of functional V gene usage within the IGHV region, ordered by regional location on the chromosome; comparison of young subjects to elderly (Nu-AGE) subjects, pre and post-intervention.

Figure 6.5 Frequency of functional V gene usage within the IGHV region, ordered by regional location on the chromosome; comparison of young subjects to elderly (Nu-AGE) subjects, pre and post-intervention. The sequencing data from 12 previously analysed control subjects were re-analysed by Peter Chovanec (Babraham Institute) using the LinkON VDJ-Seq pipeline analysis workflow used for the Nu-AGE data to allow comparison of the baseline elderly sequences to a young control group. After aligning all genes represented within both subject cohorts in regional order of location on the chromosome, comparisons were made between gene usage by the elderly and young groups of subjects; graphs produced in RStudio by Peter Chovanec. Nu-AGE subjects n=10, young subjects n=12, blue= young subjects, green= pre-intervention and red= post-intervention.

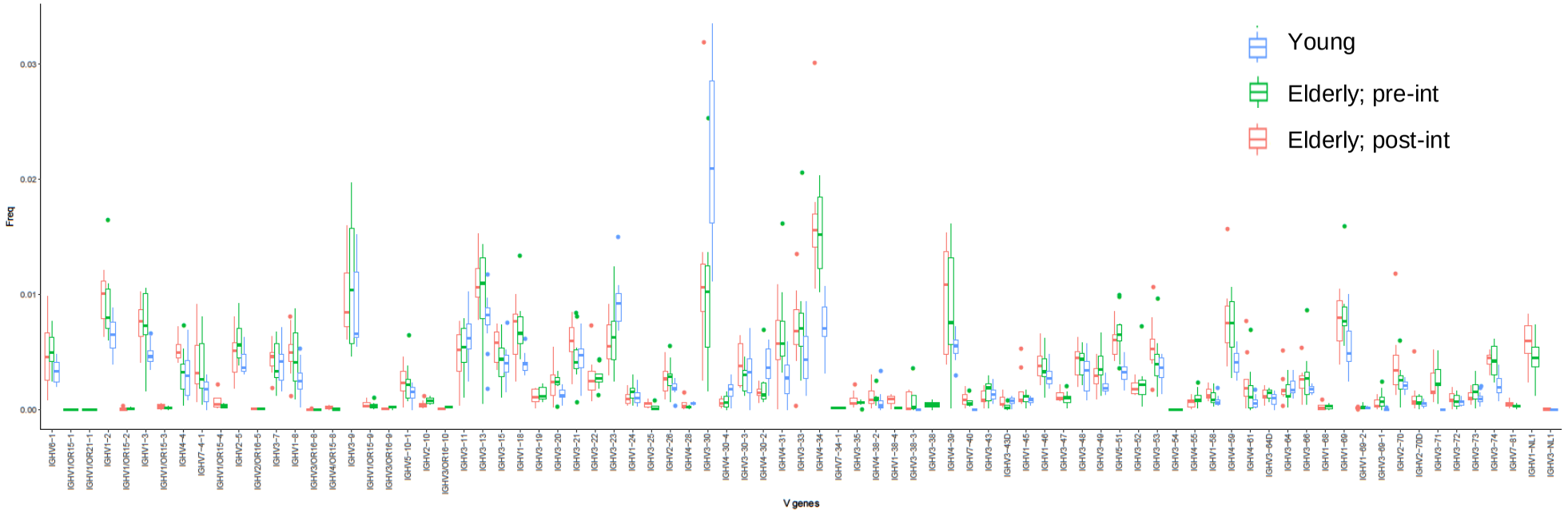


Figure 6.6 Frequency of non-functional V gene usage within the IGHV region, ordered by regional location on the chromosome; comparison of young subjects to elderly (Nu-AGE) subjects, pre and post-intervention.

Figure 6.6 Frequency of non-functional V gene usage within the IGHV region, ordered by regional location on the chromosome; comparison of young subjects to elderly (Nu-AGE) subjects, pre and post-intervention. The sequencing data from 12 previously analysed control subjects were re-analysed by Peter Chovanec (Babraham Institute) using the LinkON VDJ-Seq pipeline analysis workflow used for the Nu-AGE data to allow comparison of the baseline elderly sequences to a young control group. After aligning all genes represented within both subject cohorts in regional order of location on the chromosome, comparisons were made between gene usage by the elderly and young groups of subjects; graphs produced in RStudio by Peter Chovanec. Nu-AGE subjects n= 10, young subjects n=12, blue= young subjects, green= pre-intervention and red= post-intervention.

Reductions in usage of non-functional IGHV3-23, IGHV3-30 and IGHV3-30-2 was observed in the elderly compared to young subjects, while usage of non-functional IGHV6-1, IGHV1-2, IGHV1-3, IGHV1-8, IGHV3-11, IGHV3-13, IGHV3-15, IGHV1-18, IGHV4-31, IGHV3-33 and IGHV4-34, were all increased in the elderly compared to young subjects (Figure 6.6). These genes were all located from the 3′ end towards the middle of the chromosome, respectively. Increases in non-functional IGHV4-59, IGHV1-69 and IGHV3-74 usage frequency were also observed in the elderly compared to the young subjects, though these genes were located at the 5′ end of the chromosome.

Impact of dietary intervention on V gene usage frequency

Comparison of pre- to post-intervention V gene usage of the individual subjects in the control group revealed that the greatest number of significant changes in gene usage were within the IGHV1 (10 changes), IGHV3 (14 changes) and IGHV4 (10 changes) subgroups. Six of the IGHV3 genes significantly increased while eight genes significantly decreased. The genes that significantly changed were V3-11, V3-15, V3-21, V3-23, V3-30, V3-33, V3-49 and V3-9 (Supplementary Figure 6.3 b, c, d, e, g, h [Appendix XIX] and Figure 6.7).

Comparison of the pre- to post-intervention V gene usage of the individual subjects in the MED diet intervention group revealed that the most significant changes in gene usage were within the IGHV3 subgroup. Seven of the IGHV3 genes significantly increased while five significantly decreased gene usage. The IGHV gene variants that significantly changed were V3-23, V3-30, V3-33, V3-43, V3-48 and V3-74 (Supplementary Figure 6.9 a, f, i, j [Appendix XIX] and Figure 6.7).

Summaries comparing the gene usage for each subject at pre- and post-intervention (Figures 6.8a-i) identify the differences in usage of functional and non-functional genes. Across subjects, most genes were present at similar frequencies at pre- and post-intervention, as points cluster on or close to the dotted line; particularly apparent for subjects 134 and 157 (Figure 6.8d and h). Functional genes were expressed at greater frequencies at both pre- and post-intervention, with non-functional genes clustering to the bottom of the plots and functional at the top, Figures 6.8e, h and j.

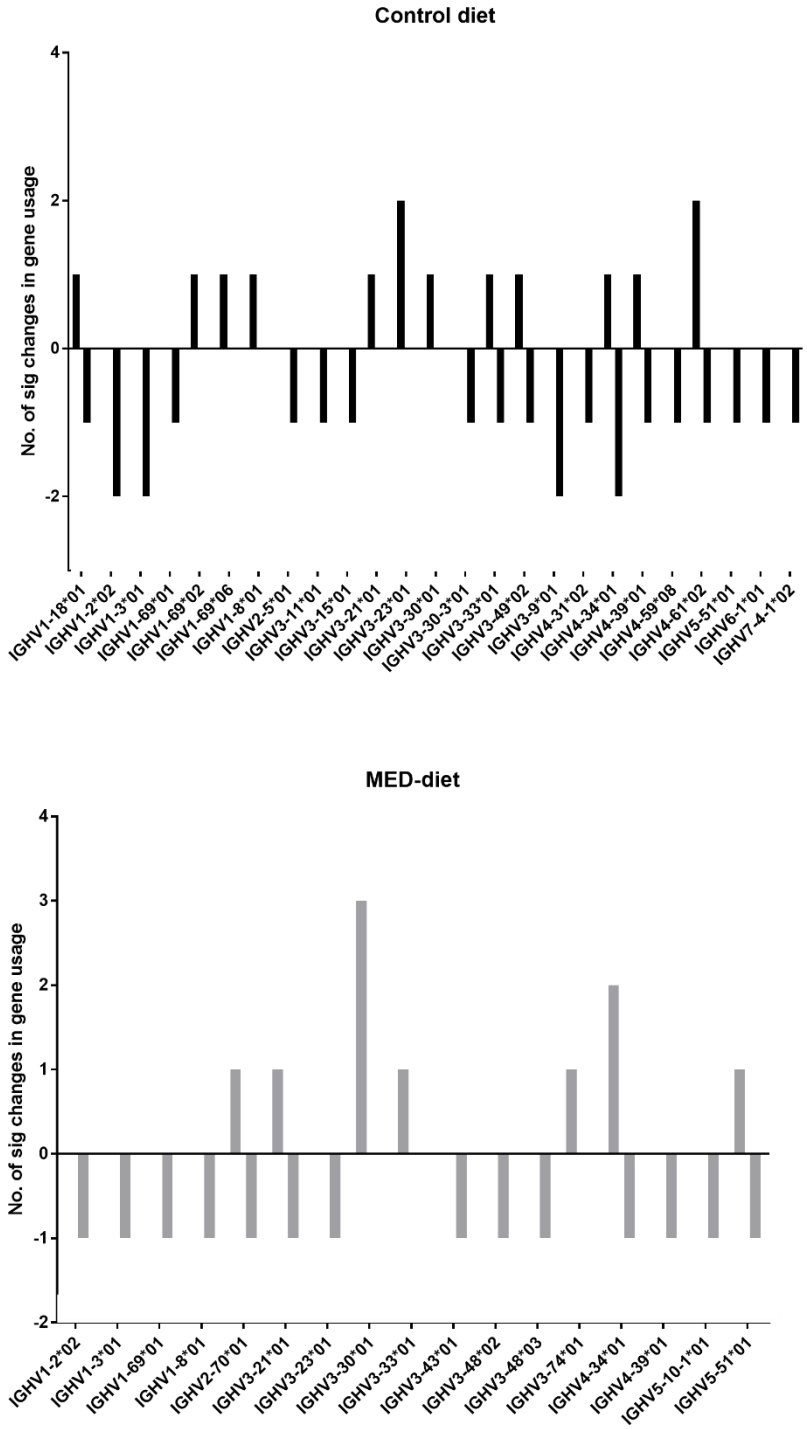


Figure 6.7. Numbers of Nu-AGE subjects with significantly changed IGHV alleles one-year post-intervention, in the control and MED-diet groups. After comparison of V allele usage between pre- and post-intervention for each individual subject (detailed in Figure 6.9), for alleles which were significantly changed, the number of observations of significant increases or decreases in each study group (control or MED diet) were plotted.

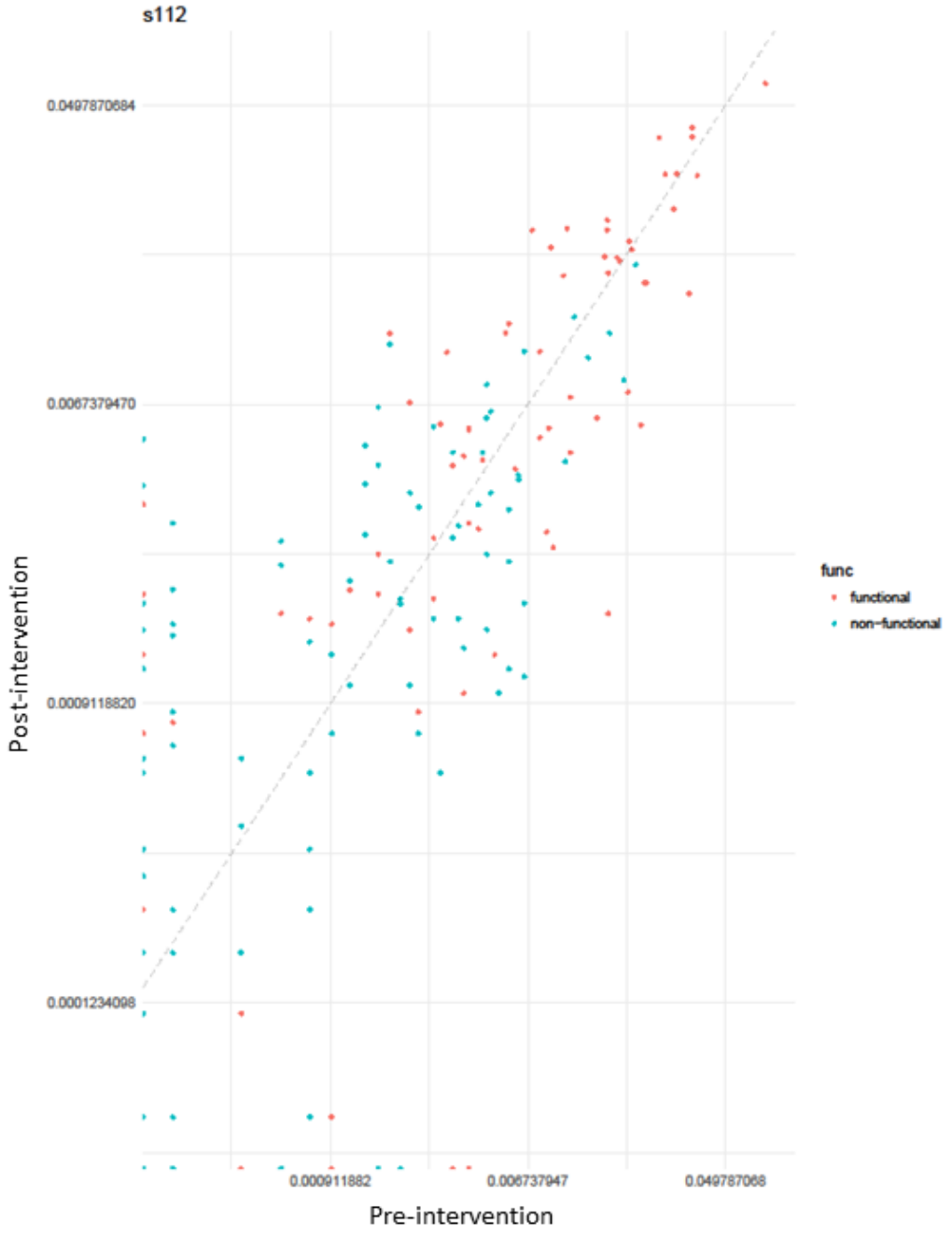


Figure 6.8a Scatter plot of IGHV gene usage for subject s112 comparing pre- to post-intervention. Frequencies of each IGHV gene, represented within the Nu-AGE sample, were plotted for functional (orange) and non-functional (blue) genes. The plot represents IGHV gene frequencies for an individual subject, with dots representing the frequency at pre-intervention (on the x axis) and post-intervention (on the y axis). The line indicates where pre- and post-intervention gene usage frequencies are the same, while dots represented on either the x or y axis indicate genes which were only represented at pre- or post-intervention, respectively. Plots produced in R studio by Peter Chovanec (Babraham Institute).

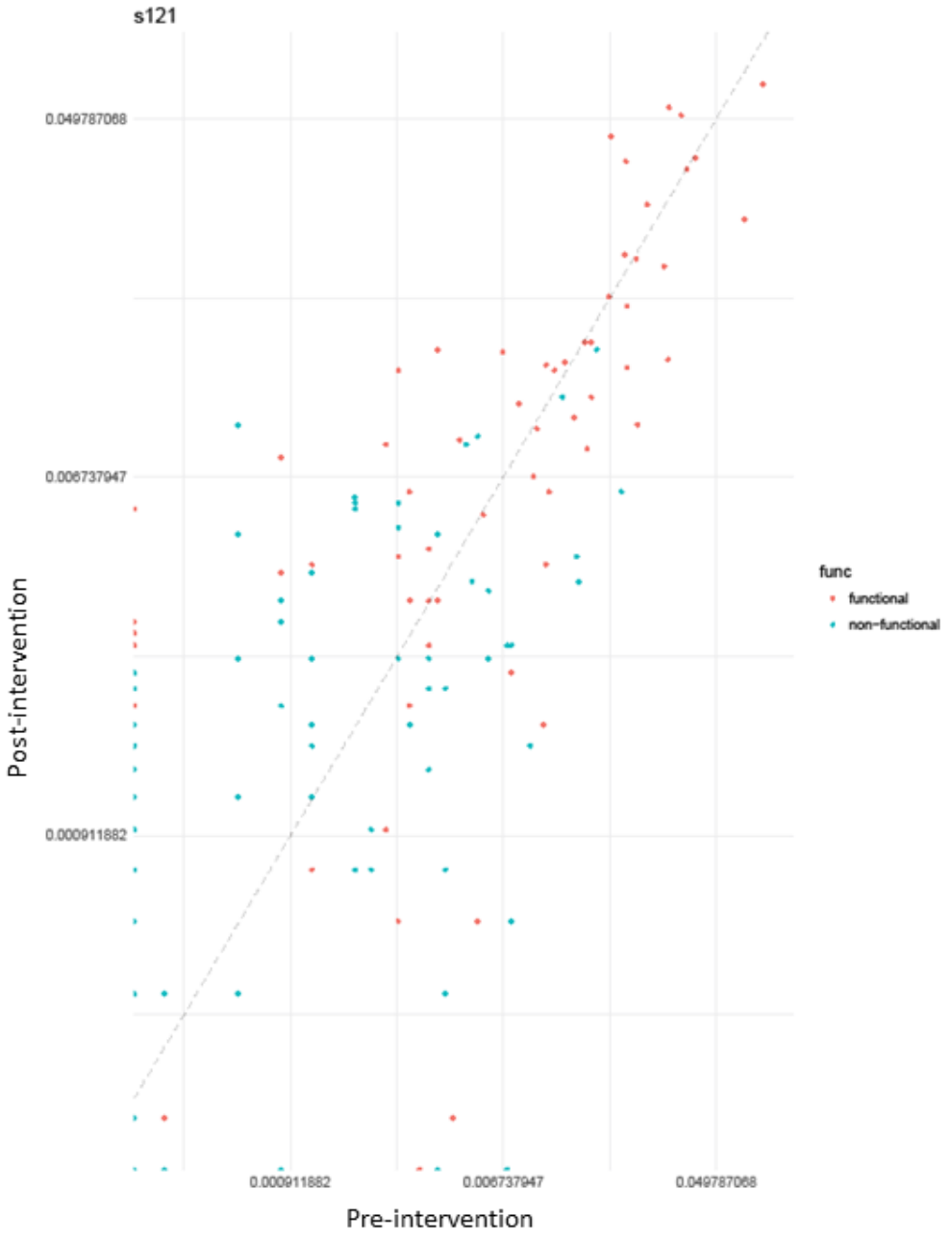


Figure 6.8b Scatter plot of IGHV gene usage for subject s121 comparing pre- to post-intervention. Frequencies of each IGHV gene, represented within the Nu-AGE sample, were plotted for functional (orange) and non-functional (blue) genes. The plot represents IGHV gene frequencies for an individual subject, with dots representing the frequency at pre-intervention (on the x axis) and post-intervention (on the y axis). The line indicates where pre- and post-intervention gene usage frequencies are the same, while dots represented on either the x or y axis indicate genes which were only represented at pre- or post-intervention, respectively. Plots produced in R studio by Peter Chovanec (Babraham Institute).

Figure 6.8c Scatter plot of IGHV gene usage for subject s127 comparing pre- to post-intervention. Frequencies of each IGHV gene, represented within the Nu-AGE sample, were plotted for functional (orange) and non-functional (blue) genes. The plot represents IGHV gene frequencies for an individual subject, with dots representing the frequency at pre-intervention (on the x axis) and post-intervention (on the y axis). The line indicates where pre- and post-intervention gene usage frequencies are the same, while dots represented on either the x or y axis indicate genes which were only represented at pre- or post-intervention, respectively. Plots produced in R studio by Peter Chovanec (Babraham Institute).

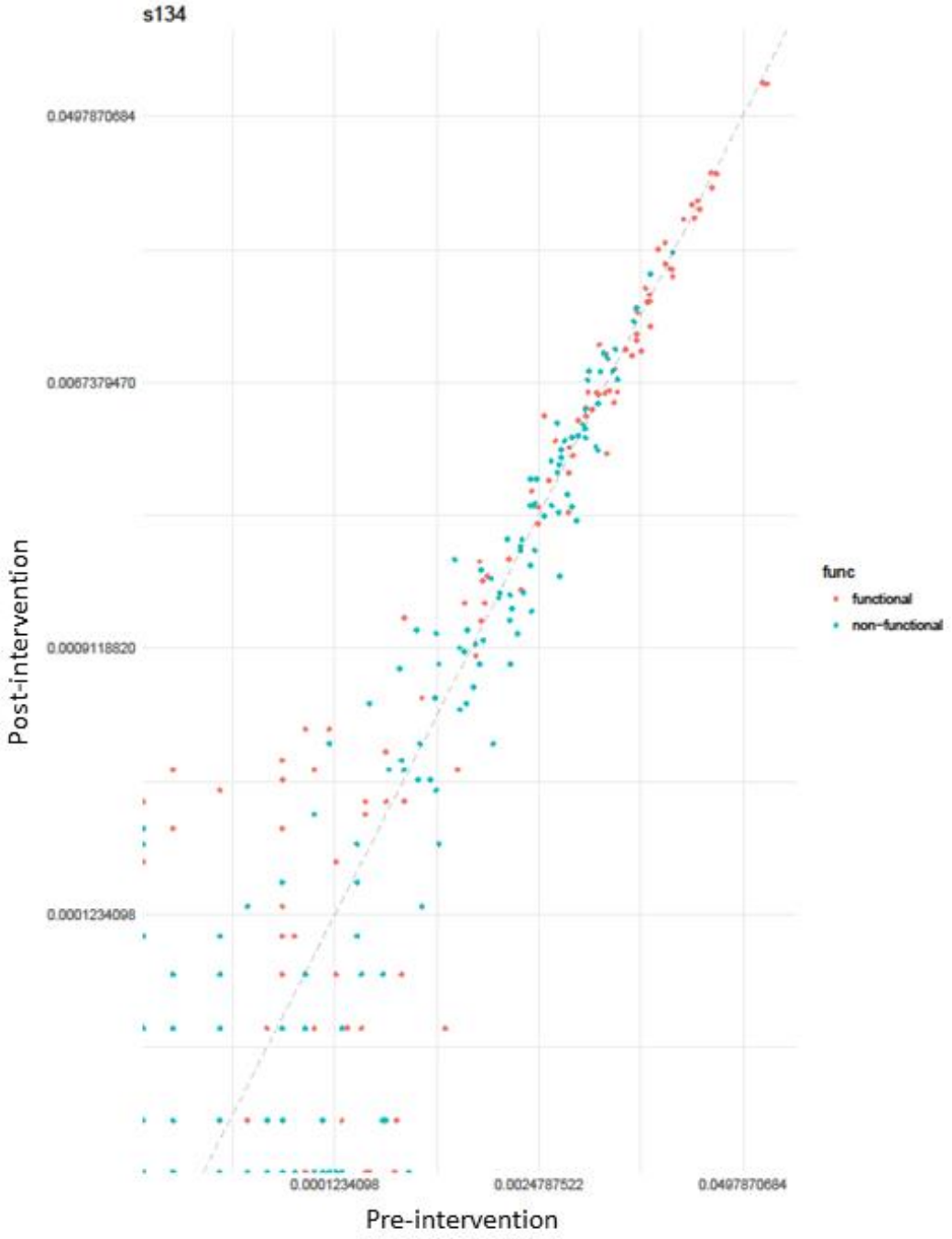


Figure 6.8d Scatter plot of IGHV gene usage for subject s134 comparing pre- to post-intervention. Frequencies of each IGHV gene, represented within the Nu-AGE sample, were plotted for functional (orange) and non-functional (blue) genes. The plot represents IGHV gene frequencies for an individual subject, with dots representing the frequency at pre-intervention (on the x axis) and post-intervention (on the y axis). The line indicates where pre- and post-intervention gene usage frequencies are the same, while dots represented on either the x or y axis indicate genes which were only represented at pre- or post-intervention, respectively. Plots produced in R studio by Peter Chovanec (Babraham Institute).

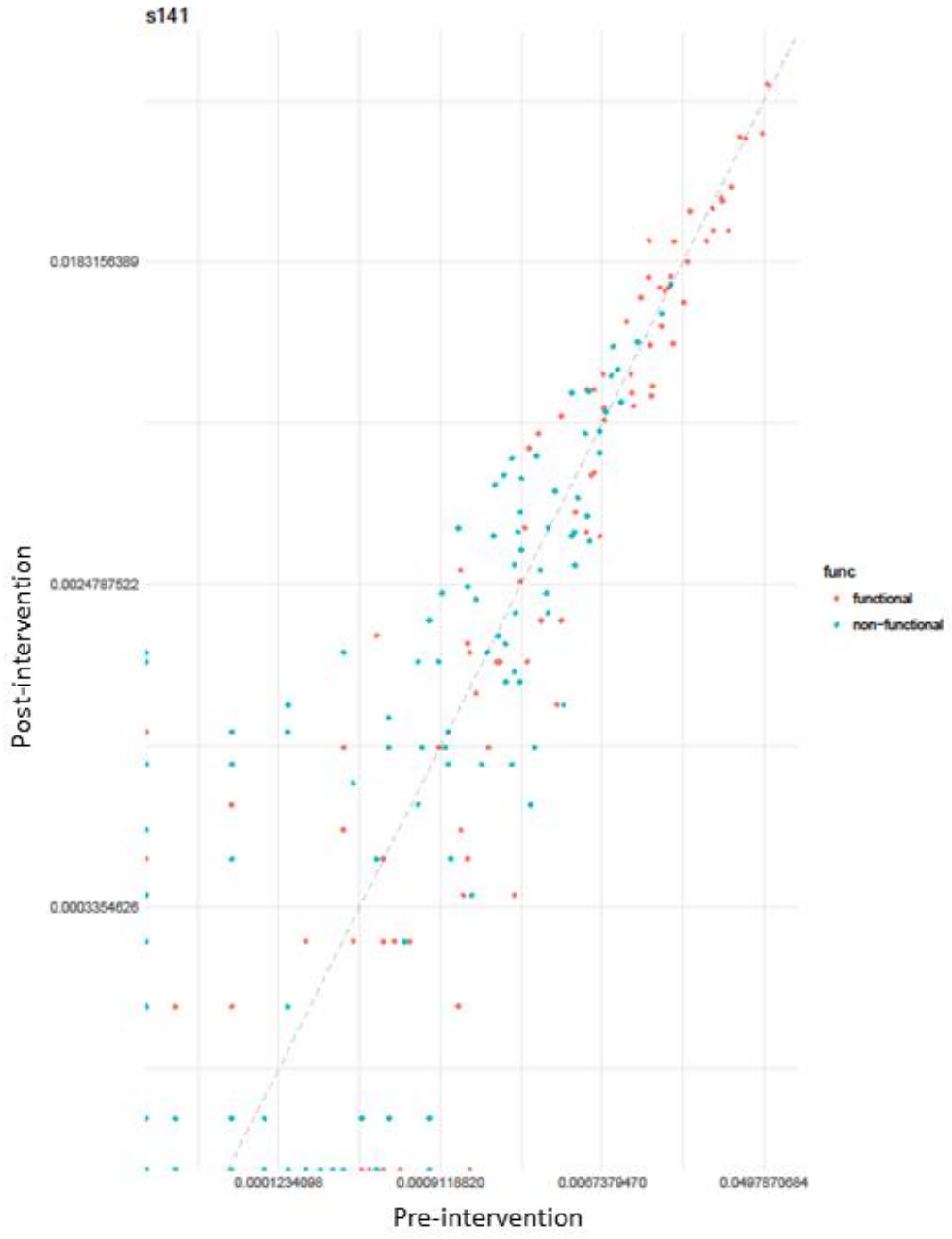


Figure 6.8e Scatter plot of IGHV gene usage for subject s141 comparing pre- to post-intervention. Frequencies of each IGHV gene, represented within the Nu-AGE sample, were plotted for functional (orange) and non-functional (blue) genes. The plot represents IGHV gene frequencies for an individual subject, with dots representing the frequency at pre-intervention (on the x axis) and post-intervention (on the y axis). The line indicates where pre- and post-intervention gene usage frequencies are the same, while dots represented on either the x or y axis indicate genes which were only represented at pre- or post-intervention, respectively. Plots produced in R studio by Peter Chovanec (Babraham Institute).

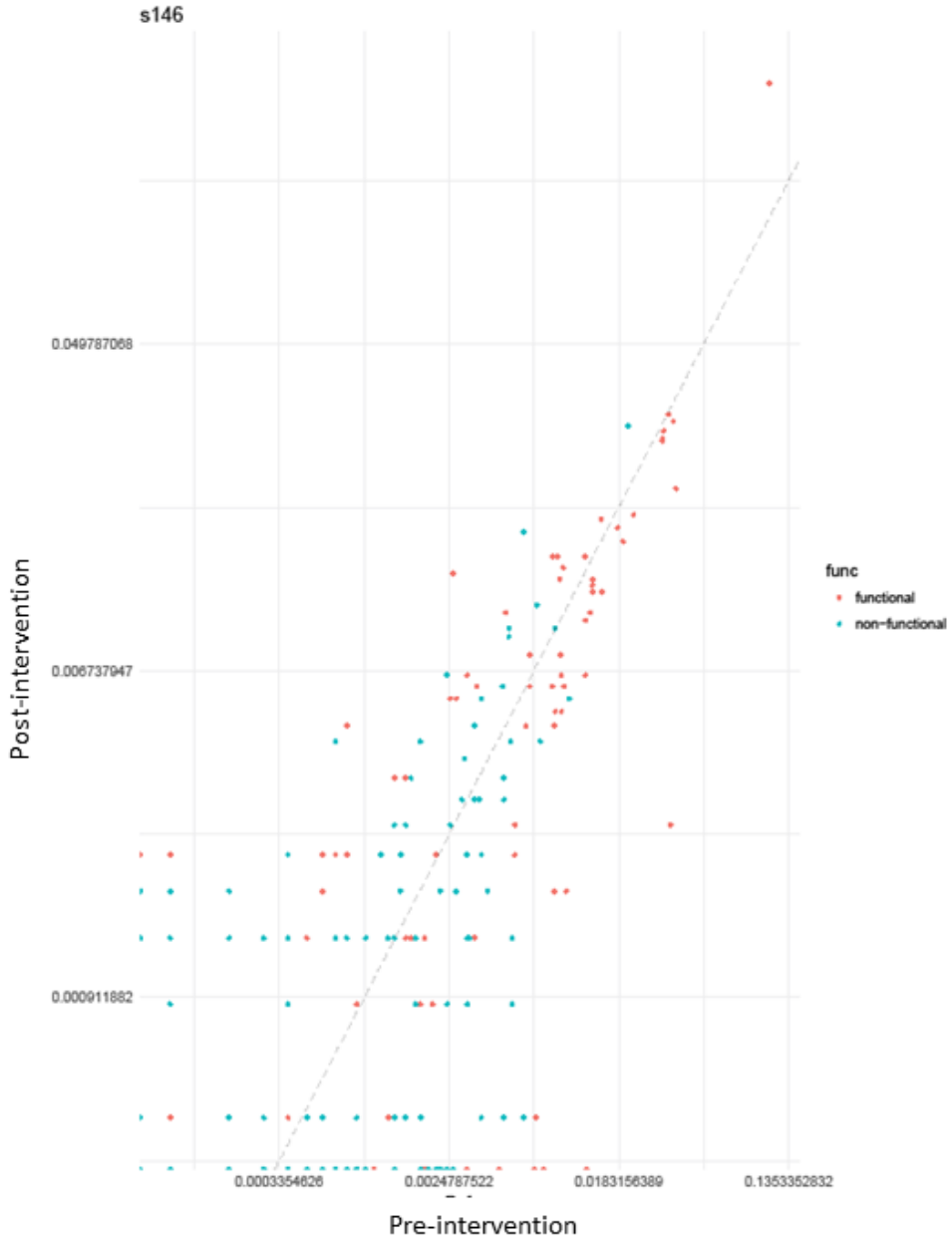


Figure 6.8f Scatter plot of IGHV gene usage for subject s146 comparing pre- to post-intervention. Frequencies of each IGHV gene, represented within the Nu-AGE sample, were plotted for functional (orange) and non-functional (blue) genes. The plot represents IGHV gene frequencies for an individual subject, with dots representing the frequency at pre-intervention (on the x axis) and post-intervention (on the y axis). The line indicates where pre- and post-intervention gene usage frequencies are the same, while dots represented on either the x or y axis indicate genes which were only represented at pre- or post-intervention, respectively. Plots produced in R studio by Peter Chovanec (Babraham Institute).

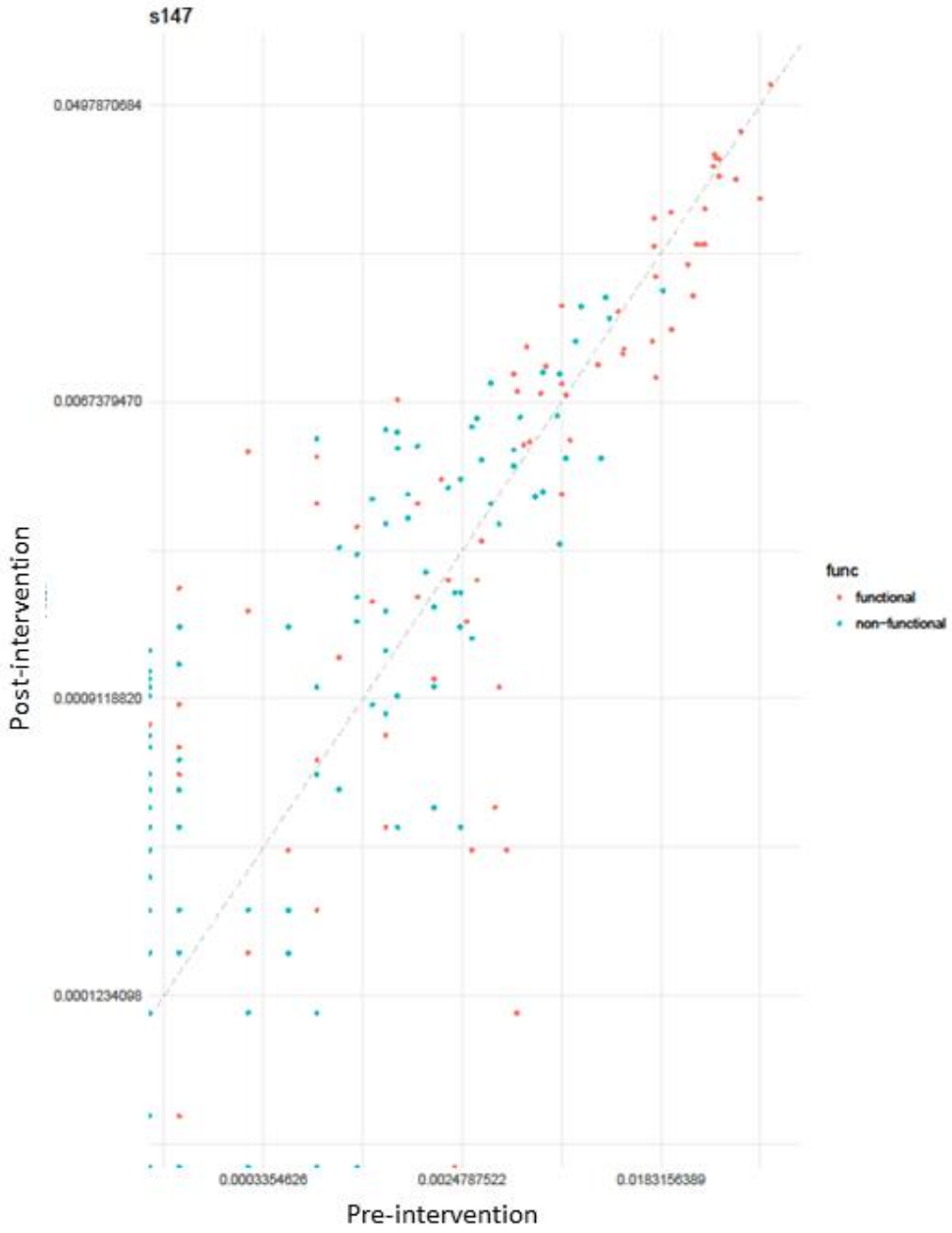


Figure 6.8g Scatter plot of IGHV gene usage for subject s147 comparing pre- to post-intervention. Frequencies of each IGHV gene, represented within the Nu-AGE sample, were plotted for functional (orange) and non-functional (blue) genes. The plot represents IGHV gene frequencies for an individual subject, with dots representing the frequency at pre-intervention (on the x axis) and post-intervention (on the y axis). The line indicates where pre- and post-intervention gene usage frequencies are the same, while dots represented on either the x or y axis indicate genes which were only represented at pre- or post-intervention, respectively. Plots produced in R studio by Peter Chovanec (Babraham Institute).

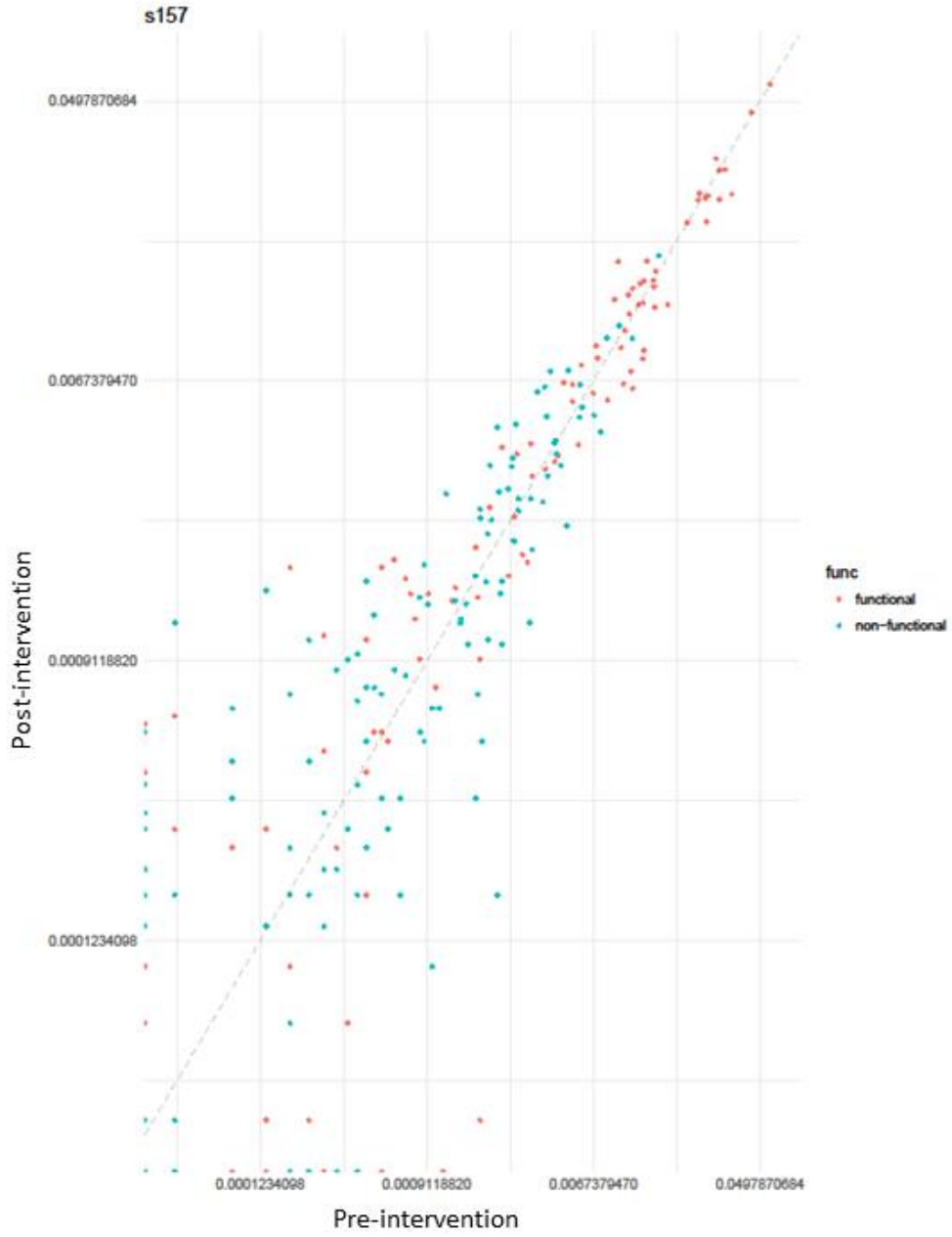


Figure 6.8h Scatter plot of IGHV gene usage for subject s157 comparing pre- to post-intervention. Frequencies of each IGHV gene, represented within the Nu-AGE sample, were plotted for functional (orange) and non-functional (blue) genes. The plot represents IGHV gene frequencies for an individual subject, with dots representing the frequency at pre-intervention (on the x axis) and post-intervention (on the y axis). The line indicates where pre- and post-intervention gene usage frequencies are the same, while dots represented on either the x or y axis indicate genes which were only represented at pre- or post-intervention, respectively. Plots produced in R studio by Peter Chovanec (Babraham Institute).

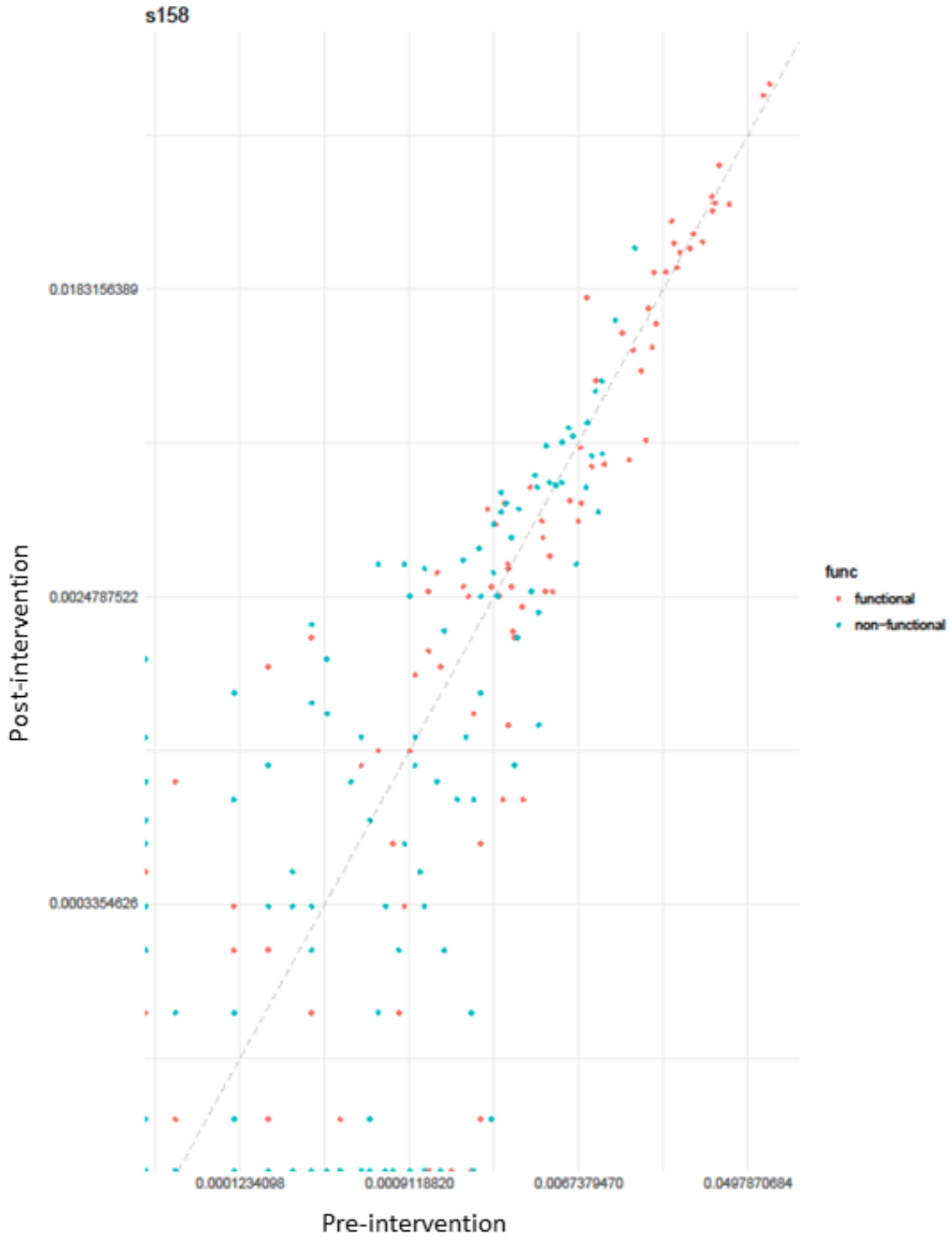


Figure 6.8i Scatter plot of IGHV gene usage for subject s158 comparing pre- to post-intervention. Frequencies of each IGHV gene, represented within the Nu-AGE sample, were plotted for functional (orange) and non-functional (blue) genes. The plot represents IGHV gene frequencies for an individual subject, with dots representing the frequency at pre-intervention (on the x axis) and post-intervention (on the y axis). The line indicates where pre- and post-intervention gene usage frequencies are the same, while dots represented on either the x or y axis indicate genes which were only represented at pre- or post-intervention, respectively. Plots produced in R studio by Peter Chovanec (Babraham Institute).

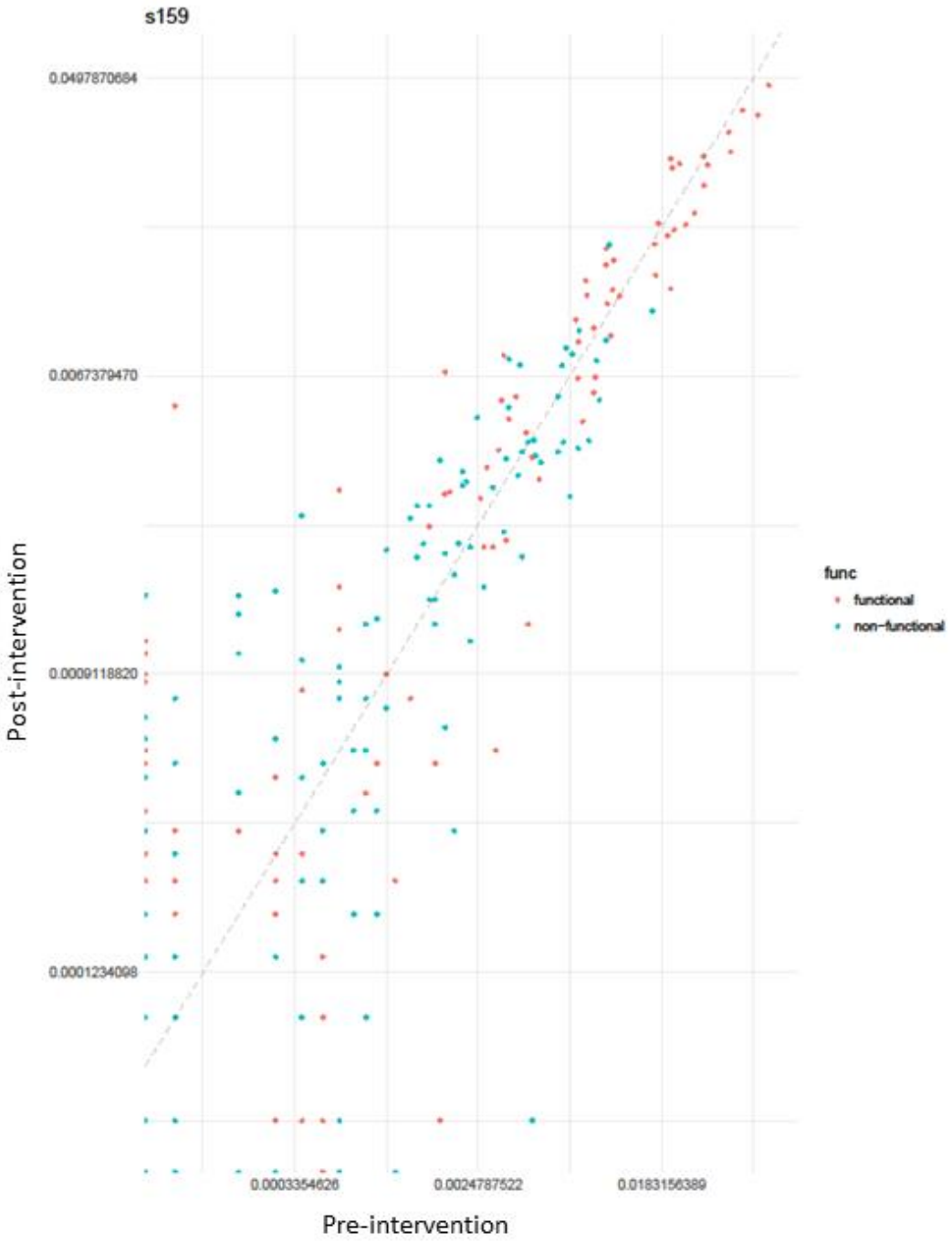


Figure 6.8j Scatter plot of IGHV gene usage for subject s159 comparing pre- to post-intervention. Frequencies of each IGHV gene, represented within the Nu-AGE samples, were plotted for functional (orange) and non-functional (blue) genes. Each plot represents IGHV gene frequencies for an individual subject, with dots representing the frequency at pre-intervention (on the x axis) and post-intervention (on the y axis). The line indicates where pre- and post-intervention gene usage frequencies are the same, while dots represented on either the x or y axis indicate genes which were only represented at pre- or post-intervention, respectively. Plots produced in R studio by Peter Chovanec (Babraham Institute).

Changes in the frequency of usage of IGHV alleles from pre- to post-intervention were determined for all alleles represented within the Nu-AGE cohort. Significantly decreased usage of IGHV1-2*02 ($p<0.0001$), IGHV3-9*01 ($p<0.0001$) and IGHV1-69*01 ($p=0.01$) was observed at post-intervention, while usage of IGHV3-30*01 increased at post-intervention ($p<0.0001$) in the control diet group (Figure 6.9). Significantly increased frequency of IGHV4-34*01 ($p<0.0001$) usage was observed at post-intervention in the MED diet group (Figure 6.9). However, overall differences in IGHV4-34*01 in the MED diet group were explained by one subject, MED146, for whom the frequency of usage of the allele IGHV4-34*01 was high at pre-intervention, and significantly increased one year after dietary intervention ($p<0.0001$). The frequency of VH4-34*01 usage by this subject was significantly greater than all other subjects analysed ($p<0.0001$) at both pre- and post-intervention (Figure 6.10).

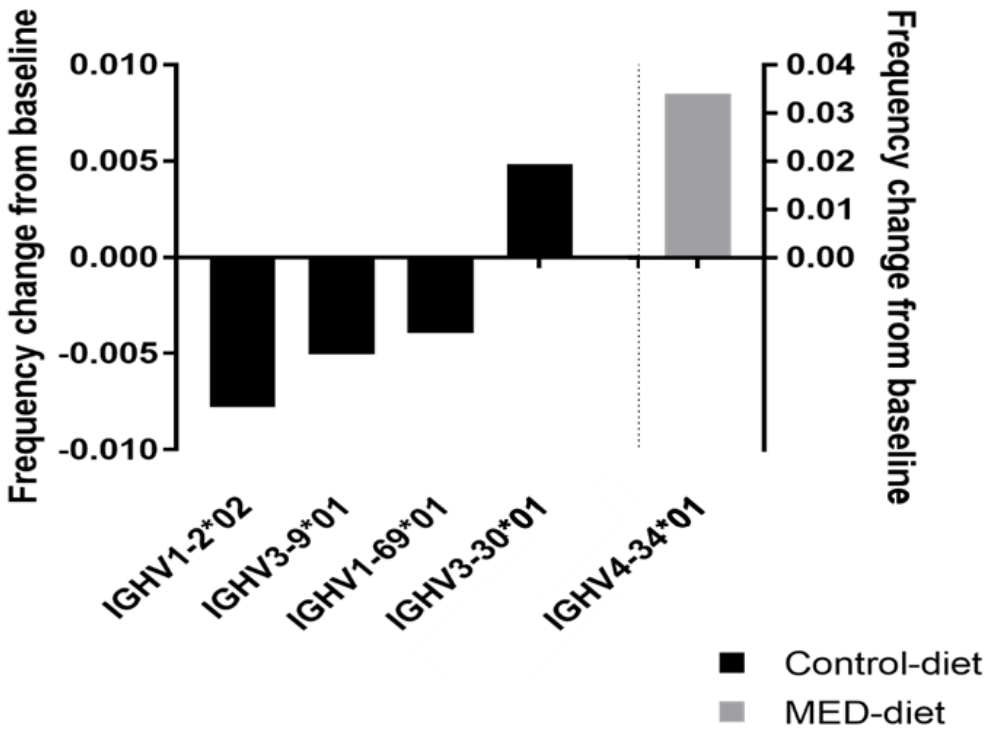


Figure 6.9 Change in frequency of usage for significantly different V alleles in IGHV of Nu-AGE subjects at pre-intervention compared to post-intervention, for both dietary groups. Mean change in frequency of IGHV allele usage from pre- to post-intervention was determined for all V genes for subjects on the control diet and the MED diet and analysed by two-way ANOVA, with *a priori* Holm Sidak's multiple comparisons tests. Differences in frequency of usage of IGHV alleles were plotted for IGHV alleles where $p<0.05$. Control diet; IGHV1-2*02 ($p<0.0001$), IGHV3-9*01 ($p<0.0001$), IGHV1-69*01 $p<0.001$), IGHV3-30*01 ($p<0.0001$). MED-diet, IGHV4-34*01 ($p<0.0001$).

Effect of dietary intervention on regional location of V genes

The regional location of functional V genes and alleles (Figures 6.5 and Supplementary Figure 6.4a-j; Appendix XX) in pre- to post-intervention showed no significant changes in IGHV gene usage in the control group. In the MED-diet group IGHV3-NL1 was significantly increased at post-intervention ($p<0.05$), while all other genes were not significantly different (all $p>0.05$). IGHV3-NL1 genes are located at the 5′ end of the chromosome and are most distal to the D/J recombination site.

Comparison of alleles showed that in the control group significant reductions were observed in the usage of IGHV1-2*02, IGHV3-9*01 and IGHV1-69*01, while IGHV3-30*01 was significantly increased (Figure 6.9). IGHV1-69*01 is located most distal to the D/J region with close proximity to the 5′ end of the chromosome. While, IGHV1-2*02 and IGHV3-9*01 are located at the 3′ end of the chromosome and IGHV3-3*01 is located in the middle of the chromosome but closer the 3′ end (Supplementary Figure 6.4a-j; Appendix XX).

6.3.3 CDR3 length

The CDR3 length was determined by the length of the nucleotide sequence and was compared from pre-intervention to post-intervention (Figure 6.11 and 6.12), for functional and non-functional genes respectively. The Kolmogorov-Smirnov (K-S) test compared the equality of the frequency distributions and showed no statistically significant changes in the distribution of CDR3 length of functional genes when comparing pre- to post-intervention derived samples from either the control or the MED diet intervention group (Figure 6.13). However, D'Agostino & Pearson normality tests on the frequency distribution data, show that the CDR3 length at pre-intervention was not normally distributed for functional gene in the control or MED diet group, nor the non-functional genes in the control group; CDR3 length was normally distributed for non-functional genes from samples on the MED diet at pre-intervention. All CDR3 length histograms for functional and non-functional genes, at post-intervention, were not normally distributed. The mean CDR3 length for the elderly subjects was 26 bp and the range was 2-103 bp across pre- and post-intervention samples. The sequence (nt) lengths of the IGH variable, diversity and joining regions, and junction length are detailed in Supplementary Table 6.1 (Appendix XXI).

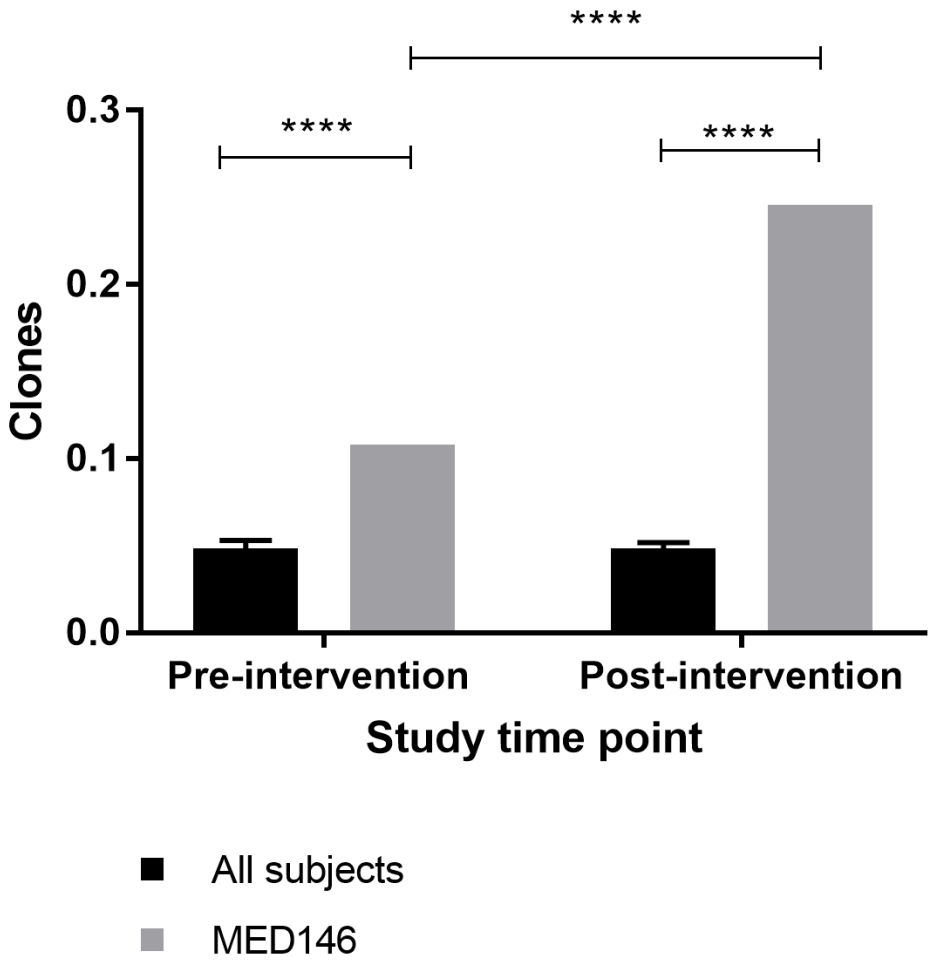


Figure 6.10 IGHV4-34*01 allele usage of one study individual compared to the remainder of the study cohort at pre- and post-intervention. Frequency of usage of the IGHV4-43*01 allele was determined after allocating gene identities using IgBLAST and IMGT. For analysis of IGHV4-34*01 usage, pre- and post-intervention frequencies, were compared for all subjects compared to the individual (MED146) using two-way ANOVA with Sidak's multiple comparisons test, using GraphPad Prism V7.02. Statistical significance was assumed at the $p<0.05$ level at the 95% confidence interval (CI). **** indicates $p<0.00001$. N= 10; all other subjects (9), compared to one individual value.

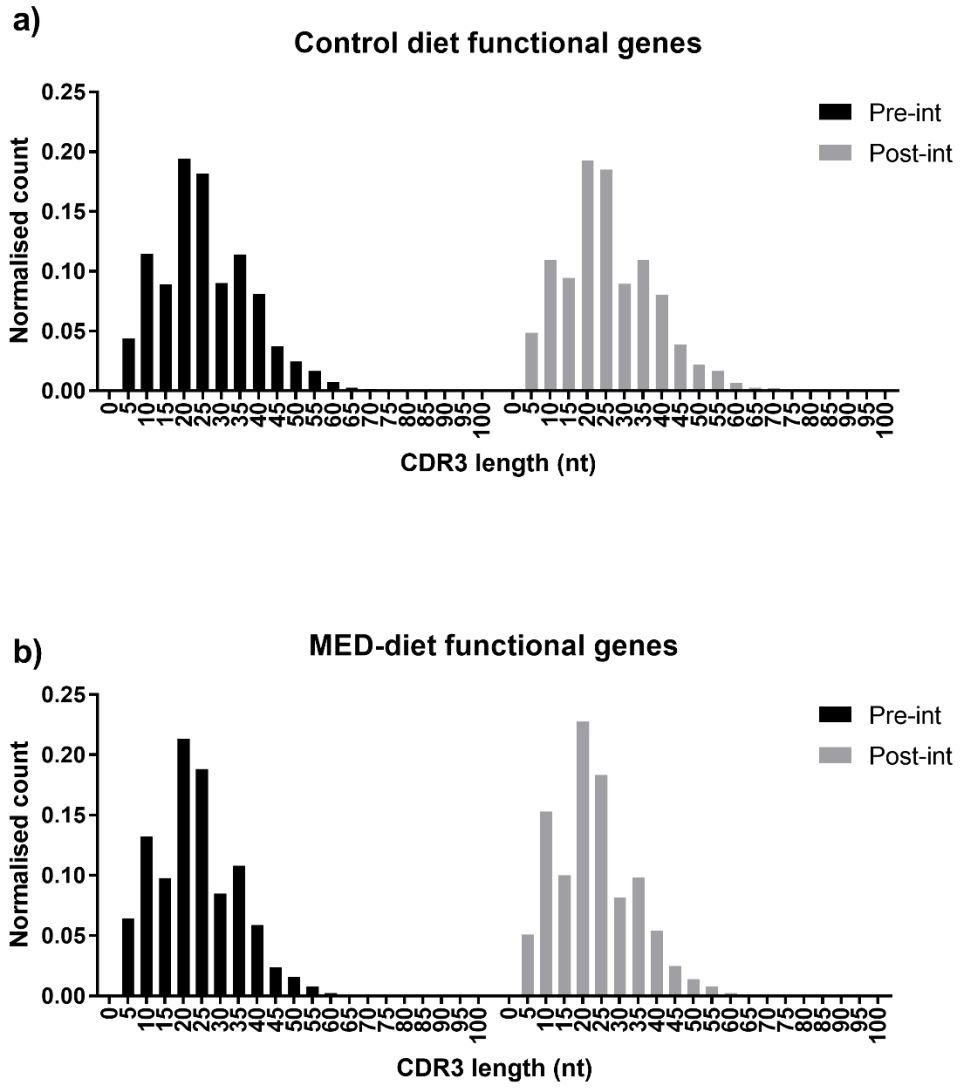


Figure 6.11. CDR3 length of nucleotide sequence for functional genes at pre- and post-intervention, for subjects on the control and MED diets. The x-axis represents the CDR3 lengths (nucleotides; nt) across all subjects, and lengths were divided into bins from 0 to 100 with a bin size of five nucleotides. The y-axis shows the frequency of CDR3 lengths which fall within each of these sizes; determined using relative frequency (fractions). Each histogram plots the normalised counts for each of these CDR3 lengths within the dataset, produced in GraphPad Prism V7.02; n=10 subjects. a) CDR3 lengths of functional genes represented in B cell DNA libraries prepared from subjects on the control diet; 6 subjects, b) CDR3 lengths of functional genes represented in B cell DNA libraries prepared from subjects on the MED diet; 4 subjects. D'Agostino & Pearson normality tests were applied to the frequency distribution data, normality tests were passed where alpha=0.05. Control group, pre-intervention (*P*=0.1546), post-intervention (*P*=0.1532), MED diet group, pre-intervention (*P*=0.1492) and post-intervention (*P*=0.0350).

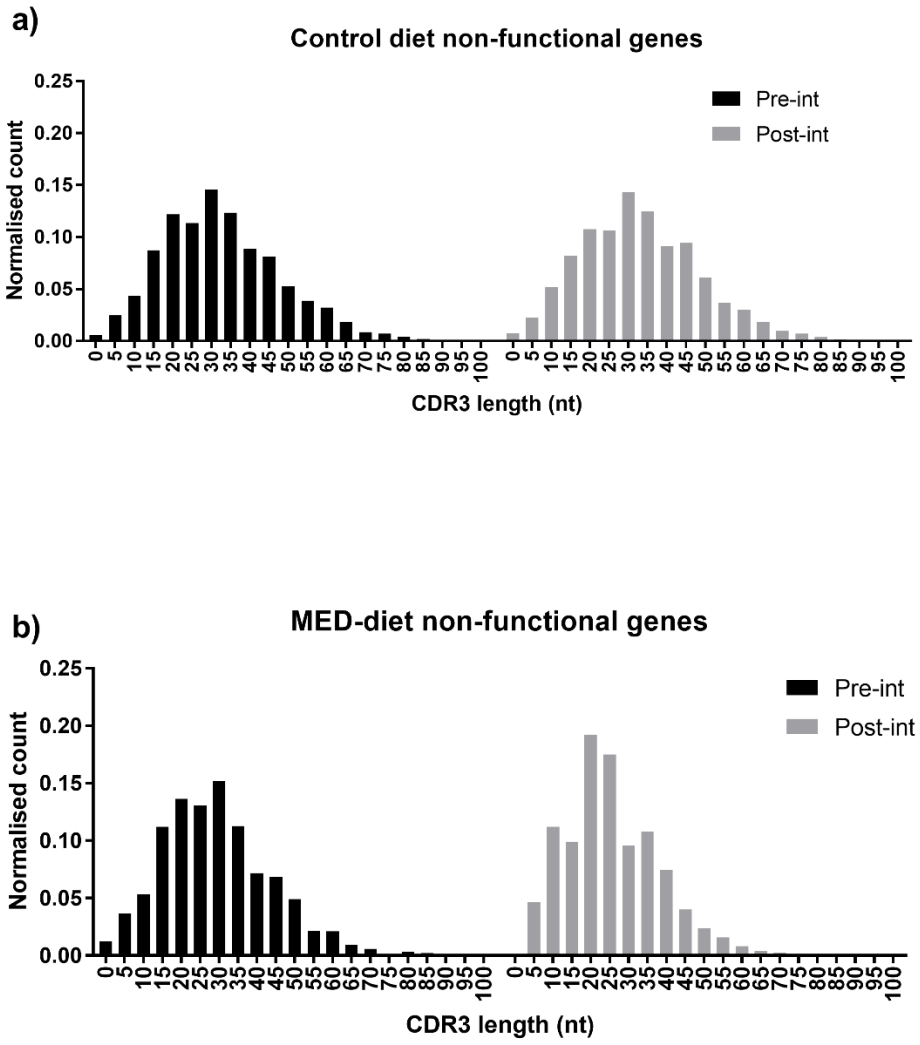


Figure 6.12. CDR3 length of nucleotide sequence for non-functional genes at pre- and post-intervention, for subjects on the control and MED diets. The x-axis represents the CDR3 lengths (in nucleotides; nt) across all subjects, lengths were divided into bins from 0 to 100 with a bin size of five nucleotides. The y-axis shows the frequency of CDR3 lengths which fall within each of these sizes; determined using relative frequency (fractions). Each histogram plots the normalised counts for each of these CDR3 lengths within the dataset; n=10 subjects. a) CDR3 lengths of non-functional genes represented in peripheral B cell derived DNA libraries prepared from blood samples from subjects on the control diet; 6 subjects, b) CDR3 lengths of non-functional genes represented in peripheral B cell DNA libraries prepared from blood samples from subjects on the MED diet; 4 subjects. D'Agostino & Pearson normality tests were applied to the frequency distribution data; normality test passed where alpha=0.05. Control group, pre-intervention (*P*=0.0431), post-intervention (*P*=0.0410), MED-diet group, pre-intervention (*P*=0.0083) and post-intervention (*P*=0.0029).

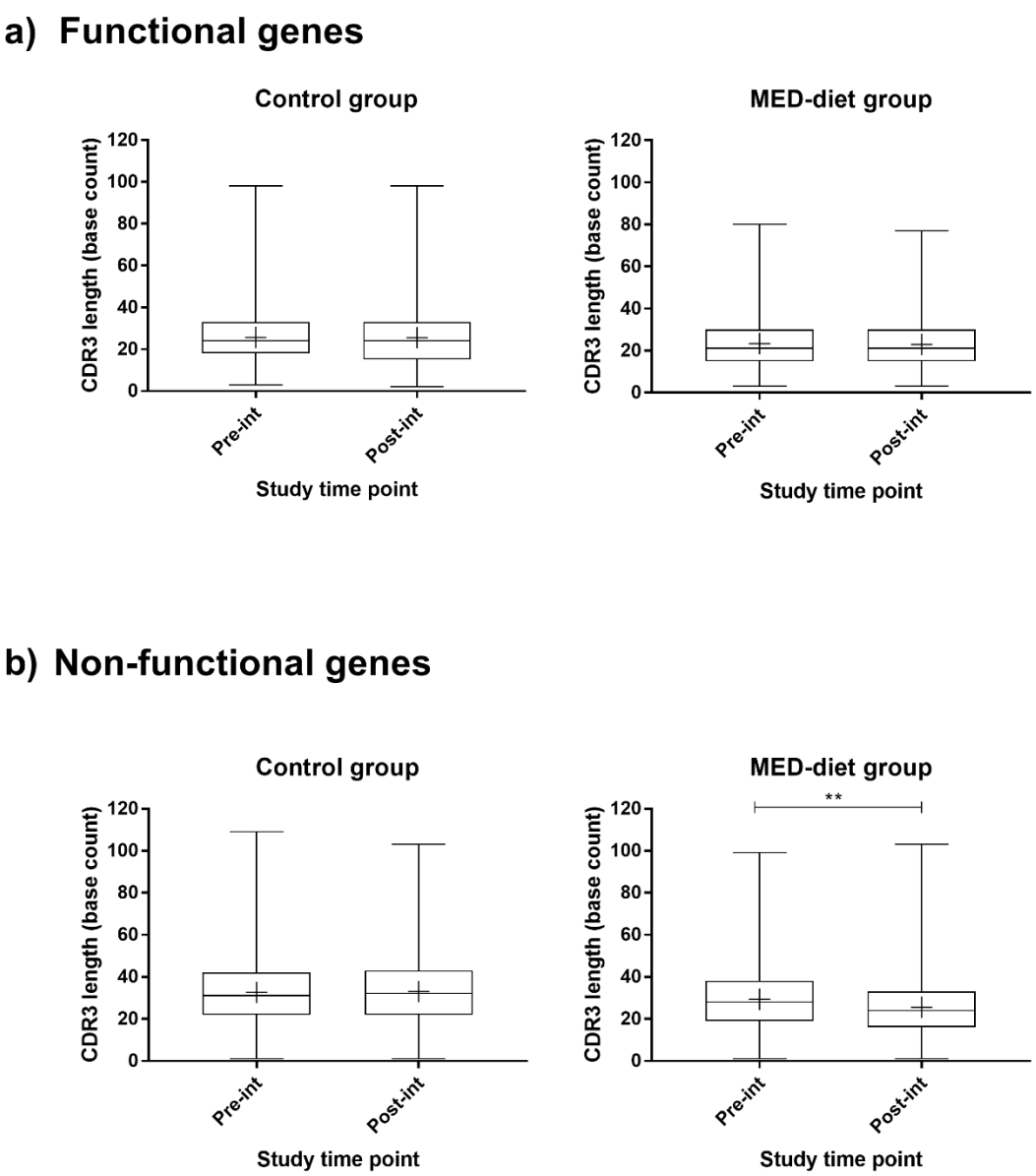


Figure 6.13. CDR3 length for functional and non-functional genes, comparison of pre- to post-intervention for subjects on the control diet or the MED diet. CDR3 length determined by count of bases within the CDR3 sequence determined using the IMGT database. Box and whisker plots with whiskers extending to the minimum and maximum values within the dataset; horizontal line through box represents median, mean represented by (+) plus sign. The Kolmogorov-Smirnov (K-S) test to compare the equality of the frequency distributions between the pre- and post-intervention data; a) control diet (*P*=0.5911; ns) and MED diet (*P*=0.9135; ns), b) control diet (*P*=0.2372; ns) and MED diet (*P*=0.0082). Tests were two-tailed, confident intervals were 95%, and statistical significance was assumed at p>0.05; ns= not significant, *p<0.05, **p<0.001, ***p<0.0001.

6.3.3 Clone analysis

Clonotypes were assigned to each gene represented for all subjects using IgBLAST and the IMGT database such that the same clonotype was assigned where the same V gene was used and the CDR3 length was the same ± one bp. The presence of different clonotypes in the VDJ-seq data from each of the libraries prepared from individual subjects' B cell DNA is represented in the tree map in Figure 6.14. Tree maps are a visualisation method which display hierarchical information in a 2-dimensional, space-filling map where 100% of the space is utilised (Johnson and Shneiderman, 1991). Information of more importance can be allocated more space within the map and the map can be divided into a collection of rectangular bounding boxes, which in turn can be divided into nodes within their bounding box (Johnson and Shneiderman, 1991). The tree maps used within this study allow display of clonoype size for study participants using nesting to subdivide each subject and colour to identify study time point (van Wijk and van de Wetering). Study subjects s134, s157, s141, s127, s147 and s121 were allocated to the control diet. With the exception of s121, the tree map for these subjects demonstrates the most unique clonotypes, as observed by the larger overall area (bounding box) for each subject and the larger size of the internal squares (nodes), representing a greater frequency of each unique clonotype. This suggests that subjects in the control group had more unique clonotypes but that the frequency of each of these clonotypes was high. While, s112, s158, s146 and s159 were allocated to the MED diet group and, with the exception of s159, these subjects had smaller areas (bounding boxes) within the tree map and the smaller size of the internal squares (nodes) suggests that while these subjects had fewer unique clonotypes, the frequency of each of these unique clonotypes was lower.

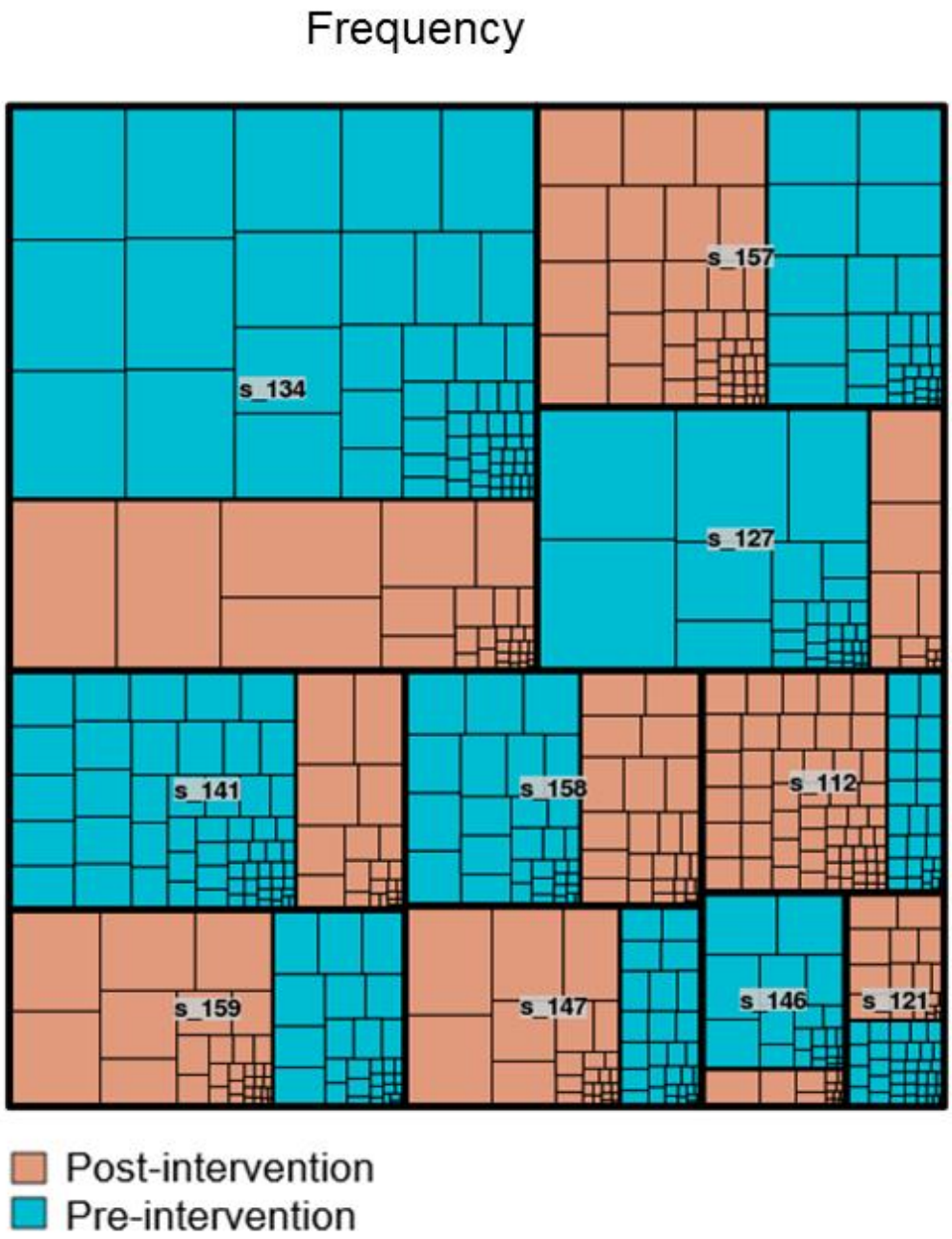


Figure 6.14. Clonotype analysis of all Nu-AGE samples comparing pre- to post-intervention. Clonotypes were assigned where the same V gene was used and the same CDR3 length, allowing one base pair (bp) difference, where different unique molecular identifiers (UMIs) and/ or start positions were observed, using IgBLAST and the IMGT database. Tree maps show the diversity of recombined VDJs for each individual subject, represented by their sample ID code. The smaller squares represent the number of unique clonotypes present within B cell derived DNA libraries from each subject, with the size of each square representing the frequency of each clonotype. Pre-intervention values are shown in orange, and post-intervention values are shown in blue. N= 10 subjects. Produced in RStudio by Peter Chovanec (Babraham Institute).

Figure 6.15 is a visual representation of the subjects clustered into their allocated dietary intervention group (control or MED diet), comparing pre- to post-intervention. The tree map shows greater frequency of unique clonotypes represented by greater overall area of each group and greater frequency of each unique clonotype within the sample group was represented by larger sizes of the internal squares. It is apparent that subjects in the control group had more unique clonotypes at both pre-intervention and post-intervention compared to the MED diet group. However, the frequency of unique clonotypes decreased in the control group after one year intervention. In the MED diet group, although there were fewer unique clonotypes at pre-intervention, the frequency remained constant after one year dietary intervention. Also, the size of the internal squares, which indicates that the subjects in the control diet group had a large number of clones of some of the clonotypes, is indicative of oligoclonality within the Ig repertoire. There were more small internal squares at post- intervention in the MED diet group suggesting that there were fewer clones of the same clonotypes and that there are more unique clonotypes one year after the dietary intervention.

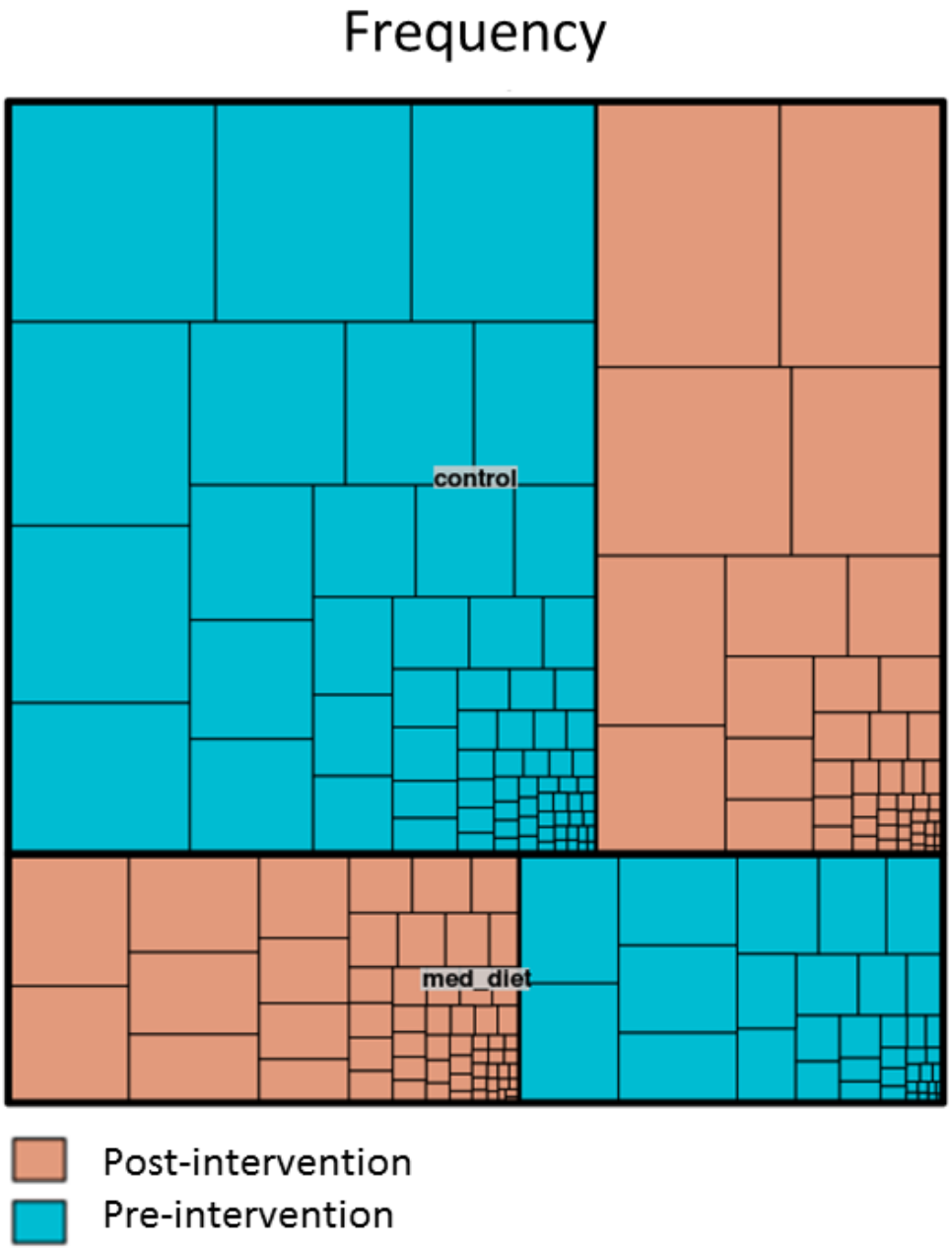


Figure 6.15. Clonotype analysis of all Nu-AGE samples, separated by dietary intervention group, comparing pre- to post-intervention. Clonotypes were assigned where the same V gene was used and the same CDR3 length, allowing one base pair (bp) difference, where different unique molecular identifiers (UMIs) and/ or start positions were observed, using IgBLAST and the IMGT database. Tree maps show the overall diversity of recombined VDJs for subjects allocated to the control or MED diet groups. The smaller squares represent the number of unique clonotypes present within B cell derived DNA libraries from each subject, with the size of each square representing the frequency of each clonotype. Pre-intervention values are shown in orange, and post-intervention values are shown in blue. N= 10 subjects. Produced in RStudio by Peter Chovanec (Babraham Institute).

6.4 Discussion

Overall, this study shows that with increased age usage of the IGHV3 gene subgroup is reduced, while usage of the IGHV1 gene subgroup is increased in elderly subjects compared to young. The age-associated genes changes observed are located at the 3′ end of the chromosome. CDR3 length is unaffected by the one year intervention with the MED diet. However, the MED diet intervention is associated with maintenance of the frequency of the unique clonotypes observed, while increased frequency of unique clonotypes is observed one year post-intervention in the control diet group.

6.4.1 IGHV3 gene subgroup usage is reduced in elderly subjects compared to young with dietary intervention having no effect on IGHV gene subgroup usage

The pattern of IGHV gene subgroup usage, from highest to lowest usage, is comparable between young and elderly subjects. With usage of IGHV3 being the most prominent gene subgroup used with the IGHV region, consistent with previous observations in peripheral blood B cells of healthy adults (Davidkova et al., 1997). However, there is a reduction in IGHV3 usage in the elderly, which is also consistent with previous findings (Martin et al., 2015; Wang and Stollar, 1999; Wu et al., 2011). Additionally, we found that elderly subjects display an increase in IGHV1 subgroup usage compare to the young subjects, though this has not been reported in previous studies.

6.4.2 Age-associated IGHV gene changes are located at the 3′ end of the chromosome

Of the IGHV gene subgroups, IGHV6 is the most D-proximal (Davidkova et al., 1997). Regional analysis of V gene usage within the IGHV region of haploid samples, allowed IGHV genes to be subdivided into three groups (Regions I-III) based on their locations (Chimge et al., 2005). Region I begins at the 3′ -end of the IGHV locus and includes genes from IGHV6-1 to IGHV2-28-1, Region II covers genes from IGHV3-29 to IGHV3-52 and Region III genes begin at the 5′-end of the IGHV locus and include IGHV3-53 up to IGHV3-52 (Chimge et al., 2005).

The regional analysis of V gene usage between young and elderly subjects reveals significant decreases in frequency of usage of the genes IGHV3-13, and IGHV3-23 which are all located within Region I, and proximal to the 3′ end of the chromosome. Significant increases in usage of IGHV3-15 is observed in the elderly compared to young, which is also located within Region I, close to the 3′ end of the chromosome.

The other significant decreases observed (IGHV3-30 and IGHV4-30-2) in functional genes are located just inside Region II of the chromosome. This is of interest since all of these genes are located in closest proximity to the D/J region and is consistent with unpublished data from the Babraham Institute where aged mice display a subtle increase in some IGHV genes, in close proximity to the 3′ end of the chromosome (Personal communication: Bolland and Matheson (2016)). However, in contrast to these provisional findings, post-pneumonia vaccination gene locus expression of IGHV3-30 was favoured in the elderly (>65 years) with a significantly greater frequency of IGHV3-07, VH3-74 (to PPS4) and IGHV3-48 and IGHV3-33 (to PPS14) usage observed in young subjects (< 30 years) (Kolibab et al., 2005). Additionally, the significant increases observed in usage of IGHV4-59, IGHV1-69 and IGHV3-74 in the elderly are all genes located at the 5′ end of the chromosome. This is of interest since IGHV1-69 gene usage has been investigated in the elderly (>75 years), in whom a small increase was observed (6%) compared to young subjects (0.8%) using IgM cDNA libraries (n=5) and single IgM B cell analysis (Potter et al., 2003). This allele is located at the 5′ end of the chromosome and is more distal to the D/J recombined region.

6.4.3 IGHV3-NL1 is significantly increased in the MED diet group post-intervention

The only significant increase in gene usage post MED-diet intervention is of IGHV3-NL1. This gene was recently discovered as a new member within the IGHV3 subgroup (Wang et al., 2011) and is the most 5′ gene on the chromosome, when considering all genes represented in samples from the Nu-AGE cohort. In addition, the significant reduction observed between pre- and post-intervention in usage of the allele IGHV1-69*01, which is also located at the 5′ end of the chromosome could be a consequence of an additional year of age and thus an artefact of ageing, however, comparison of IGHV1-69 gene usage between young and elderly subjects showed increased not decreased usage with age (Potter et al., 2003).

Significantly high usage of the allele IGHV4-34*01 is observed in one subject, the frequency of which significantly increases one year post-intervention. IGHV4-34 genes encode antibodies that are intrinsically autoreactive (IgM cold-agglutinins) due to their recognition of conserved carbohydrate self-epitopes on red blood cells (Potter et al., 2002; Pugh-Bernard et al., 2001; Silberstein et al., 1996). This is of relevance because IGHV4-34 gene usage has been shown to be a feature of autoimmune diseases such as systemic lupus erythematosus (SLE) or infection with Epstein-barr herpes virus (EBV) or Mycoplasma pneumoniae (IJspeert et al., 2016; Pugh-Bernard

et al., 2001), while reduced usage in IGHV regions is observed in antigen experienced (switched memory) compared to naïve B cells (Wu et al., 2010). Therefore, the remainder of the subjects, with lower frequencies of IGHV4-34 usage, may have repertoires more typical of antigen-experienced B cells, a common hypothesis for the reduced response to vaccinations and increased susceptibility to infections observed in the elderly. While this one individual may be displaying a greater expression of IgM cold agglutinin autoantibodies, since expression of IGHV4-34 is very high, indicating multiple clones of heavy chains expressing this gene, the highly significant changes observed in IGHV4-34 gene usage are unlikely to be a result of dietary intervention, both due to the very small sample sizes for the two dietary groups and the lack of dietary compliance observed from the diet diary analysis.

6.4.4 CDR3 length and the MED diet

Previous spectratyping based studies have shown that B cell populations of healthy young individuals typically display CDR3 spectratypes in the range of 57–117 bp (Gibson et al., 2009), with earlier reports identifying CDR3 ranges of 3-28 amino acids (9-84 nt) (Kirkham and Schroeder, 1994). The range of CDR3 lengths observed in the Nu-AGE cohort is 2-103 nt with a mean length of 26 nt. This is shorter than the reported values for young healthy subjects (Gibson et al., 2009), but in agreement with Pickman et al. (2013) where CDR3 length distributions were positively skewed toward shorter CDR3s. Similarly, comparison of young and elderly peripheral B cells show that those derived from the elderly are significantly shorter than those from young subjects, with 28 analysed sequences recorded for IgM naïve B cells from the elderly, compared to 37 sequences for the cells isolated from young subjects (Chong et al., 2003). This may be due to the elderly having greater numbers of memory B cells, with shorter CDR3s (Pickman et al., 2013). Similarly, heavy chain CDR3 repertoire diversity was significantly reduced in IgG compared to IgM from peripheral blood B cells of healthy volunteers after hepatitis B vaccination (HBV) (Ma et al., 2017). Longer CDR3 lengths have been associated with autoantibodies which display self reactivity, which in healthy individuals are mostly removed at the checkpoints during B cell developments (IJspeert et al., 2016; Kaplinsky et al., 2014; Wardemann et al., 2003). The shortened CDR3 length associated with age, is not influenced by dietary intervention as there are no significant differences in equality of distribution when comparing pre- to post-intervention.

The histograms of the CDR3 lengths for the functional genes are not normally distributed, which has been observed in spectratyping of CDR3 lengths of peripheral B cells from elderly subjects (Gibson et al., 2009). In addition, oligoclonality is

observed more frequently in the elderly, than in the young subjects (Kolibab et al., 2005). While, CDR3 length distribution from healthy, young mice follow a normal Gaussian distribution (Kaplinsky et al., 2014). The CDR3 length histogram for the non-functional genes from the MED diet intervention group is normally distributed at pre-intervention but not post-intervention, which may imply a reduction in clonality of CDR3 regions after one year of dietary intervention. However, as the frequency distribution of lengths were highly variable, (2-103 nt) this difference could have been due to an outlier.

6.4.5 Frequency of each unique clonotype was maintained post-MED diet intervention but decreased after the control diet

The lower frequency of each unique clonotype for three of the MED-diet subjects suggests that these subjects have B cells with less oligoclonality as compared to the subjects in the control group, where each unique clonotype is present in a greater frequency of Igs. This finding indicates that there are more B cells with the same random VDJ recombination, since identification of the same cell again was prevented by the use of UMIs. This has previously been observed as a feature of increased age (Gibson et al., 2009). This analysis cannot, however, be used in isolation as a measure of Ig diversity, since it does not take into consideration the amount of DNA used. The B cells utilised for the DNA library preparation were isolated from peripheral blood and were not stimulated in anyway before or after blood draw, therefore only naïve B cell repertoires are compared.

The maintenance of the clonotype profile pre- compared to post-intervention for the MED diet group suggests that the dietary intervention may have prevented, or at least delayed, the age-associated increase in oligoclonality. High MUFA diets and CLA (3 g/day) increased lymphocyte proliferation in older subjects and healthy volunteers (Han et al., 2012; Nugent et al., 2005), while fish oils decreased mitogen induced lymphocyte proliferation (Bechoua et al., 2003; Meydani et al., 1991). In addition to reduced accumulation of MHC II and PKC-θ at the immunological synapse, binding of antigen and the downstream signalling resulting in IL-6 secretion (Gurzell et al., 2015; Rockett et al., 2013; Rockett et al., 2012), there is potential for FA intake to influence B cells during development of the Ig. BM adiposity via adipokine production, which increases with increased age, could influence the B cells during development as adiponectin can inhibit early B cell precursors in murine BM and human cord blood cultures (Yokota et al., 2003). These findings were attributed to the production of PGE_2 and cyclooxygenase-2 (COX-2), which both inhibited lymphopoiesis, but was restored with addition of COX-2 inhibitor to adiponectin-containing cultures (Yokota

et al., 2003). Additionally, high fat diet induced obesity in mice, promoted the production of IgG autoantibodies in the spleen and serum, while reducing production of spontaneous IgM (Winer et al., 2011). Since diet can influence CSR outcomes in mice and the production of early B cell precursors is thought to be influenced by the pro-inflammatory eicosanoids PGE_2 and COX-2 then dietary (particularly dietary fat) modification could also influence antigen binding sites of antibodies during VDJ recombination, as this occurs in early precursor B cells.

It has been demonstrated that BM adiposity is strongly, positively correlated with serum total cholesterol, LDL cholesterol and triglyceride levels (Slade et al., 2012). Therefore, to determine whether BM adiposity may be influencing these results blood lipids profiles for the study cohort were analysed to determine whether high levels of total or LDL cholesterol or triglycerides were present within the subjects (Figure 5.1, Chapter 5). The mean total and LDL cholesterol concentrations within plasma of the Nu-AGE subjects are higher than the recommendations of <4 mmol/l and <2 mmol/l, respectively, by the British Heart Foundation (BHF, 2017). Mean HDL cholesterol and triglyceride concentrations are in accordance with the BHF recommendations of >1 mmol/l and <1.7 mmol/l, respectively (BHF, 2017). As this study cohort have high total and LDL cholesterol levels, there is potential that BM adiposity may have contributed to the age-associated alterations in the IGHV regions in this study. Additionally, BMD was negatively correlated with BM adiposity (Slade et al., 2012), so the DXA results of the study cohort (Table 4.5, Chapter 4) were investigated. The results show that the while the mean BMD is just in the normal range, a proportion of the subjects classify as having osteopenia (<1.06, >0.98 g/cm^2) or osteoporosis (<0.98 g/cm^2) confirmed by the range of T scores, which are typically used to determine presence of these conditions (between -1 and -2.5, osteopenia; ≤-2.5, osteoporosis) (Ramos et al., 2012).

Significant reductions of adipocyte differentiation and expression of genes involved in adipogenesis have been observed after *in vitro* supplementation of human stem cell derived BM cultures with the olive oil polyphenol, oleuropein (Santiago-Mora et al., 2011). Additionally, inhibition of BM adiposity has been observed with a PPARγ antagonist, which suppresses mRNA gene expression of PPARγ2, and the mature adipocyte marker (aP2) in bones of diabetic mice (Botolin and McCabe, 2006). BM adipocyte numbers of mice treated with the PPARγ agonist were also reduced compared to untreated mice, though this did not reach statistical significance (Botolin and McCabe, 2006). These results suggest a potential role for PPARγ in BM adiposity and that PPARγ could be a target for reducing this and potentially the observed

defects in B cell production and antibodies in previous studies. As discussed in Chapter 5, increases in PUFA intake have demonstrated downregulation of PPARα in PBMCs (Bouwens et al., 2009) and PPARγ in DCs (Zapata-Gonzalez et al., 2008; Zeyda et al., 2005) showing that dietary intervention has potential to induce such changes.

6.4.6 Impact of findings

Our VDJ-seq has produced similar observations in young compared to elderly subjects comparable to existing methodologies and previously published studies. The key difference between this study and previous studies in the use of J-specific oligonucleotides whereas previous methodologies have relied on cocktails of V-specific primers which could not represent all 95 V genes present in the human IgH (Boyd et al., 2010; Wood et al., 2013) and therefore result in incomplete or biased detection of some recombined products (Bolland et al., 2016). The data from this study are therefore representative of recombined IGH-VDJ and IGH-DJ sequences which are unbiased since V gene primers were not used. Consequently, this and future studies utilising this methodology would provide more comprehensive data as a result of the improved methodological approach. Since the published work using VDJ-Seq utilised murine samples (Bolland et al., 2016), this study provides proof of concept for its use in human studies using B cell DNA and is therefore novel and innovative.

Additionally, this method has been shown to be sensitive enough to detect potential markers or predisposition for autoimmune conditions such as systemic lupus erythematosus (SLE) (Pugh-Bernard et al., 2001). While this study cannot extrapolate any clinical relevance of this finding, future investigations and development of this methodology could result in the possibility of screening patients to identify “at risk” patients earlier, allowing time to intervene to reduce the associated risks or potentially even disease outcomes.

There have been several observations of reduced B cell output from the BM with increased age, and thus a subsequent accumulation of antigen experienced B cells (Banerjee et al., 2002). If the observed age-associated BM adiposity, which has been shown to reduce production of early precursor B cells, is reversible, as some studies have shown (Botolin and McCabe, 2006; Rahman et al., 2011; Santiago-Mora et al., 2011), then this could be a potential target for improving the deficits of ageing on humoral immune function. Production of pro-inflammatory eicosanoids such as PGE_2, as a consequence of the *n*-6 PUFA synthesis as opposed to *n*-3 PUFA, have been

demonstrated to induce defects in B cells. Therefore, since subjects within this study, on average, have high cholesterol levels, which is associated with increased age, dietary modification or provision of cholesterol lowering medications, such as statins, could be an area to target. This could contribute to ameliorating the adiposity associated inhibition of early B cell precursor development; where VDJ recombination of Ig heavy and light chains occurs.

Chapter 7

Conclusions

7.1 Summary

The aim of this PhD project was to identify age-associated numerical and functional changes in peripheral blood DCs and determine whether dietary intervention could improve DC function. In addition, the Ig repertoire was investigated to identify whether dietary intervention could alter VDJ recombination events that occur during the development of precursor early B cells in the BM. This discussion summarises the findings of this work and the impact of these findings for the field and their wider implications. Finally, future directions and recommendations for further work, which could be conducted as a consequence of this work, will be discussed.

7.2 Thesis outcomes & implications

7.2.1 Overall outcomes

The main findings from this thesis are that the elderly have significantly fewer peripheral mDCs than younger subjects. LPS/R848 stimulated-PBMCs derived from elderly subjects secrete significantly higher levels of resistin compared to those from younger subjects. Other cytokines including MCP-1, TNF-α, IL-8, IL-6 and IL-1β are significantly reduced with age. The usage of the IGHV3 gene subgroup within Igs from naïve B cells of elderly subjects is reduced, while usage of the IGHV1 gene subgroup is increased compared to those of younger subjects. Frequency of usage of IGHV3-15 increases while frequency of usage of IGHV4-28, IGHV3-23, IGHV4-30-2, IGHV3-30 and IGHV3-13 decreases in IgH of naïve B cells of elderly compared to younger subjects. These genes are located proximal to the 3′ end of the chromosome, in closer proximity to the DJ recombination product. MED diet intervention has no observed effect on DC number. LPS/R848 stimulated-PBMCs from elderly subjects secrete significantly less resistin after the 12 month MED diet intervention. The highest quantity of changes in frequency of gene usage is within the IGHV3 gene subgroup in both the control and MED diet groups. The significant increase in IGHV3-NL1 gene usage is only observed in the MED diet group; which is located at the 5′ end of the chromosome. More unique clonotypes are observed in naïve B cells of subjects in the control diet group, but at one year post-intervention the frequency of each unique clonotype increases, suggesting greater oligoclonality, while the frequencies of each unique clonotype are maintained post-MED diet intervention.

Assessment of urinary biomarker (HTS) analysis demonstrates compliance to the study in terms of phenolic intake, so the post-interventional changes observed can be partially attributed to the study intervention. However, analysis of the 7DD data show that the control group and the MED diet group have similar MED diet scores, which do not increase at post-intervention in the MED-diet group, as would be expected.

7.2.2 Implications of DC subset data and VDJ-Seq data

With increased age the mDC subset is reduced in number and in secretion of IL-1β, IL-6 and IL-8 when compared to younger subjects. The published data at present is conflicting (Agrawal et al., 2007; Della Bella et al., 2007; Jing et al., 2009; Pérez-Cabezas et al., 2007; Shodell and Siegal, 2002) so our findings add to the current literature and are in agreement with Della Bella et al. (2007). Since our methodological approach is specific to DCs and omits additional sample processing, which could induce cell losses, this study may encourage the use of the same methodology in future DC studies, since, while no differences were observed post-dietary intervention, the numbers were consistent after one year highlighting the reproducibility of the method. This would benefit the field since accurate comparisons between study cohorts from different studies, using consistent methods, are lacking; particularly in clarification of age-associated effects on DCs. However, this study lacks the use of an additional method to phenotypically identify and count DCs in order to compare different methodologies with our chosen method; this would have confirmed that our numbers are accurate. Compared to published reference values for a healthy cohort (11 and 28.2 cells/µl; mDCs, pDCs) using the same method with PBMCs (Narbutt et al., 2004), our mean DC counts are lower for both subsets, and our proportion of mDCs is higher than pDCs for both young and elderly subjects; however the reference data from Miltenyi Biotec (2008) is more consistent with our proportion of mDCs to pDCs (15.6 and 11.2 cells/µl; mDCs, pDCs) while our values are still lower. Our approach complies with the recommendation approved by the Nomenclature Committee of the International Union of Immunological Societies (Ziegler-Heitbrock et al., 2010). As we only show reductions in mDC counts it seems unlikely that this is a result of defective DC precursors with increased age, since both pDCs and mDCs are derived from macrophage and DC precursors (MDPs) and differentiate into common DC precursors (CDPs) (Schraml and e Sousa, 2015).

Additionally, the VDJ-Seq data comparing IgH from naïve B cells of younger and elderly subjects show comparable IGHV gene subgroup and gene usage to previous studies (Martin et al., 2015; Wang and Stollar, 1999; Wu et al., 2011), with the addition of observations not seen previously. This suggests that while previous methods of

DNA-library preparation, for high-throughput sequencing, have identified some of the age-associated changes, their use of differing combinations of V-specific primers to amplify VDJ regions – due to the lack of a primer to identify all genes within each of the seven V gene subgroups (Wood et al., 2013) – may have been insufficient to identify all changes in VDJ recombination events. Therefore, the VDJ-Seq method could provide a more comprehensive approach, since it uses six J-specific primers, as there are only six human J genes; compared to the 95 V genes in the human genome (Wood et al., 2013). This may allow further studies to be conducted to determine IGHV gene usage, CDR3 lengths and assignment of clonotypes which identify all changes in VDJ recombination events, with applications not just for ageing but also in particular diseases such as systemic lupus erythematosus (SLE) or rheumatoid arthritis (RA) (Pugh-Bernard et al., 2001; Samuels et al., 2005). This could have major implications for the development of treatments to alleviate symptoms or potentially to reduce risk of certain immunological disorders, with a current SLE treatment (rituximab) inducing $CD20^+$ B cell depletion (Anolik et al., 2004), while if specific genes could be identified, more targeted approaches might become available.

7.2.3 Implications of increased resistin secretion

The concentration of resistin secreted by PBMCs in response to *ex vivo* stimulation is significantly increased in cells derived from elderly subjects compared with younger subjects. This is of potential importance since it is becoming increasingly apparent that adipokine secretion increases with age (Arai et al., 2011; Ostan et al., 2013). Resistin suppresses secretion of IL-6, IL-12 and TNF-α by LTA-stimulated MoDCs (Son et al., 2008). Study of the BM has shown that secretion of IL-6 and a proliferation-inducing ligand (APRIL), which are important for maintenance of long-lived plasma cells, decrease with age and after incubation with ROS (Pangrazzi et al., 2017). Therefore the age-associated reductions observed in cytokine secretion (IL-6, IL-8 and IL-1β) but increases in the adipokine, resistin, could be due to an inflammatory state, typical of inflammaging.

Ageing is accompanied by replacement of BM with fat (Justesen et al., 2001) and BM adiposity is strongly positively correlated with serum lipid profiles (Slade et al., 2012). Our observations of high serum total and LDL cholesterol levels in study subjects therefore imply that elderly Nu-AGE subjects may have increased BM adiposity. Early B cell precursor and Ig production occurs in the BM and therefore increased BM adiposity could impair production, particularly since adiponectin inhibits the establishment of early B cells in cultures derived from murine BM ($CD19^-CD45R^-$

CD11b/Mac-1$^-$ cells) and human cord blood cultures (CD34$^+$CD38$^-$ cells) (Yokota et al., 2003). The observation that resistin secretion is increased in LPS/R848 stimulated-PBMCs from elderly but not younger subjects implies an age-associated, potentially adipocyte induced, defect in these cells, with additional observations of increased resistin levels in plasma, and secretion from PBMCs, associated with adiposity, CVD, insulin resistance and MetS (Arai et al., 2011; Gencer et al., 2016; Ostan et al., 2013). However, our results only imply the presence of BM adiposity in our subjects, in order to confirm this it would be necessary to take BM biopsies or carry out more in depth scans of subjects. BM biopsy analysis at the iliac crest has been utilised previously (Cohen et al., 2012; Cohen et al., 2015; Verma et al., 2002) and allows determination, by microscopy, of total adipocyte area, perimeter and percentage adipocyte volume within marrow, and has been compared to the less invasive, proton magnetic resonance spectroscopy (^{1}H-MRS) imaging technique but this only correlated at the spine, not at the proximal femur (Cohen et al., 2015). Therefore, we would need to confirm our findings using BM biopsies from the Nu-AGE subjects. Additionally, while we show DXA scan results for our elderly subjects, we do not have these data for the younger subjects, this would have been valuable to compare whether there are differences in BMD between the two groups, since Slade et al. (2012) report that BMD is negatively associated with BM adiposity. Our data provide additional evidence for altered cytokine and adipokine secretion associated with age, but further investigation is required to demonstrate BM adipocyte accumulation. This may encourage further investigation into the relationship between adipokine secretion and immune cell responses in the elderly, with further studies including clinically validated methods, such as BM biopsies, to determine whether BM adiposity is increased.

7.2.4 Implications of MED diet consumption

High fat diet promotes accumulation of large and small pre-B cells, immature and mature B cells in BM in mice (Trottier et al., 2012) in addition to promotion of inflammation and insulin sensitivity via production of autoantibodies (Shaikh et al., 2015; Winer et al., 2011). High fat diet feeding in mice also increases BM adiposity and consequently inhibits CD34$^+$ derived B cell production (Adler et al., 2014; Doucette et al., 2015).

PPARs, which are members of the nuclear receptor family, have suggested roles in nutrient sensing and regulation of the metabolism of carbohydrates and lipids (Semple et al., 2006). Both resistin expression and BM adiposity are shown to be regulated by PPARγ (Botolin and McCabe, 2006; Patel et al., 2003). Since increases

in MUFA and PUFA intakes downregulate PPARγ in DCs and PBMCs (Bouwens et al., 2009; Zapata-Gonzalez et al., 2008; Zeyda et al., 2005), this suggests that the MED diet intervention, with significantly reduced SFA intake, could reduce both BM adiposity and associated adipokine secretion (resistin), potentially reducing the age-associated inflammatory state. Additionally, cytokine secretion, induced by PPARγ receptors on DCs, is inhibited by PUFA treatment, in terms of IL-6, IL-10, IL-12, TNF-α and IFN-γ (Marion-Letellier et al., 2008; Zapata-Gonzalez et al., 2008; Zeyda et al., 2005), using *in vitro* models and *ex vivo* human PBMCs. This complements our findings of reduced IL-6^{+} DCs compared to increased IL-8^{+} DCs at post-dietary intervention. However, other studies contradict these findings (Kew et al., 2004; Stelzner et al., 2016) with one conflicting observation showing that MUFA upregulates PPARγ (Bechoua et al., 2003) suggesting that the SFA: MUFA or SFA: PUFA ratio could be more important that absolute MUFA or PUFA intakes.

Drugs target PPARγ to increase insulin sensitivity in T2D patients (thiazolidinediones; TZD) (He et al., 2015) by increasing circulating adiponectin levels, preventing increased circulating free FAs and decreasing pro-inflammatory cytokine secretion (Lefterova et al., 2014). Undesirable side effects including weight gain and increases in osteopenia and thus bone fractures (Cariou et al., 2012) reduces their use clinically (Soccio et al., 2014). However, recent observations show a novel TZD which does not to induce bone loss in mice but still decreases serum insulin levels and increases mRNA expression of adiponectin, and while increases in BM adipogenesis were still apparent, the increase was more than two-fold lower than that observed by the traditional TZD, rosiglitazone (Fukunaga et al., 2015). However these observations are independent of PPARγ (Fukunaga et al., 2015). Therefore, considering our finding that dietary FA modulation by reducing SFAs in favour of MUFA and PUFA can reduce adipokine secretion (resistin), this suggests that increased consumption of phenolic-rich foods may provide a useful and cost-effective method of reducing age-associated adipokine secretion, given that the net cost of prescription items (including TZDs) for diabetes cost £956.7 million between 2015–2016 (NHS, 2016). Circulating levels of adipokines are emerging as potential therapeutics or biomarkers for assessment of treatment responses (Blüher and Mantzoros, 2015), however since some of these adipokines were only discovered in the past 20 years, it is not known at present, whether reduced levels may also have negative health implications.

Similarly, production of B cells is regulated by prostaglandins and cyclooxygenases (Yokota et al., 2003), the series (anti- or pro-inflammatory) of which is influenced by FA intake. Production of the Ig occurs during B cell production and therefore, as no

change in frequency of each unique clonotype is observed in the present VDJ-Seq data, the one year dietary intervention may have prevented this. However, it is not possible to speculate this from our present findings since the clonotype analysis is only a qualitative measure, based on visualisation of the data within a tree plot. Additionally, this analysis cannot be used as an independent measure of Ig repertoire diversity since the amount of DNA used in library preparations is not taken into consideration. Therefore, to be able to interpret this data more accurately it may be appropriate to normalise the data to account for any variations in starting quantities of DNA. Since, our dataset for the VDJ-Seq analysis only includes four subjects randomised to the MED diet and six randomised to the control diet, it is not possible to infer any dietary effect of these results, as a much larger sample number is required.

7.3 Recommendations for future work

To further validate and expand on the outcomes of the present study, the next steps include testing immune response to antigens, directly in elderly subjects, as opposed to *ex vivo*. This could be achieved by taking blood samples, including serum, pre- and one month post-vaccination (Frasca et al., 2010; Saurwein-Teissl et al., 2002) (with the seasonal influenza vaccine) in addition to pre- and post-MED diet intervention for one year, with the inclusion of a much more stringent control diet group. The serum samples from subjects could then be tested for influenza specific antibodies using an ELISA (specific to the strain of the influenza vaccination) such as that discussed by Alvarez et al. (2010). Further B cell DNA-libraries could be prepared for samples pre- and post-vaccination, and pre- and post-dietary intervention in order to demonstrate whether the IGHVs are influenza-specific. This would add to the IGHV gene usage and clonotype repertoire information and determine whether these observations correspond with biological outcomes post-vaccination. Also, phenotypic analysis of B cells could be performed by multi-colour flow cytometric analysis, using fluorochrome conjugated mAbs to determine B cell subsets. This would give an indication of the proportions of naive, transitional and memory B cells (class-switched and non-class-switched), plasmablasts and plasma cells, using the markers CD19, CD20, CD24, CD27, CD38, IgD, IgM and CD138 (Caraux et al., 2010; Kaminski et al., 2012). In order to determine whether MED diet intervention could increase the production of naïve B cells, since reduced naïve and increased memory B cells have been observed with increased age (Lin et al., 2016; Macallan et al., 2005).

To further test DC function the phagocytic ability of DCs could be investigated using polystyrene beads or microparticles coated in protein to act as antigen, to visualise phagocytic activity and particle presentation on MHC II complexes *ex* vivo in peripheral DCs by confocal microscopy. A similar study design was utilised by Thiele et al. (2001) where it was noted that greater size of the microparticles (4.5 compared to 1.0 μm) and the surface coating (poly-L-lysine compared to bovine serum albumin) enhanced phagocytic ability. This would indicate whether the age-associated reductions in cytokine secretion by DCs were a result of impaired phagocytosis and thus a reduction in peptide presentation via MHC II complexes to engage in the peptide-MHC II: TCR immunological synapse, since this and co-stimulatory molecule interactions between DCs and T cells induce cytokine secretion (Guermonprez et al., 2002; Kambayashi and Laufer, 2014).

Furthermore, considering the overall study design, the Nu-AGE study had some weaknesses which could be addressed in future nutritional intervention studies. As the control group were ineffective in this study it would be beneficial to monitor dietary intake of the subjects more stringently and to provide more of the food components to the subjects within the study centre to both groups, to encourage and monitor changes in dietary intake. For example participants were not asked to adopt a low-phenolic or low-MED diet prior to the study, so, as observed in urinary HTS concentrations, any phenolic consumption prior to baseline was detected in pre-intervention urine samples, which may have skewed the results. However Miro-Casas et al. (2003) demonstrated that even strict dietary control prior to blood sampling could not clear HT from biological fluids. Therefore, the use of HTS as the only biological marker is another limitation to this study and the use of multiple biomarkers would be more informative to demonstrate adherence to the whole diet, not just olive oil, and potentially red wine, consumption. Previous studies assessing the MED diet have assessed plasma levels of oleic acid (olive oil), ALNA (nuts) (Mena et al., 2009), EPA, DHA (fish) and carotenoids (fruit and vegetables), in addition to the presence of SCFAs in faecal samples (wholegrain) (Vincent-Baudry et al., 2005), a combination of these approaches would be a much more valuable measure of compliance. Additionally, the collection of more frequent blood samples would have been advantageous, as the data for this thesis rely on just one blood sample at pre-intervention and one at post-intervention, whereas additional blood draw throughout the year intervention, at three monthly increments, would have provided more reliable data and demonstrated reproducibility of the results.

Overall, the data presented within this thesis provide evidence for age-associated impairments in both DCs and the Ig repertoire, with the observation that dietary intervention with a MED diet reversed the age-associated increases in resistin secretion. This is the first study to investigate the impact of dietary intervention in relation to IGHV gene usage and clonotypes. This data provides both methodological improvements and novel findings in the field of immunonutrition and as such provides the basis for further investigation of the MED diet for immunomodulation, particularly in terms of increased adipokine secretion and altered IGHV gene usage in Ig repertoires, with potential pharmaceutical implications.

Appendices

I. Im-AGE NRES ethical approval

NHS
Health Research Authority
NRES Committee South West - Cornwall & Plymouth
Level 3
Block B
Whitefriars
Lewins Mead
Bristol
BS1 2NT

Telephone: 01173421390
Fax:01173420445

25 February 2015

Miss Sarah Jayne Clements
Postgraduate research student
University of East Anglia
Institute of Food Research
Norwich Research Park, Colney Lane
NR4 7UA

Dear Miss Clements

Study title:	**Investigating the impact of ageing on the immune system (Im-AGE)**
REC reference:	**15/SW/0038**
Protocol number:	**N/A**
IRAS project ID:	**165272**

Thank you for your letter of 24th February 2015, responding to the Proportionate Review Sub-Committee's request for changes to the documentation for the above study.

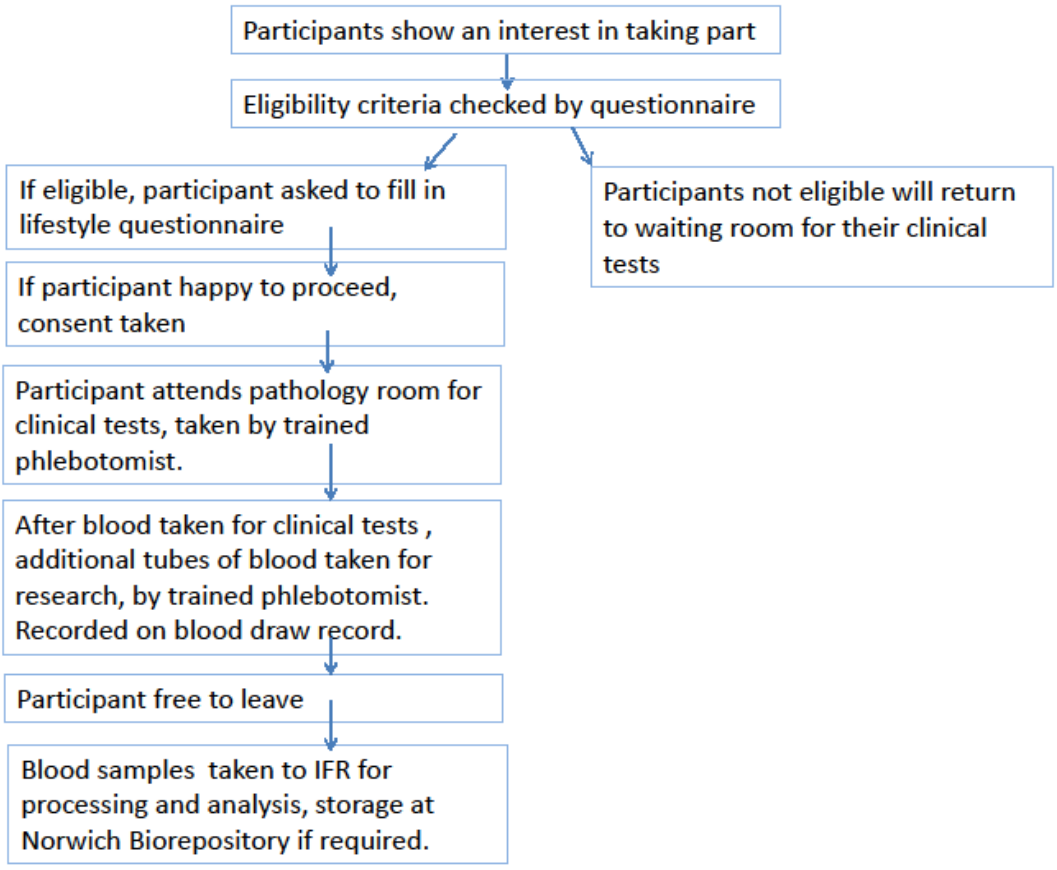
The revised documentation has been reviewed and approved by the sub-committee.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this favourable opinion letter. The expectation is that this information will be published for all studies that receive an ethical opinion but should you wish to provide a substitute contact point, wish to make a request to defer, or require further information, please contact the REC Manager Mrs Kirsten Peck, nrescommittee.southwest-cornwall-plymouth@nhs.net. Under very limited circumstances (e.g. for student research which has received an unfavourable opinion), it may be possible to grant an exemption to the publication of the study.

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

II. Im-AGE study flowchart



III. Im-AGE Consent Form

IFR Institute of Food Research | University of East Anglia | Norfolk and Norwich University Hospitals NHS Foundation Trust

Complete the following details:

Donor's name.....

Date of birth.....

Address.....

.....

Investigating the impact of ageing on the immune system (Im-AGE):

Consent form

I agree (please initial small box) that the following sample(s) may be used for research, including genetic (DNA and/or RNA) studies and for the possible development of commercial products for the improvement of patient care, from which I would receive no financial benefit: ☐

Sample(s) to be used:

.....

These samples become the property of the Norfolk & Norwich University Hospitals NHS Foundation Trust ("The Trust") ☐

The trust may store these samples in a tissue bank/ Biorepository ☐

The trust may use these samples at its discretion in properly approved research programmes ☐

The trust may pass on these samples to other approved tissue banks and/or companies, which may be in this country or abroad, in properly approved research programmes Yes ☐ No ☐

My genetic material and donated sample(s) may be stored for an indefinite amount of time for future research projects, which may include whole genome sequencing ☐

Information about my lifestyle may be collected and used to help the researcher to understand results or be able to group anonymised samples according to particular lifestyle traits ☐

Information about my case may be kept on the Norwich Biorepository database, including information collected from the lifestyle questionnaire ☐

Anonymous data derived from my sample(s) may be placed in an international database for future research ☐

Such information may be passed in an anonymous form to persons outside the Trust in connection with research and may be published with any research findings ☐

I agree that appropriately qualified staff employed by the Trust may review my hospital records, including case notes, as appropriate, for the purposes of research using the donated samples. ☐

I confirm that:

1) **I have read and understand the Im-AGE Information Sheet for Version 1, dated 05 January 2015**

2) **The issues have been explained to me, and that I have had the opportunity to ask questions**

Signed _____ **(Donor)** **Date**_____

I have explained the request for sample for research purposes and have answered such questions as the donor has asked.

Signed _____ **print name** _____

Doctor / nursing practitioner/ researcher/ other _____

(Please delete as appropriate/ indicate other status)

Date _____

IV. Nu-AGE NRES ethical approval

NHS
Health Research Authority
NRES Committee East of England - Norfolk
Victoria House
Capital Park
Fulbourn
Cambridge
CB21 5XB

Telephone: 01223 596906

Sent by email 2 may 2012

02 May 2012

Professor Susan J Fairweather-Tait
Professor of Mineral Metabolism
University of East Anglia
Department of Nutrition, Norwich Medical School
University of East Anglia
Norwich Research Park, Norwich
NR4 7TJ

Dear Professor Fairweather-Tait

Study title:	**New dietary strategies addressing the specific needs of the elderly population for healthy ageing in Europe (NU-AGE)**
REC reference:	**12/EE/0109**
Protocol number:	**R18963**

Thank you for your letter of 13 April 2012, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Vice-Chair.

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, subject to the conditions specified below.

Ethical review of research sites

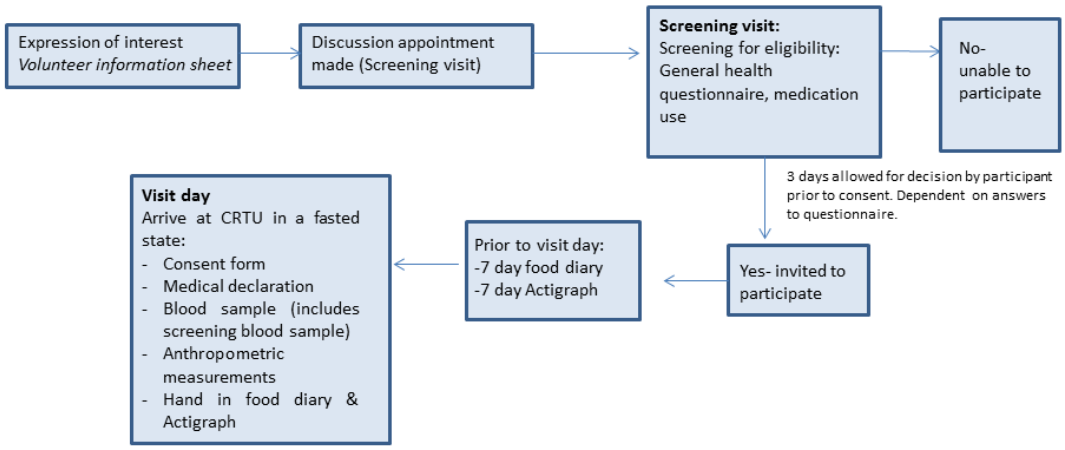
NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Non-NHS sites

The Committee has not yet been notified of the outcome of any site-specific assessment (SSA) for the non-NHS research site(s) taking part in this study. The favourable opinion does not therefore apply to any non-NHS site at present. We will write to you again as soon

V. Nu-AGE study flow chart



VI. **Nu-AGE Consent form**

NU-AGE CONSENT FORM

Title of Project: New dietary strategies addressing the specific needs of the elderly population for healthy ageing in Europe (NU-AGE)

Name of Researchers: Prof Sue Fairweather-Tait, Prof Aedin Cassidy, Prof Anne Marie Minihane

Volunteer Identification Number for this trial: _____

Please initial box

1. I confirm that I have read and understand the Volunteer Information Sheet dated ..... (version .....) for the above study.

2. I confirm that I have had the opportunity to discuss the study and ask questions, and that all of my questions have been answered in a satisfactory manner.

3. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, and without affecting future participation in other research studies.

4. I am aware that personal information will be held confidentially by responsible members of the research team at the University of East Anglia, and that anonymised data will be shared with other NU-AGE project partners.

5. I understand that my anonymised samples will be shared with other NU-AGE partners and may be stored and analysed beyond the scope of the current project.

6. I understand that the study includes DXA scans involving the direction of a low level of X-ray radiation, exposing me to a level of radiation which is equivalent to about one to two days of environmental or background exposure.

7. I agree to my GP being informed of my participation in the study.

   Name and address of GP

   .....

   .....

   .....

   .....

   .....

   .....

8. I agree for the results of the cognition/mood assessments to be sent to my GP.
   YES/NO*

9. I understand that some genetic information will be assessed during the study and that this information will not be clinically significant and will not be passed on to me or my GP.

10. A) I volunteer to give a faecal sample at the start and end of the study

YES / NO*

B) I volunteer to give an additional 23mL of blood for immune characterisation at the start and end of the study YES / NO*

11. I agree to take part in the above study.

_____ _____

_____

Name of Volunteer Date

Signature

_____ _____

_____

Name of Researcher Date

Signature

**Please delete as appropriate*

A copy of the signed consent form must be given to the volunteer.

VII. Seven-day food diary

New dietary strategies addressing the specific needs of the elderly population for healthy ageing in Europe

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NU-AGE 7 DAY FOOD

To be filled in by NU-AGE staff

Subject Code ☐☐☐☐☐☐ S C

Diary start date (dd/mm/yy): _____

Diary end date (dd/mm/yy): _____

The home visit is scheduled for :_____

Intervention time: Code ☐

As part of your participation in our research, we would like to know more about your dietary habits. For this, we ask you to keep a food diary for seven consecutive days.

INSTRUCTIONS

Carry the diary with you and write down everything you eat or drink immediately after doing so to avoid forgetting any items. Do not forget to write down things that you eat or drink in between meals (e.g. apples, nuts, cups of

tea etc.) and things you have during the night (e.g. a few sips of water). Meals outside the home should also be recorded.

The diary should be recorded in the following way:

- Please write down the date and day of the week in the space provided at the top of the page at the start of each day that you record your diary.
- Start each day with the page titled 'before breakfast'.
  There are 8 sections available for each day; before breakfast, breakfast, during the morning, lunch, during the afternoon, evening meal, evening snack, during the night.
- If nothing is eaten or drunk during one of these sections, draw a line through that section.
- Fill in the diary using the headings provided; time, place, description of foods and drinks and portion size.

Time: In this box write the time you ate or drank the item/meal.

Place: Write down the place where you ate the item or meal (e.g. home, restaurant, cafeteria at work, friend's house etc.)

Description of foods and drinks: Write down a clear description of the food or beverage that you have consumed. It is important to use exact names and descriptions and whenever the product has a **brand name**, please, write that down too (e.g. Tropicana smooth orange juice, Tesco light choices cottage cheese, Hovis wholemeal farmers loaf). Also write down any additions you add to the food or drink such as sugar or salt.

When describing a dish, write down the method of preparation (e.g. boiled or fried or grilled). If fat was used in the preparation, write the type of fat used (e.g. meatballs fried in vegetable oil). Also write down whether the food is home-made or bought ready-made.

If you make a meal that involves a recipe please make a precise note of the recipe including all the ingredients, their quantities and the main cooking methods involved. It is important to give full details of ingredients (e.g. chicken breast, no skin). Please also note the number of portions that the recipe served and indicate how much you ate from this. If someone else made the meal, ask

them for the details. There is additional space for recipe notes at the end of each day.

Meals eaten outside the home should also be recorded. When possible ask the cook or a member of the restaurant/canteen staff for information about the dish including the main ingredients and approximate quantities.

Amounts / portion sizes: Write down the portion sizes of the foods and beverages as you list them. Please weigh your food or use household measures such as coffee mugs, tablespoons, millilitres or grams to help you with this.

Use the following to help you estimate portion sizes:

Beverages: state the volume if known (e.g. 300ml) or describe using a description of the size of the glass, cup, mug etc. (e.g. large mug). Milk or cream added to coffee or tea should be measured in teaspoons or tablespoons. If you had a drink from a bottle, carton or can make a note of the size stated on the packaging, and how much you drank (e.g. “250ml bottle. Drank all”).

Bread: write the number of slices of bread eaten. Describe the loaf as small, medium or large, or give the total loaf weight. Describe the slice as thin, medium, thick or extra thick (most pre-sliced loaves state the loaf size and the slice thickness on the packaging). (e.g. “2 medium slices, 800g loaf”).

Breakfast cereal: describe the portion using small, medium or large bowl sizes or use tablespoons. Describe the amount of milk had on cereal using pints or millilitres or say if it was a ‘large’, ‘medium’ or ‘small’ amount. (e.g. 4 tablespoons of cornflakes with ¼ pint skimmed milk).

Cheese: for hard cheeses write down the number and thickness of slices. Use tablespoons to measure grated cheese. For soft cheeses such as Philadelphia measure in teaspoons or tablespoons.

Confectionary (sweets and chocolate): describe using the weight on the packet, bar weight, number of pieces or individual sweets.

Fruit: record the number of whole fruits, segments or slices. For canned fruit give the can weight and the proportion of which you ate.

Ice cream, cream and dairy desserts: use scoops or tablespoons. Where whole items are eaten give the pot or packet weight.

Meat and fish dishes: record the number or weight of meat or fish portions (e.g. “one chicken breast”, “3 rashers of unsmoked back bacon” or “1/4 of 515g pack, lean beef mince, raw weight”). Remember to state if you are recording the weight as the cooked or raw weight. Also note if the meat or fish includes skin or fat and if this was eaten.

For roast meats and cold cuts of meat state the number and thickness of slices (e.g. 2 slices of Tesco wafer thin cooked ham).

Oils, butter and margarine: use teaspoons or tablespoons. When spreading on bread or toast state if the layer was thin, medium or thick.

Pasta, spaghetti: Describe the weight using a proportion of the packet weight (e.g. 1/10 of 1kg packet of dried wholemeal fusilli) or measure in tablespoons. Remember to state if you are giving the dried weight or cooked weight.

Puddings and desserts: use tablespoons, or slices with a description of small, medium or large. For commercial items describe as a proportion of the packet weight (e.g. Sainsbury’s strawberry cheesecake 530g. Ate 1/5).

Rice: Describe the weight using a proportion of the packet weight or use tablespoons. Remember to state if you are measuring cooked or raw rice.

Sauces, gravy and dressings: use teaspoons or tablespoons. Note that even the amount of meat sauces such as Bolognaise sauce should be estimated this way (e.g. “one teaspoon of sweet chilli sauce”).

Soups: use bowls to describe the size of the portion as a small, medium or large bowl. Or if you are using canned or carton soups note the size marked on the packaging and state how much you ate (e.g. “440g can, ate half”).

Sugar: use teaspoons or tablespoons or if the sugar is cubed state the number of cubes. Remember to mention sugar sprinkled on top of cereal and sugar in tea etc.

Vegetables and salad items: use whole vegetables or salad items, slices or tablespoons as measures (e.g. “4 cherry tomatoes, 5 thin slices of cucumber and 2 tablespoons lettuce”).

Other foods: use the information given on the packaging whenever possible. The weight should be included on the packet information. If you don’t eat the whole packet note the total weight of the packet followed by the amount you think you ate (e.g. if you had a packet of walnuts, “100g packet, ate ¼ of the packet”).

If in doubt about how to describe a portion, write as much detail as possible. The portion can then be further discussed with the research assistant once the diary is complete.

Leftovers: We want to know the amount that was actually eaten, this means leftovers need to be taken into account. This can be done in two ways:

1. If any leftovers remained on your plate from the originally stated portion in your food diary, please make a note of this (e.g. “1/4 of lasagne recipe, *3 small boiled potatoes*. *Only ate 1 of the boiled potatoes”).*

2. Alternatively, you can just record the actual amount eaten (e.g. “1/4 of lasagne recipe, *1 small boiled potato*”).

Labels/wrappers: Labels are a very useful source of information for us. When possible please save any labels or packaging that shows the product information for an item you have consumed. This is especially useful for foods or brands you record which are perhaps lesser known or uncommon.

Comments: At the end of each day there is space to write any comments you might feel are relevant. For example this may be to inform us if the day was not a typical day or if there was any reason why you might have eaten more or less than usual.

The dietary records will be discussed with the research assistant during your visit to the UEA to be sure that you haven't forgotten anything and to verify whether you have given enough detail.

Example day

EXAMPLE			
Date: 07/05/2012		Day of the week: Monday	
BEFORE BREAKFAST			
Time	Place	Description of foods and drinks	Portion size
07:30	Home	Cup of Nescafe instant coffee semi skimmed milk	1 large mug 1 tablespoon
BREAKFAST			
Time	Place	Description of foods and drinks	Portion size
08.00	Home	Kellogg's fruit n fibre with Semi skimmed milk Tesco orange juice from concentrate	30g 1/5 pint 150ml
DURING THE MORNING			
Time	Place	Description of foods and drinks	Portion size

09.30 11.00	Friend's House Home	Cup of Tetley decaffeinated tea with semi-skimmed milk Water	Medium mug tablespoon Tall glass, 250ml
LUNCH			
Time	Place	Description of foods and drinks	Portion size
13.00	Home	Chicken salad sandwich: Hovis wholemeal medium sliced,800g loaf. Flora light low fat vegetable spread Tesco sliced roast chicken (240g pack) Tesco salad tomatoes Tesco baby leaf and rocket salad (90g pack) Banana Robinson orange squash, no added sugar diluted with tap water	 2 slices 1 teaspoon 1 slice 1 tomato 1/6 of packet 1 medium 50ml 200ml
DURING THE AFTERNOON			
Time	Place	Description of foods and drinks	Portion size

15.00	Starbucks coffee shop	Green tea Blueberry muffin	Regular Ate half
EVENING MEAL			
Time	Place	Description of foods and drinks	Portion size
18.30	Home	Wholemeal pasta Homemade bolognaise sauce (see recipe) Grated cheddar cheese Tap water Strawberries Tesco low fat natural yogurt (500g tub)	150g (cooked weight) 1/8 of recipe 1 tablespoon Large glass, 300ml 6 medium 2 heaped tablespoons
EVENING SNACK			
Time	Place	Description of foods and drinks	Portion size
20.30	Home	PG tea with Semi-skimmed milk	Medium mug 1 tablespoon
DURING THE NIGHT			
Time	Place	Description of foods and drinks	Portion size

Recipe notes

Please write the recipe or list the ingredients of any dishes that may require more information to be given. This might include home-made dishes, take-away meals etc that you have mentioned but not described previously. Where applicable please list amounts of ingredients and brand names. Please indicate the amount or proportion actually consumed by you.

Name of dish: Bolognaise sauce
Number of portions the recipe serves (if applicable): 8 portions

Ingredient	Amount	Ingredient	Amount
Tesco lean beef mince	500g	Napoli chopped tomatoes	400g can
Garlic	2 cloves	Tesco tomato puree	1 tablespoon
Red onion	1 medium	Tesco mild olive oil	1 tablespoon
Red pepper	1 medium	Dried mixed herbs	2 teaspoons
Yellow pepper	1 medium		
Courgette	1 medium		

Brief description of the cooking method:
Fry onion & garlic in oil, add mince and fry until brown.
Add peppers, courgette, tomatoes, puree & herbs.
Simmer for 30 minutes

Any additional comments:

I ate 1 portion from the above recipe.

END OF EXAMPLE

Additional space for notes and comments that may be useful to the researchers:

Thank you for completing your 7 day food diary

VIII. Im-AGE Lifestyle Questionnaire

Investigating the impact of ageing on the immune system (Im-AGE)

Lifestyle questionnaire

Complete the following details:
Donor's name.....
Date of birth.....

Thank you for expressing an interest to provide samples for biomedical research. Please ensure that you read the information sheet so that you understand what this means and involves.

In order to help the research scientists understand your blood results for this study, please can you answer the following questions (Circle yes or no):

1) Do you know your weight? **YES/NO** If YES please record here_____
2) Do you know your height? **YES/ NO** If YES please record here_____
3) Has your GP told you that you have high blood pressure? **YES/NO/DON'T KNOW**
4) Do you smoke? **YES/ NO**

If YES how frequently?

_____

_____

5) How often do you consume alcohol? **NEVER/ ONCE A MONTH/ ONCE A WEEK/ 2-3 TIMES A WEEK/ 4-5 TIMES A WEEK/ DAILY**
   Please indicate what type of alcohol you mostly consume
   _____
6) Are you taking any medications? **YES/ NO**

If YES, please list here

_____

_____

_____

7) Are you taking any vitamins, minerals or other supplements? YES/ NO

If YES, please list here

_____

_____

IX. Control group dietary advice

BDA THE BRITISH DIETETIC ASSOCIATION

Food Fact Sheet

Healthy Eating

A good diet is important for good health. Eating a variety of foods can help you manage your weight, improve general wellbeing and reduce the risk of conditions including heart disease, stroke, some cancers, diabetes and osteoporosis (thin bones). All you need to do is eat sensibly, choosing a range of foods in the correct proportions.

Food groups

The Food Standards Agency (FSA) Eatwell Plate is made up of five food groups – simply choose a variety of foods from each group.

2. Five portions - have *at least* five portions of fruit and vegetables each day.

Remember:

- **fresh**
- **frozen**
- **dried**
- **canned**
- **juices**

all count towards your total. Also remember to include the vegetables you add to cooked dishes, for example as onions in a stew or casserole and tomatoes in a pasta sauce or tomato soup.

The eatwell plate

Use the eatwell plate to help you get the balance right. It shows how much of what you eat should come from each food group.

In practical terms this means:

1. Plan your meals/snacks around starchy foods such as bread, chapattis, breakfast cereals, potatoes, rice, noodles, oats and pasta. Aim to include one food from this group at each meal time.

3. Variety – eat a variety of different types and colours of fruit and vegetables. This is because different coloured fruit and veg contain their own combination of vitamins and minerals, so try to include a variety of colours in your diet.

> "Choose a variety of foods from each of the five food groups on the eatwell plate."

4. Meat, fish, eggs and pulses (beans and lentils) should be eaten in moderate amounts. Choose lean cuts of meat or remove excess fat and remove the skin from chicken. Avoid frying where possible. Try to include two portions of fish each week, one of which should be an oily fish, for example: mackerel, trout, sardines, kippers or fresh tuna.

5. Dairy - aim for a pint of milk a day, or the equivalent. For example the following contain the same amount of calcium as 1/3 pint of milk:

- a small pot of yoghurt
- a small matchbox size piece of cheese
- six and eight cups of tea or coffee, with milk a day

Try to choose reduced fat versions where you can – for example semi-skimmed milk, low fat yoghurt, cottage cheese and half fat Cheddar cheese or Edam.

6. Sugar and fat should be limited so watch your intake of foods high in fat and sugar, choosing low fat or reduced sugar foods where possible. When using a spreading fat choose one that is low in saturated fat. Saturated fat increases the low density (LDL) lipoproteins or 'bad' cholesterol in your blood which can lead to heart disease. Instead choose unsaturated fats which contain high density lipoproteins (HDL) or 'good' cholesterol that is beneficial for your body.

Saturated fat (BAD)	Unsaturated fat (GOOD)
Butter ghee and lard coconut oil and palm oil.	Polyunsaturated fat Sunflower, soya, corn or safflower oil/soft spreads/ margarines, and fish oil. Monounsaturated fat Olive and rapeseed oil.

> "Choose low fat or reduced sugar foods where possible."

Summary

Eat a range of foods from the five food groups to make sure you have a balanced diet. Eat the right amount of food for how active you are. Most of all – enjoy your food!

Other Food Fact Sheets on similar topics such as Fruit and Veg – How to Get Five a Day and Cholesterol can be downloaded at www.bda.uk.com/foodfacts

X. MED-diet intervention group dietary advise sheets

Alcohol

Nu-Age goal: Maximum 1-2 glasses (3-4 units) per day for men and 1 glass (2-3 units) per day for women

Why should you drink alcohol?

There is some evidence that drinking 1-2 units of alcohol per day may help to protect against heart disease. It is unclear why alcohol may benefit health but the antioxidants contained in some alcoholic drinks are thought to be one possible explanation. Alcohol does also have its health risks, therefore if you do not usually drink alcohol it is not necessary to start including it in your diet.

Drinking above the recommended limits can lead to serious health problems. Patterns of drinking are also important to consider; consuming large amounts of alcohol in one day has negative effects on health, it is therefore advised to consume small amounts across a number of days.

How much and what types of alcoholic drinks should I consume?

If alcohol is consumed red wine is preferable due to its higher levels of antioxidants.

If you do not usually consume alcohol maintain your current drinking habits.

Dairy

Nu-Age goal: Aim to have 500ml (17.5 fl oz) of dairy each day, including the low fat cheese recommendations (equivalent to approximately 30ml)

Why should you eat dairy?

Milk and dairy products are an important source of protein, calcium and vitamin B12. The calcium in dairy foods is absorbed easily by the body helping to keep bones and teeth healthy and strong.

How can I consume enough and the right type of dairy?

While providing essential nutrients milk and dairy products can also contain high amounts of saturated fat. It is this type of fat which can contribute to raised cholesterol and increased risk of cardiovascular disease. It is therefore important to try and select low-fat dairy options with no added sugar; weight for weight they have slightly more calcium, and other important nutrients than full fat dairy products, but less fat and calories.

- Ideally choose skimmed milk, fat free yogurt, and low fat cheese products
- Semi skimmed milk and low fat yogurt are a good compromise if you are making a dietary change from whole milk and full fat dairy products
- Where possible choose calcium enriched dairy products

Ideas to help you reach this goal:

- Drink a glass of skimmed or semi skimmed milk
- Have milk or yogurt with your cereal or make your porridge with milk instead of water
- Drink milk or yogurt based smoothies
- Have fat free or low fat yogurt as a snack
- If you like to eat desserts, try low fat milk based desserts, such as rice pudding

Useful information

The average amount of milk had on cereal is 100ml (3.5 fl oz)

A tablespoon of low fat yogurt is approximately 43ml (1.5 fl oz)

The average amount of milk in a cup or mug of tea or coffee is 35ml (1.2 fl oz)

Cheese

Nu-Age Goal: Aim to eat 30g of cheese per day (this is included as part of your total dairy intake)

Why should I eat cheese?

Cheese is rich in calcium and nutrients that help to keep bones and teeth healthy and strong. It is also a great source of protein. However, cheese is also high in saturated fat and salt which can contribute to the risk of cardiovascular disease and high blood pressure.

How can I consume enough and the right type of cheese?

30g of cheese is approximately:

- 1 matchbox size cube
- 4 dice sized cubes, or
- 3 tablespoons of grated cheese

Choose low fat or half fat and reduced salt cheeses where possible.

In general softer, less aged cheeses contain less salt than harder, aged cheeses

Cheese ideas:

- If you think you are eating too much cheese try switching to a stronger flavoured cheese, you will probably find you need less of it
- Stir some low fat cream cheese in with warm wholegrain pasta and mushrooms for a quick tasty snack or meal
- A cube of cheese with fruit makes a great snack. Apples and grapes go especially well with cheese
- Cheese in salads works really well. Slices of mozzarella with tomatoes is a popular choice
- A slice of cheese in a sandwich
- Low fat cream cheese in a baked potato

Eggs

Nu-Age Goal: Aim to eat 2-4 eggs per week

Why should you eat eggs?

Eggs are a good source of high quality protein, they also contain vitamins and minerals including vitamin D, A and B2.

How can I consume enough eggs and how should I cook them?

Though eggs contain cholesterol, the cholesterol contained in foods has a smaller effect than saturated fat in foods on the cholesterol in our blood. Therefore there is no limit on the number eggs people should eat, but it is important to remember to eat them as part of a healthy well balanced diet.

Aim to eat 2-4 eggs per week

Healthy ways to eat your eggs:

- Boiled eggs
- Scrambled eggs
- Poached eggs
- Baked eggs
- Omelette
- Egg salad
- Egg sandwiches
- Eggs in recipes

Avoid frying or adding fat when cooking eggs

Fat and Oil

Nu-Age goal: consume 20g of oil and 30g of low fat margarine per day (maximum of 50g of fat per day)

Why should you eat fats and oils?

Fat is a source of energy, vitamins A, D and E and essential fatty acids. Fat isn't by definition unhealthy; your body needs fat in order to function properly. However the type of fat is important; different types of fat are made up of different proportions of saturated, monounsaturated and polyunsaturated fat. In addition, some fats, and products made with these fats, contain trans fats which have a stronger effect on cholesterol than saturated fat.

A diet high in saturated and trans fat can cause the level of cholesterol in the blood to build up, increasing the risk of cardiovascular disease, while unsaturated fat can help lower cholesterol. It is therefore important to choose foods which are higher in unsaturated fats and lower in saturated fat and contain little or no trans fats.

How can I consume the right types of fats?

Try to eat foods that are higher in mono and polyunsaturated fats, and lower in saturated fat and trans fats

How to achieve this...

- Traditional cooking fats such as butter, lard, shortening and hard block margarine should be avoided

  Replace with,
- Soft non hydrogenated margarine and vegetable oils like olive, canola, sunflower and soybean oil

Fish and Seafood

Nu-Age goal: consume two 125g portions of fish a week, including at least 1 portion of oily fish

Why should you eat fish?

Fish and seafood is a good source of protein and contains many vitamins and minerals. Being higher in unsaturated (good) fat and lower in saturated (bad) fat, fish is a good alternative to meat. Different types of fish contain different levels of these good fats; oily fish is high in omega-3 fats (EPA and DHA), which may help to prevent heart disease.

Which types of fish counts as oily fish?

Oily fish:

- Salmon
- Trout
- Anchovies
- Sardines
- Mackerel
- Herring
- Fresh tuna
- Pilchards

Non oily fish:

- Haddock
- Plaice
- Coley
- Sea bream
- Sea bass
- Pollack
- Cod
- Tinned tuna
- Skate
- Hake
- Other white fish
- Crab
- Prawns

– Steamed, baked or grilled fish makes a healthy choice. Avoid eating battered or fried fish.
– Canned and frozen fish is just as good as fresh and it stores for longer

Useful Information:

Average salmon steak 100g
1 canned sardine 25g
Average smoked mackerel fillet 75g
Average haddock fillet 120g

Some ideas...

- Fish and prawns are great in or with salads
- Fish pie makes a great wholesome winter meal
- Sardines or mackerel on wholemeal toast makes a tasty snack

Fluid

Nu-Age goal: 1.5 litres or approximately 8 glasses per day

Why should you drink fluids?

Water makes up about two-thirds of the weight of a healthy body and is required for our body to function properly, aiding the absorption of nutrients in the bowel, transport of nutrients and waste products in the body, and the regulation of body temperature.

How can I consume enough and the right types of fluids?

Though most fluid is obtained from drinks, foods can contribute to fluid intake too.

A healthy choice of drinks include water, skimmed milk, diluted fruit juice and diluted sugar free squash but not alcohol

- With age, you may lose your sense of thirst so it's easy to forget to drink enough fluids. Try and get into the habit of drinking a glass of fluid, preferably water, with each meal or snack.
- If you are taking medication this can act as a good reminder to drink a glass of water
- Drink a glass of milk each day; this is also an excellent source of calcium and contributes to your daily dairy intake.
- Have a glass of fruit juice, this is great for fluid intake and it counts as one of your portions of fruit for the day (only one glass of fruit juice per day is recommended).
- If fruit juice is too sweet dilute it down with water
- Vegetable juice is also a good source of fluid and nutrients
- Try and avoid caffeinated beverages and alcohol. They act as diuretics, meaning that they drive water from the body rather than hydrating.
- If you are drinking caffeinated beverages try and alternate with decaffeinated beverages throughout the day
- Soups are also a good source of fluid
- Your urine should be pale yellow. If its bright or dark yellow, you need to drink more liquids
- Talk with your doctor if you have trouble controlling your urine; there are ways to help bladder control problems. **Don't stop drinking liquids.**

Fruit

Nu-Age goal: consume at least 2 portions of fruit per day

Why should I eat fruit?

Fruits are a great source of vitamins and minerals and an excellent source of dietary fibre. They help reduce risk of cardiovascular disease, cancer and chronic conditions.

How can I consume enough and the right type of fruits?

Different fruits contain different nutrients and in different quantities, a healthy dietary pattern therefore should contain a variety of fruits of differing colours. Fresh fruits are the ideal choice as fruits that have undergone some form of processing, such as juicing or blending, lose some of their nutrients, especially vitamin C and fibre.

What is a portion?*

A fruit portion is approximately 80g fresh fruit or 30g dried fruit:

- 1 medium sized fruit e.g. 1 apple, 1 banana, 1 pear, 1 nectarine
- 2 or more small fruits e.g. 2 plums, 2 satsumas, 2 kiwi fruit, 7 strawberries
- ½ a grapefruit, one slice of melon (5cm), 1 large slice pineapple
- A heaped tablespoon of dried fruit
- 150ml of unsweetened 100% fruit juice also counts as 1 portion (fruit juice can only contribute 1 of your portions per day)
- Smoothies are dependent on the amount of fruit they contain. They can contribute a maximum of 2 fruit portions per day

Meat and Poultry

Nu-Age goal: consume four 125g portions of meat or poultry a week

Why should you eat meat?

Meat and poultry is a good source of protein, iron, vitamin B12 and vitamin D.

Protein is made up of amino acids which are important to help the body with growth and repair. The body can make some amino acids itself, but others must be obtained from foods. These are called essential amino acids. Animal proteins, in contrast to vegetable proteins, contain sufficient amounts of essential amino acids.

Meat, especially red meat is a good source of iron. Iron is important for the formation of haemoglobin, the protein found in red blood cells, which is required for oxygen transport in the blood. Iron can be found in foods as either heme or non heme iron. Heme iron, only found in animal products, is more available for use by the body.

How can I consume the right amount and type of meat?

A portion is 125g, this is approximately:

- A medium chicken breast
- 3 medium thick slices of roast meat

Some meats are high in fat, especially the unhealthy saturated fat that is associated with cardiovascular disease. Therefore the type of meat you choose and the way in which it is prepared and cooked can make a big difference to its nutritional quality.

Nuts

Nu-Age goal: consume 20g of nuts 2 times per week

Why should you eat nuts?

Nuts are a good source of iron, vitamin E, B vitamins, protein and unsaturated fat. Being high in mono and polyunsaturated fats which are beneficial to health, there are strong indications that nuts may be beneficial against cardiovascular disease.

How can I consume enough of the right types of nuts?

A 20g portion of nuts is approximately:

- 20 pistachio nuts
- 6 walnut halves
- 6 whole brazil nuts, or
- 9 whole almonds

Nuts are very good for you, but should be eaten in moderation

Avoid nuts that are fried in oil or salted

Nutty ideas...

- Add a small handful of nuts to your breakfast cereal
- Nuts are great in salads. Try adding walnuts, hazelnuts or pecans
- If you bake your own wholegrain bread try adding some nuts to the recipe; walnuts are especially tasty in freshly baked bread
- Add nuts to some low fat yogurt to make a great snack
- Add nuts to meat dishes for an extra crunch of flavour. Sliced almonds go nicely with chicken
- If you're making a fruit smoothie try adding some nuts before blending, this will add flavour and texture

Potatoes, rice and pasta

Nu-Age goal: aim to eat 150g of potato 4-5 times a week and 150g cooked wholegrain rice or pasta at least twice a week

Why should you eat potatoes, rice and pasta?

Starchy foods are a good source of energy and also contain a range of nutrients including fibre, iron and B vitamins. Fibre is important for good bowel function; aiding bowel movement, preventing constipation, haemorrhoids and bulges of the colon. A high fibre diet is also important in the prevention of obesity. Fibre contains few calories, gram for gram containing less than half the calories of fat, yet it gives the feeling of fullness, thus reducing the likelihood of overeating. Some fibres also have beneficial effects on blood pressure and cholesterol levels and may lower the risk of cardiovascular disease.

How can I consume enough and the right type of potatoes, rice and pasta?

A 150g portion is approximately equivalent to:

- 4 boiled new potatoes
- 1 baked jacket potato
- 3 tablespoons of mashed potato
- 4 tablespoons cooked rice
- 5 tablespoon of cooked pasta

Salt

Nu-Age goal: reduce salt intake to 5g (equivalent to 2g sodium) per day

Why should you eat salt?

Salt contains the mineral sodium, which has an important role in regulating the fluid balance of the body and is required for proper functioning of muscle cells and neurons. However, too much salt in the diet can raise your blood pressure, increasing risk of health problems such as heart disease and stroke. Excessive salt intake can also cause renal diseases.

How can you limit your consumption of salt?

Cutting down on the amount of salt you add to foods during cooking and at the table is a good start, however, 75% of the salt we eat is already contained in foods, this is something that should be considered when adding salt to the food we eat.

Some foods are high in salt because of the way they are made or processed, such as:

- Bacon
- Cheese
- Gravy granules
- Stock cubes
- Smoked meat and fish
- Salted and dry roasted nuts
- Soy sauce
- Olives
- Ham
- Yeast extract

Other foods contribute a lot of salt to our diet, not necessarily because they are high in salt, but because we tend to eat a lot of them. Some of these foods can vary in salt content from brand to brand so it is worth checking the nutrition label and comparing products before buying them.

Sugar

Nu-Age goal: limit the use of sugar and sugary drinks

Sugar occurs naturally in foods such as fruit and milk. These sugars are fine to include in our diet. Sugar that is added to foods is the sugar that we need to limit in our diet. Sugar is added to foods such as sweets, biscuits, cakes, chocolate, and fizzy drinks.

Sugary foods are not an ideal way to obtain necessary calories, they often lack in other nutrients and can cause tooth decay. Fruit juice, although healthy should also be limited to one glass per day. The sugars in the fruit have been released during the juicing process meaning they are no longer contained within the fruits structure which usually provides protection against the action of sugars on our teeth.

How can I cut down on sugar in my diet?

- Swap cakes and biscuits for a currant bun or fruit loaf
- If you eat tinned fruit make sure it is tinned in juice and not syrup
- Choose whole grain breakfast cereals that aren't coated in sugar or honey
- Preferably drink water or unsweetened fruit juice instead of fizzy drinks and sugary juice drinks
- If you usually drink fizzy drinks try diluting a little fruit juice with sparkling water as an alternative
- If you usually add sugar to your hot drinks and cereal, gradually cut down until you find you can cut it out altogether.
- Try to avoid jams and marmalades on toast, or use reduced sugar options
- If using artificially sweetened alternatives as a way to cut down on sugar e.g. diet fizzy drinks or sweeteners in hot drinks, these are a better alternative to sugar however they should be used in moderation

Vegetables and legumes

Nu-Age goal: at least 300g (approximately 4 portions) of vegetables a day and 200g of legumes (approximately 2.5 portions) per week

Why eat vegetables?

Vegetables are a great source of vitamins and minerals and an excellent source of dietary fibre. They help reduce the risk of cardiovascular disease and certain forms of cancer. It is also suggested that vegetables protect against other diseases, such as eye and respiratory disorders, and being high in fibre they are great for making us feel fuller with fewer calories.

Consuming the right type of vegetables?

There is not one specific vegetable that can provide all nutrients, therefore eating a variety of different coloured vegetables is key. The way in which you prepare your vegetables has a big influence on the quantities of nutrients they contain. Vegetables can lose 20-50% of vitamins when cooked. This means eating a combination of cooked and raw vegetables is best.

- Choose fresh, frozen or canned (in unsalted water) vegetables in a variety of colours
- Vegetable puree and vegetable juice are a good alternative to help increase your vegetable intake
- Vegetables in sauce also contribute to your overall intake. However, depending on the sauce they are contained in this may not be the healthiest way to reach your 300g target

What is a portion of vegetables?

- A vegetable portion is approximately 80g*
  - 2 heaped tablespoons cooked spinach
  - 2 broccoli spears
  - A medium tomato or 7 cherry tomatoes
  - 150ml of unsweetened 100% vegetable juice

Vitamin D

Nu-Age goal: take one 10 µg vitamin D supplement daily

Why do we need vitamin D?

We need vitamin D so that our body is able to absorb calcium and phosphate from our diet. These minerals are essential for healthy, strong bones. A deficiency in vitamin D can lead to a loss of bone mass or bone density; this makes bone more prone to breaks.

How can I obtain an adequate vitamin D intake?

Most of our vitamin D is made by our skin as a result of exposure to sunlight, but we also get some vitamin D from our diet. People aged 65 and over are at higher risk of having vitamin D deficiency as the body becomes less efficient at synthesising the vitamin, and the absorption of dietary vitamin D may be reduced. Therefore it is recommended that those aged 65 and over should take a daily vitamin D supplement.

Together with the daily vitamin D3 supplement (10µg) we are providing it is important to try and consume vitamin D through your diet where possible.

Food sources of vitamin D include:

- Eggs
- Oily fish e.g. salmon, mackerel, sardines
- Fortified fat spreads and milk
- Fortified breakfast cereals

Fibre and wholegrains

Nu-Age goal: 4-6 servings of wholegrains per day

What is a wholegrain?

A large variety of cereal crops are grown for food throughout the world. Grains are the seeds of these cereal plants. Wholegrain refers to the **entire** grain—this includes the bran, germ and endosperm (starchy part). Often during the processing of products, for example when grinding whole grains to flour or when peeling rice to produce the white rice we often see in shops, the bran and germ are removed. With the grain stripped of these important components a lot of the fibre content is lost. With wholegrain products, these fibre rich layers of the grain are not removed.

Finding wholegrain foods can be a challenge. Some foods only contain a small amount of wholegrain but the product will say it contains wholegrain on the front of the packet. When choosing cereal products and grains, read the ingredient list and look for sources of wholegrains as the **first** ingredient on the list.

Why should you eat wholegrains / fibre?

Wholegrain foods are high in fibre, a nutrient which is important for good bowel function; aiding bowel movement, preventing constipation, haemorrhoids and bulges of the colon.

A high fibre diet is also important in the prevention of obesity. Fibre contains few calories, gram for gram containing less than half the calories of fat, yet it gives the feeling of fullness, thus reducing the likelihood of overeating. Some fibres also have beneficial effects on blood pressure and cholesterol levels and may lower the risk of cardiovascular disease and type 2 diabetes. These fibres are those found in fruits and wholegrain products.

The beneficial effects of eating wholegrain foods are likely not to result from their fibre content alone, but as a result of the combined effect of the variety of nutrients they contain; B vitamins, folic acid, omega 3, magnesium, zinc, phosphorus, iron, vitamin E, selenium and copper.

What makes a portion of wholegrain?

1 serving is equal to:

- 28g (1oz) of 100% wholegrain food
  e.g. uncooked brown rice, wholegrain pasta or other grain
  OR 65-70g (2 ¼ -2 ½ oz) of cooked brown rice or pasta
- A slice of wholegrain bread or 1 ½ - 2 Ryvita crispbreads
- 120g cooked wholegrain cereal, such a oatmeal

Try to include the following in your meals:

Wholegrain cereal	Wholegrain bread	Quinoa
Whole oats / oatmeal	Bulgur	Wholegrain pasta
Brown rice	Whole barley (hulled, hulless or dehulled barley)	
Wild rice		

Use whole-wheat flours when baking and cooking such as:

Whole-wheat flour	Whole rye flour	Buckwheat flour

Fibre can be obtained from other food sources too:

Try and consume 30-40g of fibre in total per day. The following provides a guide as to how to achieve this:

4-6 portions of wholegrain per day e.g. wholemeal bread, wholegrain pasta, brown rice

150g cooked brown rice, wholegrain pasta or potatoes per day

2 portions of fruit per day

300g mixed vegetables per day

and

200g legumes per week

How to be sure you're getting the whole grain

Check for the following ingredient names: wholegrain wheat, brown rice, oatmeal, oats, whole rye, wholegrain corn, bulgur wheat, quinoa, wild rice, buckwheat, whole barley.

Some products advertise the fact that they are wholegrain; look out for wholegrain symbols on food packaging.

When checking the ingredients list...

If the first ingredient listed contains the word **"whole"** (such as "whole wheat flour" or "whole oats"), it is likely, but not guaranteed, that the product is predominantly wholegrain.

Be careful...

Words like 'multigrain' and 'wheat' do not guarantee a product to be wholegrain. The key word to look out for is **'whole'.**

Multigrain bread may well contain no wholegrain at all; carefully check the ingredients list!

XI. Urine collection information sheet

Urine collection information for volunteers

On the **day prior to both of your study day appointments** you will be required to collect all of your urine for a 24 hour period. This should include only one first urine of the day.

You have been provided with a collection pot in a plastic bag. You should collect all of your urine into this pot. If you find it difficult to use the pot directly, it is recommended that you use a suitable smaller container (e.g. plastic measuring jug or small plastic bowl) to collect your urine, and then carefully transfer it into the collection pot each time you go to the toilet.

You should try to keep the collection pot as cool as possible whilst you are collecting your urine. Please try to find a cool place in your home to store it, but do not put it in a refrigerator that contains food items and please keep it away from children. It may be practical to store it in the bathroom during the collection period to remind you to collect your urine during this time.

Please remember that you need to collect **ALL** of the urine you produce in a 24 hour period. Therefore, if you go out during the day of collection you must take your collection pot with you, or another smaller container to collect your urine whilst you are out. This can then be transferred into the main collection pot later on. When scheduling your first study day the study team will remind you of this and will try to make sure that the preceding day is suitable for such a collection.

On the morning of the day before your study day appointment do not collect the first urine of the day – this should be discarded (not collected). Then, collect all the subsequent urine you pass during the rest of the day into the collection pot. Please include any urine passed during the night and collect the first sample of the following morning (i.e. the morning that

you are coming in to the CRTU). The collection stops at this point and you should return your collection pot to the plastic carrier bag and remember to bring it with you to your study day appointment.

Please try not to alter your normal drinking patterns as a result of this collection. If you feel that you may need an additional sample pot please let the study team know.

Please remember, you are also asked to avoid heavy exercise and alcohol the day prior to your first study day.

If you have any questions about the urine collection, please contact the study team.

XII. Supplementary Tables 3.1

IL-8						
Standards	**Expected**	**Plate 1**	**Plate 2**	**Plate 3**	**Plate 4**	**Plate 5**
0.000000	0	0	0	0	0	0
0.000244	2.44	2.11	2.5	2.52	2.53	2.68
0.000977	9.77	12.79	9.31	8.86	9.43	8.68
0.003906	39.06	32.38	40.32	44.03	39.31	40.93
0.015625	156.25	166.27	155.77	144.07	154.99	157.1
0.062500	625	608.81	622.18	645.01	647.2	617.37
0.250000	2500	2540.57	2494.59	2482.39	2378.84	2480.98
1.000000	10000	9953.91	10029.16	10008.8	10262.04	10119.52

Adipsin						
Standards	**Expected**	**Plate 1**	**Plate 2**	**Plate 3**	**Plate 4**	**Plate 5**
0.000000	0	0	0	0	0	0
0.000244	12.21	8.12	12.6	14.45	12.75	14.71
0.000977	48.83	57.65	47.48	39.36	47.55	43.76
0.003906	195.31	184.35	199.07	223.23	197.11	201.41
0.015625	781.25	801.44	766.47	713.99	776.59	770.15
0.062500	3125	3260.87	3155.6	3264.29	3156.81	3078.47
0.250000	12500	10829.3	13100.47	13694.98	12203.06	15600.65
1.000000	50000	57279.68	45839.43	41811.71	51753.27	33871.74

RBP4						
Standards	**Expected**	**Plate 1**	**Plate 2**	**Plate 3**	**Plate 4**	**Plate 5**
0.000000	0	0	0	0	0	0
0.000244	12.21	N.d	12.31	12.3	12.29	12.31
0.000977	48.83	N.d	47.44	47.03	47.91	47.87
0.003906	195.31	N.d	210.76	217.48	202.4	201.55
0.015625	781.25	N.d	708.39	681.85	752.04	760.93
0.062500	3125	N.d	2973.97	3084.34	3048.2	2951.86
0.250000	12500	N.d	16089.27	15248.82	14222.74	15335.29
1.000000	50000	N.d	42612.14	44121.15	45294.13	42816.66

IL-1β						
Standards	Expected	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5
0.000000	0	0	0	0	0	0
0.000244	2.44	2.07	2.54	2.64	2.37	2.51
0.000977	9.77	11.1	9.33	8.43	10.04	9.52
0.003906	39.06	36.6	40.28	44.41	38.68	39.41
0.015625	156.25	162.71	153.74	144.45	155.82	156.57
0.062500	625	601.23	614.84	637.31	634.36	614.12
0.250000	2500	2582	2646.96	2592.47	2418.93	2600.44
1.000000	10000	9873.97	9569.96	9594.33	10366.4	9653.05

TNF-α						
Standards	Expected	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5
0.000000	0	0	0	0	0	0
0.000244	2.44	2.33	2.65	2.54	2.62	2.65
0.000977	9.77	11.09	8.66	8.54	8.9	9.04
0.003906	39.06	33.39	40.1	45.77	41.11	39.43
0.015625	156.25	172.83	166.2	143.79	153.34	160.46
0.062500	625	601.22	585.48	629.78	628.7	603.62
0.250000	2500	2514.09	2557.98	2531.9	2493.83	2571.2
1.000000	10000	10003.36	10004.58	9943.82	10014.44	9885.35

Resistin						
Standards	Expected	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5
0.000000	0	0	0	0	0	0
0.000244	2.44	2.98	2.76	2.94	3.51	3.27
0.000977	9.77	10.69	9.03	7.77	8.45	9.12
0.003906	39.06	32.79	38.2	43.61	38.26	36.33
0.015625	156.25	166.75	162.8	147.81	156.68	163.76
0.062500	625	625.67	616.99	649.95	641.01	615.16
0.250000	2500	2394.29	2427.85	2425.59	2363.03	2436.55
1.000000	10000	10376.53	10299.13	10131.05	10453.77	10344.57

IL-10						
Standards	**Expected**	**Plate 1**	**Plate 2**	**Plate 3**	**Plate 4**	**Plate 5**
0.000000	0	0	0	0	0	0
0.000244	2.44	2.48	2.57	2.39	2.5	2.51
0.000977	9.77	13.35	8.77	9.38	9.21	9.35
0.003906	39.06	32.7	42.4	45.07	41.42	40.1
0.015625	156.25	153.86	155.91	140.63	151.48	155.81
0.062500	625	614.18	589.7	615.03	626.51	618.95
0.250000	2500	2710.65	2651.73	2655.94	2530.94	2518.83
1.000000	10000	9617.67	9781.54	9731.84	9908.36	9989.2

IP-10						
Standards	**Expected**	**Plate 1**	**Plate 2**	**Plate 3**	**Plate 4**	**Plate 5**
0.000000	0	0	0	0	0	0
0.000244	2.44	2.69	2.68	3.38	2.85	2.65
0.000977	9.77	10.23	8.66	7.3	9	10.02
0.003906	39.06	34.13	40.99	40.7	38.9	36.55
0.015625	156.25	172.35	158.69	159.39	158.08	163.51
0.062500	625	618.07	609.91	637.97	634.66	619.94
0.250000	2500	2223.52	2433.44	2277.07	2329.07	2324.47
1.000000	10000	11183.52	10387.93	10793.73	10648.16	10825.09

Adiponectin						
Standards	**Expected**	**Plate 1**	**Plate 2**	**Plate 3**	**Plate 4**	**Plate 5**
0.000000	0	0	0	0	0	0
0.000244	48.83	28.33	32.71	65.64	40.74	56.19
0.000977	195.31	271.43	296.33	126.96	241.78	178.76
0.003906	781.25	717.3	612.64	954.35	691.62	765.06
0.015625	3125	3122.14	3335.74	2918.76	3224.21	3259.02
0.062500	12500	12568.92	12338.1	12756.43	12510.97	12106.46
0.250000	50000	49857.39	50071.28	49628.1	49414.89	51046.49
1.000000	200000	200134	199940.4	200430.2	201687.7	198221

IFN-γ						
Standards	**Expected**	**Plate 1**	**Plate 2**	**Plate 3**	**Plate 4**	**Plate 5**
0.000000	0	0	0	0	0	0
0.000244	2.44	4.29	2.82	2.62	3	4.66
0.000977	9.77	13.45	9.95	7.91	10.3	7.48
0.003906	39.06	22.56	33.02	47.93	36.43	31.04
0.015625	156.25	159.74	167.49	138.61	151.41	167.79
0.062500	625	657.85	631.15	648.72	662.18	660.44
0.250000	2500	2468.37	2465.19	2481.46	2360.74	2363.6
1.000000	10000	10009.52	10037.56	10006.86	10312.35	10219.52

IL-6						
Standards	**Expected**	**Plate 1**	**Plate 2**	**Plate 3**	**Plate 4**	**Plate 5**
0.000000	0	0	0	0	0	0
0.000244	2.44	1.86	2.45	2.25	2.46	2.48
0.000977	9.77	13.6	9.38	9.7	9.51	9.5
0.003906	39.06	33.45	41.48	45.59	40.69	39.88
0.015625	156.25	161.24	152.36	138.51	149.45	155.13
0.062500	625	611.66	607.16	627.06	652.8	621.38
0.250000	2500	2600.29	2577.01	2663.52	2406.63	2534.27
1.000000	10000	9661.45	9500.44	9537.42	10210.19	9905.72

Leptin						
Standards	**Expected**	**Plate 1**	**Plate 2**	**Plate 3**	**Plate 4**	**Plate 5**
0.000000	0	0	0	0	0	0
0.000244	2.44	3.77	2.76	3.43	5.21	2.7
0.000977	9.77	11.22	8.64	8.18	10.32	9.04
0.003906	39.06	31.68	40.74	39.5	33.26	40.18
0.015625	156.25	166.16	155.42	157.04	161.8	155.03
0.062500	625	609.84	620.28	621.88	622.48	637.58
0.250000	2500	2531.64	2562.53	2513.9	2370.07	2278.74
1.000000	10000	9975.05	9633.23	9963.81	10717.64	10990.67

MCP-1						
Standards	**Expected**	**Plate 1**	**Plate 2**	**Plate 3**	**Plate 4**	**Plate 5**
0.000000	0	0	0	0	0	0
0.000244	2.44	2.3	2.5	2.61	2.7	2.81
0.000977	9.77	10.5	9.31	8.49	8.91	8.74
0.003906	39.06	36.72	40.32	44.48	40.9	40.88
0.015625	156.25	166.31	155.77	148.51	153.67	156.11
0.062500	625	588.66	622.18	611.06	613.51	582.24
0.250000	2500	2545.29	2494.59	2504.42	2515.18	2598.88
1.000000	10000	10124.72	10029.16	10258.14	10257.33	10428

Supplementary Table 3.1 Concentrations of serial diultion of standards across plates all plates run, for all 13 analytes.

XIII. PE1 Adapter ligation

Mix 1:

```
5′ ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNGACTCG*T 3′

            3′ [SpcC3]C*GAGAAGGCTAGANNNNNNCTGAGC[Phos] 5′
```

Mix 2:

```
5′ ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNCTGCTCC*T 3′

            3′ [SpcC3]C*GAGAAGGCTAGANNNNNNGACGAGG[Phos] 5′
```

*Adapter mix = 2 oligos: “PE1 adaptor F1/F2” & “Short adaptor R1/R2-block”, annealed together to create asymmetric PE1 adaptors.

* = phosphorothioate bond; short R adaptors should be phosphorylated at 5’ end and have a Carbon-3 spacer incorporated at 3’ end to prevent it being used to prime in later reactions.

XIV. **Biotinylated primers**

Enrichment of VDJ recombined fragments

Thermal cycler conditions: 95°C 4 min, then 6-8 cycles of (95˚C 30s, 59°C 5 min, 72°C 2 min), 4°C pause

* A mixture of 6 biotinylated primers are used, Tms between 62.4°C to 64.2°C:

Hu J1 R Bio	CCAGACAGCAGACTCACCTG
Hu J2 R Bio	TGCAGTGGGACTCACCTG
Hu J3 R Bio	AGAAGGAAAGCCATCTTACCTG
Hu J4 R Bio	CAGGAGAGAGGTTGTGAGGACT
Hu J5 R Bio	AGGGGGTGGTGAGGACTC
Hu J6 R Bio	CCATTCTTACCTGAGGAGACG

Incorporation of PE2 adapter by PCR

Place on the PTC100 using the following conditions:

Thermal cycler conditions: 94°C 2mins, X cycles of: 94°C 15 secs, 61°C 30 secs, 72°C 45 secs, followed by 72°C 5mins, and 4°C pause.

X= number of cycles dependant on starting amount of DNA; 12-15 cycles to Nu-AGE samples.

* = phosphorothioate bond

* Primer sequences, Short PE1 primer Tm 68.5°C, J primers Tm range 70.1-73°C

Short PE1 primer	ACACTCTTTCCCTACACGACGCTC*T
Hu J1 PE2 PCR	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGTGCCCTGGCCCCAGT*G
Hu J2 PE2 PCR	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGTGCCACGGCCCCAGAG*A
Hu J3 PE2 PCR	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACCATTGTCCCTTGGCCCCA*G
Hu J4 PE2 PCR	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGACCAGGGTYCCYTGGCCC*C
Hu J5 PE2 PCR	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCAGGGTTCCYTGGCCCCAG*G

Hu J6 PE2 PCR.1 GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCTTTGCCCCAGACGTCCATGTAG*T

Hu J6 PE2 PCR.2 GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTKSCCCCAGACGTCCATACCG*T

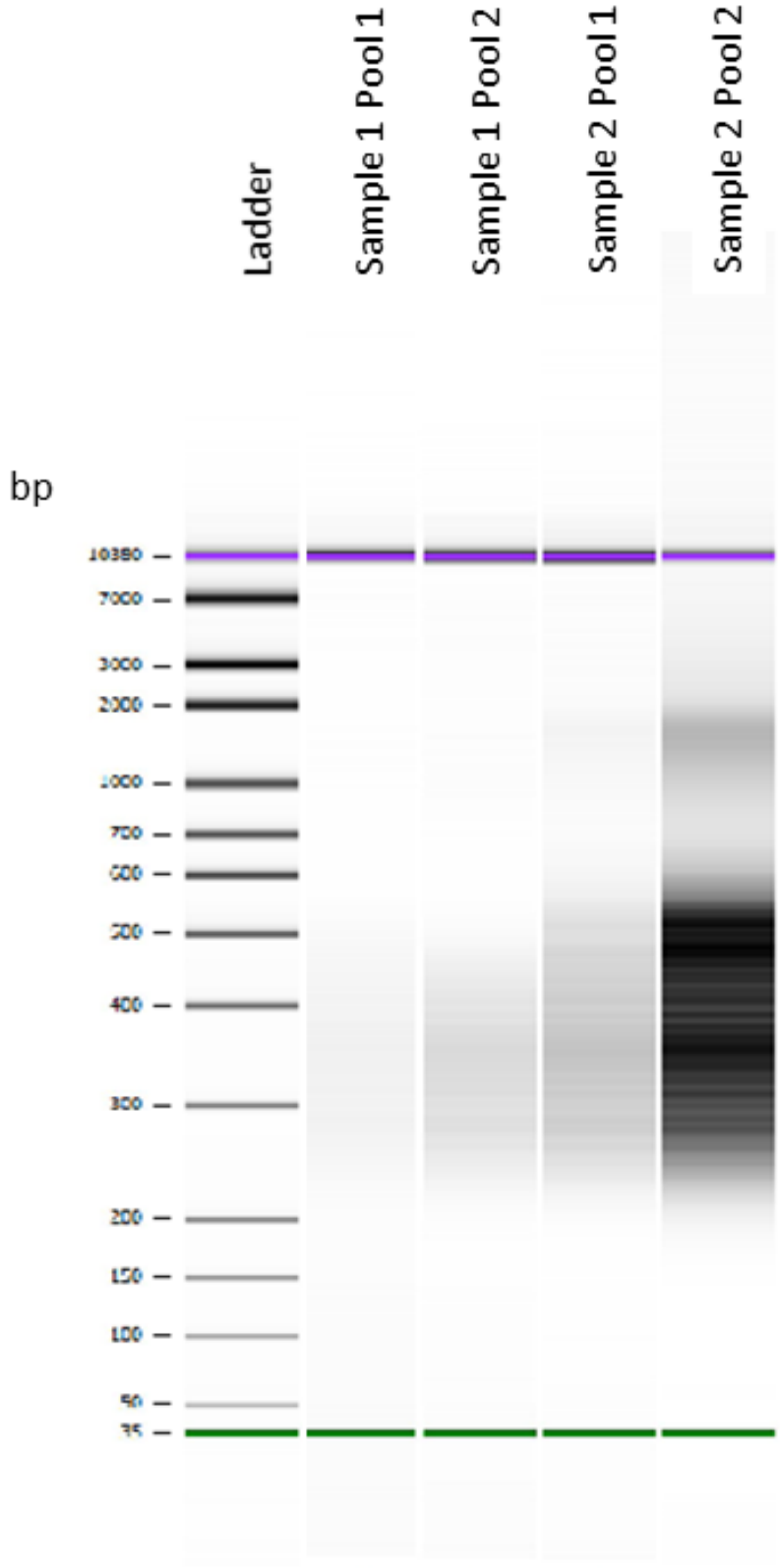
Incorporation of flowcell binding & barcoding sequences by PCR

Thermal cycler conditions: 94°C 2mins, 5 cycles of: 94°C 15 secs, 55°C 30 secs, 72°C 45 secs, followed by 72°C 5mins, and 4°C pause.

Tms of regions annealing to target are 58.2°C - PE1, and 61.9°C PE2.

Flowcell PE1	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC
Flowcell PE2 Index 1	CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCAGACGTGT
Flowcell PE2 Index 2	CAAGCAGAAGACGGCATACGAGATACATCGGTGACTGGAGTTCAGACGTGT
Flowcell PE2 Index 3	CAAGCAGAAGACGGCATACGAGATGCCTAAGTGACTGGAGTTCAGACGTGT
Flowcell PE2 Index 4	CAAGCAGAAGACGGCATACGAGATTGGTCAGTGACTGGAGTTCAGACGTGT
Flowcell PE2 Index 5	CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTGGAGTTCAGACGTGT
Flowcell PE2 Index 6	CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTCAGACGTGT
Flowcell PE2 Index 7	CAAGCAGAAGACGGCATACGAGATGATCTGGTGACTGGAGTTCAGACGTGT
Flowcell PE2 Index 8	CAAGCAGAAGACGGCATACGAGATTCAAGTGTGACTGGAGTTCAGACGTGT
Flowcell PE2 Index 9	CAAGCAGAAGACGGCATACGAGATCTGATCGTGACTGGAGTTCAGACGTGT
Flowcell PE2 Index 10	CAAGCAGAAGACGGCATACGAGATAAGCTAGTGACTGGAGTTCAGACGTGT
Flowcell PE2 Index 11	CAAGCAGAAGACGGCATACGAGATGTAGCCGTGACTGGAGTTCAGACGTGT
Flowcell PE2 Index 12	CAAGCAGAAGACGGCATACGAGATTACAAGGTGACTGGAGTTCAGACGTGT

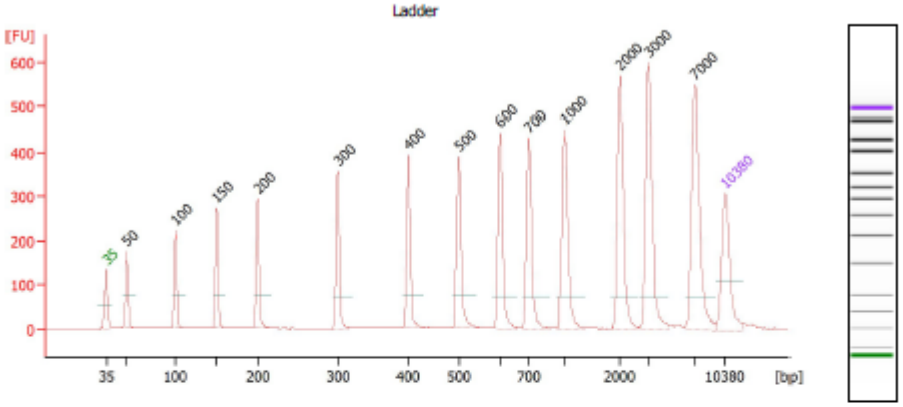
XV. **Bioanalyser results**



Assay Class: High Sensitivity DNA Assay
Data Path: S:\...gh Sensitivity DNA Assay_DE13804912_2016-11-08_15-42-15.xad

Created: 08/11/2016 15:42:15
Modified: 09/11/2016 09:23:42

Electropherogram Summary



Overall Results for Ladder

Noise: 0.3

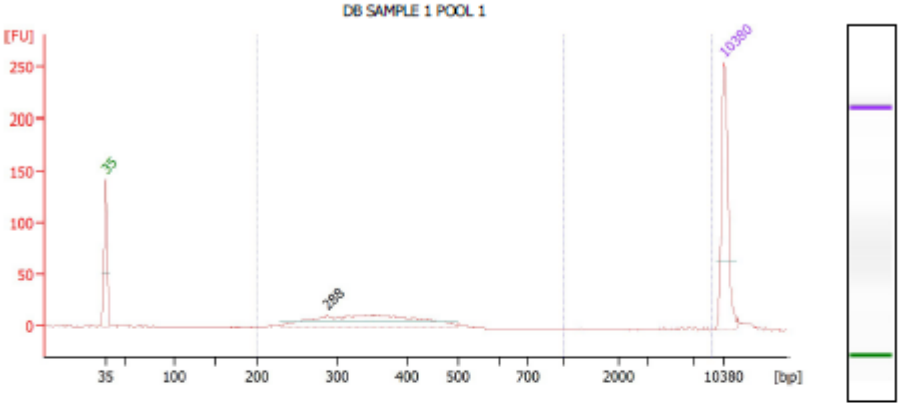
Peak table for Ladder

Peak	Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
1	35	125.00	5,411.3	Lower Marker
2	50	150.00	4,545.5	Ladder Peak
3	100	150.00	2,272.7	Ladder Peak
4	150	150.00	1,515.2	Ladder Peak
5	200	150.00	1,136.4	Ladder Peak
6	300	150.00	757.6	Ladder Peak
7	400	150.00	568.2	Ladder Peak
8	500	150.00	454.5	Ladder Peak
9	600	150.00	378.8	Ladder Peak
10	700	150.00	324.7	Ladder Peak
11	1,000	150.00	227.3	Ladder Peak
12	2,000	150.00	113.6	Ladder Peak
13	3,000	150.00	75.8	Ladder Peak
14	7,000	150.00	32.5	Ladder Peak
15	10,380	75.00	10.9	Upper Marker

Assay Class: High Sensitivity DNA Assay
Data Path: S:\...gh Sensitivity DNA Assay_DE13804912_2016-11-08_15-42-15.xad

Created: 08/11/2016 15:42:15
Modified: 09/11/2016 09:23:42

Electropherogram Summary Continued ...



Overall Results for sample 5 : DB SAMPLE 1 POOL 1

Number of peaks found:	1	Corr. Area 1:	246.4
Noise:	0.3	Corr. Area 2:	2.8

Peak table for sample 5 : DB SAMPLE 1 POOL 1

Peak	Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
1	35	125.00	5,411.3	Lower Marker
2	288	145.18	762.8	
3	10,380	75.00	10.9	Upper Marker

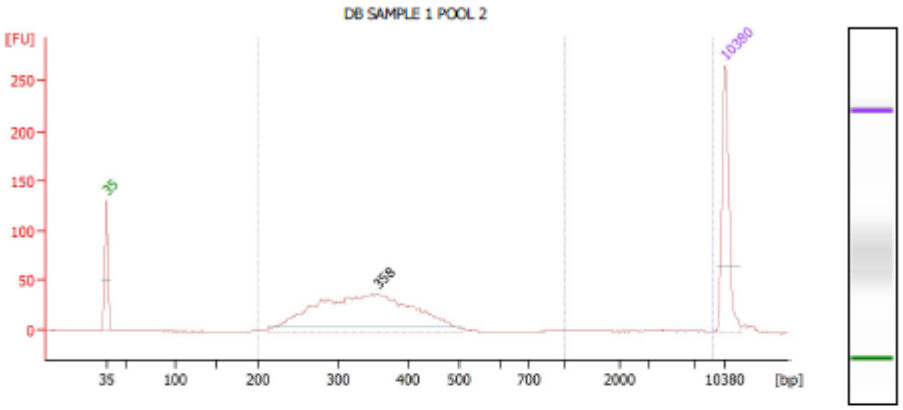
Region table for sample 5 : DB SAMPLE 1 POOL 1

From [bp]	To [bp]	Corr. Area	% of Total	Average Size [bp]	Size distribution in CV [%]	Conc. [pg/µl]	Molarity [pmol/l]	Color
200	1,000	246.4	91	353	20.0	142.77	649.2	
1,000	9,000	2.8	1	6,355	23.2	1.12	0.3	

Assay Class: High Sensitivity DNA Assay
Data Path: S:\...gh Sensitivity DNA Assay_DE13804912_2016-11-08_15-42-15.xad

Created: 08/11/2016 15:42:15
Modified: 09/11/2016 09:23:42

Electropherogram Summary Continued ...



Overall Results for sample 6 : DB SAMPLE 1 POOL 2

Number of peaks found:	1	Corr. Area 1:	690.0
Noise:	0.4	Corr. Area 2:	11.5

Peak table for sample 6 : DB SAMPLE 1 POOL 2

Peak	Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
1	35	125.00	5,411.3	Lower Marker
2	358	363.93	1,541.6	
3	10,380	75.00	10.9	Upper Marker

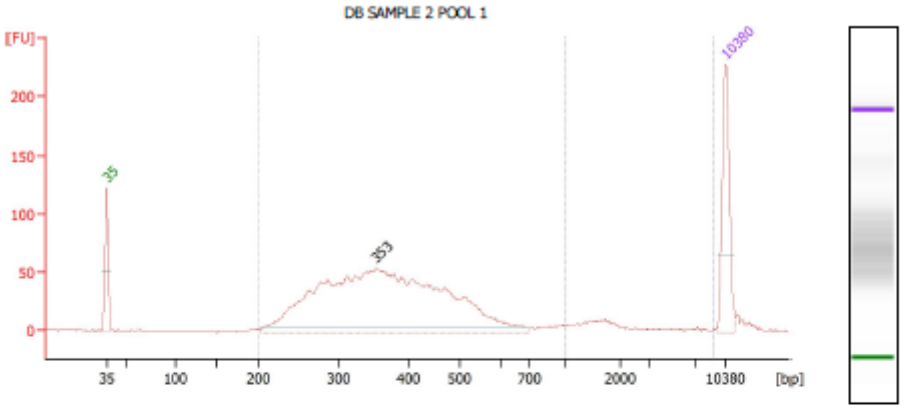
Region table for sample 6 : DB SAMPLE 1 POOL 2

From [bp]	To [bp]	Corr. Area	% of Total	Average Size [bp]	Size distribution in CV [%]	Conc. [pg/µl]	Molarity [pmol/l]	Color
200	1,000	690.0	97	343	20.2	360.53	1,673.5	
1,000	9,000	11.5	2	3,738	59.5	4.23	2.7	

Assay Class: High Sensitivity DNA Assay
Data Path: S:\...gh Sensitivity DNA Assay_DE13804912_2016-11-08_15-42-15.xad

Created: 08/11/2016 15:42:15
Modified: 09/11/2016 09:23:42

Electropherogram Summary Continued ...



Overall Results for sample 7 : DB SAMPLE 2 POOL 1

Number of peaks found:	1	Corr. Area 1:	1,255.6
Noise:	0.3	Corr. Area 2:	70.5

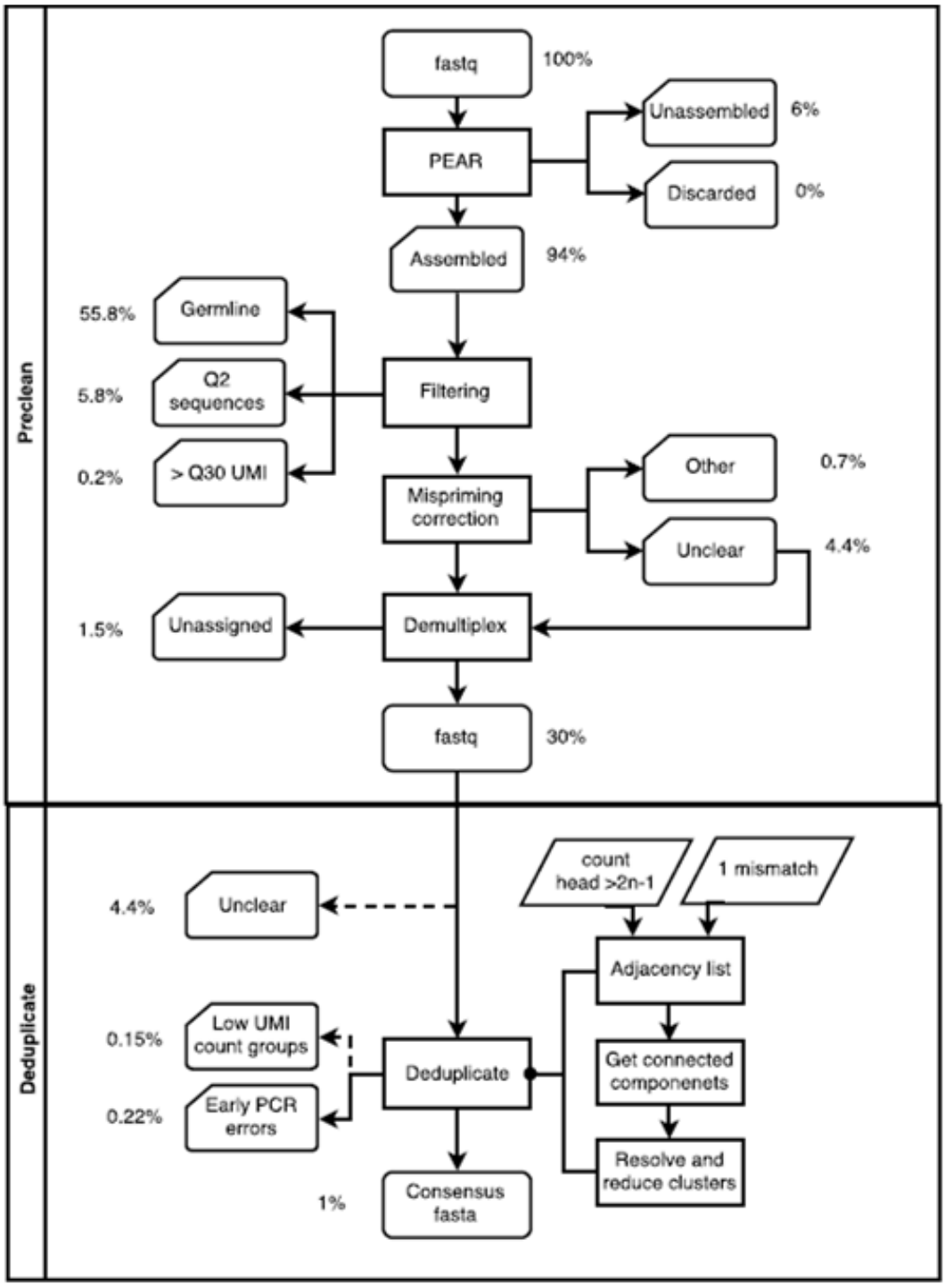
Peak table for sample 7 : DB SAMPLE 2 POOL 1

Peak	Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
1	35	125.00	5,411.3	Lower Marker
2	353	751.78	3,222.5	
3	10,380	75.00	10.9	Upper Marker

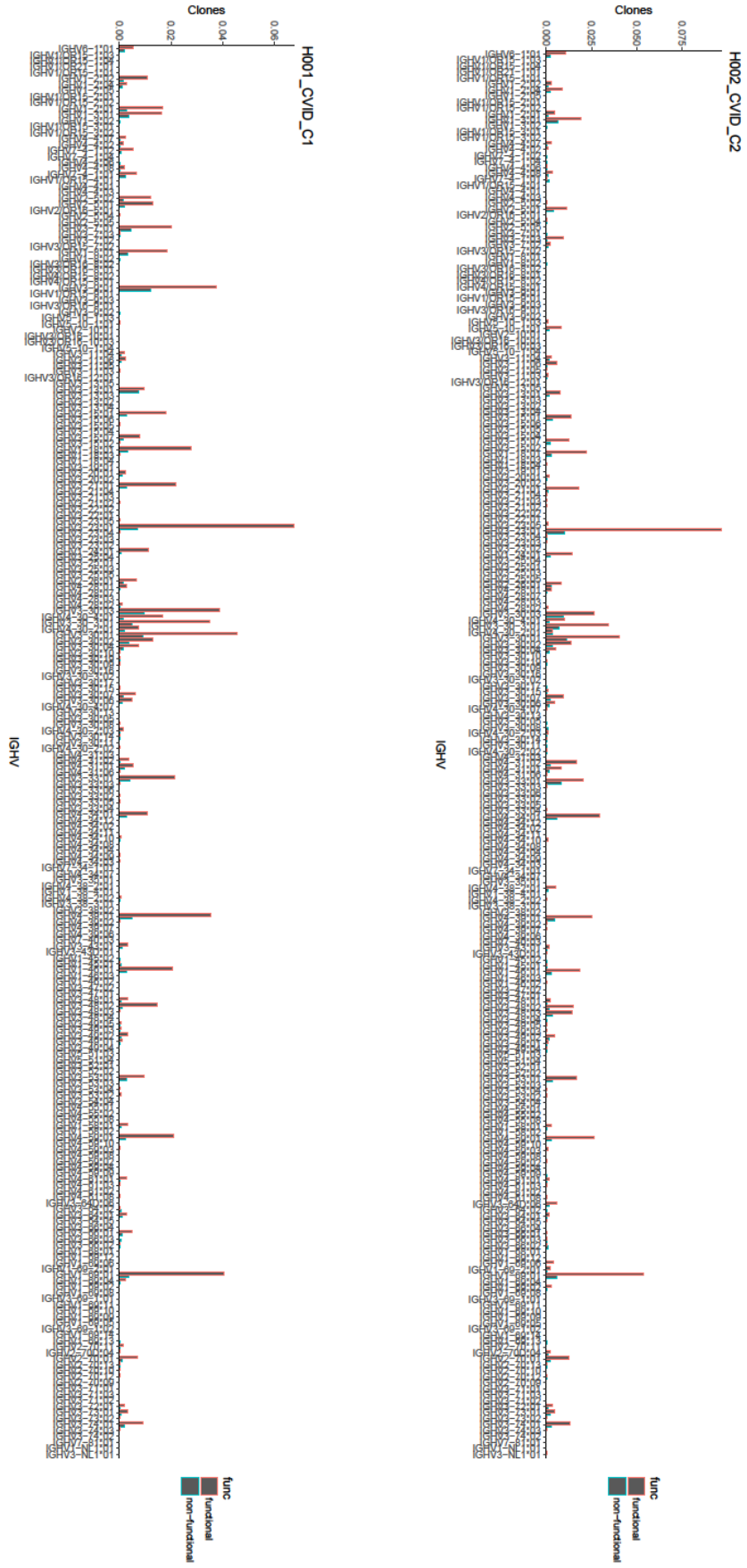
Region table for sample 7 : DB SAMPLE 2 POOL 1

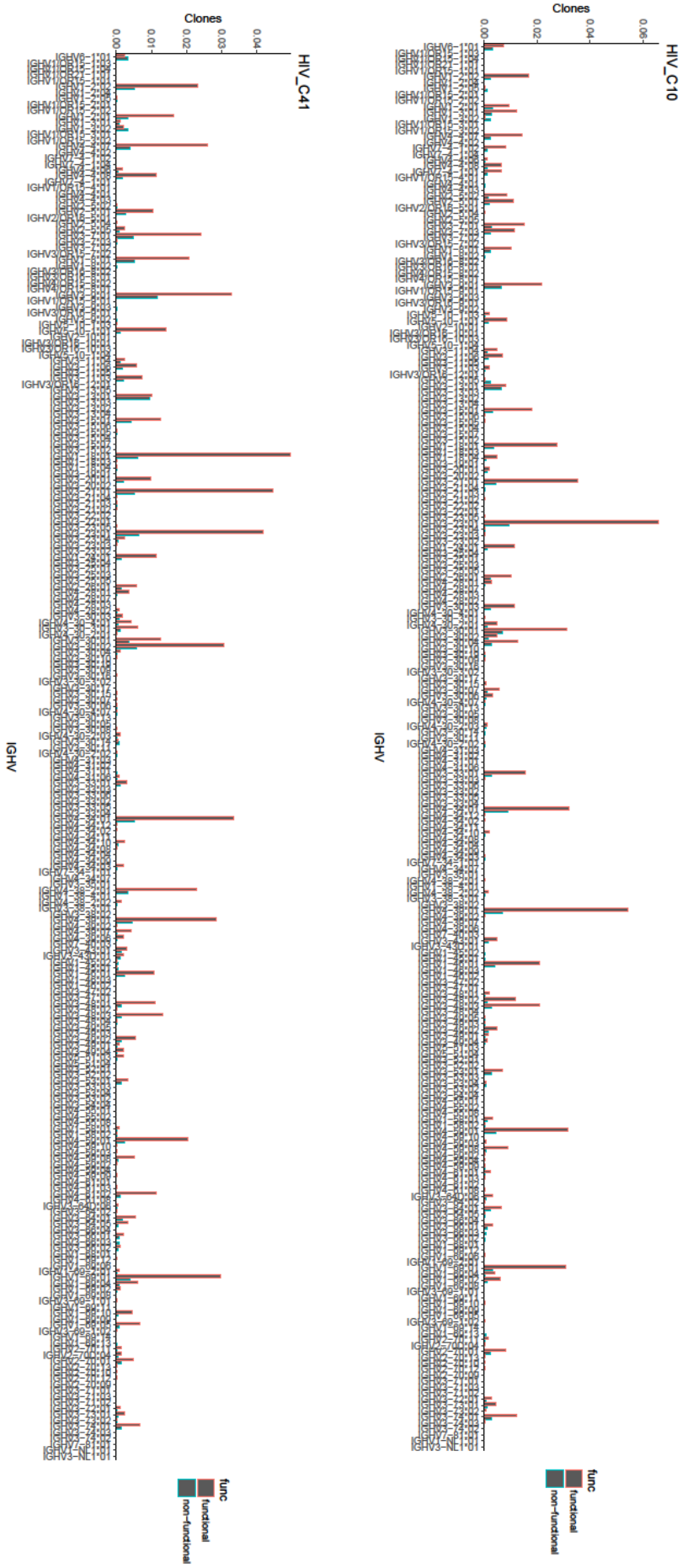
From [bp]	To [bp]	Corr. Area	% of Total	Average Size [bp]	Size distribution in CV [%]	Conc. [pg/µl]	Molarity [pmol/l]	Color
200	1,000	1,255.6	93	385	29.2	724.87	3,157.5	
1,000	9,000	70.5	5	2,506	71.9	29.54	24.0	

XVI. Babraham LinkOn pipeline analysis flow chart



XVII. **Supplementary Figure 6.1. IGHV allele usage for all young subjects after ordering alleles according to regional location on the chromosome.**




HIV_C41
Clones
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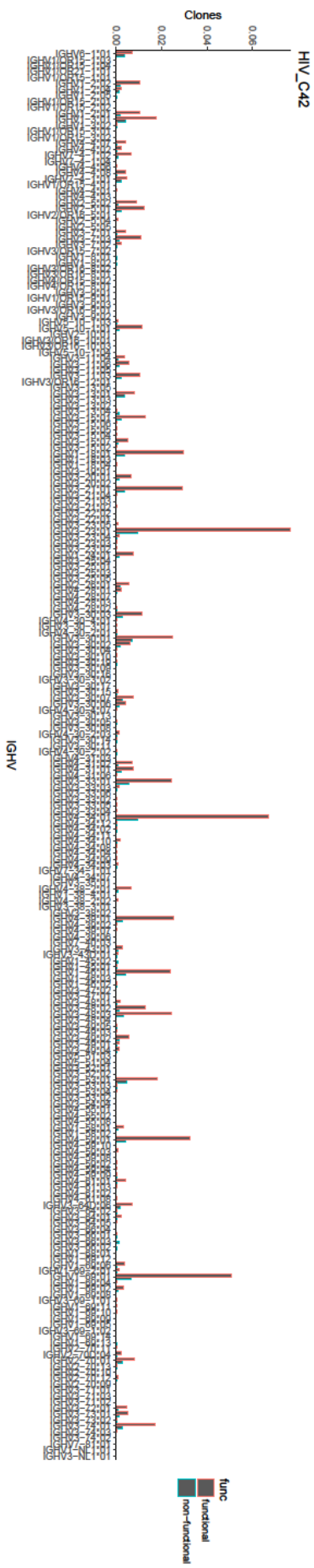
0.01
0.02
0.03
0.04
IGHV
func
functional
non-functional
HIV_C10
Clones
0.00
0.02
0.04
0.06
IGHV
func
functional
non-functional


HIV_C42
Clones
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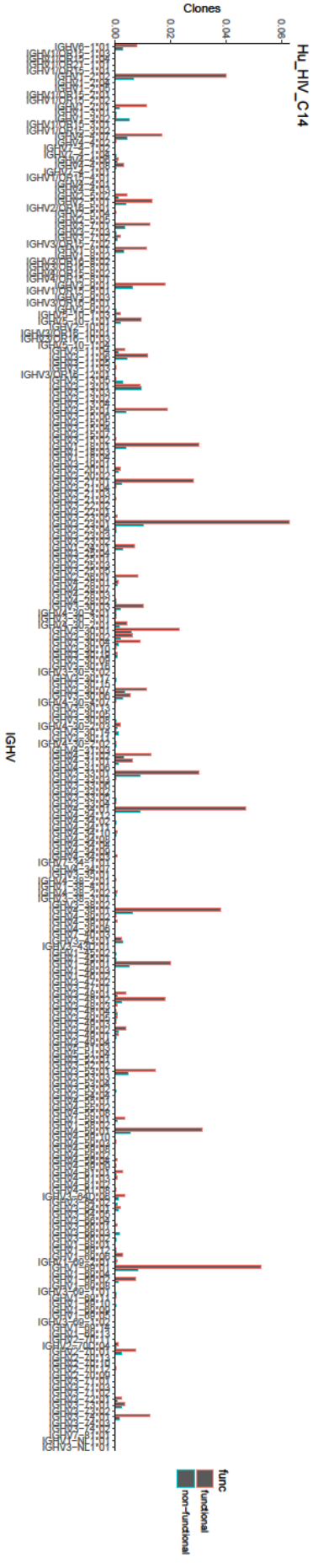
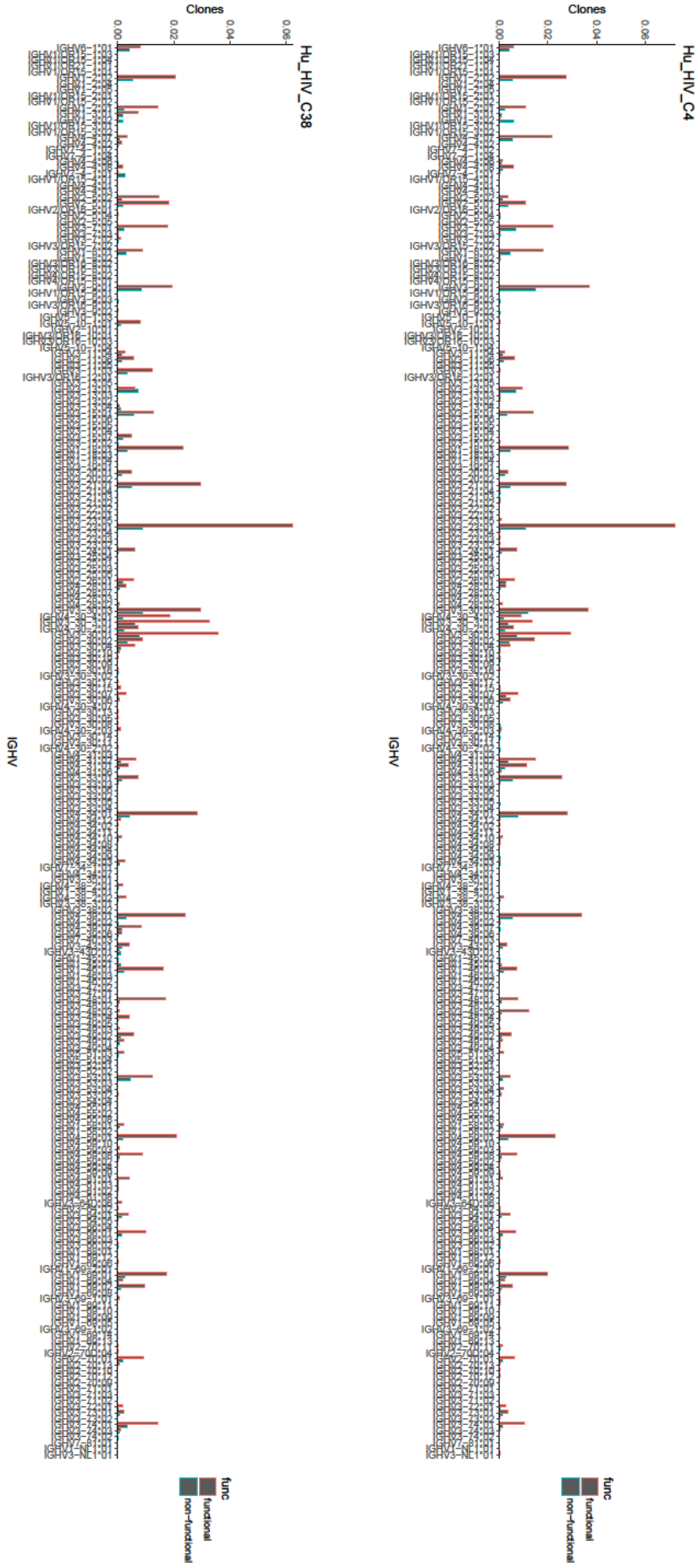
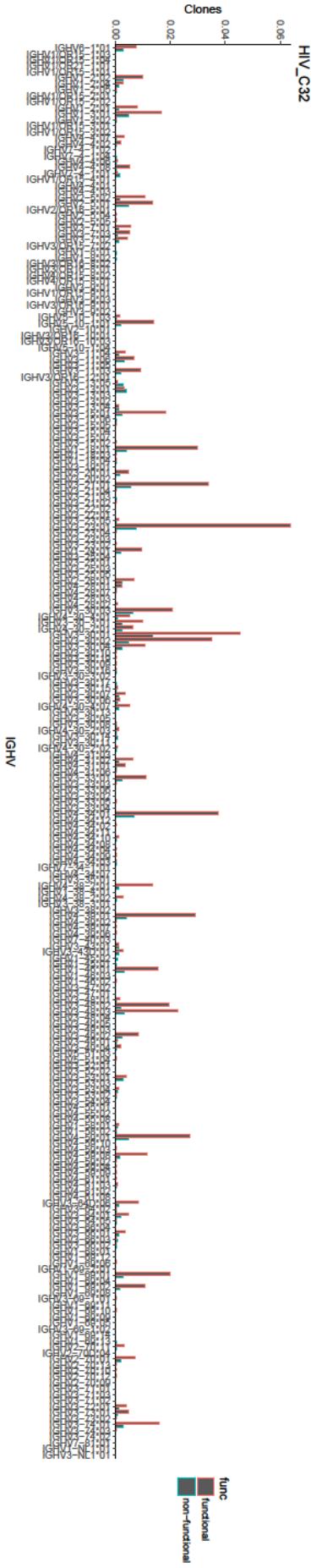
0.02
0.04
0.06
IGHV
func
functional
non-functional


Hu_HIV_C14
Clones
0.00
0.02
0.04
0.06
IGHV
func
functional
non-functional


Hu_HIV_C4
Clones
0.00
0.02
0.04
0.06
IGHV
func
functional
non-functional
Hu_HIV_C38
Clones
0.00
0.02
0.04
0.06
IGHV
func
functional
non-functional


HIV_C32
Clones
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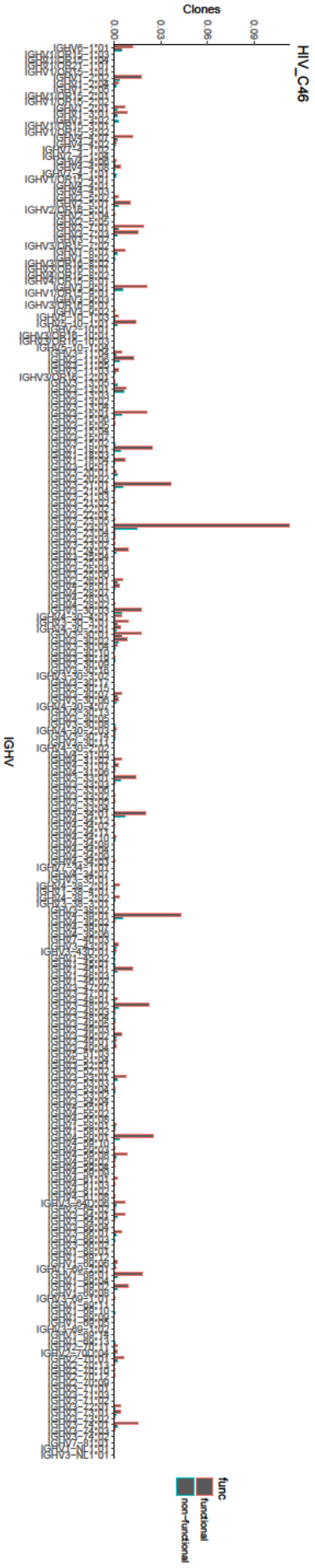
0.04
0.06
IGHV
func
functional
non-functional


HIV_C46
Clones
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IGHV
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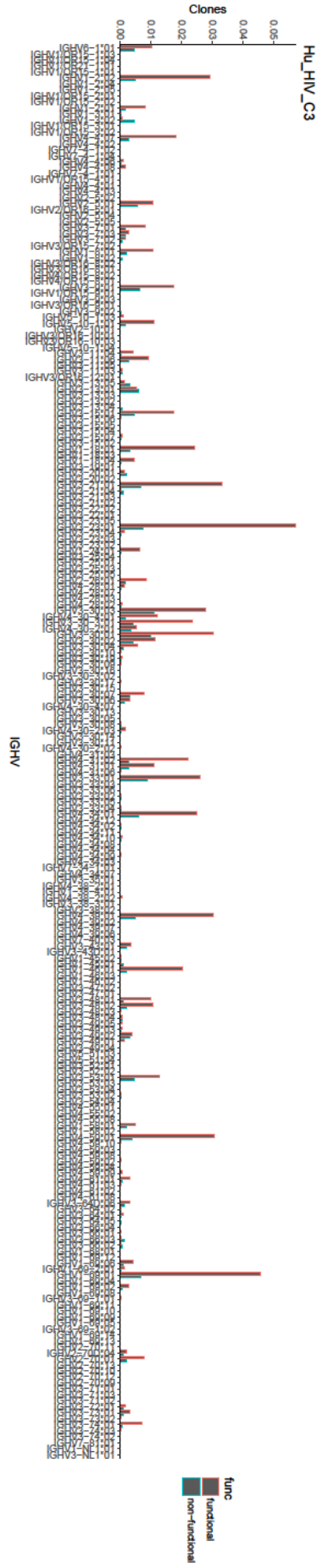
functional
non-functional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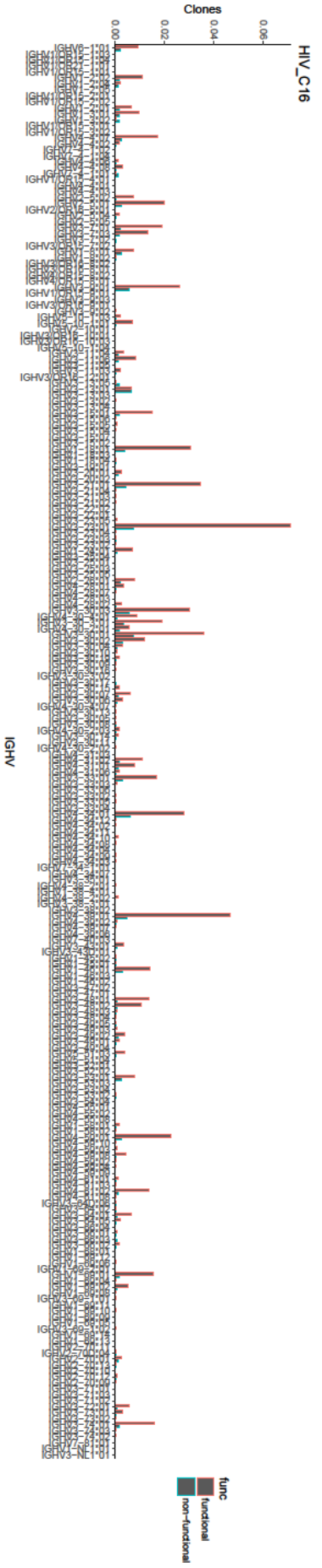
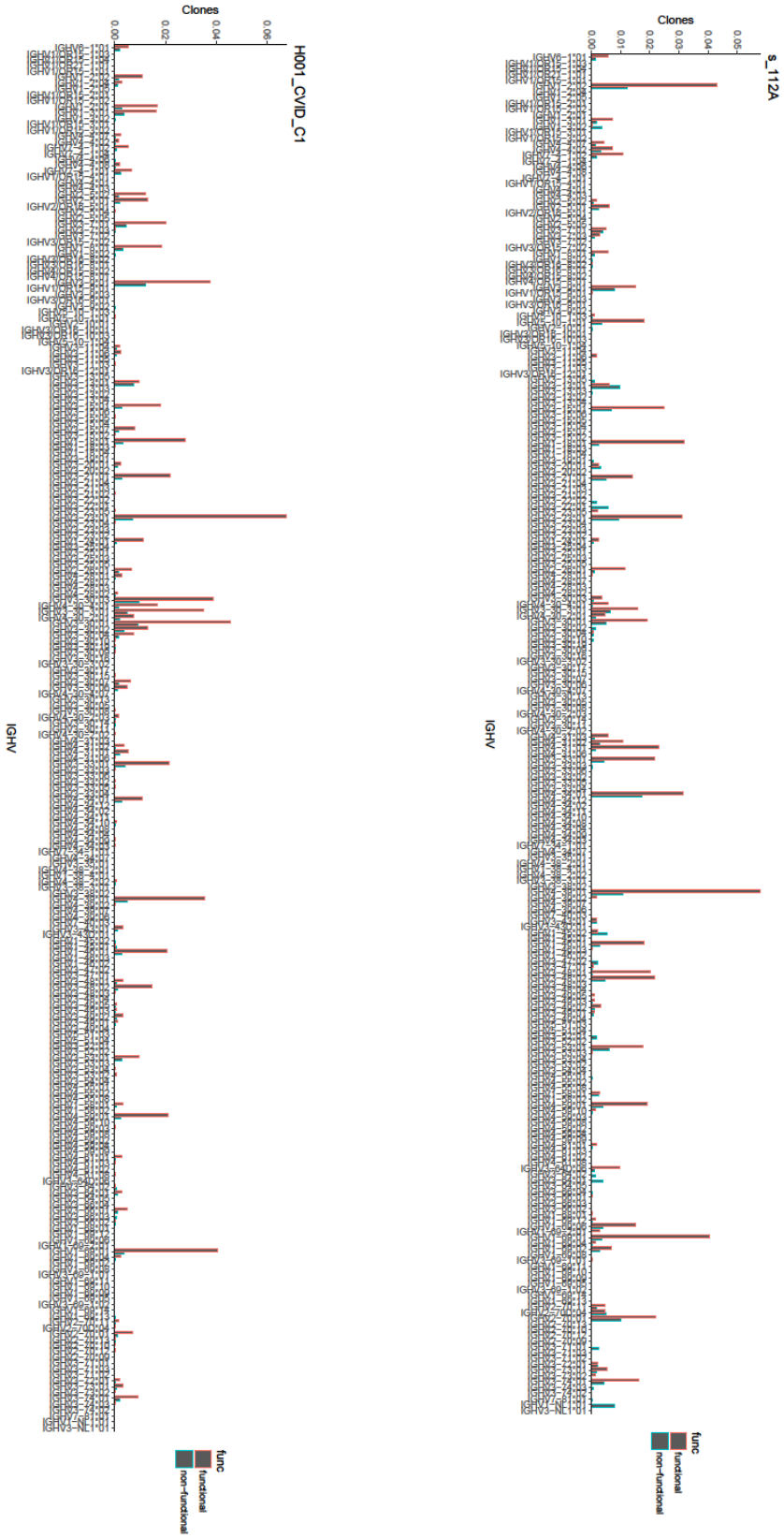
0.05
IGHV
func
functional
non-functional


HIV_C16
Clones
0.00
0.02
0.04
0.06
IGHV
func
functional
non-functional

Supplementary Figure 6.1. IGHV allele usage for all young subjects after ordering alleles according to regional location on the chromosome. The sequencing data from 12 previously analysed control subjects were re-analysed by Peter Chovanec (Babraham Institute) using the LinkON VDJ-Seq pipeline analysis workflow used for the Nu-AGE data to allow comparison of the baseline elderly sequences to a young control group. All alleles represented within the subject cohort were aligned in regional order of location on the chromosome. Each graph represents data from one individual subject. Functional genes (orange), non-functional genes (blue).

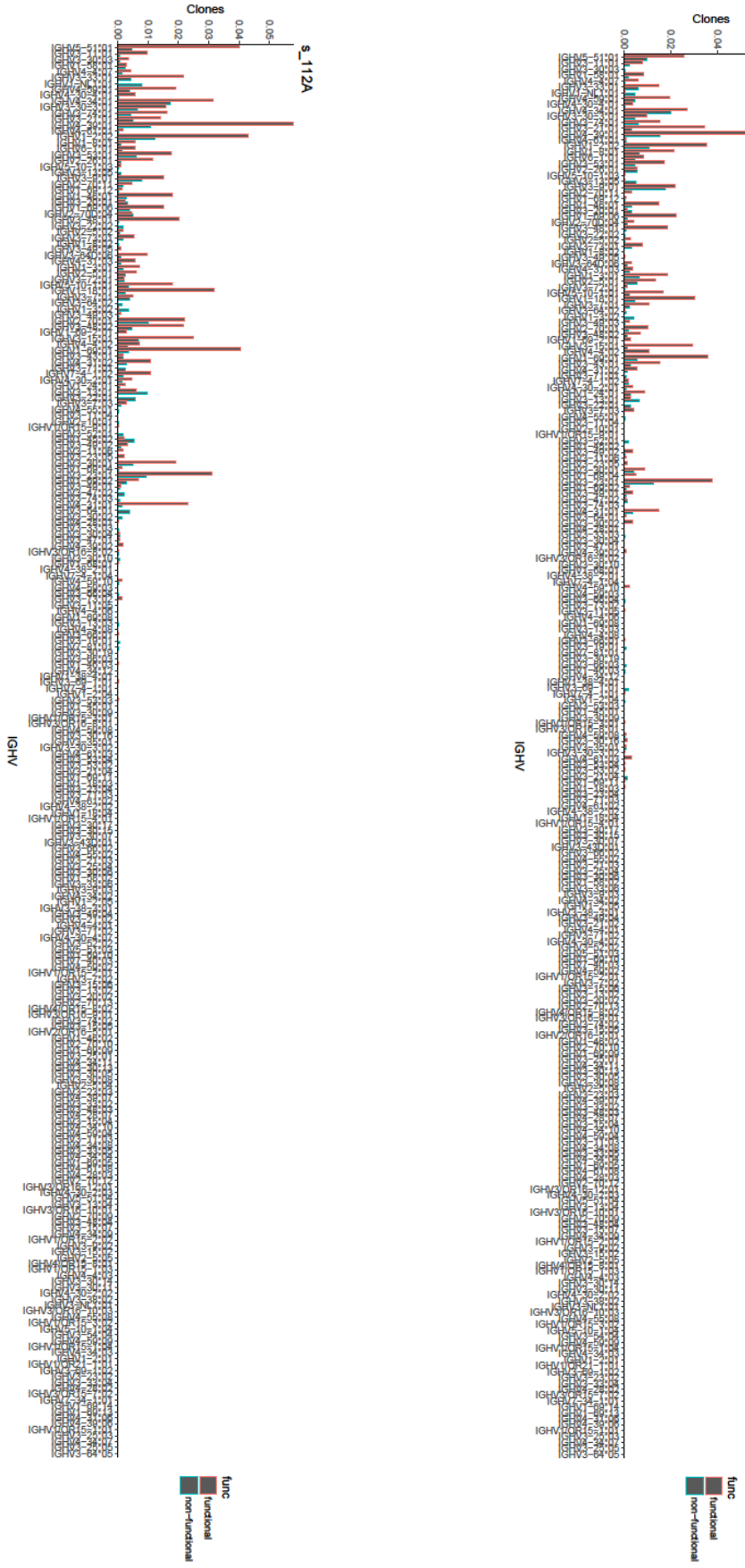
XVIII. **Supplementary Figure 6.2. Representative example of ordered IGHV allele usage of one young (H001_CVID_C1) and one elderly subject (s112) at baseline, according to regional location on the chromosome.**



Supplementary Figure 6.2 Representative example, ordered IGHV alleles of one young subject (H001_CVID_C1) and one elderly subject (s112) at baseline, according to regional location on the chromosome. **The sequencing data from 12 previously analysed control subjects were re-analysed by Peter Chovanec (Babraham Institute) using the LinkON VDJ-Seq pipeline analysis workflow used for the Nu-AGE data to allow comparison of the baseline elderly sequences to a younger control group. All alleles represented within both subject cohorts were aligned in regional order of location on the chromosome, to allow comparisons between elderly and younger subject cohorts.**

XIX. **Supplementary Figure 6.3. Variable allele usage within the IGHV for each subject.**

a)



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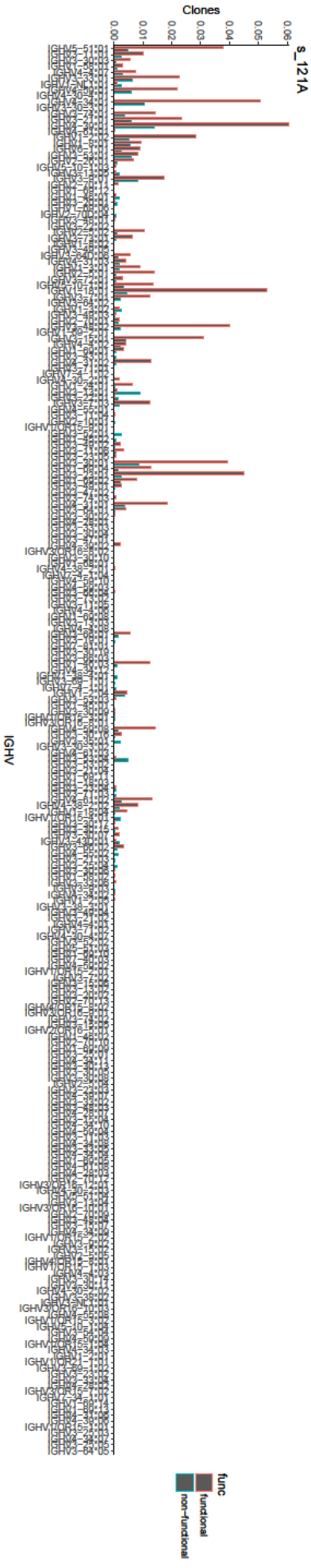
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Clones
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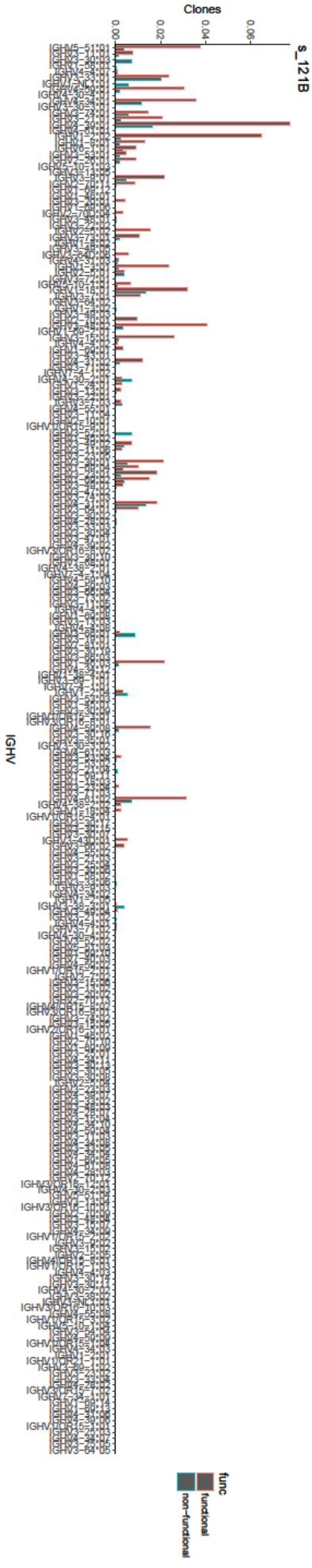
0.03
0.04
0.05
0.06
IGHV
func
functional
non-functional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Clones
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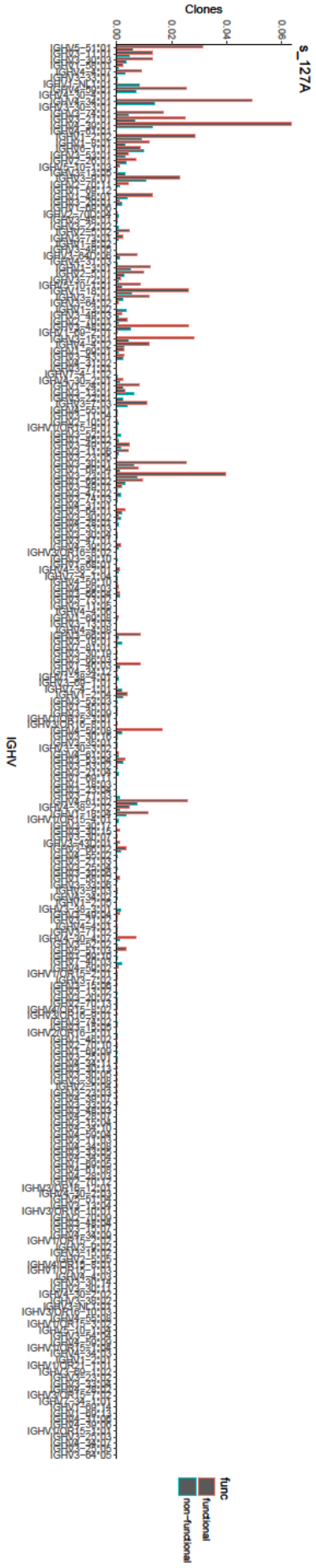
0.04
0.06
IGHV
func
functional
non-functional

c)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0.06
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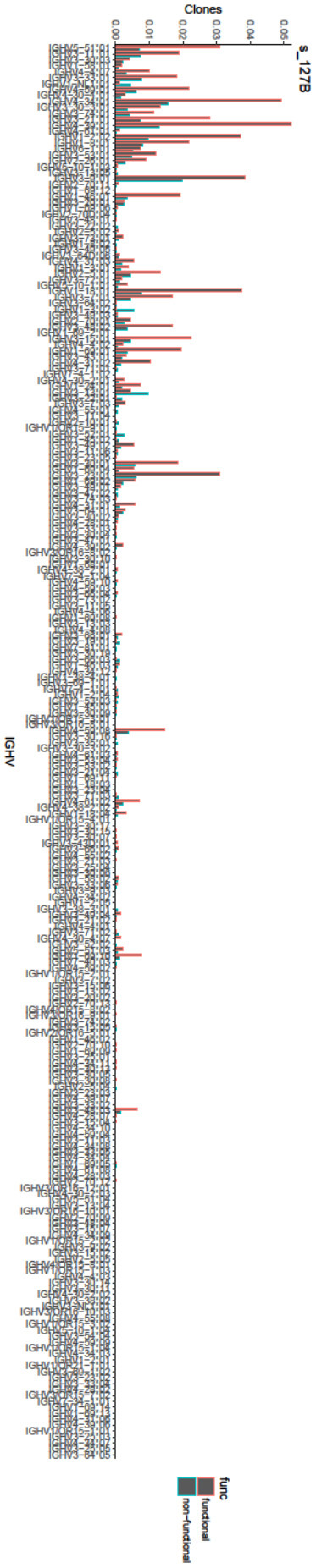
IGHV
func
functional
non-functional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0.05
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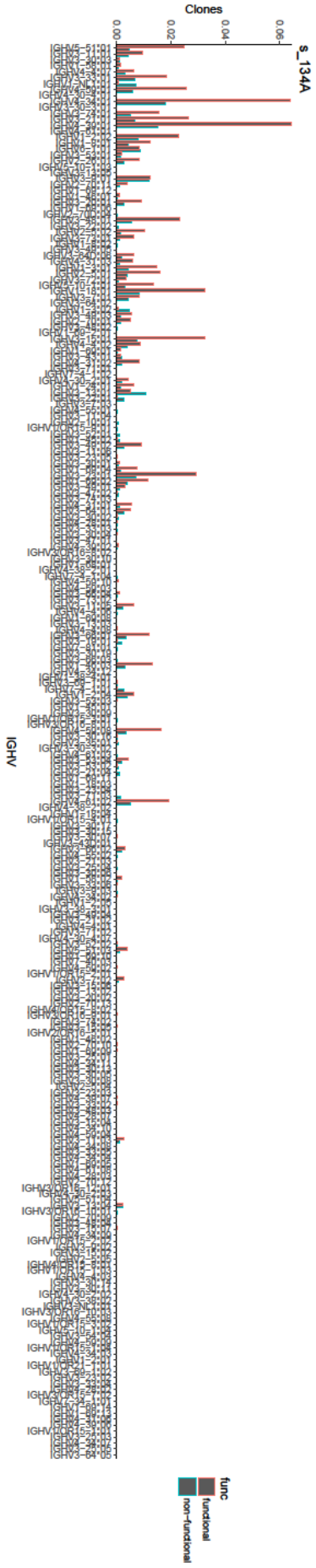
IGHV
func
functional
non-functional

d)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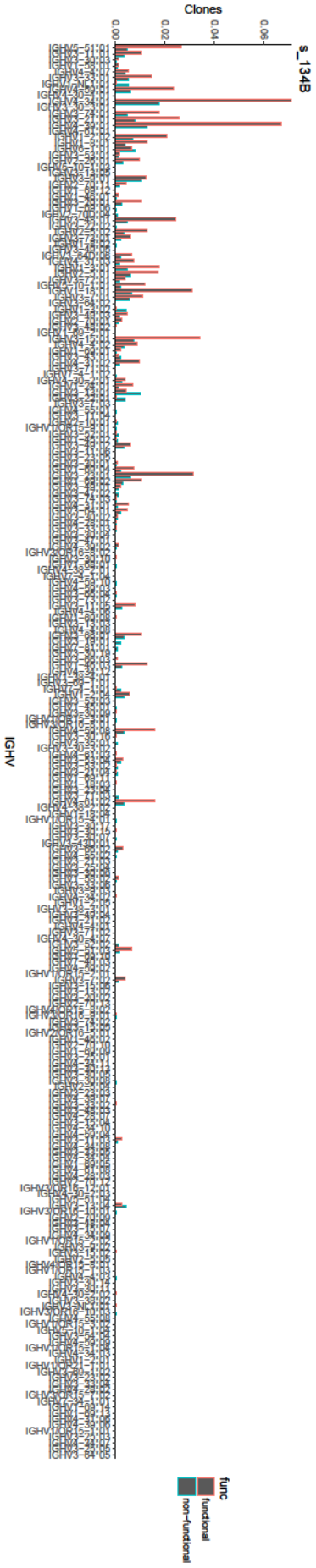
IGHV
func
functional
non-functional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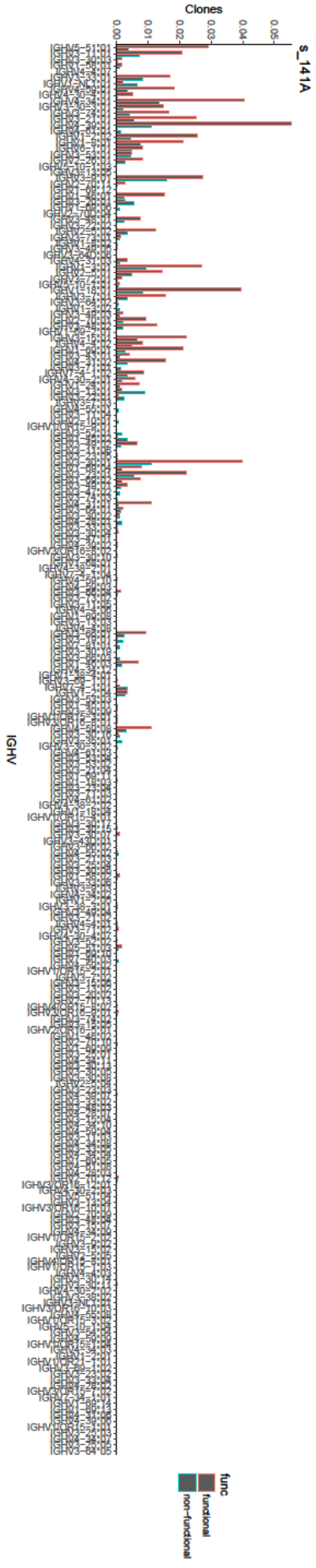
func
functional
non-functional

e)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0.03
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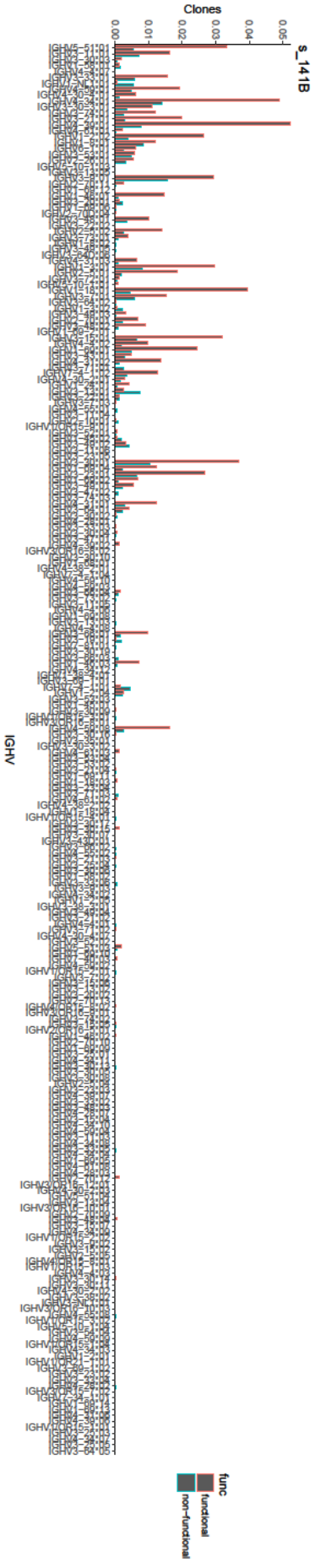
0.05
IGHV
func
functional
non-functional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Clones
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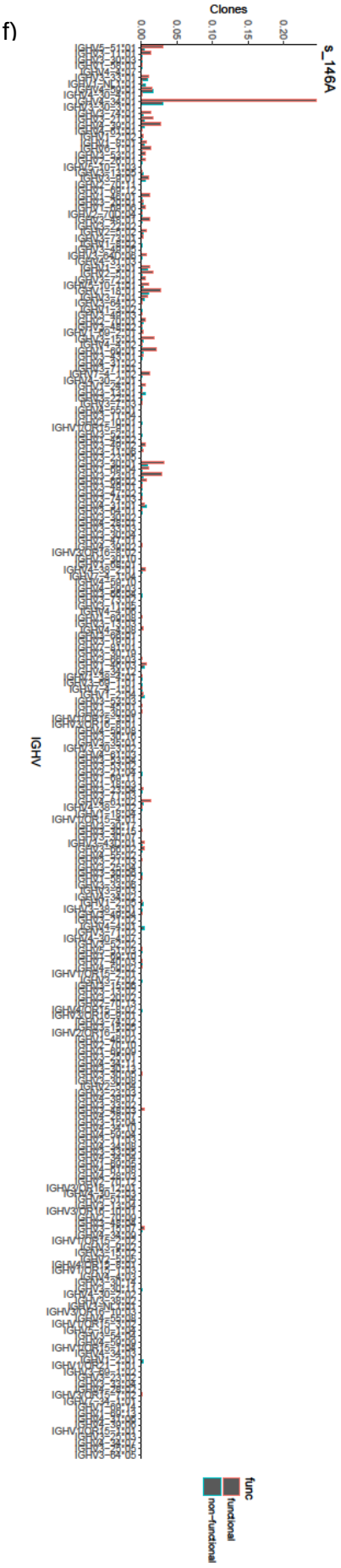
IGHV
func
functional
non-functional

f)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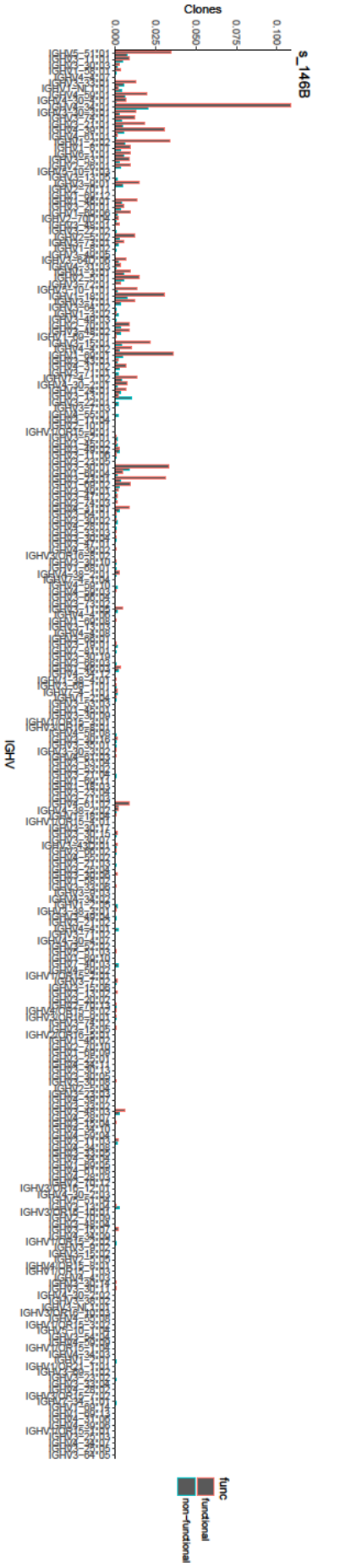
IGHV
func
functional
non-functional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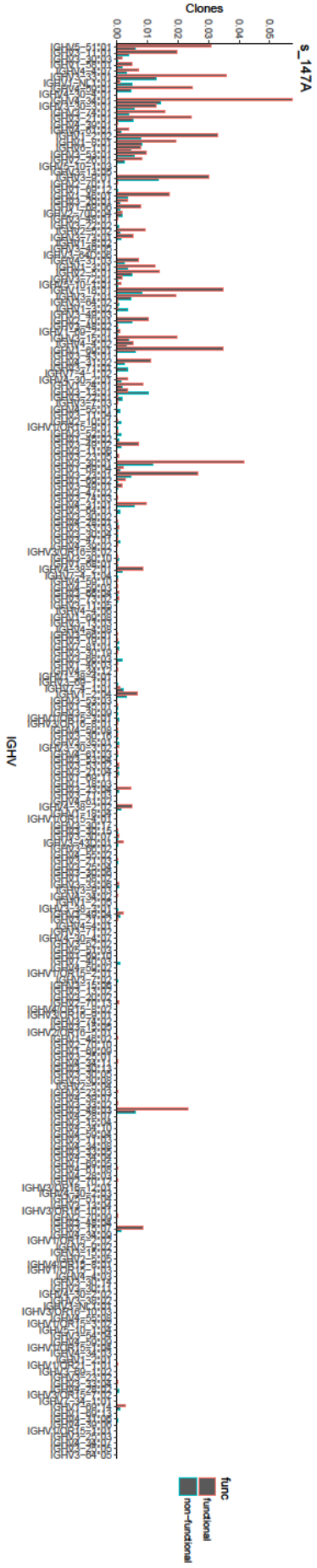
s_146B
IGHV
func
functional
non-functional

a)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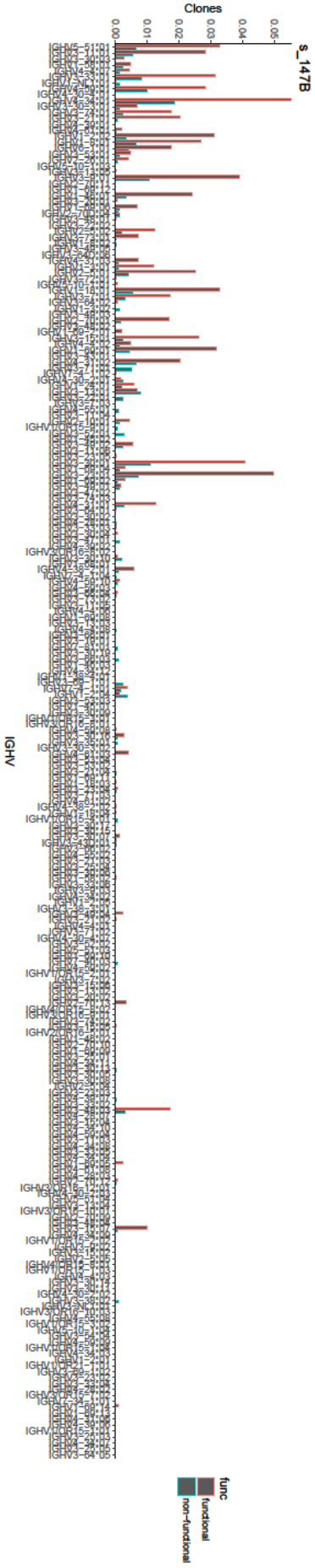
functional
non-functional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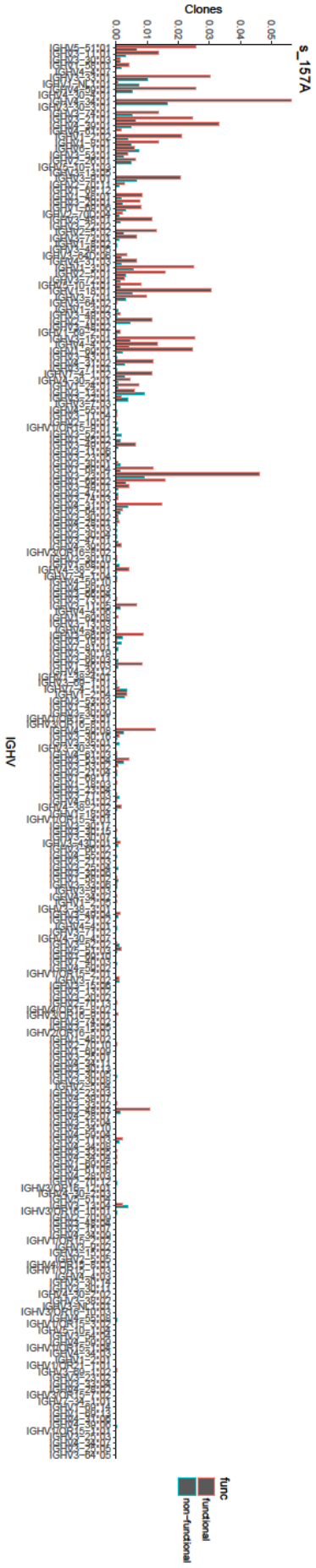
func
functional
non-functional

h)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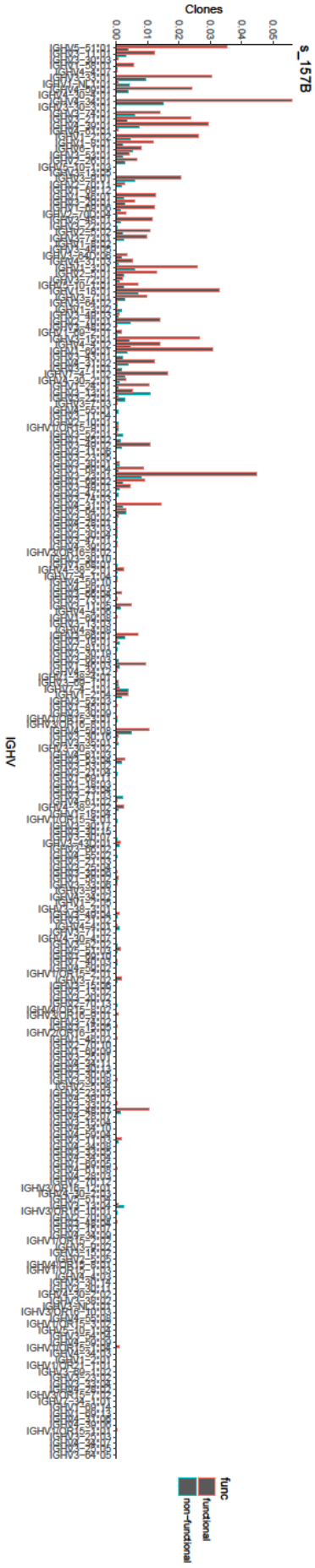
IGHV
func
functional
non-functional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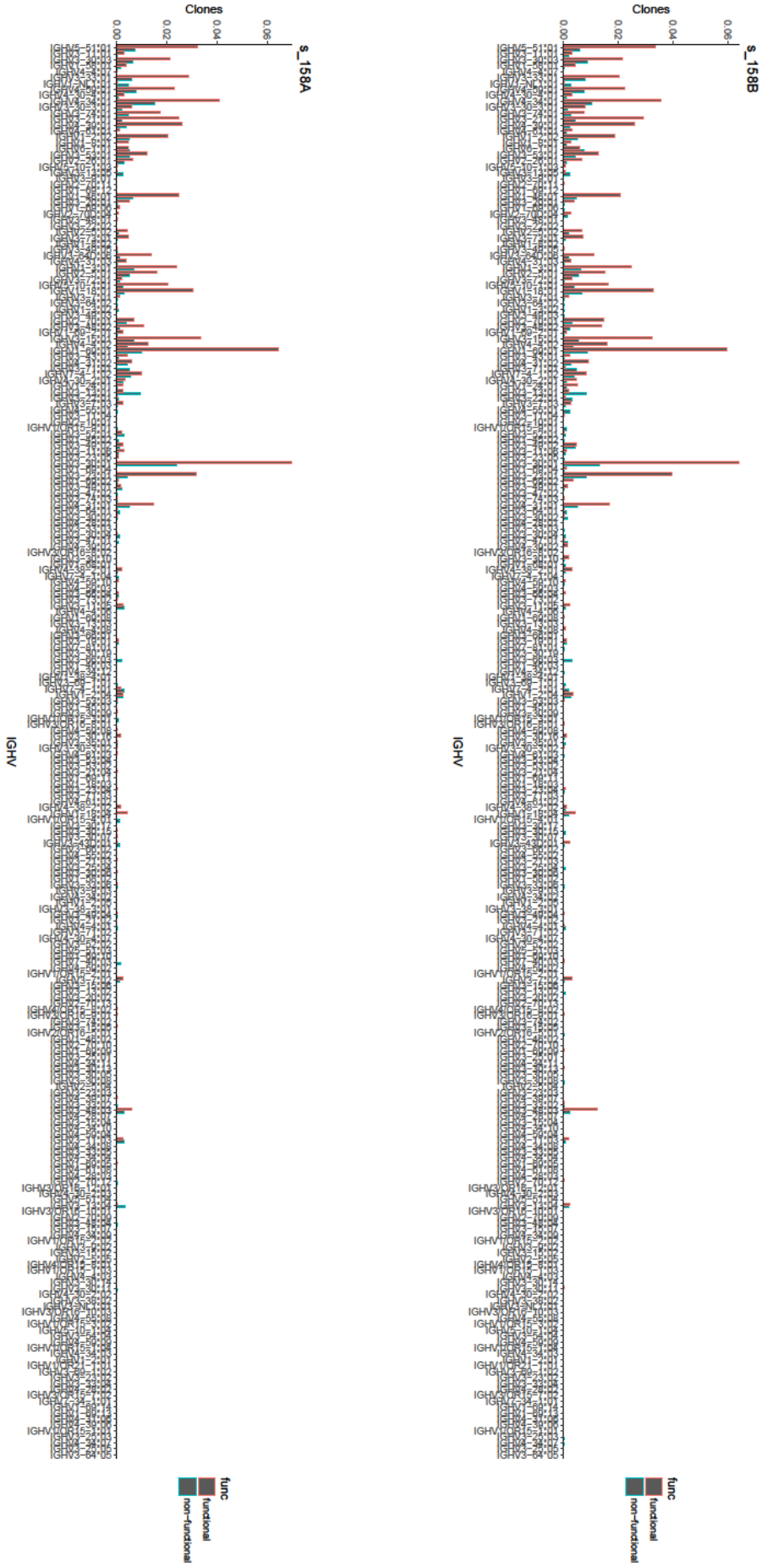
functional
non-functional

i)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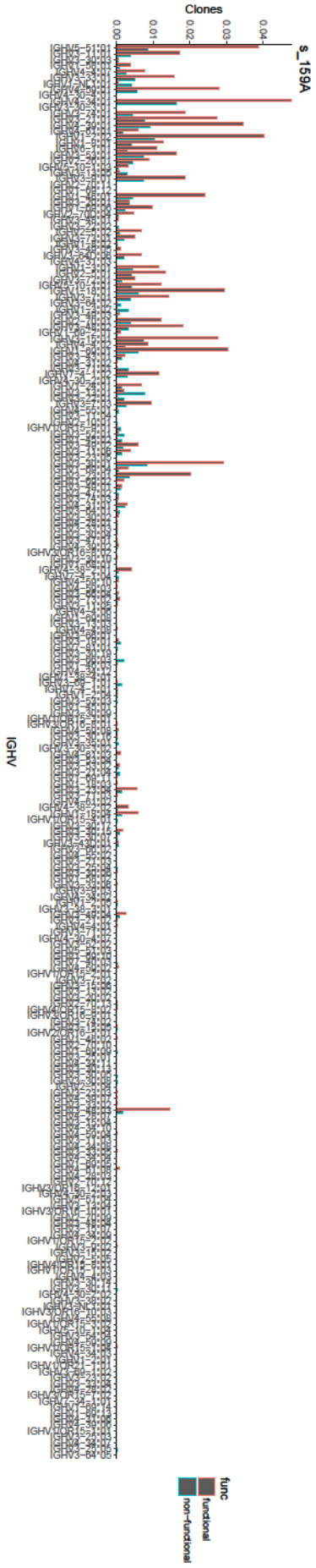
func
functional
non-functional

i)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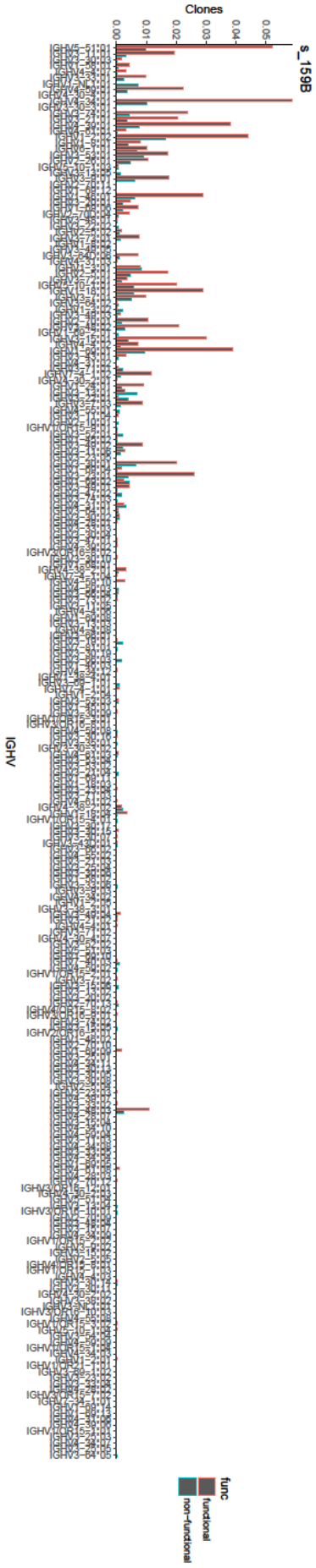
functional
non-functional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0.04
0.05
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IGHV
func
functional
non-functional

Supplementary Figure 6.3 Variable allele usage within the IGHV for each subject. Frequency of usage of V alleles by each subject, pre- (B) and post-intervention (A), all alleles shown are representative of the alleles expressed in DNA-libraries derived from samples across the entire study cohort; n=10. Plots produced in RStudio by Peter Chovanec (Babraham Institute). Clones represent the frequency of expression. Non-function (blue) and functional (orange) genes. For comparison of V allele use between baseline and post-intervention, frequencies for all alleles, represented across all Nu-AGE subjects, were compared for each subject by two-way ANOVA with Sidak's multiple comparisons test, using GraphPad Prism V7.02. This allowed comparison of each of the 246 alleles, which were classified as functional or non-functional. MED diet (Figure 6.9 a, f, i and j), Control diet (Figure 6.9 b, c, d, e, g and h).

XX. **Supplementary Figure 6.4. Variable allele usage within the IGHV ordered by regional location on the chromosome for each subject**

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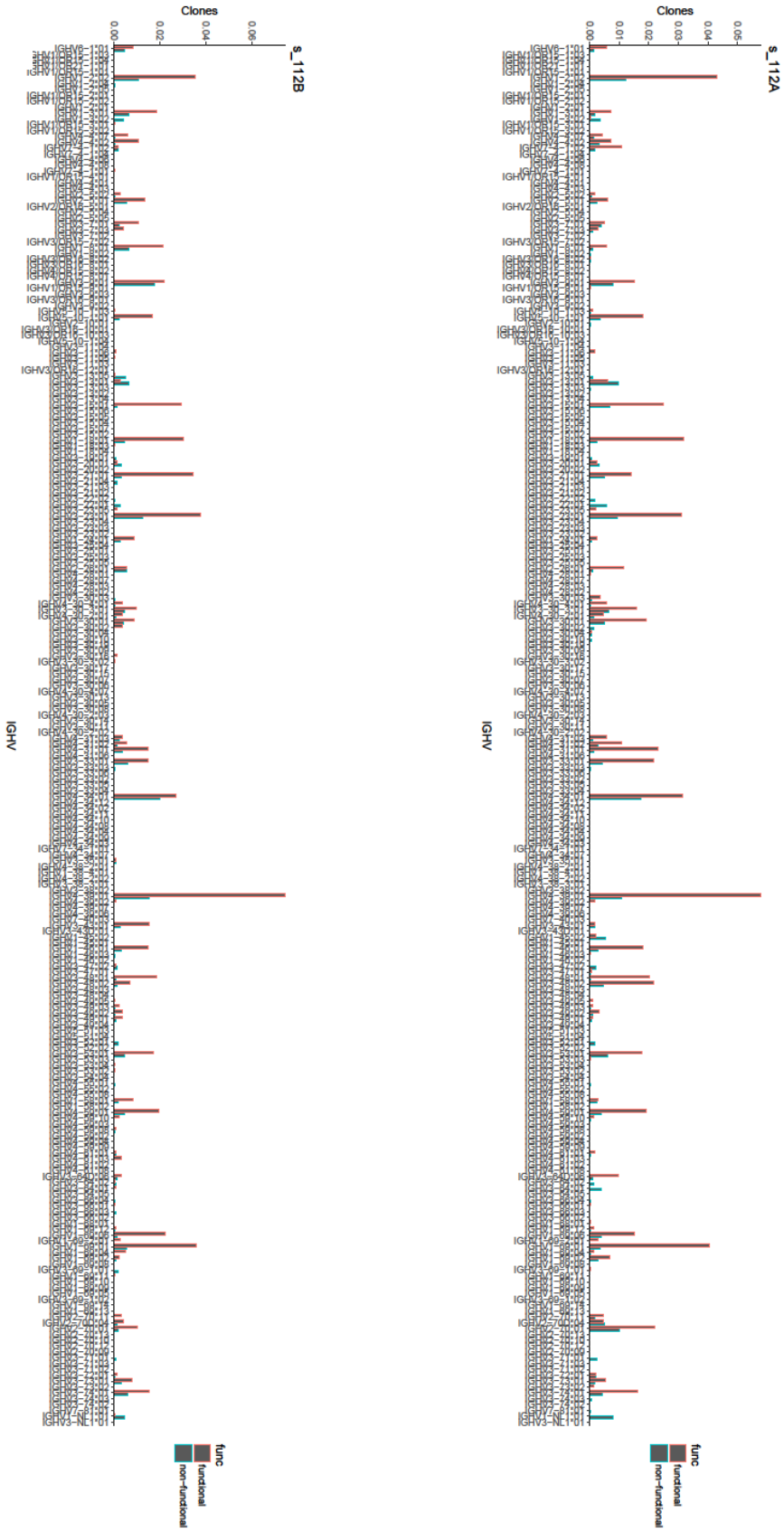

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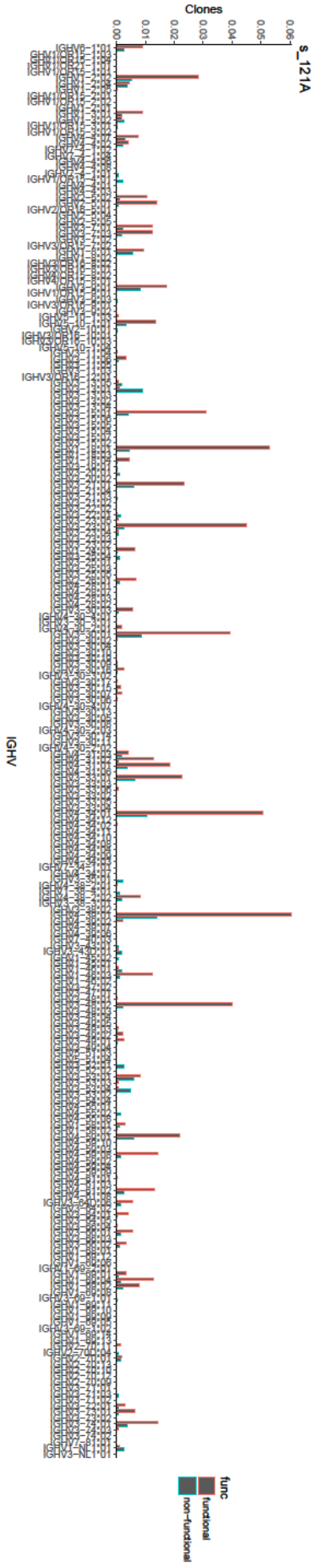
Clones
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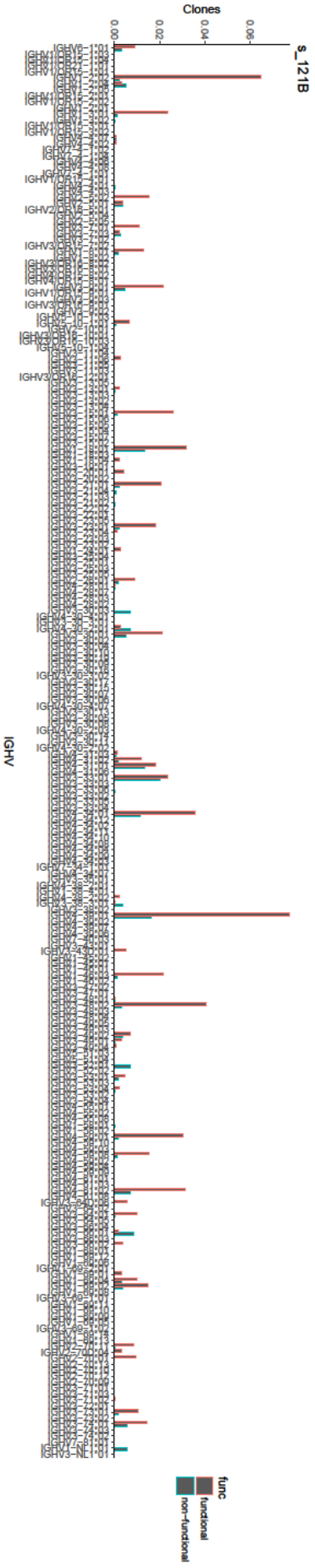
0.05
0.06
s_121A
IGHV
func
functional
non-functional


Clones
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IGHV
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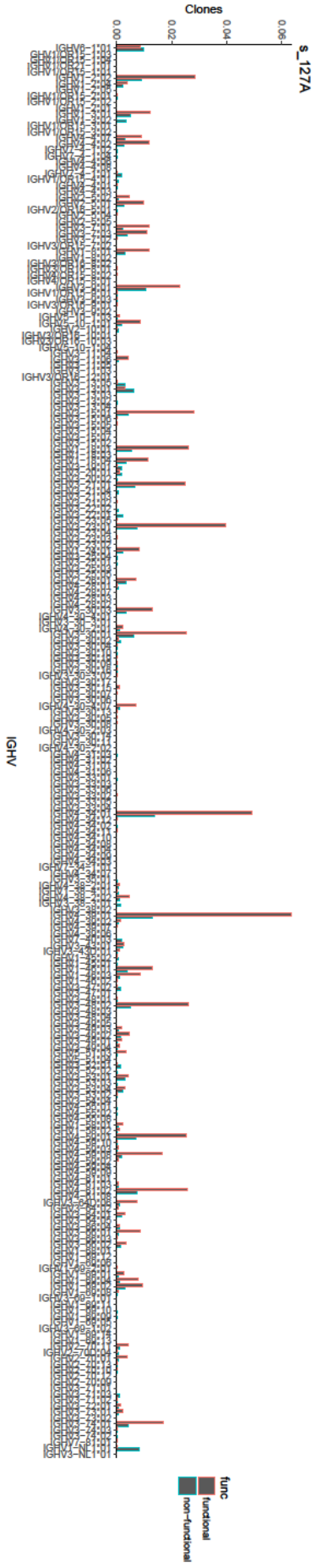
functional
non-functional

c)


Clones
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0.06
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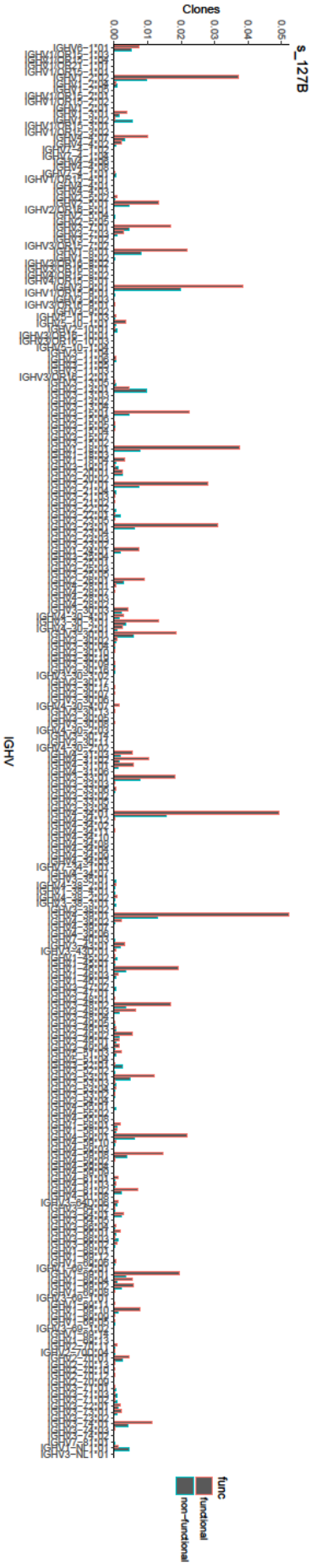
IGHV
func
functional
non-functional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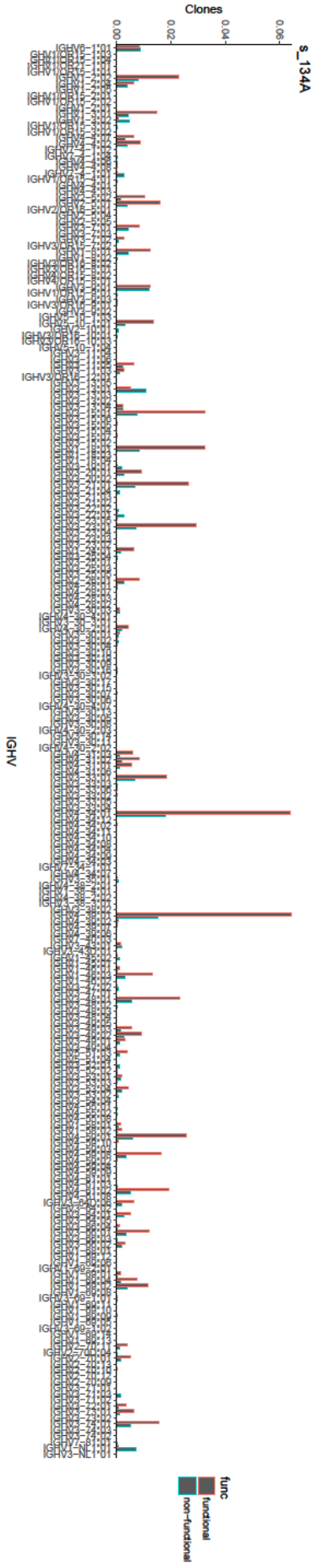
IGHV
func
functional
non-functional

d)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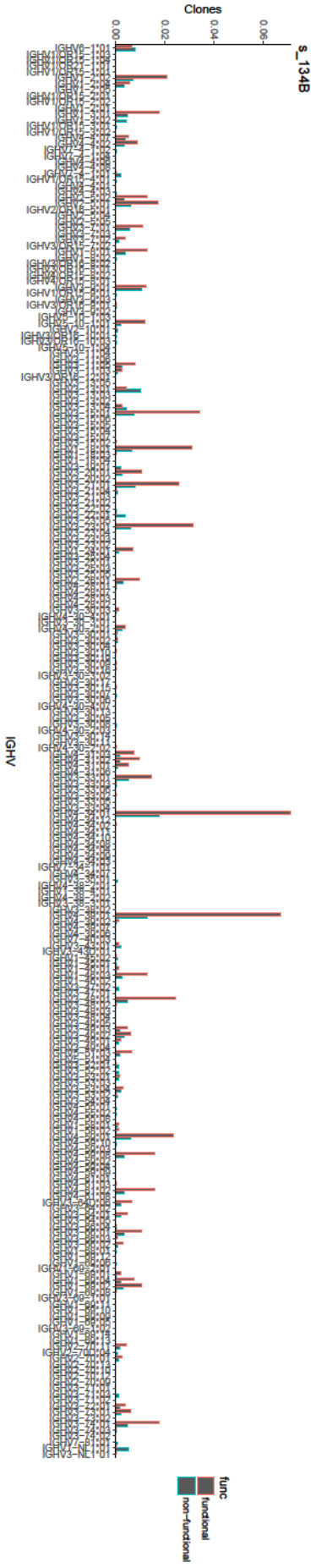
functional
non-functional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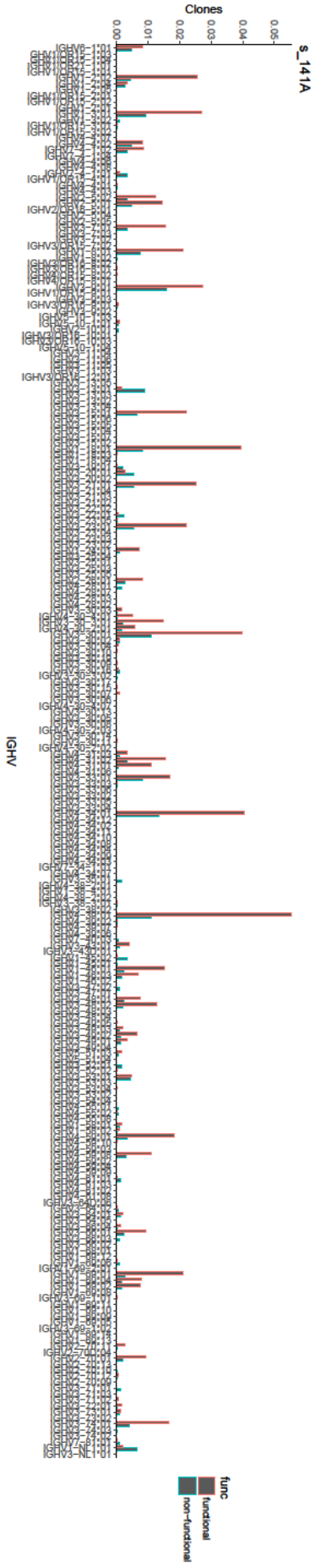
func
functional
non-functional

e)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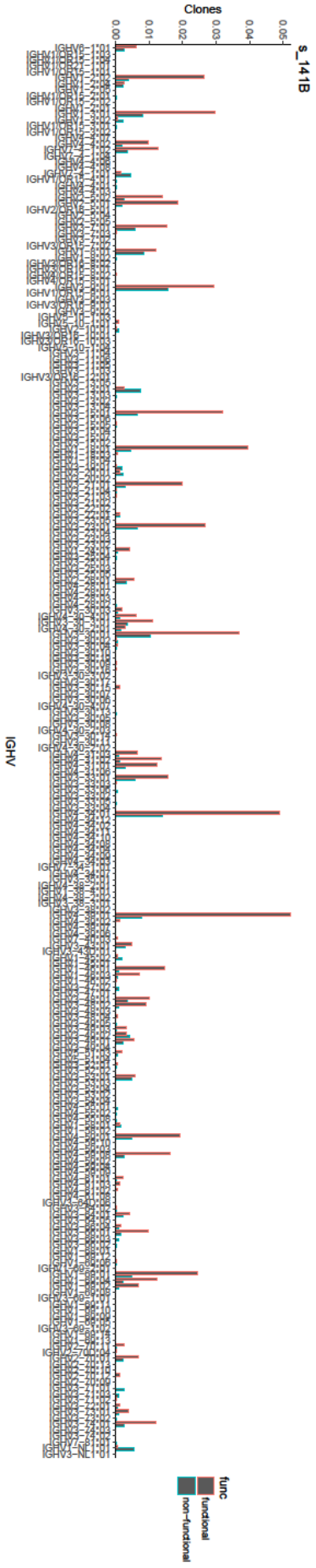
0.05
s_141A
IGHV
func
functional
non-functional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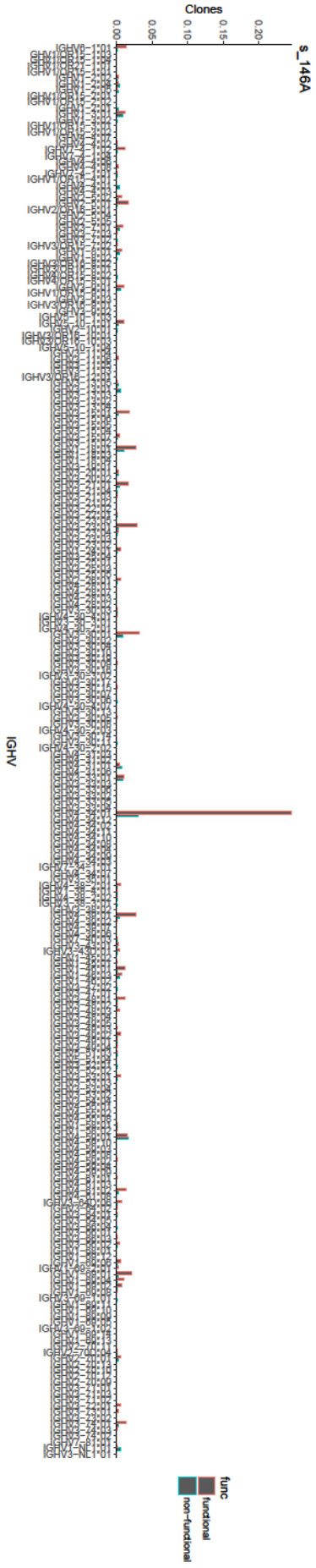
IGHV
func
functional
non-functional

f)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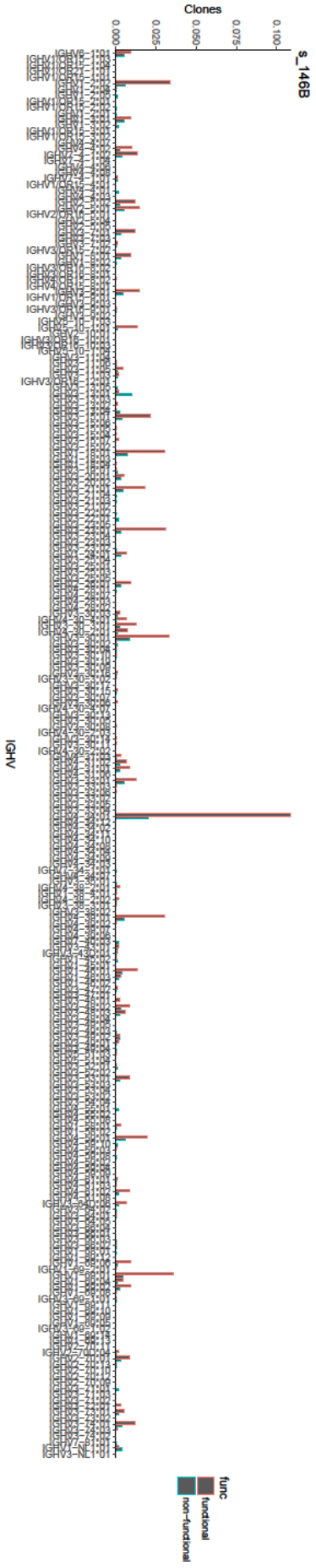
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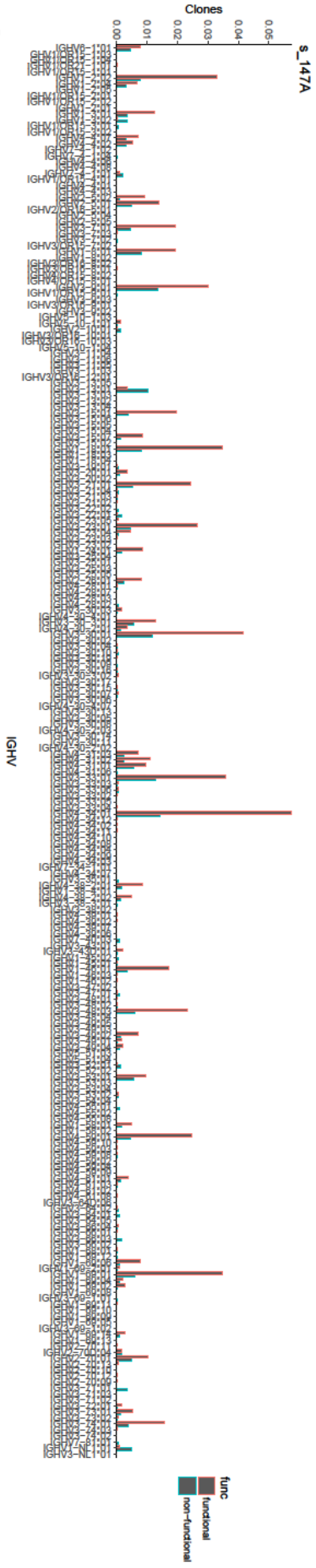
func
functional
non-functional

g)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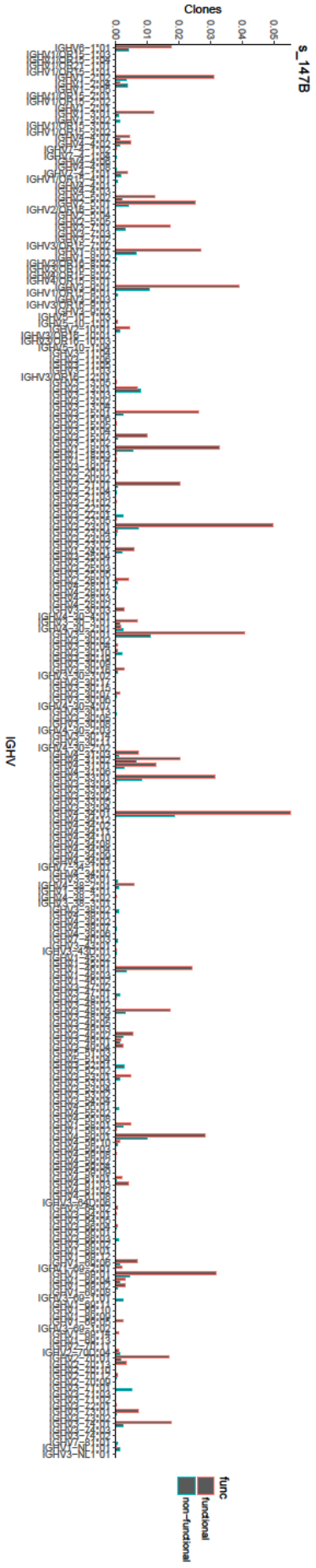
s_147A
IGHV
func
functional
non-functional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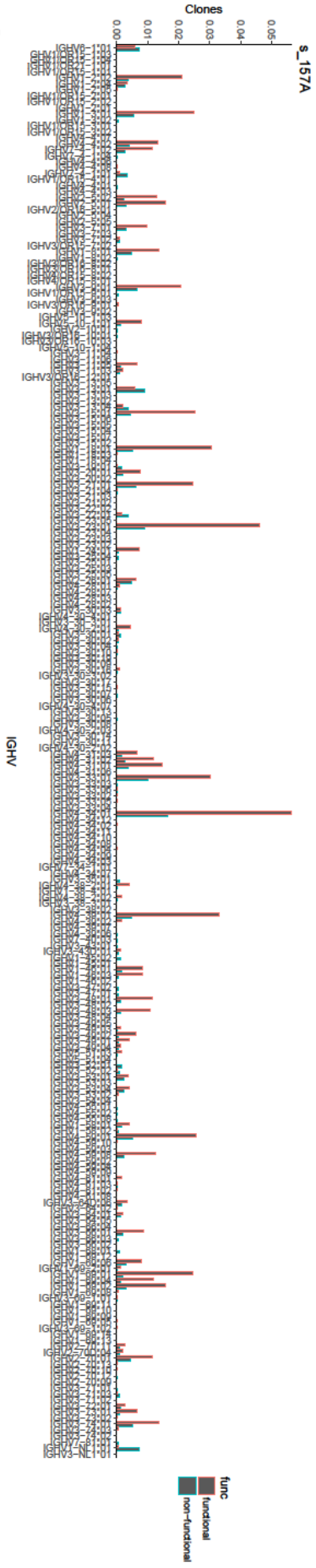
s_147B
IGHV
func
functional
non-functional

h)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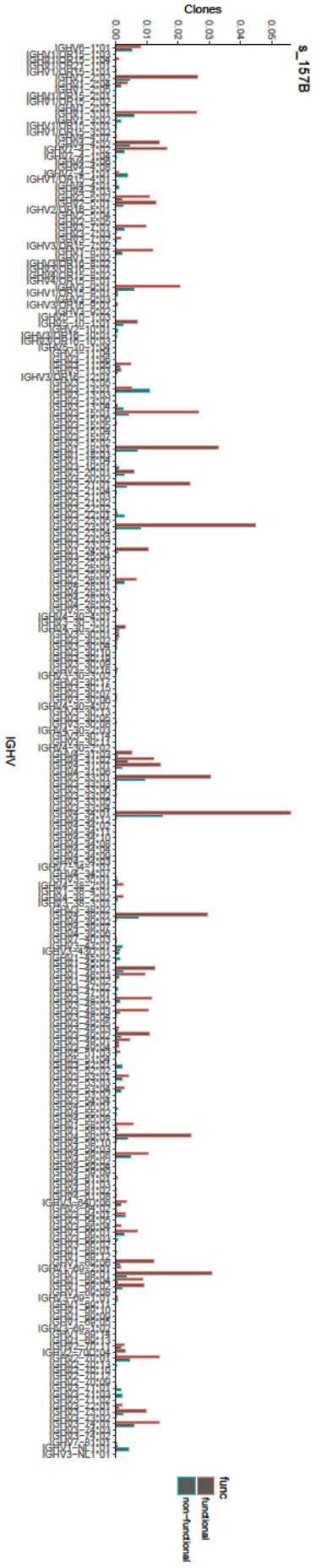
s_157A
IGHV
func
functional
non-functional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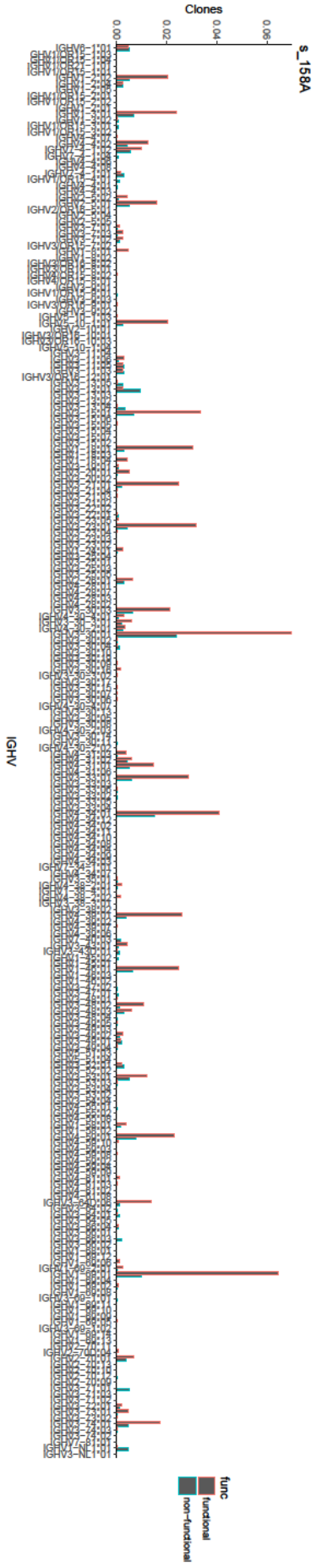
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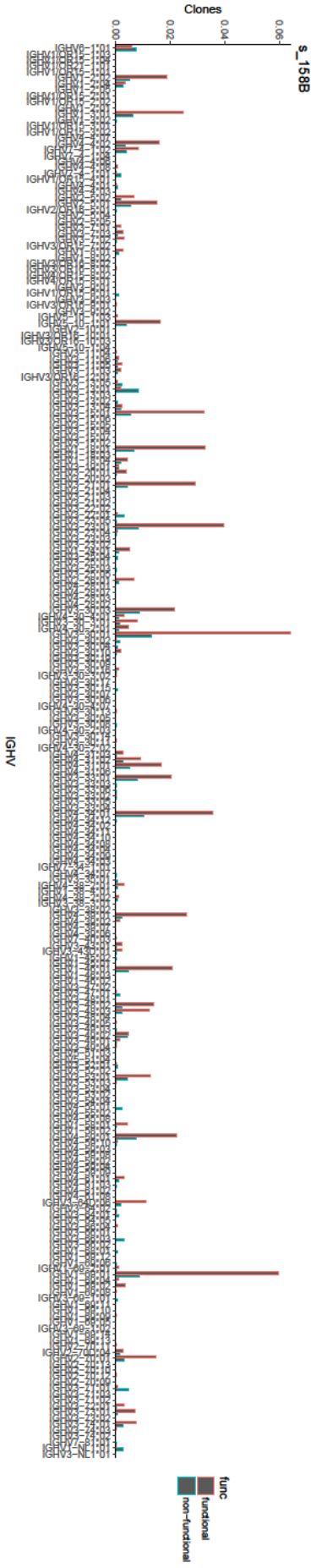
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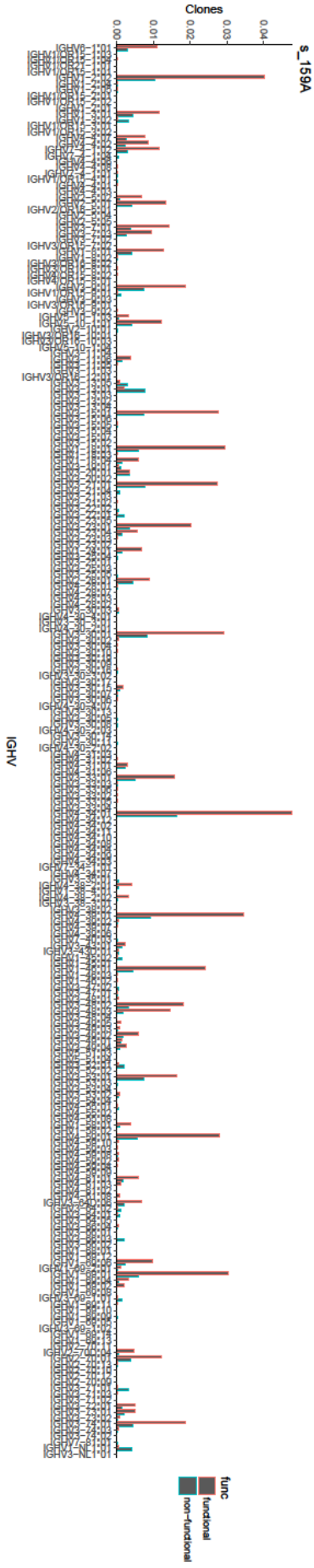
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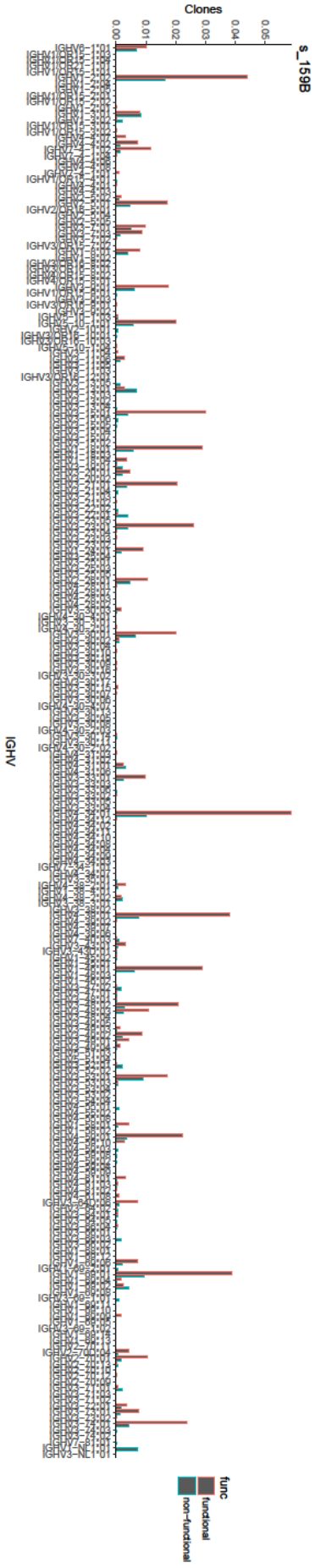
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Supplementary Figure 6.4 (a–j) Variable allele usage within the IGHV ordered by regional location on the chromosome for each subject. Regional positions of the V alleles were determined by ordering alleles according to location on the chromosome, from the 3′ to the 5′ end; carried out by Peter Chovanec (Babraham Institute) using the IMGT database. Frequency of usage of V alleles by each subject, pre- (B) and post-intervention (A), all alleles shown are representative of the alleles expressed in DNA-libraries derived from samples across the entire study cohort; n=10; graphs produced in RStudio by Peter Chovanec. Clones represent the frequency of expression. Non-function (blue) and functional (orange) genes. For statistical analysis of V allele usage, baseline and post-intervention frequencies for all alleles, represented across all Nu-AGE subjects, were compared for each subject by two-way ANOVA with Sidak's multiple comparisons test, using GraphPad Prism V7.02. This allowed comparison of each of the 246 alleles, which were classified as functional or non-functional. MED- diet group (Supplementary Figure 6.4 a, f, i and j), control diet group (Supplementary Figure 6.4 b, c, d, e, g and h).

XXI. Supplementary Table 6.5 Average sequence lengths of IGH regions

Sample	V SEQ LENGTH	D SEQ LENGTH	J SEQ LENGTH	JUNCTION LENGTH
112-PRE	142.3517	15.05936	27.37651	30.55901
112-POST	142.3784794	14.85278484	27.76518288	30.05952381
121-PRE	138.6917	13.37422	27.56166	30.25307
121-POST	137.5994356	14.07118386	28.565381	29.56274694
127-PRE	160.8628	14.91129	30.06739	35.18643
127-POST	158.5040573	13.65429853	29.84236277	33.02159905
134-PRE	170.4771	15.84149	29.56878	34.43026
134-POST	163.6021548	16.03053113	30.09228315	36.96242253
141-PRE	135.3157	14.52862	28.56405	30.95315
141-POST	138.6234283	14.81081081	28.73197648	31.30357304
146-PRE	130.976	15.33764	29.24033	31.97629
146-POST	136.7730645	14.91192299	28.5764071	31.62114967
147-PRE	141.6585	13.90832	28.2058	30.22146
147-POST	172.2778539	14.58261785	29.71529567	32.59692004
157-PRE	139.5441	15.15375	29.00526	30.92206
157-POST	148.7399057	14.99435864	28.38057662	30.66424131
158-PRE	130.969	15.1715	28.9257	31.00561
158-POST	135.177394	15.31282351	29.30422209	31.17061885
159-PRE	136.2603	13.87854	28.82845	29.09689
159-POST	171.7949604	13.89075012	29.54511962	30.29546075

Supplementary Table 6.5 Average sequence lengths of V, D, J and Junction regions of sequenced IGH of peripheral B cell derived DNA-libraries from Nu-AGE subjects, pre- and post-intervention.

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