

Effects of Broccoli-Derived Sulphur Compounds on the Prostate Microenvironment

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Abstract

Epidemiological studies suggest a negative association between prostate cancer risk and dietary intake of cruciferous vegetables. These vegetables have a characteristic sulphur metabolism, delivering specialised compounds including glucosinolates, such as glucoraphanin, and S-methyl cysteine sulfoxide (SMCSO). An interim analysis of ongoing clinical trials at Quadram Institute Bioscience suggested an increase in inorganic sulphate, adenosine 5'-diphosphate (ADP) and potential antioxidant capacity of prostate tissue following broccoli consumption.

Sulforaphane, a hydrolytic product of glucoraphanin, influences multiple pathways relevant to prostate cancer prevention. Furthermore, degradation of SMCSO produces reactive intermediates, which induce apoptosis in prostate cancer cells, and ultimately yields high proportions of inorganic sulphate in human metabolism. Sulphate is likely to drive synthesis of phosphoadenosine 5'-phosphosulphate. Adenosine 5'-triphosphate (ATP) fuels this process, producing ADP and phosphate. As cancerous cells are unable to adjust their metabolism, depleting ATP by diet could prove an effective strategy for cancer prevention.

A high-dose, randomised, parallel-unblinded broccoli-intervention study was carried out in men awaiting trans-perineal prostate biopsies. Global metabolomic and targeted metabolite analyses were undertaken on prostate, adipose and urine samples to test the biological availability and activity of sulphur-containing metabolites from the study diet. *In vitro* experiments were undertaken to investigate the effects of sulforaphane and SMCSO on real-time prostate bioenergetics and redox status of proteins relevant to prostate cancer.

SMCSO was present at significantly higher levels ($p < 0.01$) in both the urine and prostates of men receiving a broccoli-enriched diet. Whereas sulforaphane and its conjugates were undetectable in tissue. Levels of ATP and sulphate were not different between study groups. At physiological concentrations neither sulforaphane nor SMCSO affected mitochondrial function or redox status *in vitro*.

SMCSO accumulation in tissue after broccoli consumption may mediate the putative effects of cruciferous vegetables towards prostate cancer prevention through its degradation to highly-reactive intermediate products.

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Abbreviations

2-DG	2-deoxyglucose
ACAC	Acetyl CoA carboxylase
ACLY	ATP citrate lyase
ADP	Adenosine 5'-diphosphate
AJCC	American Joint Committee on Cancer
AMACR	α -methylacyl-CoA racemase
AMP	Adenosine 5'-monophosphate
AMPK	Adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
APS	Adenosine phosphosulphate
ATCC	American Type Culture Collection
ATP	Adenosine 5'-triphosphate
BBSRC	Biotechnology and Biological Sciences Research Council
BCA	Bicinchoninic acid
B-ITC	Mixed isothiocyanates
BMI	Body mass index
BPH	Benign prostatic hyperplasia
BPTES	Bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulphide
BSA	Bovine serum albumin
CPT-1	Carnitine palmitoyltransferase-1
CVFFQ	Cruciferous vegetable food frequency questionnaire
DCFH-DA	2',7'-dichlorofluorescein diacetate
DHEA-S	Dehydroepiandrosterone sulphate
DRE	Digital rectal examination
ECACC	European Collection of Authenticated Cell Cultures
ER-NAC	Erucin-N-acetyl cysteine
ESCAPE	Effect of Sulforaphane on prostate CAncer PrEvention
ESI	Electrospray ionisation

ETC	Electron transport chain
FADH ₂	Flavin adenine dinucleotide reduced
FASN	Fatty acid synthase
FCCP	Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone
FCS	Foetal calf serum
FDG	Fluorodeoxyglucose
GPCR	G-protein coupled receptor
GSH	Glutathione reduced
GSTM1	Glutathione S-transferase Mu 1
GSTP1	Glutathione S-transferase pi
GSTT1	Glutathione S-transferase theta-1
HBSS	Hank's balanced salt solution
HG-PIN	High-grade prostatic intraepithelial neoplasia
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High-performance liquid chromatography
HRA	Health Research Authority
HRGC	Human Research Governance Committee
IFR	Institute of Food Research
IRAS	Integrated research application system
ITC	Isothiocyanate
Keap1	Kelch-like ECH-associated protein 1
LC-MS/MS	Liquid chromatography tandem mass spectroscopy
LIMS	Laboratory information management system
MMAC1	Mutated in multiple advanced cancers
MMTSI	Dimethyl disulphide sulfoxide
MMTSO	Dimethyl disulphide sulfone
mTORC1	Mammalian target of rapamycin complex 1
NAD ⁺	Nicotinamide adenine dinucleotide

NADH	Nicotinamide adenine dinucleotide reduced
NICE	National Institute for Clinical Excellence
NNUH	Norfolk and Norwich University Hospital
NRES	National Research Ethics Services
Nrf-2	Nuclear factor erythroid 2-related factor 2
PAPS	3'-phosphoadenosine 5'-phosphosulphate
PBS	Phosphate buffered saline
PCF	Prostate Cancer Foundation
PK1	3-phosphoinositide dependent kinase 1
PEITC	Phenethyl isothiocyanate
PET	Positron emission topography
PI3K	Phosphoinositide 3-kinase
PIP2	phosphatidylinositol-4,5 bisphosphate
PIP3	Phosphatidylinositol 3,4,5 triphosphate
PIS	Participant Information Sheet
PMSF	Phenylmethanesulfonyl fluoride
PSA	Prostate specific antigen
PTEN	Phosphatase and tensin homologue
PVDF	Polyvinyl difluoride
QC	Quality control
QIB	Quadram Institute Bioscience
REC	Research Ethics Committee
ROS	Reactive oxygen species
RSD	Relative standard deviation
RTK	Receptor tyrosine kinase
SAP	Sulphate Accumulation in Prostate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SELECT	SELenium and vitamin E Cancer prevention Trial

SF-Cys	Sulforaphane-cysteine
SF-GSH	Sulforaphane-glutathione
SF-NAC	Sulforaphane-N-acetyl cysteine
SHBG	Sex hormone binding globulin
SMCSO	S-methyl-L-cysteine sulfoxide
TCA	Tricarboxylic acid
TNM	Tumour node metastasis
TPB	Trans-perineal template prostate biopsy
TRUS	Trans-rectal ultrasound scan
UEA	University of East Anglia
UPLC	Ultra-performance liquid chromatography
USDA	United States Department of Agriculture
WHO	World Health Organisation

1 General Introduction

1.1 The Prostate Gland

The prostate gland is an exocrine organ of the male genitourinary tract, located at the base of the bladder, anterior to the rectum and encompassing the urethra. The primary function of the prostate is to secrete prostatic fluid during ejaculation, which contributes 30% of the total volume to semen [1]. Enzymes, proteins and nutrients within this alkaline fluid provide support for the survival of spermatazoa in the acidic conditions of the vagina [2]. Prostate-specific antigen (PSA), a protease that liquefies semen by breaking down high molecular weight proteins, is a constituent of this fluid that facilitates free motility of sperm. PSA is also detectable in serum and is used as a common laboratory test to aid prostate cancer diagnosis [3, 4].

The prostate is divided into three major zones; central, transitional and peripheral. The central zone surrounds the ejaculatory ducts, which run from the seminal vesicles to the prostatic urethra. The transitional zone surrounds the urethra as it descends from the bladder. It is the most common site for development of benign prostatic hyperplasia (BPH), a nodular enlargement of the prostate that compresses the urethra causing symptomatic impairment to urinary flow. The peripheral zone is the largest zone of the prostate and is the most easily palpable during digital rectal examination (DRE) [5]. 70-80% of all prostate cancers originate in the peripheral zone [6, 7].

1.1.1 Prostate Cancer

Prostate cancer is the second most commonly diagnosed cancer in the UK. Men carry a lifetime risk of 1 in 8, with incidence being strongly linked to age (Figure 1-1) [8]. Worldwide, the highest incidences of prostate cancer are seen in Australia and New Zealand, and the lowest in South Central Asia [9]. The aetiology, however, remains relatively poorly understood. Geographical variation in the incidence of prostate cancer cannot be explained by known risk factors such as age, race and family history. This implicates environmental factors, including diet, in the risk of developing prostate cancer. There is also growing evidence to suggest that multifocal tumours arising within the same prostate gland have discrete mutational profiles with varying malignant potential [10]. Multifocal prostate cancer could, therefore, be susceptible to metabolic changes induced by dietary interventions that generate a hostile environment against the emergence and proliferation of cancerous clones.

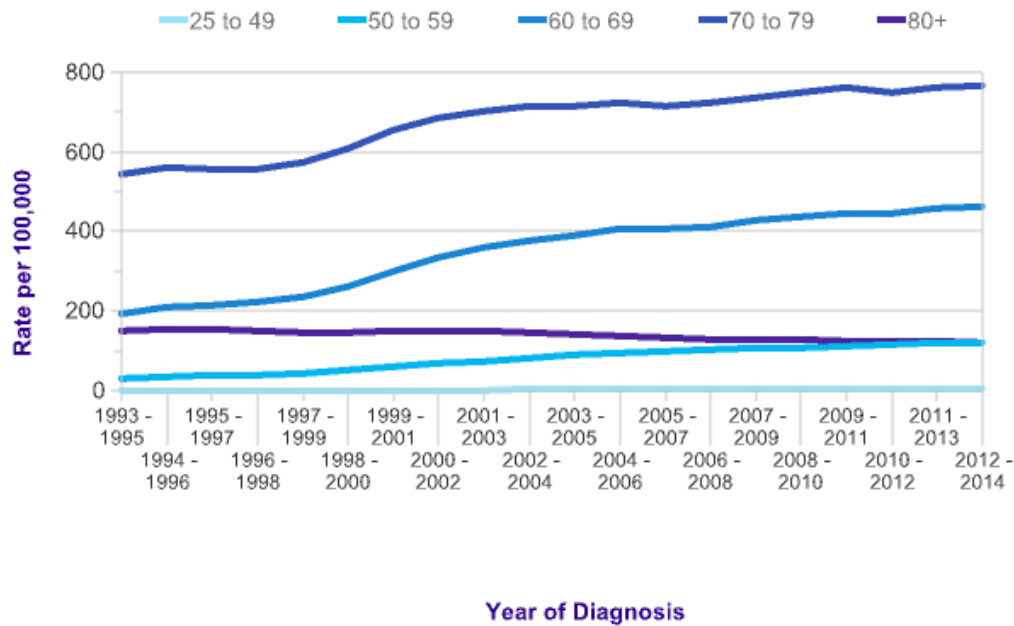


Figure 1-1 Cancer Research UK statistics for prostate cancer incidence by age in UK males, 1993-2014 [8].

1.1.2 Prostate Cancer Diagnosis

There is no current screening programme for prostate cancer in the UK. Although men can request a PSA level to be checked by their General Practitioner, uptake between the ages of 45-89 is currently only 6%, with little change over the last decade [11]. Prostate cancer is predominantly diagnosed by trans-rectal ultrasound (TRUS) and needle biopsy once the findings of either an abnormal DRE or elevated serum PSA have been established. Standard practice for first biopsy is to take 10-12 needle biopsy cores through the rectum, under local anaesthetic and with appropriate antibiotic prophylaxis. Magnetic Resonance Imaging (MRI) is not routinely used in screening for prostate cancer. However, it is recommended by the National Institute of Clinical Excellence (NICE) for staging the disease after diagnosis, or for additional information prior to a second prostate biopsy if the first was benign and clinical suspicion remains high [12].

Trans-perineal template biopsy of the prostate (TPB) is indicated to increase the diagnostic yield of a second biopsy following an initial negative or inconclusive TRUS biopsy. Sampling of the anterior prostate is improved by this technique, a larger number of cores are taken and in some cases suspicious lesions detected by MRI may be specifically targeted (Figure 1-2) [13]. All prostate tissue samples from participants in the human intervention studies described in this thesis (ESCAPE and SAP, Section 1.3 and Chapter 2 respectively) were obtained during TPB.

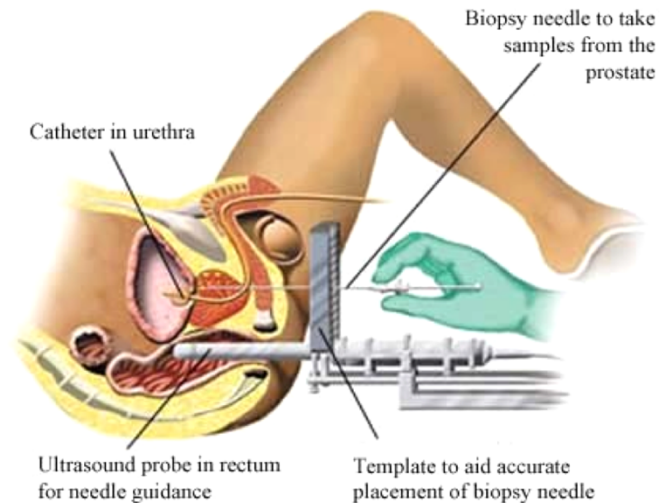


Figure 1-2 The procedure for trans-perineal template prostate biopsy. Image from the British Association of Urological Surgeons' patient information sheet on trans-perineal prostate biopsies [14].

1.1.3 Prostate Cancer Grade and Stage

The histopathological grade of prostate cancer is determined by the appearance of the tissue under a microscope. The most commonly used scoring system was developed by Donald Gleason and colleagues in the 1960s, categorizing the architectural patterns from 1 to 5. A well-differentiated appearance is scored 1 and the least differentiated is scored 5, with separate scores being assigned to both the primary and secondary patterns observed. Combining both primary and secondary patterns provides a Gleason Sum between 2 and 10 (e.g. Gleason 3+4=7), a higher score corresponding to progressive loss of differentiation and therefore worsening prognosis [15]. The system was revised in 2005, reallocating the groups to which certain morphologies were assigned and improving the overall performance [16]. As Gleason patterns 1-2 are considered normal, the minimum Gleason Sum to amount to cancer is 6 (Gleason 3+3). For this reason a simplified 'Grade Group' system was implemented by the World Health Organisation (WHO) in 2016, reclassifying cancers into groups from 1 to 5 according to prognostic risk and modified Gleason score (Figure 1-3) [17].

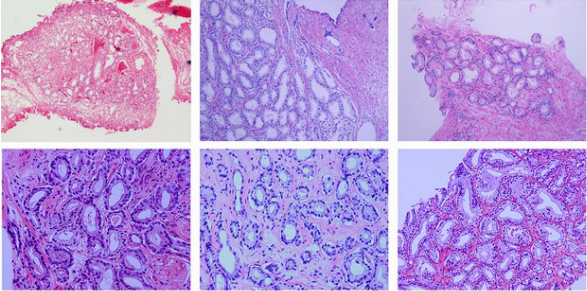
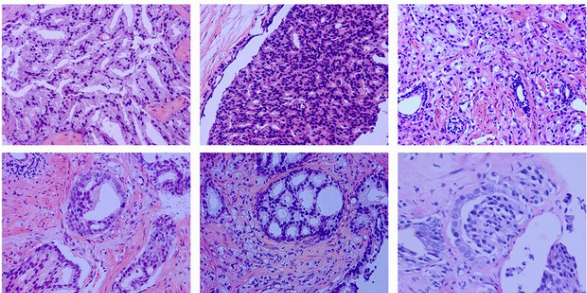
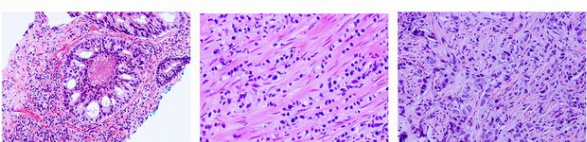
	Gleason patterns 1-3 distinct, discrete, individual glands	Gleason score ≤ 6	Grade group I
	Gleason pattern 4 fused, cribriform, or poorly-formed glands, or glomerular	Gleason score 3+4=7	Grade group II
	Gleason pattern 5 comedo necrosis, cords, sheets, solid nests, single cells	Gleason score 4+3=7 4+4=8 3+5=8 5+3=8	Grade group III
		Gleason score 4+5=9 5+4=9 5+5=10	Grade group IV
			Grade group V

Figure 1-3 The histological appearance, Gleason pattern, Gleason score and corresponding WHO Grade Group of prostate cancer described by Chen and Zhou, 2016 [18].

The TNM classification system, as described by the American Joint Committee on Cancer (AJCC), is used to describe the stage of the tumour according to the primary tumour (T), spread to regional lymph nodes (N) and distant metastases (M) [19]. The grade, stage and PSA are used to stratify the risk from prostate cancer and to inform decisions about how each patient is managed.

1.1.4 The Metabolism of Prostate Cancer

Normal cells utilise glucose via enzymatic breakdown in glycolysis to produce pyruvate. In aerobic conditions, pyruvate enters the mitochondria where it is converted to acetyl CoA by pyruvate dehydrogenase and subsequently to citrate to fuel the tricarboxylic acid (TCA) cycle [20]. Adenosine 5'-triphosphate (ATP) is produced by oxidative phosphorylation as a universal energy source [21]. Prostate epithelial cells have a characteristic metabolism that facilitates the import of zinc and excretion of citrate into prostatic fluid as an energy source for sperm [22]. The high concentration of zinc inhibits aconitase, preventing enzymatic conversion of citrate to iso-citrate in the TCA cycle. Citrate exits the mitochondria and is either excreted into prostatic fluid from the cytosol or converted back to acetyl CoA by ATP citrate lyase (ACLY) for lipid and cholesterol synthesis [23]. Thus, prostate cells are highly glycolytic, rely on aspartate and glutamine to replace intermediates of the TCA cycle and utilise more fatty acids through beta oxidation for energy production than other cell types [24].

Cancers at most tissue sites tend to adjust their metabolism in a process called the Warburg Effect; utilising glucose via glycolysis and producing lactate even when oxygen is abundant [25]. Prostate cancer, however, demonstrates a range of

relatively exclusive characteristics. High glucose dependency is not a typical feature, which accounts for the poor accumulation of ^{18}F -fluorodeoxyglucose (FDG) [26]. The radio-labelled isotope is detectable by positron emission topography (PET) and commonly used in staging other cancer types, but has limited clinical value in prostate cancer [27]. Instead, prostate cancers reverse the usual excretion of citrate and rely heavily on fatty acid beta oxidation. Loss of zinc accumulation and downstream aconitase inhibition by cancerous cells redirects citrate back into the TCA cycle. Subsequent production of ATP via oxidative phosphorylation meets the high demands of the proliferating cancer [28]. Cytosolic citrate is also more readily converted to acetyl CoA by ACLY, an enzyme that is commonly upregulated in prostate cancer [29]. Acetyl CoA is a central substrate for cholesterol and fatty acid synthesis and histone acetylation, all of which have close links to prostate cancer. Increased *de novo* fatty acid synthesis is dependent on the catalytic activity of both acetyl CoA carboxylase (ACAC) and fatty acid synthase (FASN). Upregulation of the latter is so common in prostate cancer it is often referred to as an oncogene [30]. Fatty acids form building blocks for cell growth and proliferation. Alternatively, they can be used as an energy source by beta oxidation, which first requires transformation of branched chains by α -methylacyl-CoA racemase (AMACR). In prostate cancer AMACR overexpression is characteristic, making it a useful marker for immunohistochemistry staining of histopathology specimens, with a sensitivity of 82-100% [31].

Normal epithelial cells of the prostate therefore have metabolic characteristics that resemble a more cancerous than healthy phenotype of other body cells. This may predispose them to malignant transformation and contribute towards the high overall incidence of prostate cancer [32]. Dysregulation of both citrate and fatty acid metabolism is typical in prostate cancer, though multiple metabolic pathways are likely to be involved in both development and prevention of the disease.

1.1.5 Prostate Exposure to Urinary Metabolites

Under normal resting conditions the detrusor muscle of the bladder is relaxed and both the internal and external urethral sphincters are contracted, allowing the bladder to fill. During micturition (voiding) the opposite occurs; the detrusor muscle contracts and both sphincters relax to allow urine to be expelled from the bladder [33]. The prostate gland in men lies directly between the internal urethral sphincter, also known as the bladder neck, and the external urethral sphincter (Figure 1-4). The prostatic and ejaculatory ducts open into the prostatic urethra between these two sphincters, posing a risk of urinary reflux if flow is restricted at any point distal to the bladder neck.

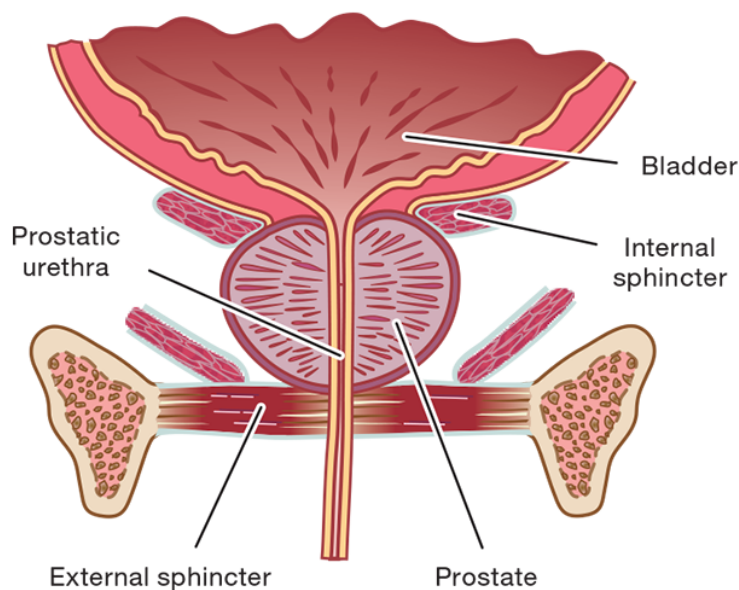


Figure 1-4 Sphincter anatomy of the prostate. Image for patient information from the Prostate Cancer Foundation (Australia) [34].

Reflux of urine into the prostatic ducts was first proven in the 1980s [35]. Several days before undergoing a trans-urethral resection of the prostate (TURP), 10 men with chronic prostatitis received an intra-vesical instillation of carbon microspheres. 70% of the resected prostate tissue was later found to contain microspheres, implicating urinary reflux in the aetiology of chronic inflammation [35]. Consistent with chemical irritation from urine exposure, the prostate secretions from men with abacterial prostatitis also contain higher concentrations of waste products urate and creatinine [36]. Other inflammatory conditions of the prostate include xanthogranulomatous prostatitis; a potential complication of intra-vesical bacillus Calmette-Guerin (BCG) used in the treatment of high-risk non-muscle invasive bladder cancer. The resultant granuloma formation is not limited to the tissue immediately adjacent to the urethra but occurs throughout all zones of the prostate gland, proving the extent to which urinary reflux occurs [37, 38].

Although the role of urinary reflux has been largely studied in the context of pathological contact with irritants, urine may also provide a valuable route of exposure to highly-concentrated dietary metabolites. Modulation of inflammation by lycopene in the diet has previously been suggested by this route [39]. However, comparison of tissue concentrations of specific diet-derived metabolites at sites with and without exposure to urine has not previously been made.

1.2 Cruciferous Vegetables and Prostate Cancer

Cruciferous vegetables belong to the family of Brassicaceae, which include a wide range of cultivars, including the most commonly consumed green-leaf vegetables (*Brassica oleracea*) such as broccoli, cabbage, brussels sprouts and cauliflower. A meta-analysis of population-based case-control and cohort studies identified a negative correlation between intake of cruciferous vegetables and prostate cancer incidence [40]. This extended to an overall reduction in the risk of locally advanced

cancer (higher T stage). Habitual intake of cruciferous vegetables is especially high in Asia where the incidence of prostate cancer is the lowest worldwide [9].

Several mechanisms have been suggested by which these vegetables exert their potential anti-cancer effects. The biological activity is primarily attributed to isothiocyanates (ITCs) derived from glucosinolates. *In vitro* studies of ITCs have demonstrated their influence on multiple mechanisms associated with chemoprevention [41]. Glucoraphanin, the most abundant glucosinolate in broccoli, is broken down to the ITC sulforaphane (1-isothiocyanato-4-methylsulfinylbutane) by myrosinase. This enzyme, however, is commonly denatured during cooking, in which case glucoraphanin is broken down by thioglucosidase activity of gut microbiota (Figure 1-5) [42]. Sulforaphane has been shown in animal models and *in vitro* studies to modulate enzymes involved in activation and detoxification of carcinogens, induce apoptosis in prostate cancer cells, and inhibit both inflammation and angiogenesis [43, 44]. The major molecular target for these biological effects is nuclear factor erythroid 2-related factor 2 (Nrf-2), a regulator of phase I and phase II detoxification enzymes, antioxidant defence and nicotinamide adenine dinucleotide phosphate (NADPH) regeneration [45]. Sulforaphane prevents Kelch-like ECH-associated protein (Keap)1 ubiquitination of Nrf-2, increasing both its nuclear abundance and activity. However, despite the multi-modal influence of sulforaphane on such a wide range of cell-signalling and metabolic pathways, human-intervention trials have so far been unable to confirm its role as either a preventive or therapeutic agent against prostate cancer [46, 47]. The significant effects of POMI-T, a polyphenol-rich dietary supplement that also included sulforaphane, on PSA rise in prostate cancer was a result of four phytochemicals in combination rather than individual ingredients [48].

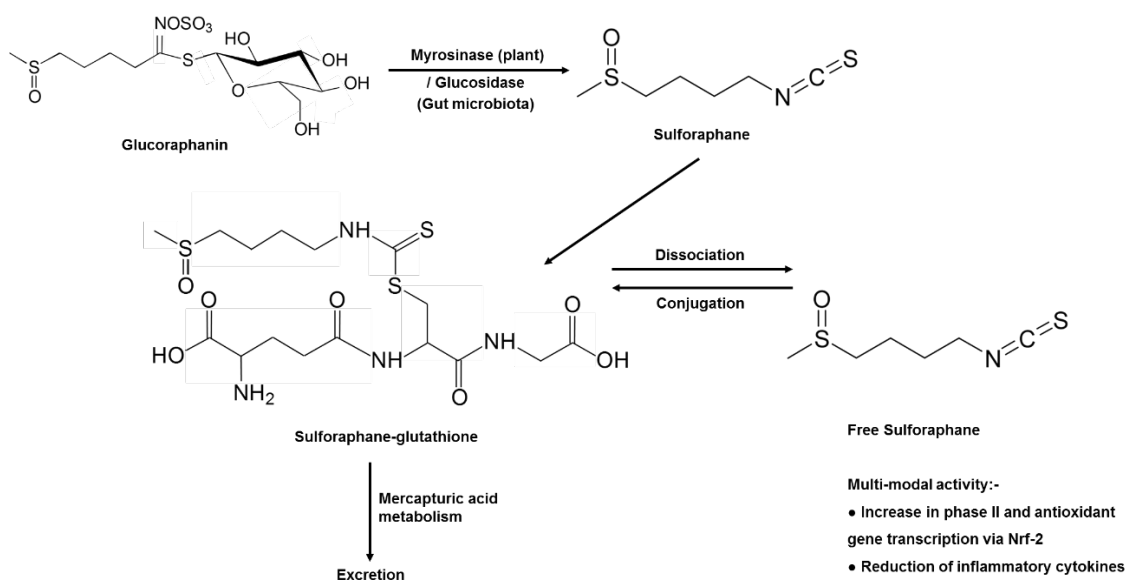


Figure 1-5 Pathway for human metabolism of glucoraphanin. Enzymatic breakdown produces sulforaphane, which is readily absorbed by the gut and conjugated with glutathione. Sulforaphane may either be cleaved from glutathione or metabolised via the mercapturic acid pathway. Redrawn from [49].

In addition to accumulation of ITCs, the unique sulphur metabolism of these vegetables allows them to deliver inorganic sulphate and S-methyl-L-cysteine sulfoxide (SMCSO), which may also offer significant biological activity [50, 51]. SMCSO is degraded by cysteine lyase to produce intermediates, including dimethyl disulphide sulfoxide (MMTSI) and dimethyl disulphide sulfone (MMTSO) (Figure 1-6). Both products provide *in vivo* protection against known carcinogens such as benzo[a]pyrene, Aflatoxin B₁ and azoxymethane in animal models of skin, bone marrow and colon cancer respectively [52-54]. MMTSI also induces apoptosis in human prostate cancer cell lines via both caspase-dependent and independent mechanisms [55]. However, the most noteworthy characteristic in the metabolic fate of SMCSO is the high yield (up to 50%) of inorganic sulphate recovered in urine after consumption of radio-labelled [³⁵S]-SMCSO [56]. The relevance of sulphate production to prostate cancer and the work conducted in this thesis are discussed below.

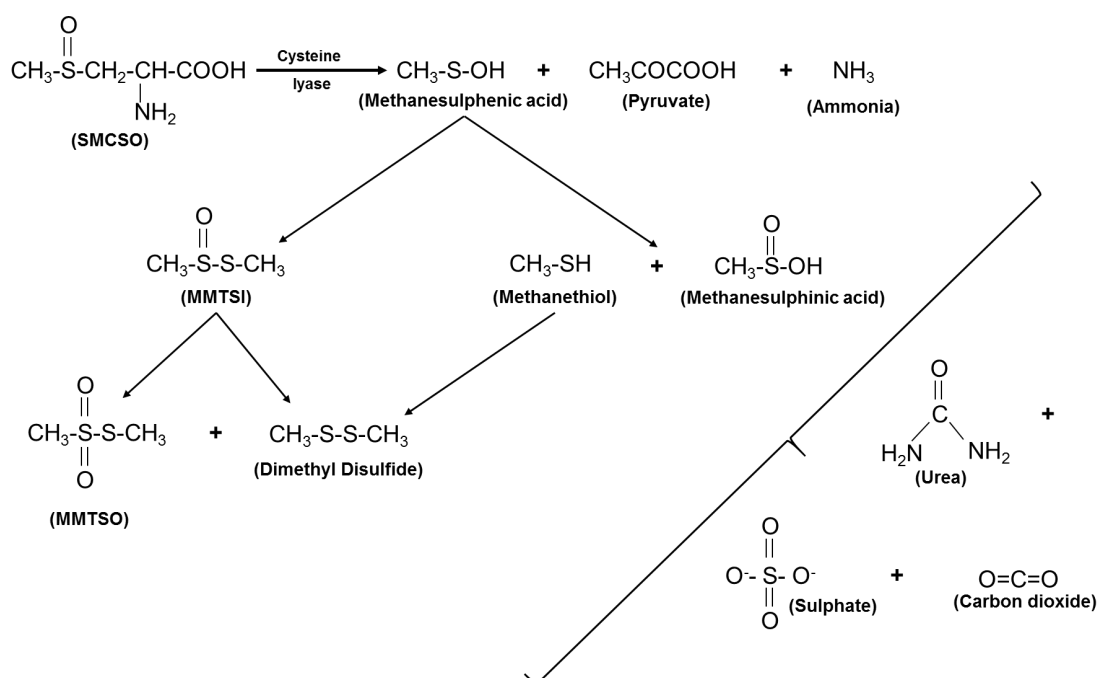


Figure 1-6 Pathway for metabolism of SMCSO by cysteine lyase. Reactive intermediates include MMTSI, MMTSO and dimethyl disulfide. Adapted from [57] to include predominant end products in urine after consumption of radio-labelled SMCSO (right).

1.2.1 Glutathione S-Transferase Mu 1 Genotype

Systematic reviews and meta-analyses have identified associations between genetic polymorphisms in glutathione S-transferases (GSTs) and the development of prostate cancer [58, 59]. GSTs are part of a broad family of phase II detoxification enzymes which have roles in both inactivating carcinogens and utilising GSH to remove oxidative metabolites that could otherwise damage DNA. Therefore, those with deficiencies in GSTs are potentially at an increased risk of developing cancers at multiple sites. Of the 8 recognised classes of GSTs, three have been intensively studied in the context of prostate cancer; glutathione S-transferase Mu 1 (GSTM1), glutathione S-transferase theta-1 (GSTT1) and glutathione S-transferase pi

(GSTP1) [60]. The consistently reported finding is that the inherited GSTM1 homozygous deletion polymorphism (GSTM1 null) genotype confers the greatest risk of developing prostate malignancy due to loss of enzyme function [58].

GSTM1 is among the many antioxidant genes regulated by Nrf-2, which accumulates in the nucleus after exposure to dietary ITCs such as sulforaphane from broccoli. Epidemiological studies have shown that the reduction in prostate cancer risk specifically due to cruciferous vegetable consumption is higher in those with a functioning GSTM1 allele [61]. The metabolism and glutathione conjugation of sulforaphane have been linked to GSTM1 genotype, and bioavailability studies show that sulforaphane and its biologically-active conjugates are more rapidly cleared (lost) from circulation in GSTM1 null individuals [62-64]. GSTM1 genotype is, therefore, considered an important explanatory variable when interpreting results from all broccoli-based dietary-intervention studies conducted at QIB.

1.3 Human Studies Evidence from High-Glucosinolate Broccoli Trials at QIB

Ongoing clinical trials at the Quadram Institute Bioscience (QIB) seek to explore the potential health benefits of high-glucosinolate broccoli. By selective breeding and introgression of a Myb28 allele from wild-type *Brassica villosa*, cultivars of broccoli have been developed on the Norwich Research Park to express three-fold (Beneforte[®]) and six-fold (Beneforte Extra) higher concentrations of glucoraphanin in their florets [65].

The Effect of Sulforaphane on Prostate Cancer Prevention (ESCAPE) study (REC 13/EE/0110; NCT01950143) is a 12-month dietary intervention study that first started recruiting in 2014. It was designed to test the effects of increasing sulforaphane concentrations on prostate metabolism by intervening with a single portion of glucoraphanin-enriched broccoli soup per week. The high-glucosinolate broccoli varieties, Beneforte[®] and Beneforte Extra, were therefore compared with a standard broccoli soup control. Men were recruited and randomised to one of the three broccoli soup diets after deciding on active surveillance for management of either low or intermediate-risk, organ-confined prostate cancer. The approach of active surveillance is to offer regular clinical follow up to monitor for any signs of disease progression, delaying or potentially avoiding the potential side effects of radical treatments such as radiotherapy or surgery [66]. In addition to regular PSA and DRE monitoring, men on the ESCAPE study underwent TPB both before and after 12 months of the dietary intervention. Prostate needle biopsy cores were taken separate to those required for histological analysis and used for dedicated research purposes; both global and targeted metabolite analysis, and next-generation RNA sequencing.

In October 2015, an interim analysis was carried out on the prostate tissue from the first 15 volunteers to complete paired 0 and 12-month sample collections. Tissue extracts were sent to a metabolomics platform for global profiling (Metabolon[®]), which was successful in matching 381 detectable metabolites to their reference database. Statistically significant changes (paired t test, $p < 0.05$) were identified in 22 metabolites, highlighting distinct changes in several metabolic pathways

(unpublished data). Modification to tissue redox status, as could be expected after exposure to ITCs, was confirmed by significant increases in the glutathione metabolites glutathione-reduced (GSH), s-methyl glutathione and cysteine-glutathione disulphide. The significant findings, however, occurred across all three arms of the study, indicating potential biological activity of the diet that was unrelated to the glucoraphanin content of the broccoli.

The most statistically significant result from this interim analysis was an increase in levels of inorganic sulphate over the 12-month period ($p=0.0006$). The levels of both adenosine 5'-diphosphate (ADP) and phosphate also increased significantly ($p=0.002$ and $p=0.005$ respectively) suggesting depletion of ATP, the vital energy source. Most importantly, both changes in sulphate and ADP were significantly associated with a reduction in prostate cancer volume, defined by the percentage of biopsy cores affected (Figure 1-7) (unpublished data). These findings introduced the possibility of a novel link between sulphate accumulation and ATP depletion in prostate tissue. As there was no significant difference between study diets, the outcome may have been due to alternative sources of sulphate in the broccoli. SMCSO, for example, represents a major pool of sulphur metabolism and degrades in humans to provide a rich source of inorganic sulphate, as previously described in Figure 1-6.

To test the hypothesis that sulphate delivered by broccoli consumption, and SMCSO degradation, was accumulating in prostate tissue, an independent study was required. The design of this study has introduced a non-intervention control arm (Chapter 2). The potential mechanistic links between sulphate, ATP and prostate cancer prevention are discussed below.

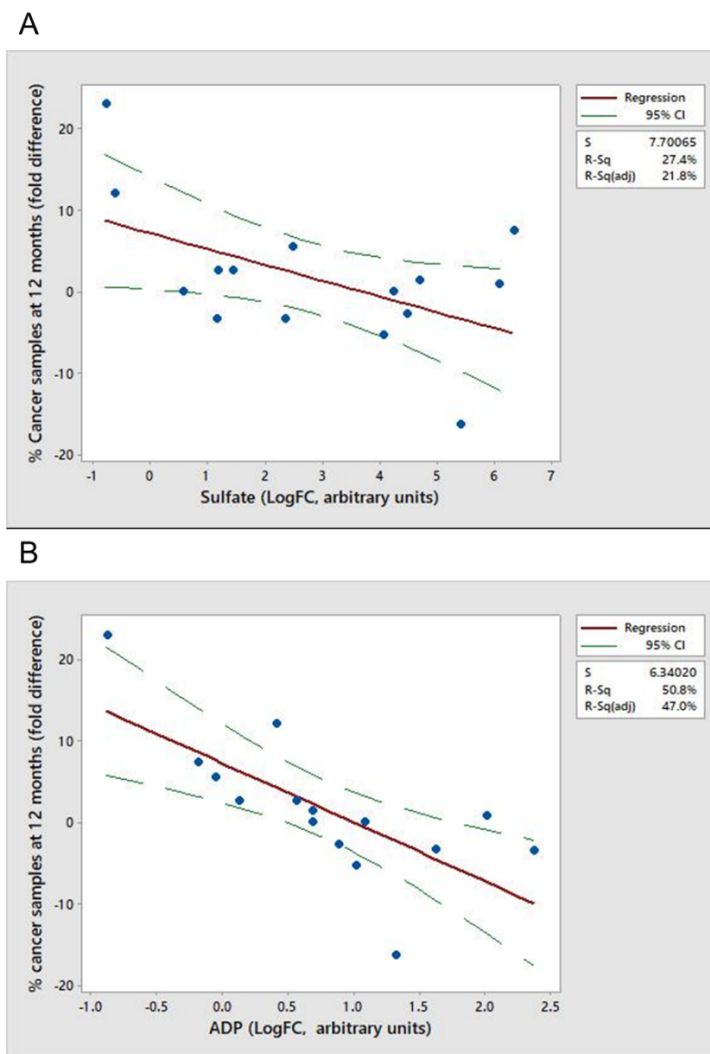


Figure 1-7 Graphs from unpublished doctoral thesis (Al Kadhi, O. 2017) showing the significant negative associations between change in cancer volume over 12 months and tissue levels of (A) sulphate ($p < 0.05$) and (B) ADP ($p < 0.01$) in the ESCAPE subgroup analysis ($n = 15$) [67].

1.4 Sulphate and Cancer

A direct link between tissue levels of inorganic sulphate and cancer has not previously been established. Increasing the level of sulphate is likely to drive synthesis of 3'-phosphoadenosine 5'-phosphosulphate (PAPS), a universal sulphate donor for sulphonation reactions [68]. The process of sulphonation via PAPS provides a vital metabolic pathway for detoxification of a wide range of xenobiotics, drugs and endogenous compounds [69, 70]. However, some dietary and environmental agents have the opposite characteristic, depending on sulphonation for activation to highly-reactive, toxic intermediates with implications towards carcinogenesis [71].

Synthesis of PAPS is limited by the intra-cellular availability of inorganic sulphate [68, 72]. Each of the two PAPS synthase enzymes (PAPSS-1 and PAPSS-2) has dual enzymatic activity, acting as both an ATP-sulfurylase and adenosine phosphosulphate (APS) kinase. A molecule of ATP is used at each step, thereby

depleting two mol ATP for each mol of inorganic sulphate that is converted to PAPS (Figure 1-8). Deficiencies in PAPS synthase have been linked to a range of disease states, although none have shown a causative relationship with cancer [73]. Although it may not translate to loss of function of PAPSS-2, copy number losses in established prostate cancer specimens have been linked to PSA recurrence after prostatectomy [74]. Interestingly, upregulation of PAPSS-1 has also been shown to protect against proliferation in other hormone-sensitive cancer models, such as breast cancer, due to increased sulphonation of oestrogens [75]. A clinically relevant approach to prostate cancer through regulation of androgens by sulphonation, however, is yet to be investigated [76].

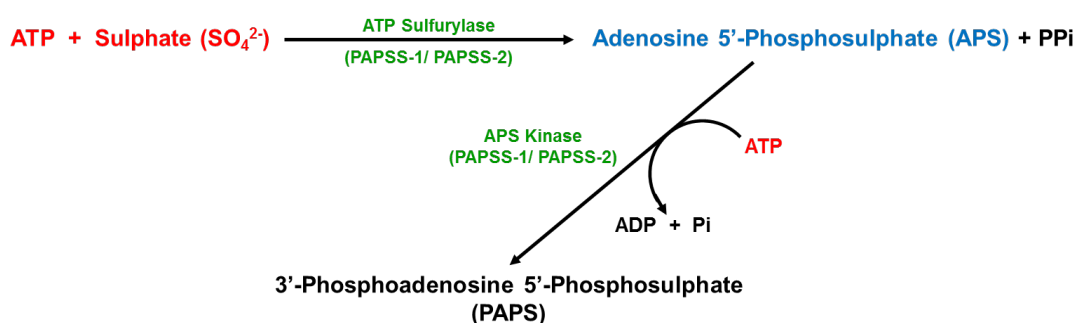


Figure 1-8 Synthesis of PAPS from inorganic sulphate. The two-step process is catalysed at both stages by PAPS synthases (PAPSS-1 and PAPSS-2), which have dual enzymatic functions. Availability of sulphate is rate limiting, so increased concentrations are likely to drive PAPS synthesis, consuming ATP as both substrate and energy source.

Animal studies of diets deficient in the sulphur-containing amino acids cysteine and methionine have demonstrated a reduction in levels of inorganic sulphate both circulating in plasma and excreted in urine. This led to reduction in tissue levels of “biologically active sulphate” (PAPS), as well as levels of the antioxidant glutathione [77]. Conversely, the unique sulphur metabolism of broccoli offers a rich source of both methionine and cysteine, in addition to the glucoraphanin, inorganic sulphate and SMCSO content [78].

1.5 ATP Depletion and Cancer

Cancerous cells have high energy requirements and are unable to adjust their metabolism according to fluctuations in ATP [79]. The response in non-cancerous cells to a rise in the adenosine monophosphate (AMP)/ATP and adenosine diphosphate (ADP)/ATP ratios is through activation of the AMP protein kinase (AMPK) pathway by LKB1. Activated AMPK restores cellular energy levels by reducing protein synthesis, increasing glycolysis and fatty acid oxidation, and inducing cell cycle arrest in proliferating cells [80, 81]. Cancerous cells have a characteristic metabolism that exhibits some of these traits under normal conditions; increased glycolysis and greater turnover of fatty acids. However, cancerous cells favour lactate production by aerobic glycolysis (the Warburg Effect), which is

relatively inefficient at producing ATP (2mol ATP/ mol glucose) compared to oxidative phosphorylation via the TCA cycle (~36mol ATP/ mol glucose) [25]. The role of activated AMPK is also complex because it is upregulated in several prostate cancer cell lines, but excess stimulation inhibits proliferation and induces apoptosis [82]. Thus, novel therapeutic agents targeting AMPK activation are under ongoing investigation in the treatment of prostate cancer [83]. The same mechanism is also proposed to account for the cancer-preventing properties of the anti-diabetic drug metformin [84].

ATP suppression by dietary intervention therefore offers a potentially attractive mechanism for cancer prevention. Global metabolomics analyses have recently been used to investigate the use of a large number of diet-derived bioactive compounds in reducing ATP levels in prostate cancer models [85]. Although SMCSO was not included, the most interesting results were observed in combinatorial treatments compared to individual compounds. This supports the use of diet intervention with food (broccoli soups) in trials undertaken at QIB because of the combined content of glucosinolates, SMCSO and other constituents of broccoli that may confer health benefit.

1.6 Thesis Objectives

This thesis describes the impact of broccoli-derived sulforaphane and SMCSO on prostate metabolism in both humans and prostate cells *in vitro*. The primary hypothesis is that the multi-modal effects of broccoli-derived bioactives induce an energy-deplete prostate microenvironment with an increased capacity to resist oxidative stress and inhibit prostate carcinogenesis. The approach includes:

- Carrying out a randomised, parallel-unblinded, high-dose broccoli intervention study in men awaiting TPB
- Investigating untargeted global metabolomic differences in tissue samples of men recruited to a high-dose broccoli intervention study
- Development and application of targeted analytical methods to quantify sulphate, ATP and SMCSO in human samples
- Investigating the effects of sulforaphane and SMCSO on real-time bioenergetics in prostate cell lines
- Testing the antioxidant capacity of sulforaphane and SMCSO in protecting the phosphatase and tensin homologue (PTEN) tumour suppressor against oxidative stress.

2 A Pre-Biopsy Window-of- Opportunity Study to Measure Sulphate Levels in Human Prostate After Broccoli Consumption

Summary

The chemopreventive effects of cruciferous vegetables are largely attributed to their ability to deliver ITCs, including sulforaphane from broccoli. This chapter describes the background, study design and working hypotheses of a high-dose, broccoli-enriched dietary-intervention study during the pre-biopsy 'window of opportunity' for men under investigation for prostate cancer. The metabolism of the prostate is uniquely adapted to support the exocrine secretion of nutrients in seminal fluid. It may also be exposed to the highly-concentrated mix of metabolites from urine as it refluxes via the prostatic ducts during voiding. The Sulphate Accumulation in Prostate (SAP) study aims to clarify the relationship between sulphur-containing compounds from broccoli and prostate metabolism. Utilising both global and targeted analytical platforms, potential mechanistic links to prostate cancer prevention will be sought, primarily via an accumulation of inorganic sulphate.

2.1 Introduction

Sulphur metabolism in cruciferous vegetables accounts for their characteristic flavour and is central to production of biologically active metabolites, including glucosinolates (sulphur-containing glycosides) [50, 78]. Major pools of sulphur also exist in Brassicaceae in the forms of SMCSO, and to lesser extents amino acids (cysteine and methionine), glutathione and sulphate [51]. Degradation products of sulphur metabolites include the putative anti-cancer agent sulforaphane, volatile intermediates and inorganic sulphate [43, 56].

2.1.1 Emerging Evidence for Sulphate Accumulation in Prostate

Interim results from a 12-month dietary intervention with broccoli soup (ESCAPE study) identified changes in several metabolic pathways. The most significant was an increase in inorganic sulphate levels in prostate tissue (unpublished data). Sulphate availability is the rate-limiting substrate for synthesis of PAPS, the universal sulphate donor for sulphonation reactions [72]. Consistent with an increased drive to synthesise PAPS, a significant increase in both ADP and phosphate was also detected in the ESCAPE subgroup analysis, suggesting depletion of ATP stores to fuel the reaction. Furthermore, the change in ADP was negatively correlated to change in number of prostate cores affected by cancer after 12 months (unpublished data). Potential depletion of ATP in human prostate tissue by dietary intervention represents a novel mechanistic link between the sulphur metabolism of cruciferous vegetables and prostate cancer prevention; cancerous clones have high energy demands and are unable to adapt to restricted availability of ATP, making them more susceptible to elimination [79, 86].

The ESCAPE study seeks to investigate the effects of varying the glucosinolate content in broccoli soups on prostate metabolism. A major advantage of upregulating the glucosinolate content within broccoli varieties, and consumption as food rather than isolated sulforaphane, is maintaining the contribution of other phytochemicals. No statistically significant differences have emerged between study diets, which could suggest a contribution of other constituents to sulphate accumulation, though the subgroup has relatively limited power (n=15). To independently test the effects of a glucoraphanin-enriched broccoli soup on

sulphate metabolism in the prostate, an additional human intervention study was required to compare those receiving the intervention to a control group continuing their normal diet. The study hypothesis was that metabolism of SMCSO in the broccoli soup, which yields high proportions of inorganic sulphate (~50%), drives PAPS synthesis and depletes ATP in prostate tissue (Figure 2-1).

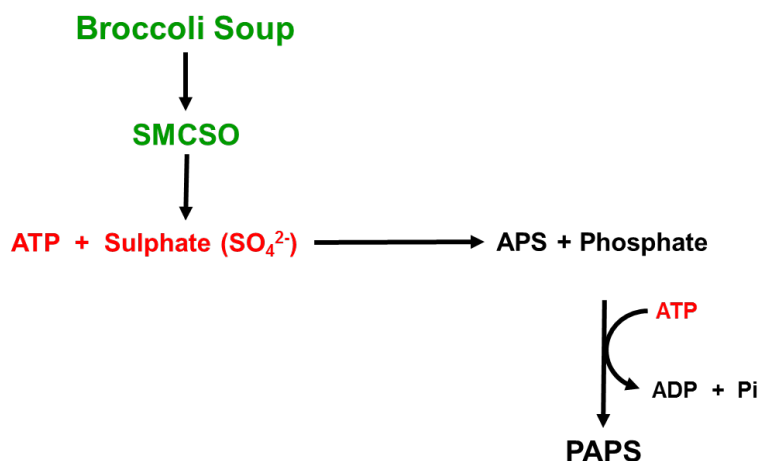


Figure 2-1 Pathway hypothesis for SAP study. Degradation of SMCSO from broccoli soup produces sulphate, driving synthesis of PAPS and depleting ATP. Modified from Figure 1-7.

2.1.2 Route of Exposure to Broccoli Metabolites

The Bioavailability of Broccoli Soups (BOBS) study (REC 14/EE/1121; NCT02300324) at QIB detected both SMCSO and glucoraphanin in urine after broccoli soup consumption in healthy subjects. Reflux of urine into the prostatic ducts during voiding may, therefore, provide an additional route of exposure to systemic circulation for the accumulation of broccoli-derived sulphur-containing metabolites in the prostate [35]. To date there is no evidence that urinary reflux into the prostate provides an additional route of exposure to dietary agents. Hence, further investigation is needed to elucidate the process. Biopsies of pelvic adipose tissue can be obtained at the same time as prostate biopsies during TPB, without extending the surgical field beyond the template grid. Utilising the same extraction and analytical methods for both prostate and adipose tissue provides a method for comparing metabolic profiles with and without urine exposure. This could represent a useful experimental tool for investigating *in vivo* whether exposure to urinary compounds drives unique metabolic changes in the prostate.

2.1.3 Modulation of Redox Status by Dietary Intervention

Increased levels of antioxidant glutathione metabolites in the ESCAPE subgroup analysis was one of the most significant metabolic pathway changes after 12 months of broccoli soups (unpublished data). The consequence of increasing capacity to resist oxidative stress could be to maintain the function of proteins regulating metabolism, such as the phosphatase and tensin homologue (PTEN). The importance of the PTEN tumour suppressor in prostate cancer is widely

recognised and will be described in greater depth in Chapter 6. In essence, the PTEN protein inhibits activation of the phosphoinositide 3-kinase (PI3k)/Akt pathway, which would otherwise promote the rapid cell growth and proliferation required for cancer progression [87]. Mutations and deletions in one or both PTEN alleles are common in prostate cancer and provide prognostic information on the likelihood of disease recurrence after radical treatment [88, 89]. However, the ability of PTEN to dephosphorylate phosphatidylinositol 3,4,5 triphosphate (PIP3) is further reduced at the protein level due to reversible inactivation by oxidative stress [90]. Upregulating the antioxidant capacity of prostate tissue by dietary intervention could restrict cancer growth by maintaining PTEN in its reduced (active) state.

2.2 SAP Study Aims

The aims of the SAP study, as specified in the original protocol (Annex 1), are detailed below. As the control diet of the ESCAPE study includes standard broccoli soup, the primary role of SAP was to compare levels of sulphate in the prostates of men on a high-dose broccoli intervention to those of men continuing their normal diets. Additional outcome measures, including an increase in ADP and phosphate, aimed to further test the hypothesis that sulphate accumulation was driving down ATP via PAPS synthesis. Comparing metabolite profiles in prostate, adipose and urine sought to establish whether diet-induced changes in the prostate were related to systemic circulation or exposure to urine refluxing via the prostatic ducts. Further mechanistic links to prostate cancer prevention were outlined through plans to assess the redox status of the PTEN tumour-suppressor protein. The impact of GSTM1 genotype on both prostate cancer risk and the bioavailability of broccoli bioactives is well established [58, 63]. The GSTM1 status of participants, therefore, remains an important explanatory variable when interpreting the results of this study. The results from all study outcomes were also interpreted in the context of habitual cruciferous vegetable intake assessed by a validated food frequency questionnaire.

2.2.1 Primary aim

- To determine whether a diet rich in broccoli will result in a difference in sulphate levels in tissue samples obtained from men scheduled for prostate biopsy.

2.2.2 Secondary aims

- To determine whether a diet rich in broccoli will result in metabolic differences such as increased ADP in prostate tissue of men scheduled for prostate biopsy
- To determine whether differences in sulphate and ADP levels induced by diet are specific to prostate tissue.
- To determine whether the metabolite differences induced by diet will affect the redox status of phosphatase proteins such as PTEN in prostate tissue.
- To determine whether the differences in metabolites induced by diet in prostate tissue are associated with metabolite differences in urine.

- To demonstrate if the differences in metabolites induced by diet are modulated by glutathione S-transferase Mu 1 (GSTM1) genotype or other relevant genotypes.

2.3 Materials and Methods

2.3.1 SAP Study Design

Figure 2-2 outlines the design of a two-arm, parallel un-blinded, dietary-intervention study, delivering a short, high-dose broccoli diet intervention to men in the pre-biopsy window before their TPB (SAP study). Eighteen men were randomised to either continue their normal diet (non-intervention controls) or receive three portions of Beneforte[®] (glucoraphanin-enriched) broccoli and stilton soup (300g) per week for a minimum of 4 weeks. The soup content from sulphur analysis at QIB is included in Table 2-1. Recruited participants completed a health questionnaire and Cruciferous Vegetable Food Frequency Questionnaire (CVFFQ). On the study day samples of whole blood, post-DRE urine, prostate and adipose tissue were collected. The routine TPB was performed to meet the patients' clinical requirements and their follow-up care was continued by the urology consultants at the Norfolk and Norwich University Hospital (NNUH). A further supply of soup was offered to all participants after completing the study for their own consumption.

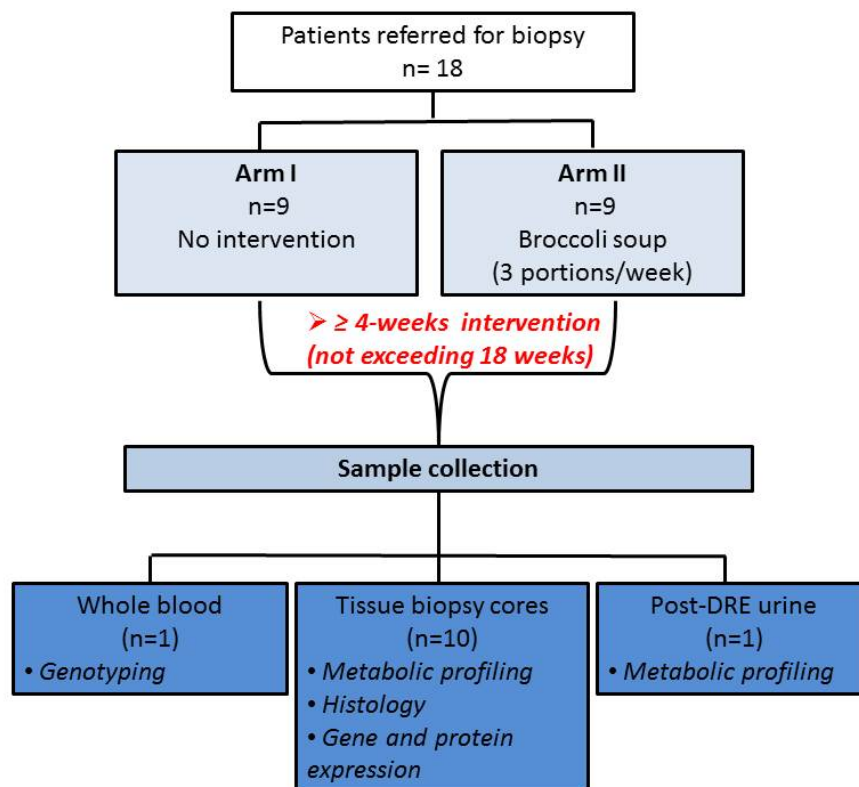


Figure 2-2 Flow diagram of the SAP study outline

Table 2-1 Results from content analysis for the batch of Beneforte® broccoli and stilton soup used in the SAP study

Beneforte Broccoli Soup	Glucoiberin (µmol/G)	Glucoraphanin (µmol/G)	SMCSO (µmol/G)	Sulphate (µmol/G)	Polyphenols (µmol/G)
Batch 1C	0.527	4.077	20.05 ± 1.36	10.43 ± 0.14	15.01 ± 0.46

2.3.2 Study Management

The SAP study was funded by the Biotechnology and Biological Sciences Research Council (BBSRC, UK) and the Prostate Cancer Foundation (PCF, USA). It was carried out in collaboration with QIB (formerly the Institute of Food Research (IFR)), the Urology and Histopathology departments at NNUH and co-investigators from the University of East Anglia (UEA). The named scientists were Professor Richard Mithen (Chief Investigator), myself and Dr Antonietta Melchini (QIB Investigators), and Mr Robert Mills (NHS Investigator). The protocol was approved by the Human Research Governance Committee (HRGC IFR 01/2016) at QIB in January 2016 for submission to the National Research Ethics Services (NRES). The East of England – Cambridge East Research Ethics Committee (REC) gave full ethical approval in February 2016 (ref: 16/EE/0054) and, in compliance with Health Research Authority (HRA) requirements, the trial was registered on a publicly-accessible database (ClinicalTrials.gov, NCT02821728).

2.3.3 Study Population

Men who were on the waiting list for TPB at NNUH as part of their routine clinical care were targeted for enrolment if they were between the ages of 18 and 80 years and had a body mass index (BMI) of between 19.5 and 35 kg/m². This included those with either a previous negative TRUS-guided prostate biopsy or those with a histologically-confirmed abnormality, including diagnosis of prostate cancer, that required further investigation or surveillance. A list of inclusion and exclusion criteria is included in Table 2-2. Importantly, it remained essential to exclude participants whose biopsy was scheduled within the 4-week dietary intervention period to avoid creating a delay in any individual's routine clinical care.

Table 2-2 Inclusion and exclusion criteria for participation in the SAP study

Basic Inclusion Criteria	Basic Exclusion criteria
Males	Those regularly taking 5 α -reductase inhibitors or testosterone replacement medicines
Scheduled for TPB as part of routine investigation or staging for prostate cancer	Those on warfarin treatment
Aged 18–80 years	Those with a diagnosis of diabetes
BMI between 19.5 and 35 kg/m ²	Those diagnosed with or suspected to be high risk for human immunodeficiency virus (HIV) and/or hepatitis
Smokers and non-smokers	Those allergic to any of the ingredients of the broccoli and stilton soups
	Those taking dietary supplements or herbal remedies that may affect the study outcome
	Those that are unable to understand English or provide informed consent
	Parallel participation in another research project that involves dietary intervention
	Any person related to or living with any member of the study team

2.3.4 Study Recruitment and Randomization

Recruitment was carried out by myself and Mr Mills (Urology Consultant) through close communication with the administrative team at NNUH. As soon as potentially eligible patients were added to the waiting list for TPB they were sent an information pack containing an invitation letter (Annex 2), participant information sheet (PIS) (Annex 3) and soup information sheet (Annex 10). After one week, or if they expressed their interest by response slip sooner, patients were contacted by telephone to ensure they had obtained all relevant information. Those who wished to take part were given a 3-day consideration period before being randomised to either arm of the study. Formal written consent was obtained before receiving the study diet during a visit to the Human Nutrition Unit (HNU) at QIB for participants in the intervention arm, or on the day of their hospital visit for TPB in the non-intervention arm. The process for recruitment and study participation is described in Figure 2-3.

Randomisation was performed by a third party with use of an online randomisation generator (www.randomization.com). The method of 'block randomisation' ensured that participants were evenly distributed between both study arms.

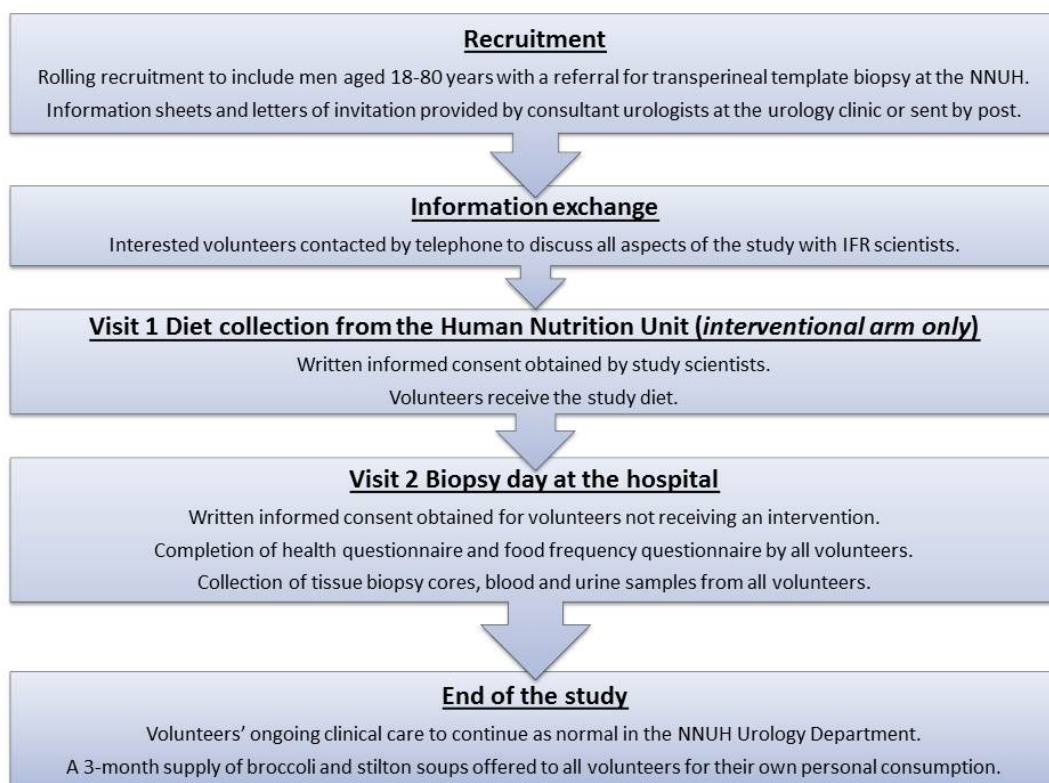


Figure 2-3 Flow diagram detailing the involvement of participants within the SAP study

2.3.5 Study Day Procedure

To participate in the study volunteers were required to complete 3 separate consent forms; trial consent (Annex 4), clinical consent for the biopsy (Annex 6) and consent for tissue banking at the Norwich Biorepository (Annex 5). The banking of biological samples at the Biorepository also ensures their availability for future research purposes. The sample of whole blood for genotyping was obtained via the intravenous cannula inserted for use by the anaesthetist, thus avoiding additional venepuncture. After prostatic massage, a first-pass urine sample was collected for both metabolomic and targeted analyses.

TPB is performed under general or spinal anaesthesia with appropriate antibiotic prophylaxis. Once anaesthetised the patient is positioned supine with their legs elevated in stirrups. A template grid is placed on the skin of the perineum and the prostate is visualised by trans-rectal ultrasound scan. After measuring the volume of the prostate, the whole gland is systematically sampled through the template grid, which has holes spaced at 5mm intervals. For the SAP study eight biopsy cores of prostate tissue were taken from a region of the prostate not known or suspected to contain cancer; one placed in methanol for metabolomics analysis, two in RNAlater for next-generation RNA sequencing and five snap frozen for targeted analyses.

An additional two cores were taken from the peri-prostatic adipose tissue outside of the prostate gland but within the scope of the template grid. One was placed in methanol and the other snap frozen.

Samples in methanol were incubated at room temperature for 24 hours as per Metabolon[®]'s protocol. Tissue samples that were snap frozen were immediately placed on dry ice and subsequently transferred for storage at -80° C.

2.3.6 PSA Testing and Histopathology

The most recent PSA level was recorded for each participant from the clinical records held at NNUH. PSA density was calculated by dividing PSA (ng/ml) by the prostate volume (cm³) measured by trans-rectal ultrasound at the start of TPB.

To minimise variation in prostate tissue sampling, all biopsies were carried out following the same procedural technique by Mr Mills at NNUH. Biopsies taken for clinical evaluation were fixed in formalin and later paraffin-embedded. Sections were stained for haematoxylin and eosin, with additional processing steps such as immunohistochemical staining performed if necessary. The tissue was analysed by a single consultant histopathologist, Professor Richard Ball, to prevent inter-observer error; a potential hazard in diagnosing and grading prostate cancer [91]. In addition to providing a Gleason score for WHO grading, the volume of cancer was reported as a percentage involvement of each tissue core affected. The prostate tissue samples that had been incubated in methanol for metabolite extraction were also sent to histopathology for analysis by Professor Ball.

2.3.7 Cruciferous Vegetable Food Frequency Questionnaire

To evaluate the habitual intake of cruciferous vegetables of all volunteers, a CVFFQ, developed by the University of Arizona, was completed during the study day. The questionnaire has been validated against other standard food frequency questionnaires and improves accuracy in correlation with urinary dithiocarbamate excretion, a marker of cruciferous vegetable consumption [92]. Data were adjusted according to both portion size and cooking method. Results are shown in grams per day and portions per day for cruciferous vegetables, and as total glucosinolate intake (mg/day) according to data from the US Department of Agriculture (USDA) Nutrient Database [93].

2.3.8 Quantification of Urinary Isothiocyanates by Liquid Chromatography-Tandem Mass Spectrometry

Sulforaphane and its conjugates sulforaphane-cysteine (SF-Cys), sulforaphane-glutathione (SF-GSH) and sulforaphane-N-acetyl cysteine (SF-NAC), as well as erucin-N-acetyl cysteine (ER-NAC), were measured in urine using a validated LC-MS/MS method [94]. A 10-point, matrix-matched calibration curve was generated by serial 2-fold dilution of standards (SF, SF-Cys, SF-GSH, SF-NAC and ER-NAC) in blank (non-intervention group) urine from 8µg/ml to 0. 100µl aliquots of study sample urine were diluted in 900µl of 0.1% ammonium acetate buffer containing mixed internal standards (B-ITC). The sample was centrifuged at 14,000g for 10 minutes at 4° C and the supernatant transferred to an HPLC vial for analysis by LC MS/MS.

Sulforaphane and its metabolites were quantified using an Agilent 6490 triple-quad LC MS mass spectrometer (Agilent technologies) with HPLC Phenomenex® Luna 3u C18 (2) (100A, 100 x 2.1 mm) column with a Phenomenex® C18 (2) 100A column guard. The system comprised a degasser, binary pump, column oven, cooled autosampler, diode array detector and 6490 mass spectrometer. The LC-MS/MS was set on a flow rate of 0.3 ml/min. The column temperature and autosampler were maintained at 40° C and 4° C respectively. Samples were injected at 5µl, 'system suitability' was injected at 1µl, and blank was injected at 20µl. Separation of metabolites was carried out with 0.1% ammonium acetate in Milli Q water plus 0.1% acetic acid (mobile phase A, pH 4) and 0.1% acetic acid in acetonitrile (mobile phase B). The LC eluent flow was sprayed into the mass spectrometer interface without splitting. Sulforaphane and conjugates were monitored using mass spectrometry Multiple Reaction Monitoring (MRM) mode in positive polarity with electrospray ionisation (ESI) source. The source parameters were: gas temperature 200° C with a gas flow of 12 l/minute, a sheath gas temperature of 400° C with a sheath gas flow of 12 l/minute, a nebuliser pressure of 60 psi and capillary voltage of 4000 V.

2.3.9 GSTM1 Genotyping

DNA was extracted from whole blood samples using the QIAamp DNA minikit following the manufacturer's instructions (Qiagen Inc.). Both the DNA and 260:280 ratio were quantified on a Nanodrop™ spectrophotometer (ThermoFisher). Genotype was determined with 20ng genomic DNA in a StepOnePlus real-time PCR system (Applied Biosystems), with GSTM1 primer (ThermoFisher) and Taqman™ universal master mix II (Applied Biosystems). A 10-minute activation period was carried out at 95°C, followed by 40 PCR cycles at 92°C for 15 seconds and 60°C for 90 seconds. Allelic discrimination of GSTM1 genotype was determined using StepOne™ v2.3 software (Applied Biosystems).

2.3.10 Statistical analysis

There are no data reporting the effect of a diet rich in broccoli on sulphate levels within prostate or at the systemic level. The sample size for the SAP study was calculated taking into consideration the preliminary data obtained from the interim analysis of the ESCAPE study. The metabolite profiling of prostate tissue from patients (n=15) randomised to a 12-month broccoli intervention has shown a significant accumulation of sulphate in prostate tissue after the intervention period compared to baseline. To detect a difference of 1.735 (normalised ion count) at a 5% significance level with 90% power and assuming a within-group standard deviation of 1.056 (normalised ion count) required a sample size of 9 individuals in each group (total 18). These sample sizes were calculated for a two-group study design (broccoli intervention vs no intervention) assuming a two-sided comparison (i.e. to detect a difference rather than a higher level).

2.4 Results

2.4.1 Recruitment

Data from the coding office at NNUH confirmed that a total of 190 TPBs were performed in 2015. Based on experience with ESCAPE, and an expected recruitment rate of 20%, a timeline was set for completion within six months. The first information packs were sent on 19th April 2016 with a target deadline for the last biopsy to be completed on 31st October 2016 to allow time for the intervention. As can be seen in Figure 2-4, recruitment completed early and the last biopsy was performed on 9th September 2016.

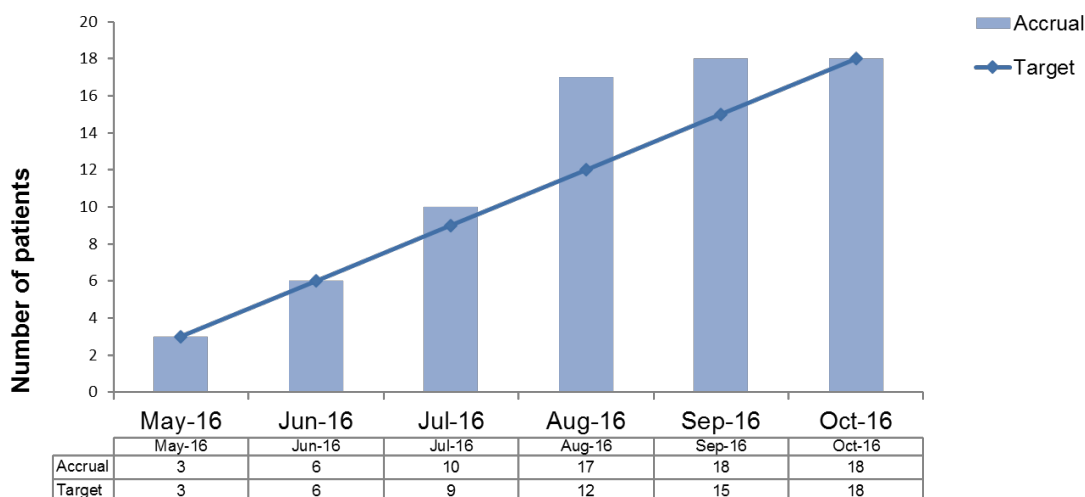


Figure 2-4 SAP study recruitment timeline. All participants had agreed to participate by August 2016. The last biopsy was performed on 9th September 2016, 7 weeks ahead of schedule.

During the recruitment period, 92 information packs were sent to patients on the waiting list for TPB (Figure 2-5). 24 potential participants returned response letters, another 27 were contacted by telephone and no response by either method was received from the remaining 41. The conversion rate to full participation was high (62.5%) amongst those who returned the response slip, primarily limited by a scheduled biopsy date that was too soon for the intervention. Those that were first contacted by telephone were less likely to meet the inclusion criteria and only three (11%) subsequently decided to enrol on the study. One volunteer in the non-intervention arm dropped out from further participation due to illness on the study day.

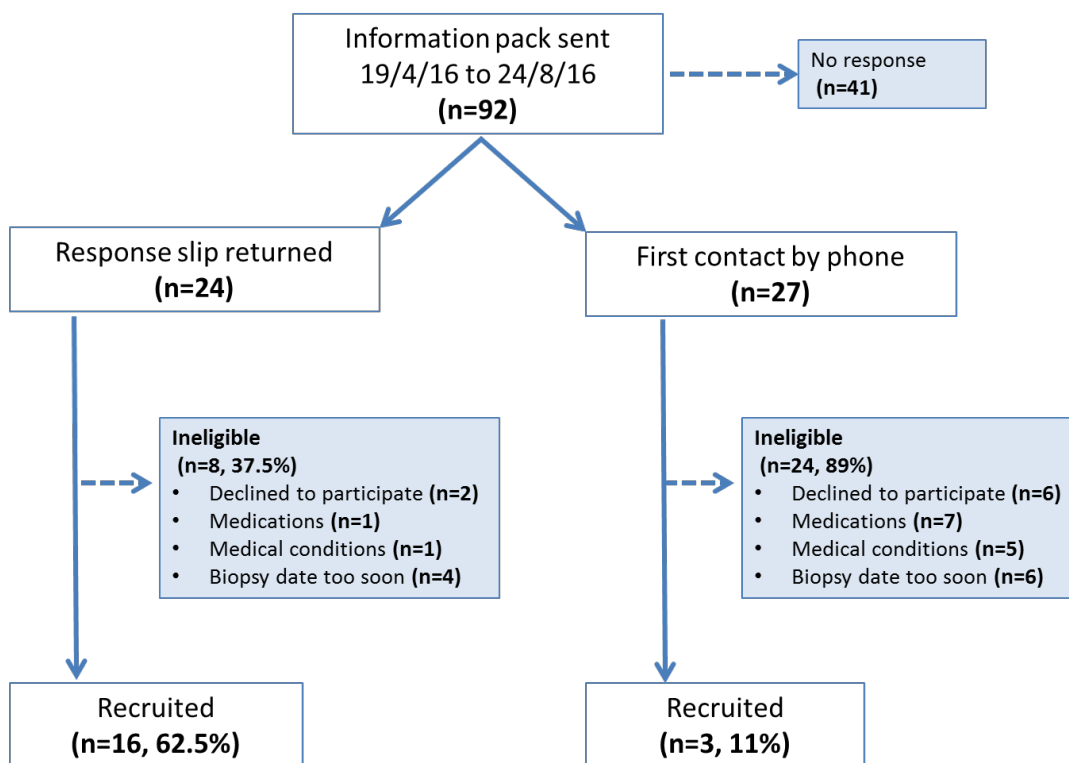


Figure 2-5 Flow chart of pathway to recruitment

2.4.2 Participant Demographics

Block randomisation distributed participants equally to both study arms, with no statistically significant difference (unpaired t-test) in age, BMI, PSA or PSA density (Table 2-3). The mean BMI for men in both arms fell into the overweight category, and as anticipated for men recommended to undergo TPB the PSA in both groups was above the age-adjusted normal range.

Table 2-3 Distribution of participants across both study arms. Data expressed as mean \pm SD.

	Non-Intervention (n=9)	Intervention (n=9)
Age (years)	64.67 \pm 5.385	68.56 \pm 6.464
BMI (kg/m ²)	26.84 \pm 3.288 (n=8)	28.05 \pm 2.575 (n=8)
PSA (ng/ml)	7.789 \pm 4.165	8.667 \pm 2.644
PSA Density (ng/ml/cm ³)	0.1426 \pm 0.1012	0.1216 \pm 0.04817

2.4.3 Participant Cancer Grade and Volume

Despite clinical suspicion of prostate cancer indicating the need for TPB, histology reports provided by Professor Ball revealed 11 of the 18 participants had benign diagnoses. There were no significant differences in the overall distribution of cancer diagnoses between the two groups (Figure 2-6). Study biopsies were taken from the right anterior quadrant of the prostate or the most remote region from any focal areas of clinical suspicion. Three volunteers were diagnosed with cancer in the quadrant sampled (2 in the intervention group and one in the non-intervention group). However, the volume of tissue affected was small (<15%) so further histological analysis of samples used for metabolite extraction is awaited from Professor Ball before any additional normalisation is performed.

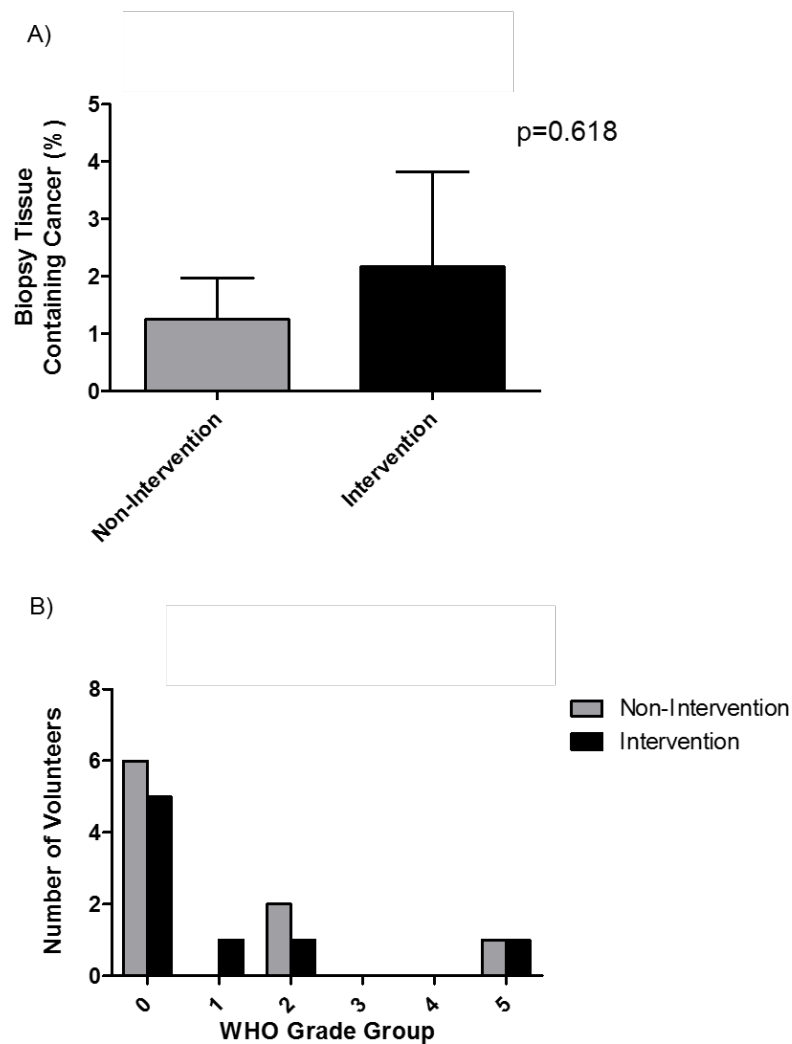


Figure 2-6 Graphs of histological findings in both study arms. A) Percentage of all tissue received for histological analysis affected by cancer (mean + SEM, $p=0.618$ by unpaired Student's t-test). B) Grade of cancer by WHO grade group.

2.4.4 Cruciferous Vegetable Intake

Only one volunteer failed to fully complete and return the Arizona CVFFQ. The data revealed a wide range in consumption between individuals. However, there was no statistically significant difference between groups for either cruciferous vegetable or glucosinolate consumption (Table 2-4, Figure 2-7).

Table 2-4 Data from the CVFFQ on daily intake of cruciferous vegetables across both study arms. Presented as mean \pm SD. Additional column with supplemental content of study diet (right).

	Non-Intervention (n=8)	Intervention (n=9)	Additional Content in Study Diet
Cruciferous vegetable intake (g/day)	72.12 \pm 54.47	66.63 \pm 52.96	36
Cruciferous vegetable intake (servings/day)	1.094 \pm 0.9241	1.098 \pm 0.9067	0.45
Glucosinolate intake (mg/day)	83.52 \pm 101.3	54.90 \pm 52.07	38.82

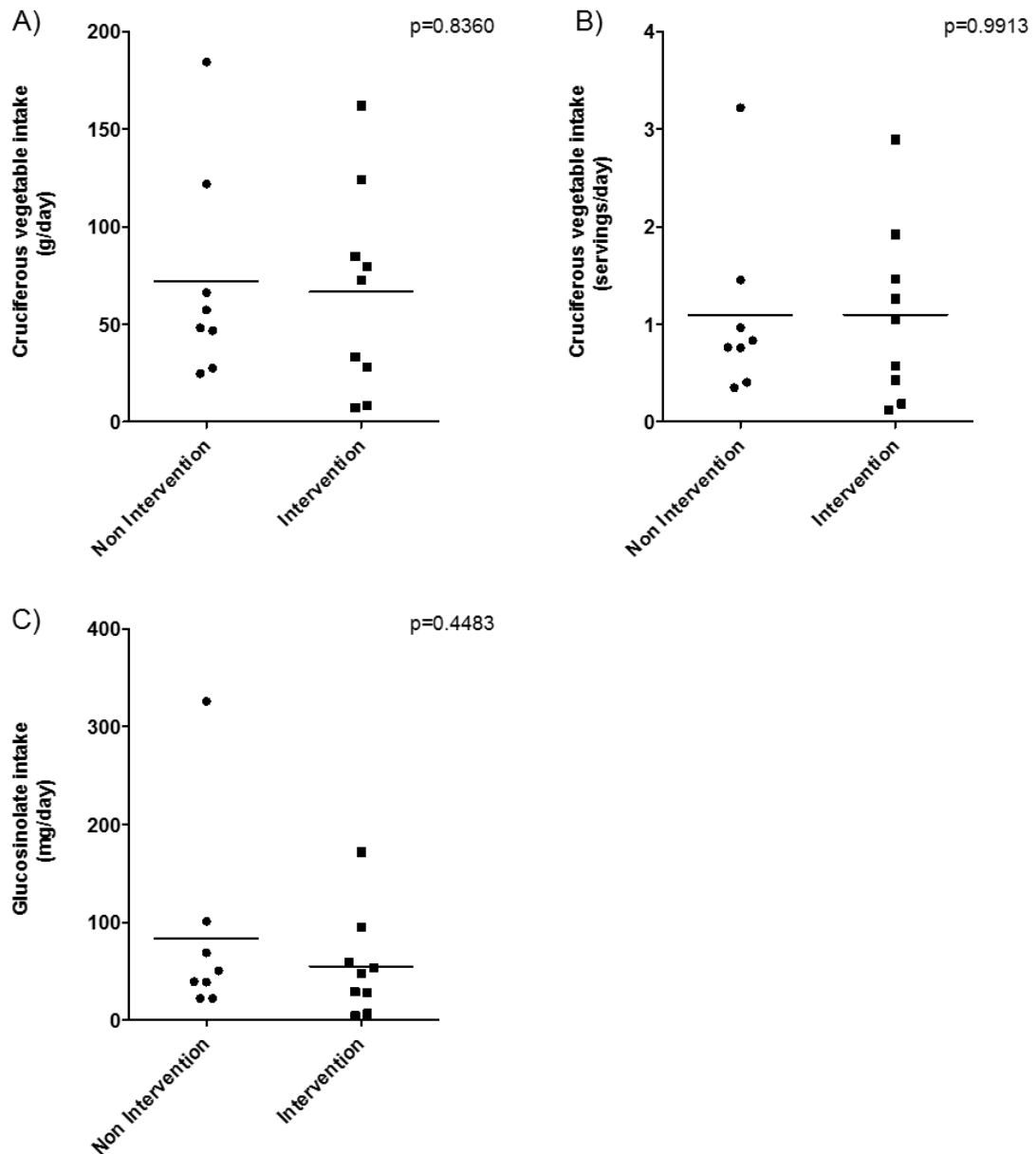


Figure 2-7 Cruciferous vegetable consumption data calculated from the Arizona CVFFQs comparing intake of cruciferous vegetables as g/day (A), servings/day (B) and glucosinolates (C). Data shown as mean + individual values to demonstrate the range in consumption. No significant difference detected between study groups (p values from unpaired Student's t tests).

2.4.5 Urinary Isothiocyanates

Sulforaphane was detectable in the urine samples of all participants in the SAP study. The conjugates SF-Cys and SF-NAC, as well as ER-NAC, were detectable in all participants in the intervention group and most of those in the non-intervention group. Similar to the findings of the CVFFQ, there was a wide range between individuals but no significant differences overall between groups (Figure 2-8). There was also a significant positive correlation between urinary ITCs and habitual intake of cruciferous vegetables and glucosinolates (data not shown).

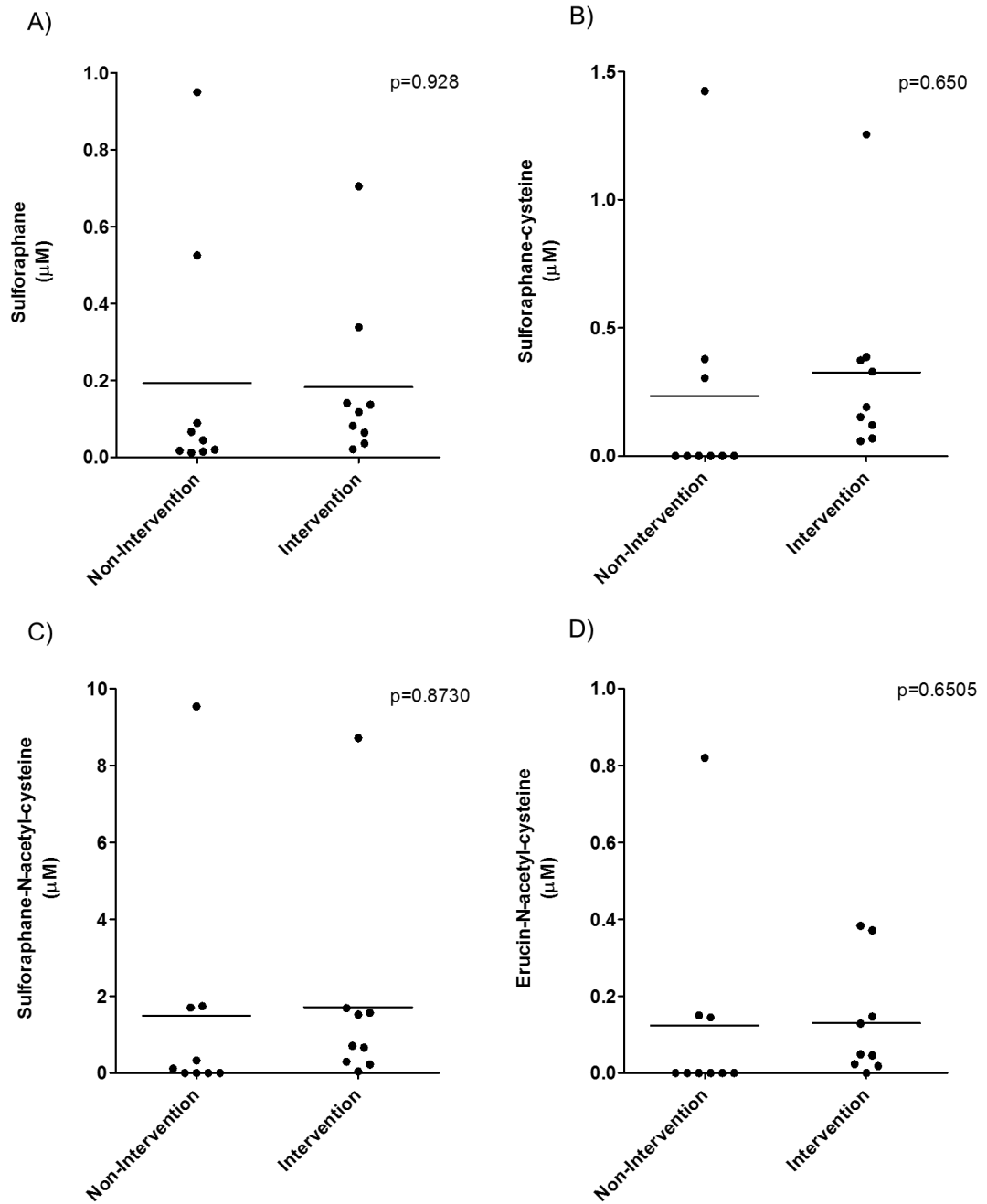


Figure 2-8 Graphs of urinary isothiocyanate concentrations between SAP study arms. Data for sulphoraphane (A) and its conjugates sulforaphane-cysteine (B) and sulforaphane-N-acetyl cysteine (C), and erucin-N-acetyl cysteine (D) shown as mean and individual concentrations. No statistically significant ($p < 0.05$) differences detected.

2.4.6 GSTM1 Genotype

Genomic DNA was extracted from all participants' blood samples with a mean 260:280 ratio of 1.54 (range 1.39 to 1.78). GSTM1 genotyping was successfully performed for all participants, revealing an overall incidence of 50% GSTM1 null, which is in line with European population studies [60]. There was no significant difference between study arms (Figure 2-9).

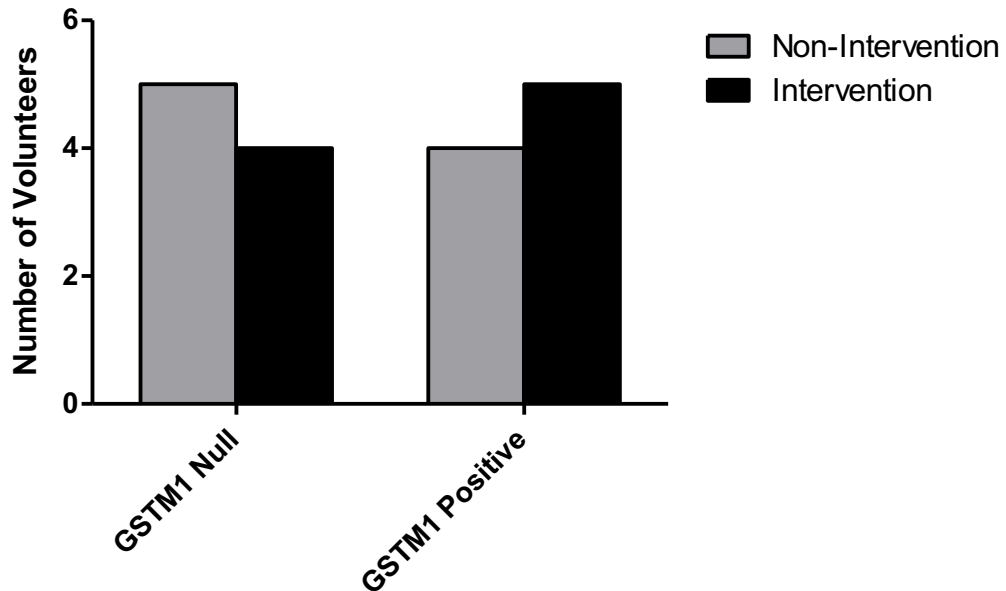


Figure 2-9 Graph of individuals within both study arms with either GSTM1 null or GSTM1 positive phenotypes.

2.5 Discussion

Recruitment to the SAP study exceeded expectations by completing ahead of schedule. Failure to recruit sufficient numbers to clinical trials represents a major problem in health research, commonly resulting in delays, inconclusive results and financial loss [95]. Only 55% of trials successfully recruit their target number, with further challenges for those working with seriously ill or cancer patients [96]. Although a typical window-of-opportunity study is carried out in the interval between cancer diagnosis and radical treatment, the pre-biopsy design offered an effective and successful model for a short-term dietary intervention [97]. Participation was readily taken up by men who were both eligible and whose biopsy date was scheduled beyond the minimum period for intervention. A single participant dropped out for reasons unrelated to the study and there were no reported complications from taking part.

Comparing age, BMI, PSA and PSA density did not reveal any significant differences between study arms (Table 2-3). Both groups were also homogenous in terms of cruciferous vegetable intake, GSTM1 genotype and histology. The fact that over 60% of study participants received a benign diagnosis is consistent with known cancer detection rates in men who proceed to TPB after an initial negative TRUS biopsy [98]. The increasing number of patients considered for TPB at NNUH during

the SAP study period reflects the rising demand for the procedure. As multiparametric MRI increases in sensitivity for detecting small target lesions, and centralisation of services means more men are being referred to tertiary cancer centres, this trend is likely to continue [99, 100].

The range of cruciferous vegetable intake was broad in both groups, replicating results of variation in habitual diet from previous dietary studies in the Norfolk population [51]. The correlation between the CVFFQ and presence of ITCs in urine is probably representative of consumption the night before the study. Bioavailability studies have shown that ITCs reach peak concentrations in urine within 6-8 hours of consumption and are almost completely cleared within 48 hours [101]. No significant difference between groups was expected as those receiving the study diet consumed their last portion of soup a minimum of 48 hours before the study samples were collected. Thus, any metabolic changes resulting from the study diet could be considered cumulative effects of the high-dose 4-week intervention.

2.6 Conclusion

The SAP study successfully employed a window-of-opportunity design, with full ethical approval, to recruit the target 18 participants on the waiting list for TPB. The men randomised to either the non-intervention group or the broccoli-enriched dietary intervention were homogenous in their baseline demographics, histology, cruciferous vegetable consumption and GSTM1 genotype. Prostate tissue, adipose and urine were obtained for global and targeted analysis and no adverse events were reported as a result of participation in the study.

3 Global Metabolomic Profiles of Men Enrolled on a Broccoli Intervention (SAP) Study

Summary

Global metabolomics have broadened our understanding of common changes that occur in prostate carcinogenesis and of the unique characteristics of the normal prostate that might predispose it to malignant transformation. Manipulating the prostate microenvironment by dietary intervention is complicated by the milieu of micronutrients in food and their influence on multiple metabolic pathways. In this chapter the use of global metabolite profiling is applied to prostate, adipose and urine samples of men recruited to the SAP study (Chapter 2). Combining univariate and multivariate pathway-enrichment analyses allows us to identify common metabolites and metabolic pathways that characterise differences in both habitual diet and a broccoli-enriched dietary intervention.

3.1 Introduction

The prostate gland is known to have a unique metabolite profile [102]. The evolution of technology with the power to investigate systems biology across multiple cellular pathways have created growing interest in the scientific 'omics' networks that relate to the development of disease. The prostate gland is no exception, with cancer research embracing the tools to study genomics, transcriptomics, proteomics and metabolomics [103]. Metabolomics is the study of small molecules and end products spanning a wide range of biological reactions. The output is a cumulative effect of upstream gene and protein expression changes, and may characterise the most relevant phenotypic changes related to disease or intervention. The use of global metabolomics in prostate tissue has already identified specific metabolites, such as sarcosine, which are associated with prostate cancer progression [104]. More recently, serum metabolites have been identified that correlate with aggressiveness of prostate cancer at diagnosis up to 20 years later [105]. Thus, targets continue to emerge with the potential for cancer prevention and treatment by lifestyle (diet/exercise) or other therapeutic interventions.

US company Metabolon[®] has built up a library of over 4500 chemicals against reference standards of known metabolite structure. Combined with the Discovery HD4[™] Platform, which employs ultra-high performance LC-MS and tandem mass spectroscopy (MS/MS), this offers some of the widest commercially-available coverage of global metabolite profiling [106].

Data utilising the Metabolon[®] platform from ongoing clinical trials at QIB suggest that a broccoli-enriched diet leads to accumulation of sulphate and ADP in the prostate, as well as upregulating capacity for antioxidant defence (ESCAPE study) (unpublished data). These findings appeared unrelated to the glucosinolate content of the broccoli, bringing into question the role of other biologically-active broccoli metabolites; no difference was seen between the three study diets, which included standard broccoli and two varieties of glucoraphanin-enriched broccoli (Beneforté[®] and Beneforte Extra). The SAP study design was described in detail in Chapter 2. Men on the waiting list for TPB were randomised to either continue their normal diet or receive a short-term, high-dose broccoli intervention before providing samples of prostate tissue, pelvic adipose tissue and urine. All three tissue types were subsequently analysed by Metabolon[®].

3.2 Aims

In addition to taking a global analytical approach to detecting diet-induced metabolomic change, the overall aims are consistent with those of the SAP study:

- To determine whether a diet rich in broccoli will result in a difference in sulphate levels in tissue samples obtained from men scheduled for prostate biopsy.
- To determine whether a diet rich in broccoli will result in metabolic differences such as increased ADP in prostate tissue of men scheduled for prostate biopsy.
- To determine whether differences in sulphate and ADP levels induced by diet are specific to prostate tissue.

3.3 Material and Methods

3.3.1 Sample Collection and Processing

As described in Chapter 2, ethical approval for the use of prostate, adipose and urine from SAP study volunteers was granted by the East of England - Cambridge East REC (ref: 16/EE/0054) in January 2016. Written informed consent was obtained for study participation, clinical consent for the biopsies and tissue banking consent at the Norwich Biorepository. Following DRE, a first-pass urine sample was collected, divided into 1ml aliquots and snap frozen on dry ice. Biopsy cores of both prostate and adipose were taken through the template grid during TPB. Metabolites were extracted according to the Metabolon[®] protocol for concurrent histopathologic and metabolomic analysis of tissue biopsies (PREF[™]: Preservation by Extraction and Fixation) based on the study carried out by Brown and colleagues (Figure 3-1) [107]. One tissue core of prostate and one of adipose from each participant was incubated in 80% methanol (HPLC grade)/20% water at room temperature for 24 hours. The tissue core was then removed and placed in formalin to be sent for histological analysis at the NNUH Histo and Cellular Pathology department (Cotman centre, Norwich). The residual extraction solvent was dried down using a centrifugal vacuum concentrator (SpeedVac[®], Thermo Fisher Scientific) and stored at -80° C before shipping to the US on dry ice. Details of the subsequent sample preparation, processing and quality control are derived from the Metabolon[®] analysis report below.

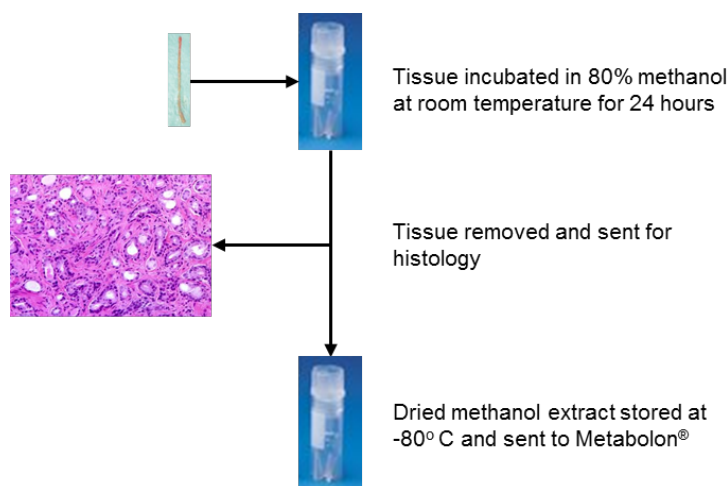


Figure 3-1 Flow diagram for metabolite extraction from needle-biopsy tissue cores. Structural integrity of tissue retained for histological assessment as previously demonstrated by Brown [107].

3.3.2 Sample Preparation

Once received by Metabolon[®], samples were prepared using the automated MicroLab STAR[®] system from Hamilton Company. Several recovery standards were added prior to the first step in the extraction process for quality control (QC) purposes. Proteins were precipitated with methanol under vigorous shaking for 2 min (Glen Mills GenoGrinder 2000) followed by centrifugation. The resulting extract was divided into five fractions: two for analysis by two separate reverse phase (RP)/ultra-performance liquid chromatography (UPLC)-MS/MS methods with positive ion mode electrospray ionization (ESI), one for analysis by RP/UPLC-MS/MS with negative ion mode ESI, one for analysis by hydrophilic interaction liquid chromatography (HILIC) /UPLC-MS/MS with negative ion mode ESI, and one sample was reserved for backup. Samples were placed briefly on a TurboVap[®] (Zymark) to remove the organic solvent. The sample extracts were stored overnight under nitrogen before preparation for analysis.

3.3.3 Quality Assurance and Control

A pooled matrix was generated by mixing small volumes of each experimental sample for analysis as technical replicates throughout. A selection of QC standards was added to every sample being analysed, avoiding interference with endogenous compounds and allowing both instrument performance monitoring and chromatographic alignment. Experimental samples were randomized across the platform run with QC samples spaced evenly among the injections.

3.3.4 Ultra-high Performance Liquid Chromatography-Tandem Mass Spectroscopy

All methods utilised a Waters ACQUITY UPLC and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyser operated at 35,000 mass resolution. The sample extract was dried then reconstituted in solvents compatible to each of the four methods. One aliquot was analysed using acidic positive ion conditions, chromatographically optimised for more hydrophilic compounds. Another

aliquot was also analysed using acidic positive ion conditions, however it was chromatographically optimised for more hydrophobic compounds. Another aliquot was analysed using basic negative ion optimised conditions using a separate dedicated C18 column. The fourth aliquot was analysed via negative ionisation following elution from a HILIC column (Waters UPLC BEH Amide 2.1x150 mm, 1.7 μm). The MS analysis alternated between MS and data-dependent MSⁿ scans using dynamic exclusion. The scan range varied slightly between methods but covered 70-1000 m/z.

3.3.5 Bioinformatics

The informatics system consisted of four major components, the Laboratory Information Management System (LIMS), the data extraction and peak-identification software, data processing tools for QC and compound identification, and a collection of information interpretation and visualisation tools for use by data analysts. The hardware and software foundations for these informatics components were the LAN backbone, and a database server running Oracle 10.2.0.1 Enterprise Edition.

3.3.6 Data Extraction and Compound Identification

Raw data was extracted, peak-identified and QC processed using Metabolon[®]'s hardware and software. Compounds were identified by comparison to library entries of purified standards or recurrent unknown entities. Metabolon[®] maintains a library based on authenticated standards that contains the retention time/index (RI), mass to charge ratio (m/z), and chromatographic data (including MS/MS spectral data) on all molecules present in the library. Furthermore, biochemical identifications are based on three criteria: retention index within a narrow RI window of the proposed identification; accurate mass match to the library +/- 10 ppm; and the MS/MS forward and reverse scores between the experimental data and authentic standards. The MS/MS scores are based on a comparison of the ions present in the experimental spectrum to the ions present in the library spectrum. While there may be similarities between these molecules based on one of these factors, the use of all three data points can be utilised to distinguish and differentiate biochemicals. More than 3300 commercially available purified standard compounds have been acquired and registered into LIMS for analysis on all platforms for determination of their analytical characteristics.

3.4 Results

3.4.1 Global Metabolomics Analysis Achieved Quality Control Criteria

The total number of metabolites detectable in each sample type are detailed in Table 3-1. Instrument and process variability checks fell within the acceptance criteria for Metabolon[®] (Table 3-2). Instrument variability was determined by calculating mean relative standard deviation (RSD) against internal standards added to each sample before injection into the mass spectrometers. Process variability was determined by calculating mean RSD of all detectable endogenous compounds in pooled samples for multiple technical replicates.

Table 3-1 Number of compounds detected in each sample type by Metabolon®

<i>Matrix</i>	<i>Named Biochemicals</i>
Prostate	482
Adipose	372
Urine	719

Table 3-2 Instrument and process variability data for sample analysis by Metabolon®

<i>QC Sample</i>	<i>Measurement</i>	<i>Median RSD</i>		
		<i>Prostate</i>	<i>Adipose</i>	<i>Urine</i>
Internal Standards	Instrument Variability	3 %	2 %	5 %
Endogenous Biochemicals	Total Process Variability	7 %	7 %	7 %

3.4.2 Univariate Analysis Identified Common Metabolites in Prostate, Adipose and Urine

Unpaired Student's t-tests were performed on the full data set for each tissue type (prostate, adipose and urine) to identify differences between study arms. The top 20 metabolites for each tissue were ranked by p-value (Table 3-3). Corresponding differences were seen throughout in tryptophan betaine, a metabolite marker of peanut consumption, and both trigonelline and quinate, which are linked to coffee consumption. All three of these metabolites were significantly higher in the non-intervention group ($p < 0.05$) (Figure 3-2).

Table 3-3 Top 20 metabolites ranked by p-value between dietary arms from global metabolomic analysis of prostate, adipose and urine. Significant results from unpaired t-tests are listed above a threshold of $p < 0.05$, and common differences shown for coffee (*) and peanut (**) metabolites.

Prostate		Adipose		Urine	
Metabolite	p value	Metabolite	p value	Metabolite	p value
1-(1-enyl-palmitoyl)-2-linoleoyl-GPC	0.006	Tryptophan betaine**	0.009	S-methylcysteine sulfoxide	0.004
Adenosine 5'-diphosphate (ADP)	0.019	Quinate*	0.017	1,3,7-trimethylurate	0.007
Trigonelline* (N'-methylnicotinate)	0.025	Choline phosphate	0.018	1,7-dimethylurate	0.015
Xanthine	0.026	Theophylline	0.021	Acesulfame	0.017
Palmitoyl ethanolamide	0.028	Significance threshold ($p < 0.05$)		Ectoine	0.022
2-hydroxy-acetaminophen sulfate	0.029	Trigonelline* (N'-methylnicotinate)	0.071	Vanillate	0.023
Tryptophan betaine**	0.029	Sphingadienine	0.077	Quinate*	0.023
Uridine 5'-diphosphate (UDP)	0.034	Androstenediol (3beta,17beta) disulfate (1)	0.079	2-ketogluconate	0.031
Quinate*	0.036	4-guanidino-butanoate	0.081	2-ketogulonate	0.031
1-(1-enyl-palmitoyl)-2-oleoyl-GPC	0.038	3-hydroxyhippurate	0.082	Malonylcarnitine	0.032
1-stearoyl-2-linoleoyl-GPE	0.040	Pyroglutamine*	0.088	N-acetyl-S-allyl-L-cysteine	0.034
Palmitoyl dihydrosphingomyelin	0.040	2-hydroxydecanoate	0.090	N-acetyl-aspartyl-glutamate (NAAG)	0.036
1-stearoyl-2-arachidonoyl-GPE	0.043	Hippurate	0.118	Tryptophan betaine**	0.037
Glycocheno-deoxycholate	0.045	Argininosuccinate	0.137	5-acetylamino-6-amino-3-methyluracil	0.038
p-cresol sulfate	0.046	2,3-dihydroxy-isovalerate	0.138	3-methylglutaryl-carnitine (2)	0.039
Inosine	0.050	Anserine	0.138	5-hydroxyhexanoate	0.041
Significance threshold ($p < 0.05$)		7-methylguanine	0.141	Tartarate	0.042
Sphingomyelin	0.051	4-acetamidophenol	0.142	Allantoin	0.043
3-hydroxyhippurate	0.051	Pimelate (heptanedioate)	0.145	Fucose	0.043
4-methylcatechol sulfate	0.055	5-oxoproline	0.152	Trigonelline* (N'-methylnicotinate)	0.047
Nicotinamide adenine dinucleotide reduced (NADH)	0.056	4-methylcatechol sulfate	0.155	Significance threshold ($p < 0.05$)	

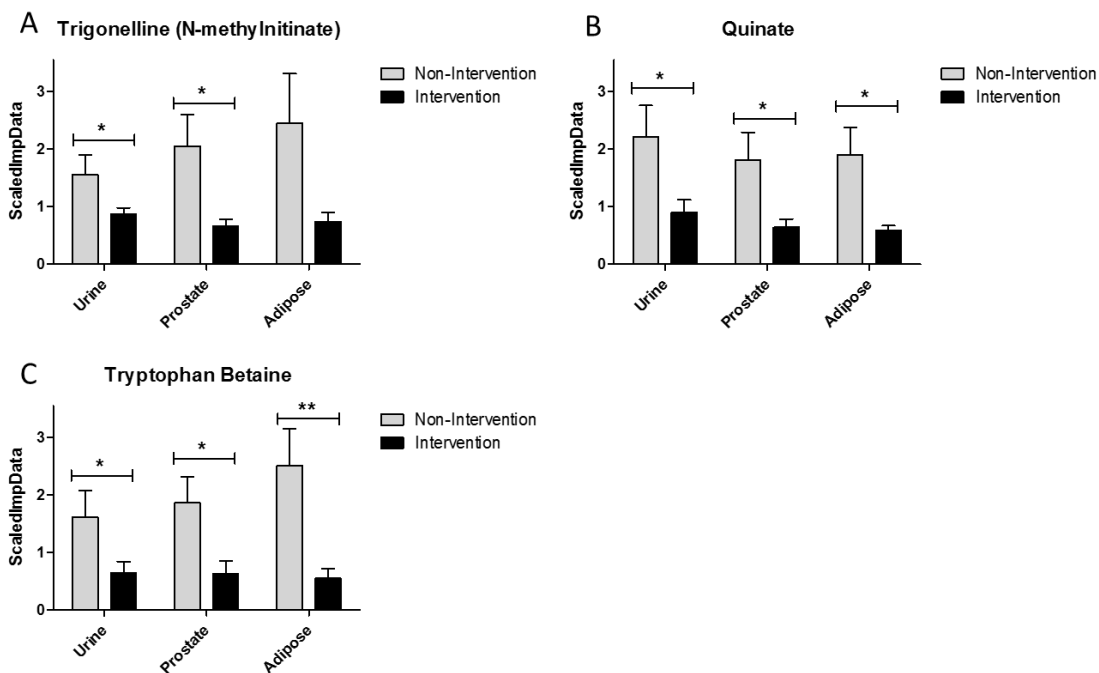


Figure 3-2 Graphs of individual metabolite differences between study arms for (A, B) dietary coffee metabolites (trigonelline and quinate respectively) and (C) peanut (tryptophan betaine) intake. Data are expressed as ScaledImpData (rescaled values to set the median equal to 1) \pm SD; unpaired Student's t-tests, *= $p < 0.05$, ** = $p < 0.01$.

3.4.3 Univariate Analysis Showed Significant Differences in Levels of SMCSO in Urine and ADP in Prostate

Although the Metabolon[®] platform was unable to detect SMCSO in either prostate or adipose tissue extracts, the increased concentration of SMCSO in urine in the dietary-intervention group represented the highest ranking metabolic difference by p-value (Figure 3-3). There was no difference in levels of inorganic sulphate in either prostate or adipose tissue because of the study diet. ADP, however, was significantly higher in prostate tissue in the intervention group, consistent with previous findings in the ESCAPE subgroup analysis (Figure 3-4).

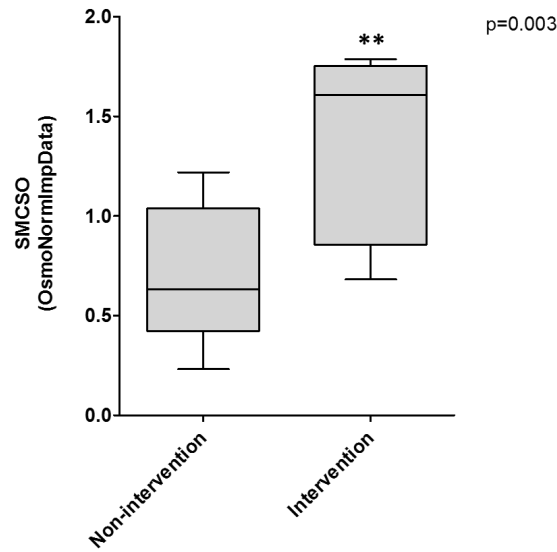


Figure 3-3 SMCSO in urine of SAP study arms. Concentrations significantly higher in the intervention group by unpaired Student's t-test, representing the most significant metabolite change in urine ($p < 0.01$). Data are expressed as OsmoNormImpData (values normalised by sample osmolality) \pm SD.

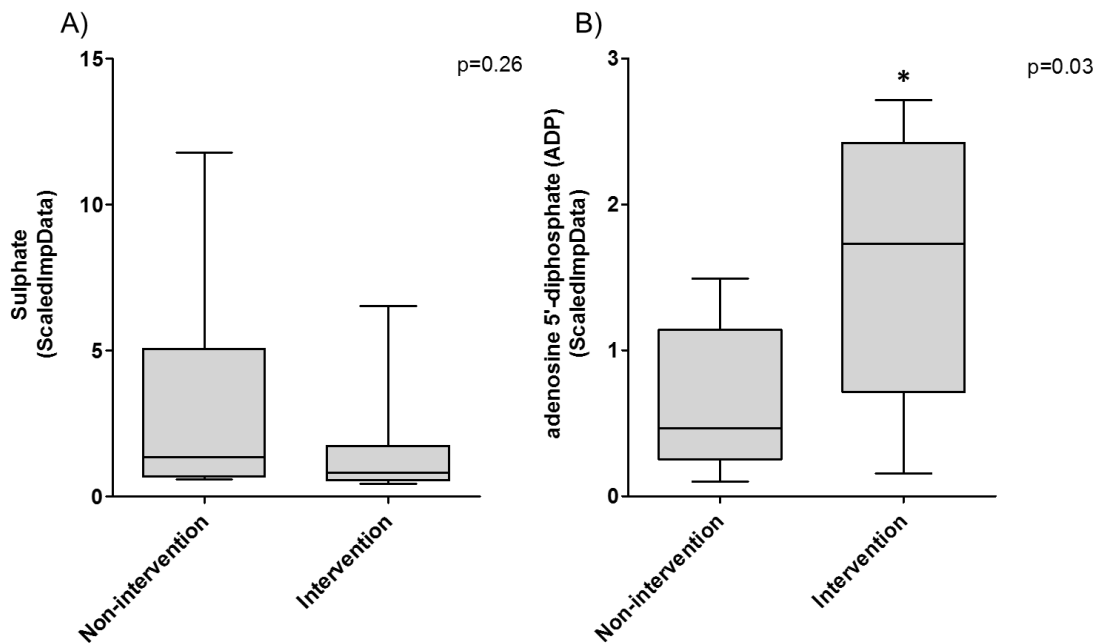


Figure 3-4 Individual metabolite data for sulphate (A) and ADP (B) in prostate tissue. No significant difference was seen by unpaired Student's t-test in sulphate levels between intervention arms ($p = 0.26$). ADP, however, was significantly higher ($p < 0.05$) in the intervention group. Data are expressed as ScaledImpData \pm SD.

3.4.4 Multiple Correction Testing

Multiple correction testing was performed using the Benjamini and Hochberg method [108]. Once corrected, none of the p-values for individual metabolites within separate tissue compartments remained significant. Ranking metabolites, however, provided useful insight into potential influences of the dietary intervention despite the relatively small study population. Comparing parallel findings in different tissue types and performing integrated pathway analyses were therefore justified to further investigate biological activity and dietary influence on prostate metabolism.

3.4.5 Integrated Pathway Analysis

All metabolomics data were uploaded by HMDB code to the Metaboanalyst 3.0 web tool [109]. Integrated pathway enrichment and topology analyses were performed on all metabolites recognised by the software; 382 in prostate, 299 in adipose and 494 in urine. No filters were applied, the data were log transformed for normalisation and underwent global testing with relative-betweenness centrality.

3.4.5.1 Prostate Tissue Pathway Analysis Showed Significant Differences in Steroid Hormone and Caffeine Metabolism

Prostate tissue pathway analysis revealed statistically significant differences in both steroid hormone biosynthesis and caffeine metabolism pathways (Table 3-4, Figure 3-5). The difference in caffeine metabolism corresponds to previously suggested differences in individual coffee metabolites identified by univariate analysis between groups (Figure 3-2). The difference in steroid hormone biosynthesis is due to consistent trends toward lower levels of both androst-5-ene diol and dehydroepiandrosterone sulphate (DHEA-S) in the intervention group, which were not significant individually. Purine metabolism, to which ADP is central, was the third highest-ranked pathway but did not reach statistical significance overall ($p=0.235$).

Table 3-4 Table of metabolic pathway differences in prostate tissue. Pathways are ranked by FDR-adjusted p-value with a significance threshold of $p < 0.05$. The 20 highest-ranked pathways are shown.

Metabolic Pathway	Match Status	p	FDR	Impact
Steroid hormone biosynthesis	3/99	0.025	0.958	0.015
Caffeine metabolism	5/21	0.037	0.958	0.344
Significance threshold ($p < 0.05$)				
Purine metabolism	20/92	0.235	0.958	0.340
Primary bile acid biosynthesis	4/47	0.243	0.958	0.081
Linoleic acid metabolism	3/15	0.244	0.958	0.656
Pyrimidine metabolism	18/60	0.257	0.958	0.403
Vitamin B6 metabolism	2/32	0.258	0.958	0.019
Arachidonic acid metabolism	2/62	0.272	0.958	0.216
Riboflavin metabolism	1/21	0.278	0.958	0.000
alpha-Linolenic acid metabolism	1/29	0.290	0.958	0.000
Phenylalanine metabolism	8/45	0.305	0.958	0.150
Glycosylphosphatidylinositol(GPI)-anchor biosynthesis	1/14	0.320	0.958	0.043
Starch and sucrose metabolism	4/50	0.352	0.958	0.257
Sphingolipid metabolism	7/25	0.355	0.958	0.547
Sulfur metabolism	4/18	0.360	0.958	0.040
beta-Alanine metabolism	11/28	0.380	0.958	0.156
Histidine metabolism	12/44	0.400	0.958	0.274
Nicotinate and nicotinamide metabolism	12/44	0.412	0.958	0.096
Pantothenate and CoA biosynthesis	8/27	0.425	0.958	0.292
Glycerophospholipid metabolism	15/39	0.448	0.958	0.581

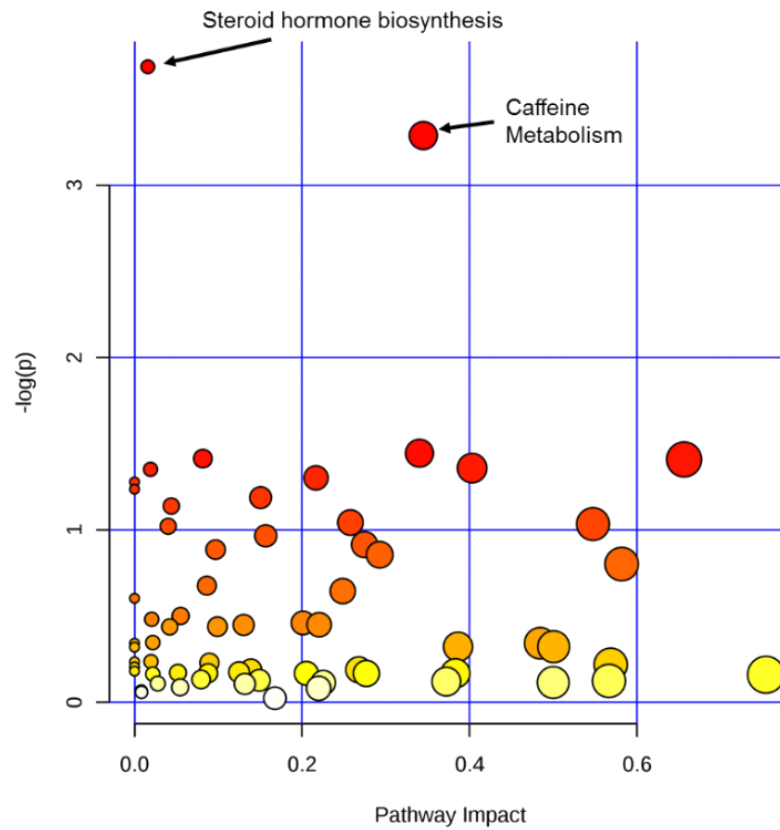


Figure 3-5 The “metabolome view” from prostate tissue pathway analysis using the Metaboanalyst software. Y-axis values are presented as $-\log(p)$ for graphical separation and X-axis values for pathway impact according to topology analysis. The match status and centrality within pathways confer higher pathway impact. Steroid hormone biosynthesis and caffeine metabolism are both significantly different ($p < 0.05$) between study arms.

3.4.5.2 Adipose Tissue Pathway Analysis Exhibited Similar Characteristics to Prostate

Similar to prostate tissue, pathway analysis for adipose identified steroid hormone biosynthesis and caffeine metabolism to be the highest-ranked pathways by p-value, although no pathway reached statistical significance in adipose by FDR-corrected p-value (Table 3-5, Figure 3-6).

Table 3-5 Table of metabolic pathway differences in adipose tissue. The 20 highest-ranked pathways by FDR-adjusted p-value are shown.

Pathway Name	Match Status	p	FDR	Impact
Steroid hormone biosynthesis	3/99	0.129	0.921	0.015
Caffeine metabolism	3/21	0.133	0.921	0.160
Nicotinate and nicotinamide metabolism	11/44	0.178	0.921	0.072
beta-Alanine metabolism	10/28	0.229	0.921	0.145
Sulphur metabolism	3/18	0.295	0.921	0.040
Arginine and proline metabolism	21/77	0.303	0.921	0.568
Valine, leucine and isoleucine biosynthesis	6/27	0.313	0.921	0.146
Selenoamino acid metabolism	1/22	0.329	0.921	0.000
Cyanoamino acid metabolism	4/16	0.334	0.921	0.000
Ether lipid metabolism	2/23	0.338	0.921	0.000
Ubiquinone and other terpenoid-quinone biosynthesis	2/36	0.351	0.921	0.036
Glutathione metabolism	9/38	0.355	0.921	0.338
Valine, leucine and isoleucine degradation	4/40	0.367	0.921	0.022
Synthesis and degradation of ketone bodies	1/6	0.393	0.921	0.000
Aminoacyl-tRNA biosynthesis	19/75	0.411	0.921	0.225
Alanine, aspartate and glutamate metabolism	11/24	0.437	0.921	0.754
Nitrogen metabolism	11/39	0.452	0.921	0.008
Tyrosine metabolism	5/76	0.478	0.921	0.047
D-Arginine and D-ornithine metabolism	2/8	0.485	0.921	0.500
Histidine metabolism	12/44	0.489	0.921	0.274

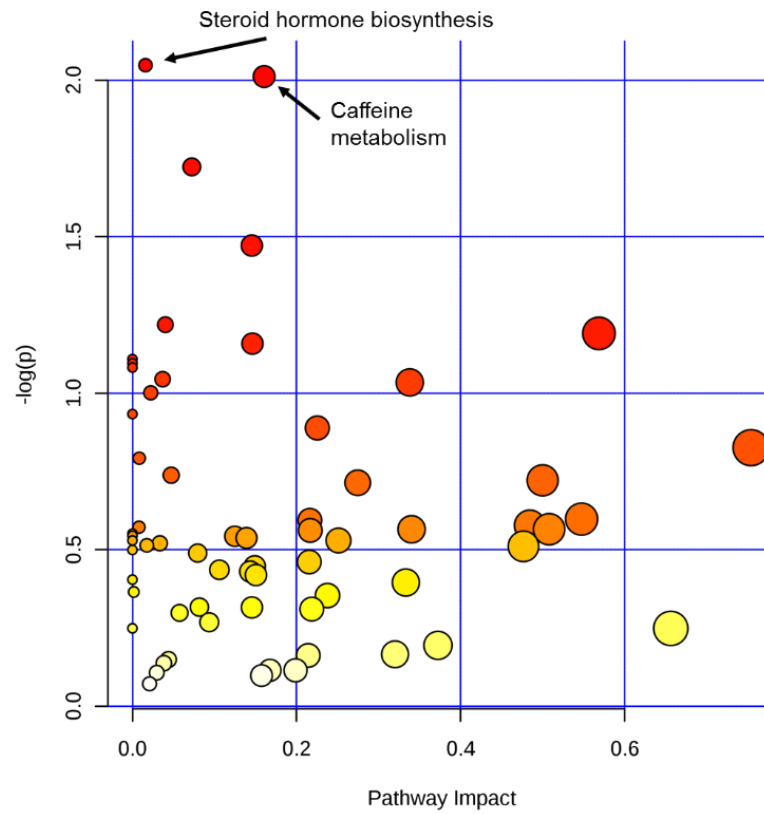


Figure 3-6 The “metabolome view” from adipose pathway analysis using the Metaboanalyst software. Y-axis values are presented as $-\log(p)$ for graphical separation and X-axis values represent pathway impact according to topology analysis. The match status and centrality within pathways confer higher pathway impact. Steroid hormone biosynthesis and caffeine metabolism are the highest-ranking pathways affected but do not reach statistical significance ($p < 0.05$).

3.4.5.3 Urine Pathway Analysis Reveals a Discrete Profile to Solid Tissues

In urine, the only pathway that reached statistical significance was glyoxylate and dicarboxylate metabolism (Table 3-6). Tartarate, glycerate and succinate appeared lower in the intervention group, although tartarate was the only metabolite to reach statistical significance ($p=0.04$).

Despite falling below the threshold for significance, differences in caffeine metabolism seen in the prostate may also be present in urine as this was the fifth highest-ranked pathway. In addition to ascorbate and fatty acid metabolism, glutathione metabolism also featured just outside the significance threshold due to a non-significant reduction in cysteinylglycine and an increase in ascorbic acid (Figure 3-7).

Table 3-6 Metabolic pathway differences in urine. Pathways are ranked by FDR-adjusted p-value with a significance threshold of $p < 0.05$. The 20 highest-ranked pathways are shown.

Pathway Name	Match Status	p	FDR	Impact
Glyoxylate and dicarboxylate metabolism	11/50	0.038	0.825	0.229
Significance threshold ($p < 0.05$)				
Ascorbate and aldarate metabolism	9/45	0.068	0.825	0.247
Fatty acid metabolism	1/50	0.082	0.825	0.000
Glutathione metabolism	10/38	0.086	0.825	0.108
Caffeine metabolism	11/21	0.105	0.825	0.637
Folate biosynthesis	2/42	0.108	0.825	0.131
Phenylalanine metabolism	17/45	0.112	0.825	0.244
Propanoate metabolism	5/35	0.114	0.825	0.003
D-Arginine and D-ornithine metabolism	3/8	0.141	0.825	0.500
Butanoate metabolism	7/40	0.144	0.825	0.145
Nicotinate and nicotinamide metabolism	11/44	0.158	0.825	0.087
beta-Alanine metabolism	11/28	0.172	0.825	0.239
Amino sugar and nucleotide sugar metabolism	6/88	0.192	0.825	0.011
Glycolysis or Gluconeogenesis	3/31	0.208	0.825	0.095
Pentose phosphate pathway	4/32	0.213	0.825	0.108
Histidine metabolism	16/44	0.247	0.825	0.345
Ether lipid metabolism	2/23	0.252	0.825	0.000
Valine, leucine and isoleucine biosynthesis	9/27	0.261	0.825	0.323
Synthesis and degradation of ketone bodies	1/6	0.267	0.825	0.000
Fructose and mannose metabolism	2/48	0.288	0.825	0.040

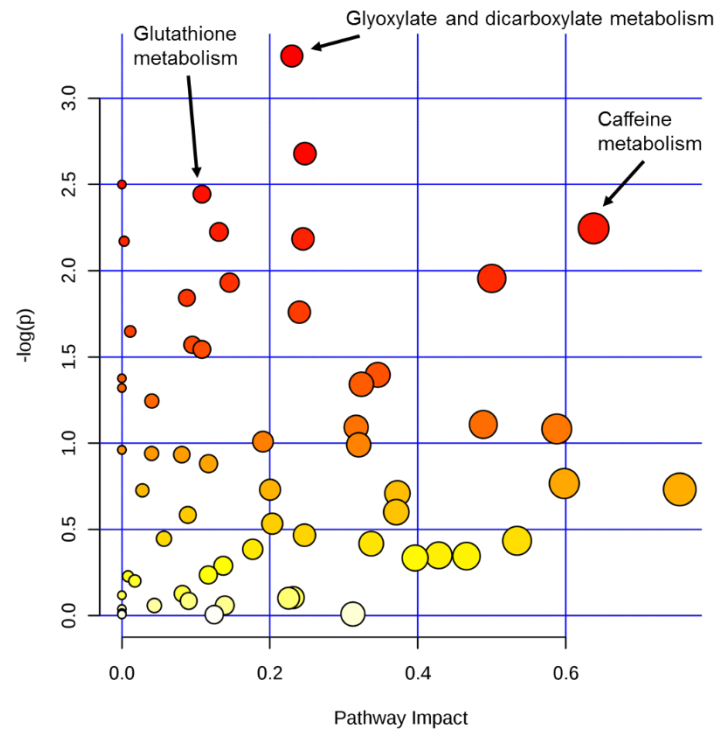


Figure 3-7 The “metabolome view” from urine pathway analysis using the Metaboanalyst software. Y-axis values are presented as $-\log(p)$ for graphical separation and X-axis values represent pathway impact according to topology analysis. The match status and centrality within pathways confer higher pathway impact. Glyoxylate and dicarboxylate metabolism is the only pathway to be significantly affected by intervention ($p=0.038$).

3.4.6 Steroid Hormone Analysis Demonstrates Common Trends Across Sample Types

The prominence of steroid hormone biosynthesis in the pathway analyses for both prostate and adipose tissue was further evaluated by comparison of the detectable hormone levels in all 3 tissue types (Figure 3-8). None reached statistical significance individually but a trend towards reduction in the intervention group explains the overall pathway impact. In prostate tissue, the levels of trigonelline (coffee metabolite) and androsterone sulphate are positively correlated, indicating a potential link between the highest ranked pathways (Figure 3-9).

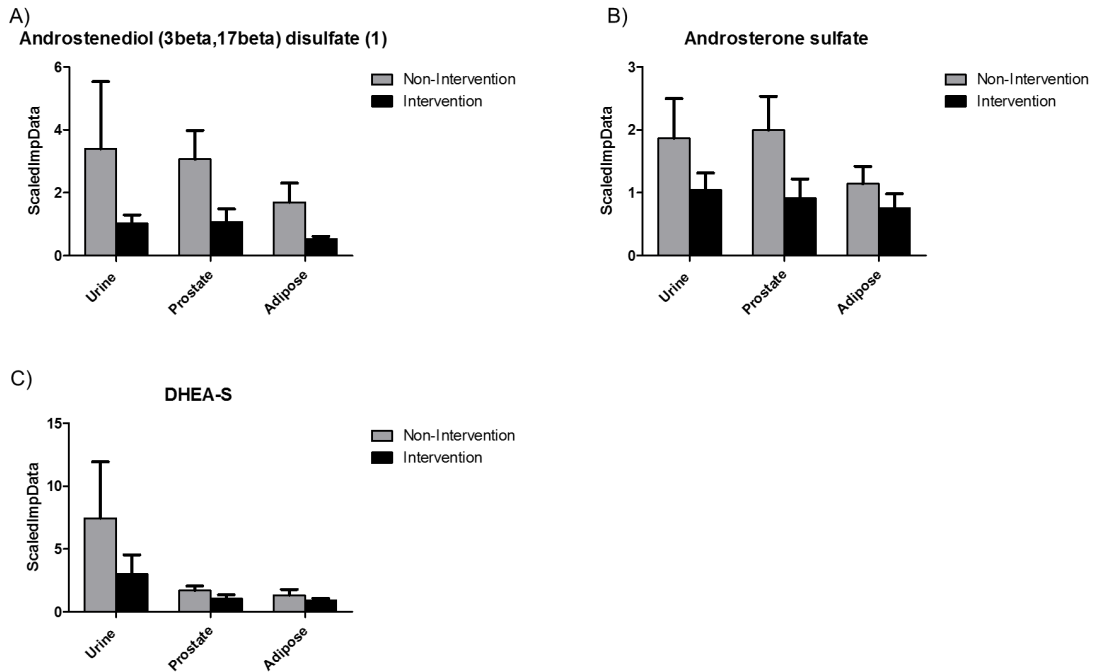


Figure 3-8 Graphs of steroid hormone levels detected by Metabolon[®] in all 3 tissue types. No statistically significant differences detected by unpaired student's t-test, but the trend towards lower levels in the intervention group accounts for impact on previous pathway analyses. Data are expressed as ScaledImpData \pm SD

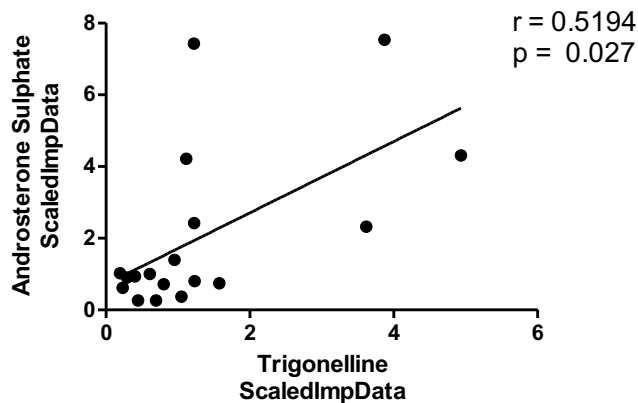


Figure 3-9 Correlation between trigonelline (coffee metabolite) and androsterone sulphate in prostate tissue. Weak but statistically significant ($p < 0.05$) positive correlation detected by Pearson correlation analysis. Data are expressed as ScaledImpData.

3.5 Discussion

3.5.1 Sulphate Levels in Prostate Tissue

No significant difference was seen in the sulphate levels of prostate tissue for men in either study arm (Figure 3-4). The range of levels in both study arms was much higher than those observed in the ESCAPE subgroup analysis (unpublished data). The SAP study was also powered for a difference between groups instead of a change from pre- to post-intervention, increasing potential sensitivity to distortion by individuals with much higher baseline sulphate levels. On review of individual health questionnaires, no participant was found to regularly take medications or supplements formulated to contain high levels of sulphate. The concentration of sulphate in tap water is known to vary nationally but will have been limited by recruiting participants from a single hospital site [110]. Other sources of dietary sulphate, in addition to the influence of colonic microbiota (sulphate-reducing bacteria) on sulphur metabolism were not controlled during the study [111]. Independent quantification of sulphate levels in the SAP study tissue will be discussed in Chapter 4, utilising an optimised protocol for targeted analysis.

3.5.2 ADP in Prostate Tissue

Ranking by p-value found higher levels of ADP in the prostate of men recruited to the intervention arm to be the second most significant metabolite difference (Table 3-3). Verification of one of the key ESCAPE study interim findings is therefore provided in an independent study delivering a broccoli-enriched dietary intervention. The link to change in cancer volume in ESCAPE, whether this is via sulphate-driven PAPS synthesis or otherwise, continues to represent a promising mechanism for cancer prevention by ATP depletion. As the Metabolon[®] platform was unable to detect ATP directly, targeted analytical methods are required to measure ATP, ADP and AMP in prostate tissue. Similarly, SMCSO could not be detected in the prostate or adipose tissue by global metabolomics. The difference in urine concentration of SMCSO between groups suggests the diet intervention is increasing its biological availability, but presence in tissue and links to cancer prevention are yet to be confirmed. Both purine and SMCSO quantification will also be described in full in Chapter 4.

3.5.3 Metabolic Pathway Differences Between Study Groups

Common to prostate, adipose and urine were higher levels of tryptophan betaine, trigonelline and quinate in the non-intervention groups (Figure 3-2, Table 3-3). In epidemiological studies, these metabolites all have specific dietary associations [112, 113]. Global metabolite profiling of participants in the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial and comparison with diet information from the National Cancer Institute's food frequency questionnaire linked tryptophan betaine to peanuts and both trigonelline and quinate to coffee consumption [113]. Habitual intake of these dietary components was beyond the scope of the CVFFQ used in the SAP study. However, it appears likely that participants in the non-intervention arm of the study were higher consumers of both coffee and peanuts in their habitual diet.

The two metabolic pathways in the prostate that were both significantly different between study arms were steroid hormone biosynthesis and caffeine metabolism.

Despite the small number of participants in the SAP study, a significant positive correlation between trigonelline and androsterone sulphate in prostate tissue could support a link between the two pathways *in vivo* (Figure 3-9). Increased coffee intake is associated with higher free and bound testosterone and sex hormone binding globulin (SHBG) levels, possibly due to stress axis stimulation by caffeine causing an increase in cortisol [114, 115].

Overall incidence of prostate cancer is considered unlikely to be affected by coffee consumption [116, 117]. The reduced risk of lethal prostate cancer reported in men who drank more than 6 cups of coffee per day in the Health Professionals Follow-Up Study was unrelated to caffeine content, suggesting a role for other bioactive constituents [118]. In contrast, a recent metabolomics study evaluated serum xanthine levels as a surrogate for coffee/caffeine consumption and found significantly higher levels in men who later developed T4-staged prostate cancer [105].

As cortisol levels were no different between arms of the SAP study it remains unclear whether coffee intake influenced hormone levels by this or another, caffeine-independent mechanism. The sample size is relatively small and these findings may be due to chance. The higher levels of tryptophan betaine from peanuts in the non-intervention group are also further suggestive of differences in habitual diet between groups. However, alternative hypotheses remain possible, including induction of detoxification pathways in response to the broccoli-enriched diet. Specific phase I cytochrome p450 enzymes are known to increase in broccoli consumption, with implications for metabolism of both caffeine and androgenic hormones [119, 120]. Combining RNA sequencing data from the SAP study with the full diet diary and tissue analysis of ESCAPE participants will hopefully provide further clarity on the influence of study versus habitual diet on these two pathways.

3.6 Conclusion

Global metabolomic analysis of prostate, adipose and urine from SAP study participants was technically successful and has highlighted several metabolite changes relevant to the study objectives. Although there was no significant difference in inorganic sulphate, participants in the intervention arm had higher levels of both SMCSO in urine and ADP in prostate tissue. To establish whether broccoli-derived metabolites are biologically available in tissue and driving ATP depletion, further targeted analysis is required (Chapter 4). Investigating whether sulforaphane or SMCSO could reduce ATP by inhibiting its production will also be considered with *in vitro* studies of their effects on prostate energy metabolism (Chapter 5). Glutathione metabolism pathways were not significantly different in any tissue analysed but ongoing work on PTEN redox status will also be described (Chapter 6).

4 Targeted Quantification of Sulphate, SMCSO and ATP Levels After Consuming a Broccoli-Enriched Diet

Summary

The beneficial effects of cruciferous vegetables on health are likely to extend beyond their content of glucosinolates to other specialised sulphur-containing metabolites, such as SMCSO. Some of the key findings from global metabolomic analysis of prostate, adipose and urine samples from participants of the SAP study were significant increases in SMCSO in urine and ADP in prostate following a broccoli-enriched diet. To test the hypothesis that these findings were linked by sulphate (derived from SMCSO) driving synthesis of PAPS and depleting ATP, increased analytical sensitivity was required to quantify the relevant metabolites in a range of biological matrices. This chapter describes the analyses of key metabolites, including ATP and SMCSO, in tissue and urine samples of men recruited into the SAP study. Extensive profiling of the metabolic changes induced by diet within the prostate gland, and at the systemic level, may prove influential in the future management and prevention of prostate cancer.

4.1 Introduction

Epidemiological studies support an inverse association between cruciferous vegetable consumption and both cancer and cardiovascular disease risk [40, 121]. The unique sulphur metabolism of cruciferous vegetables confers potential health benefits that include accumulation of glucosinolates, the hydrolysis breakdown products of which have proven biological activity in cell and animal models of prostate cancer [122]. Like many health foods, however, cruciferous vegetables are a rich source of other minerals and compounds which may contribute to their health-promoting properties. SMCSO, another sulphur-containing metabolite from broccoli, was the single highest-ranking difference in urine by global metabolomic profiling of men receiving a high-broccoli diet during the SAP study (Chapter 2).

By comparison to glucosinolate breakdown products, experimental evidence for the biological activity of SMCSO is sparse [57]. Human metabolism of SMCSO predominantly yields inorganic sulphate [123]. Availability of sulphate is the rate-limiting step in synthesis of PAPS, the universal sulphate donor, a process that consumes ATP [69]. SMCSO was, therefore, hypothesised to be responsible for the significant accumulation of both sulphate and ADP in the prostates of men receiving a broccoli-enriched diet for 12 months (ESCAPE study). Global metabolomic analysis of tissue obtained from the SAP study revealed no significant difference in sulphate levels between those in the intervention arm and those receiving their normal diet (Chapter 3). ADP, however, was significantly higher in the intervention arm ($p < 0.05$), suggesting loss of ATP either by (i) depletion (dephosphorylation) to ADP and AMP, or (ii) restricted production.

A validated LC-MS/MS method for targeted sulphate and SMCSO analysis in freeze-dried powder of plant material had previously been developed at QIB [124]. However, taking into consideration the limitations of quantitative analysis by LC-MS in terms of matrix effects [125], further work was required to optimise not only extraction conditions in human tissue and urine samples but also chromatography and detection parameters. Furthermore, a novel analytical method was developed for ATP quantification in different biological matrices, which is likely to provide a

useful tool for a wider range of applications in the future. Commercially-available bioluminescence assays for ATP are restricted by their ability to only measure one metabolite, and risk co-precipitation of ATP during extraction from protein-rich biological samples [126].

ATP is considered the “energy currency” of organisms, as well as playing vital roles in signal transduction, coenzyme function and as a precursor for RNA synthesis. The reactions that dephosphorylate ATP to ADP, and subsequently AMP, provide the energy to fuel most cellular functions [127]. The levels of ATP relative to ADP and AMP therefore provide a useful insight into the metabolic status of the cell [21]; a rise in AMPK resulting in a cascade of metabolic changes to conserve ATP [128].

The availability of ATP also has important implications for cancer cells, which establish metabolic characteristics that support rapid growth and proliferation but are unable to adapt their metabolism according to changes in ATP levels [79]. Therapeutic agents that drive ATP depletion or inhibit production in prostate cancer cells are currently under investigation, making accurate quantification of ATP, ADP and AMP a critical analytical process [129].

4.2 Aims

- To develop highly-accurate LC-MS/MS methods for quantification of sulphate, SMCSO and ATP in human tissues and urine
- To quantify sulphate, SMCSO and ATP levels in human tissues and urine from men enrolled on the SAP study

4.3 Materials and Methods

All LC-MS/MS methods applied after extraction of metabolites were optimised by Dr Shikha Saha (QIB Senior Analytical Chemist). Urine sample preparation followed the protocol developed by Dr Tharsini Sivapalan for the analysis of samples collected as part of The Bioavailability Of Sulforaphane From Broccoli Soups Study (BOBS, NCT02300324) (unpublished doctoral thesis).

4.3.1 Sample Collection and Processing

4.3.1.1 Ex Vivo Prostate Tissue

Prostate tissue was accessed via the Norwich Biorepository with ethical approval issued by the Faculty of Medical and Health Sciences Research Ethics Committee at the University of East Anglia in November 2015 (ref: 20152016 – 30HT). Men undergoing radical prostatectomy for confirmed prostate cancer consented to tissue banking with the Norwich Biorepository Adult Information Sheet for Patients and Consent Form (version 15, 21 February 2014). Immediately following extraction of the prostate, needle-biopsy tissue cores were taken from regions remote from the known cancer. Tissue cores were snap frozen on dry ice and stored at -80°C until required for analysis. No impact was made on the histological assessment of the remaining specimen, as confirmed by Professor Ball (NNUH histopathology consultant).

4.3.1.2 *In Vivo* Prostate, Adipose and Urine Samples from the SAP Study

As described in Chapter 2, ethical approval for the use of prostate, adipose and urine from SAP study participants was granted by the East of England - Cambridge East REC (ref: 16/EE/0054) in January 2016. Written informed consent was obtained for study participation, clinical consent for the biopsies and tissue banking consent at the Norwich Biorepository. Following DRE, a first-pass urine sample was collected, divided into 1ml aliquots and snap frozen on dry ice. Biopsy cores of both prostate and adipose were taken through the template grid during TPB. Those to be used for targeted analysis were immediately snap frozen on dry ice to minimise ischaemia time. All samples were stored at -80° C until required for analysis.

4.3.2 Sulphate and SMCSO Extraction from Tissue

Snap-frozen tissue cores were individually weighed on a Cubis® high-sensitivity balance (Sartorius) and transferred to screw-top tubes. 200µl of cold Milli-Q® water and 300µg of autoclaved, acid-washed 710 to 1180µm glass beads (Sigma®) were added to each tube. The tissue was completely homogenised using a DNA Fast-Prep® (MP Biomedicals) at 4.0m/s for 3 cycles of 60 seconds each. The samples were then placed on a revolving shaker for 15 minutes at 4° C. The tubes were centrifuged at 17,000g for 10 minutes at 4° C and 50µl of supernatant transferred to a new Eppendorf. 10µl of 50% trichloroacetic acid (TCA) were added to each sample to precipitate proteins. The centrifugation step was repeated and 50µl of supernatant transferred to HPLC insert vials for analysis by LC MS/MS.

4.3.3 Sulphate and SMCSO Extraction from Urine

Aliquots of urine were thawed on ice, vortexed briefly and 100µl was transferred to a new Eppendorf tube. A 10-fold dilution in 5% TCA was performed, the sample was vortexed again and then incubated on ice for 10 minutes to precipitate proteins. The sample was centrifuged at 14,000g for 10 minutes at 4° C and the supernatant transferred to an HPLC vial for analysis by LC-MS/MS.

4.3.4 Quantification of Sulphate and SMCSO by LC-MS/MS

Quantification was performed by matrix-matched calibration curve. Matrix matching was achieved by pooling samples from participants in the non-intervention arm of the SAP study. Stock solutions of 1mg/ml sulphate (Sigma®) and SMCSO (LKT laboratories Inc) were generated by dilution of weighed powder into Milli-Q® water. Serial 5-fold dilutions were performed in the relevant matrix to produce 6-point calibration curves from 10µg/ml to 0 on the day of analysis.

Sulphate was quantified using an Agilent 6490 triple-quad LC MS mass spectrometer (Agilent technologies) with Thermo Scientific Hypercarb, Porous Graphitic Carbon (PGC) (3 x 50mm, 3 µm) column. The system comprised a degasser, binary pump, column oven, cooled autosampler (4° C), diode array detector and 6490 mass spectrometer. 2µl were injected from each sample, with separation by 1% formic acid in Milli-Q® water (mobile phase A) and 1% formic acid in methanol (mobile phase B). The gradient started at 8% mobile phase B, increasing to 70% over 5.5 minutes and returning to 8% mobile phase B for re-equilibration over the last 9.5 minutes. The column temperature was maintained at 60° C and the flow rate at 0.3ml/min. The LC eluent flow was sprayed into the mass spectrometer interface without splitting. Inorganic sulphate was monitored using

mass spectrometry in MRM mode ($m/z = 97/80$ transition) with electrospray ionisation in the negative polarity. The mass spectroscopy source parameters were: gas temperature 200° C, gas flow 16 l/min, sheath gas temperature 400° C, sheath gas flow 12 l/min, capillary voltage 3000 V and nozzle voltage 1000 V.

Quantification of sulphate was performed by peak area against the matrix-matched standard curve, and identification by retention time and product ions. Table 4-1 summarises the optimal parameters for sulphate detection.

Table 4-1 The optimised LC MS/MS parameters for detection of the sulphate anion

Analyte	Retention time (mins)	Precursor ion (m/z)	Product ion (m/z)	Collision energy	Cell accelerator energy	Polarity
Sulphate	2.0	96.99	80.0	24	4	Negative

SMCSO was quantified with the same Agilent system described above. Samples were injected at 2 µl eluted at a flow rate of 0.3 ml/min on an Agilent SB-AQ 1.8µM (100 x 21mm) C18 column. Separation was carried out using 10mM ammonium acetate + 0.05% heptafluorobutyric acid in Milli-Q® water (mobile phase A) and 10mM ammonium acetate + 0.05% heptafluorobutyric acid in 90% methanol (mobile phase B). The gradient started at 2% mobile phase B, increasing over 2 minutes to 5% B and returning to 2% mobile phase B for re-equilibration over the last 2 minutes. The column was set at 20° C due to the instability of SMCSO at high temperatures. The LC eluent flow was sprayed into the mass spectrometer interface without splitting. SMCSO ion was monitored by mass spectrometry in MRM mode ($m/z = 87.9$) in positive polarity with electrospray ionisation. The source parameters were: gas temperature 200° C, gas flow 16 l/minute, sheath gas temperature 300° C with a sheath gas flow of 11 l/minute, a nebuliser pressure of 50 psi and capillary voltage 3500 V. Quantification was performed by peak area against the matrix-matched standard curve, and identification by retention time and product ions. Table 4-2 summarises the optimal parameters for SMCSO detection.

Table 4-2 The optimised parameters for detection of SMCSO ions by LC-MS/MS

Analyte	Retention time (mins)	Precursor ion (m/z)	Product ion (m/z)	Collision energy	Cell accelerator energy	Polarity
SMCSO	0.98	152.19	87.9	4	4	Positive
SMCSO	0.98	152.19	69.9	16	4	Positive
SMCSO	0.98	152.19	42.1	20	4	Positive

4.3.5 ATP, ADP and AMP Extraction from Prostate Tissue

Labelled (^{15}N) internal standards for ATP (purity 95%), ADP (purity 90%) and AMP (purity 90%) were purchased from Sigma[®]. 1mg/ml stock solutions were generated from dried powder in Milli-Q[®] water and stored at -20°C . On the day of analysis stock solutions were serially diluted in Phenol-TE (Sigma[®]) to a final concentration of mixed standards of $1\mu\text{g/ml}$. Snap-frozen tissue cores were individually weighed on a high-sensitivity balance and transferred to screw-top tubes. 200 μl of cold Phenol-TE containing internal standards and 300 μg of autoclaved, acid-washed 710 to 1180 μm glass beads (Sigma[®]) were added to each tube. The tissue was completely homogenised using a DNA Fast-Prep[®] (MP Biomedicals) at 4.0m/s for 3 cycles of 60 seconds each. The samples were then placed on a revolving shaker for 15 minutes at 4°C . The tubes were centrifuged at 17,000g for 10 minutes at 4°C . The supernatant was serially diluted to 1000-fold in Milli-Q[®] water before being transferred to an HPLC vial for analysis by LC-MS/MS.

Due to the highly labile nature of ATP, meticulous care was taken to ensure samples were maintained below 4°C at all stages of extraction and analysis [130].

4.3.6 Quantification of ATP, ADP and AMP by LC-MS/MS

ATP, ADP and AMP were quantified with the same Agilent system described above. Samples were injected at 2 μl eluted at a flow rate of 0.3 ml/minute on an Agilent SB-AQ 1.8 μm (100 x 2.1mm) C18 column. Separation was carried out using 50mM ammonium acetate in Milli-Q[®] water (mobile phase A) and 50mM ammonium acetate in methanol (mobile phase B). The gradient started at 2% mobile phase B, increasing over 5 minutes to 20% mobile phase B and finally re-equilibrated to 2% mobile phase B for 2 minutes. The column temperature was set at 40°C . The LC eluent flow was sprayed into the mass spectrometer interface without splitting. ATP, ADP and AMP ions were monitored using mass spectrometry in MRM mode, in positive polarity with electrospray ionization. The source parameters were kept the same as described for SMCSO. Quantification was performed by peak area against matrix-matched calibration curve. Identification was achieved based on retention time and product ions. Table 4-3 summarises the monitored ions and the optimised MS operating parameters of the analytes.

Table 4-3 The optimised parameters for detection of ATP, ADP and AMP ions by LC-MS/MS

Compound Name	Retention Time (mins)	Precursor ion (m/z)	Product ion (m/z)	Collision energy	Cell accelerator energy	Polarity
15N5 ATP	1.34	513.16	141.2	40	4	Positive
ATP1	1.34	508.15	136.1	48	4	Positive
ATP1	1.34	508.15	39.2	80	4	Positive
15N5 ADP	1.586	433.18	141	28	4	Positive
ADP1	1.586	428.2	136.2	24	4	Positive
ADP1	1.586	428.2	119	80	4	Positive
15N5 AMP	2.339	353.13	141	12	4	Positive
AMP	2.339	348.13	136	16	4	Positive
AMP	2.339	348.13	119.2	68	4	Positive

4.4 Results

4.4.1 Sulphate Quantification in Prostate Tissue Required Strict Experimental Controls

Baseline population levels of inorganic sulphate in the prostate were quantified during optimisation of the analytical technique. Tissue cores obtained from prostatectomy specimens were of comparable size and weight (2-5mg per core) to those obtained during TPB, depending on the size of the prostate gland. The method for sulphate extraction previously included a 95° C heating step after the tissue had been homogenised (data not shown). However, further optimisation revealed that the most reliable results were achieved with cold extraction and deproteinisation, which allowed preservation of the same sample for further analysis.

A total of 11 prostatectomy specimens were analysed to establish variability between individuals, which were quantified in 2 batches. Analysis of variance testing revealed no significant difference in the first 8 prostatectomy specimens ($p = 0.178$, $F = 1.975$) (Figure 4-1), which were run as a single batch of 4 biopsy tissue cores per patient. Analysis of a further 3 prostatectomy samples, which were extracted and run as a second batch revealed significant variability from the first ($p < 0.0001$, $F = 25.14$) (Figure 4-2). Interestingly, we observed inter-day variability in inorganic sulphate concentrations in the Milli-Q® water used for the LC-MS/MS analysis. Therefore, normalisation of samples analysed in separate batches was not possible due to the varying sulphate content of the water used as both extraction solvent and in generating standard curves. All SAP study samples were later analysed in a

single batch using the same original stock of Milli-Q® water as the first 8 prostatectomy specimens.

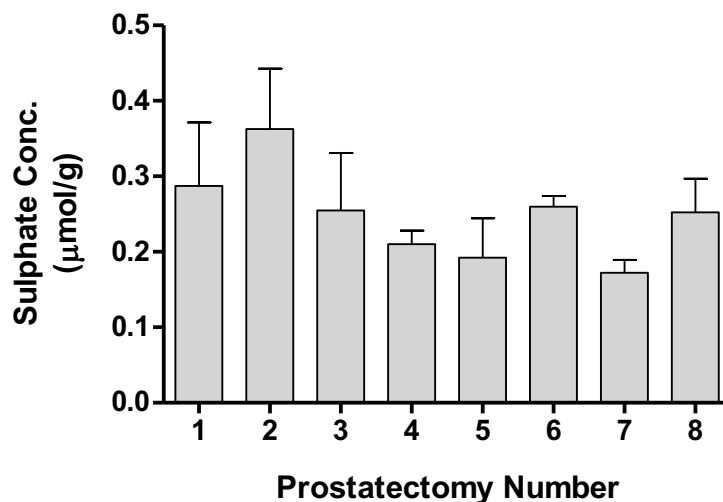


Figure 4-1 Sulphate levels ($\mu\text{mol/g}$ tissue) analysed as a single batch from 4 prostate biopsy cores per patient in men undergoing radical prostatectomy. Mean range 0.173 to 0.363 $\mu\text{mol/g}$ frozen tissue weight. Data shown as mean \pm SD. No significant difference between individuals by one-way ANOVA ($p = 0.178$, $F = 1.975$).

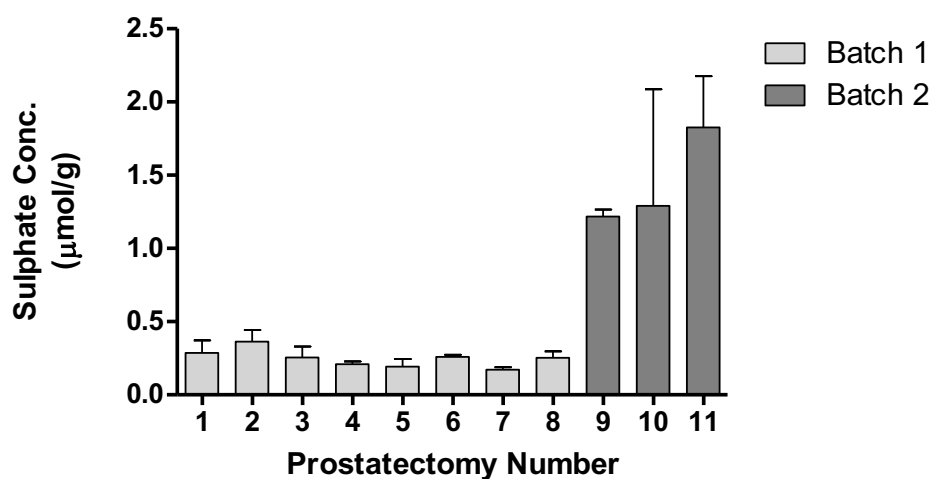


Figure 4-2 Sulphate levels ($\mu\text{mol/g}$ tissue) in prostate biopsy cores, indicating significant variability between batches due to inter-day variability of sulphate content in the Milli-Q® water used for sulphate extraction and generation of standard curves. Data shown as mean \pm SD. Significant difference between batches by one-way ANOVA ($p < 0.0001$, $F = 25.14$).

4.4.2 Sulphate Levels in the Prostate Did Not Significantly Increase Following a Broccoli-enriched Diet

A single snap-frozen prostate biopsy core was analysed per participant enrolled in the SAP study. All samples were extracted and analysed in parallel as a single batch with a single stock of Milli-Q[®] water used for both extraction and generation of calibration curves. Two participants in the non-intervention group were found to have very high levels of sulphate, which were out of keeping with the rest of the cohort, as well as previous results from prostatectomy samples (Figure 4-3). Grubbs' test, also called extreme studentised deviate test, was performed on both results and they were found to be significant outliers ($p < 0.05$). The two outliers were removed from further analysis. Although there was no statistically significant difference between groups, an apparent bi-modal separation emerged in individuals receiving the dietary intervention as part of the SAP study (Figure 4-4).

Quantification of sulphate in the adipose tissue of SAP study participants revealed no significant difference between groups (Figure 4-5).

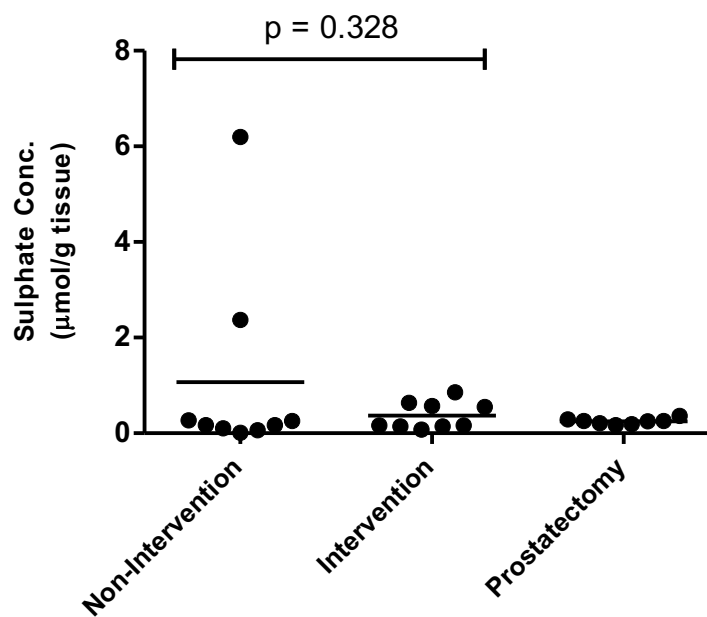


Figure 4-3 Graph of sulphate levels ($\mu\text{mol/g}$) in prostate tissue comparing both study arms of the SAP study to previous results from prostatectomy specimens. Data are shown as mean + individual concentrations for visual separation. By carrying out a Grubb's test, two outliers were identified in the non-intervention group and subsequently removed from further analysis.

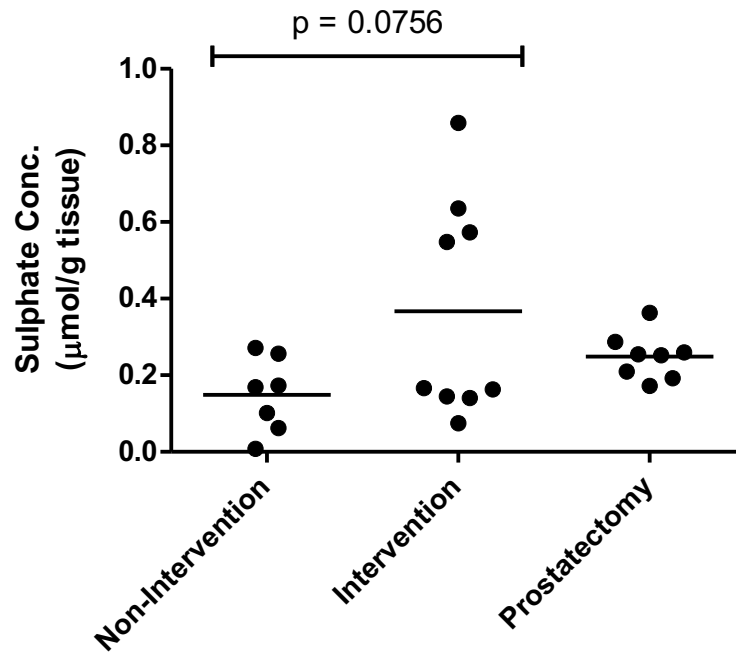


Figure 4-4 Graph of sulphate levels ($\mu\text{mol/g}$) in prostate tissue comparing both study arms of the SAP study to previous results from prostatectomy specimens (outliers removed). Data are shown as means + individual concentrations for visual separation. No statistically significant difference was observed by unpaired Student's t-test between groups, but there is a trend towards clear separation of the individuals in the intervention arm of the SAP study.

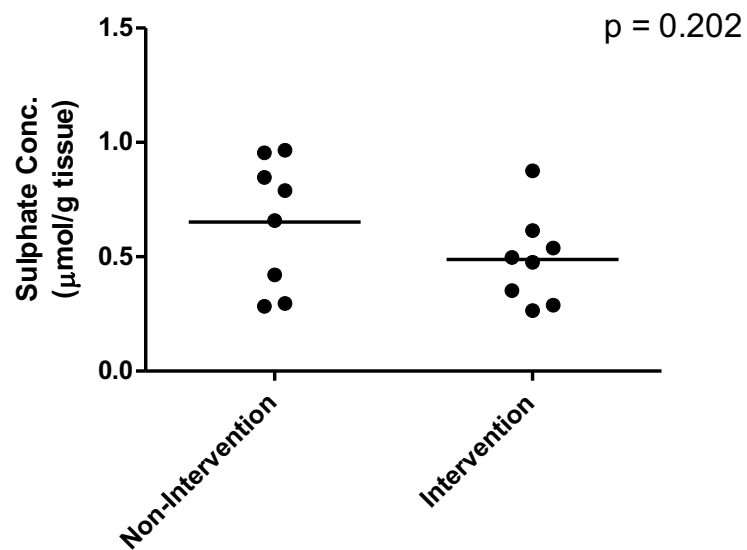


Figure 4-5 Graph of sulphate levels ($\mu\text{mol/g}$) in adipose tissue of SAP study participants. Data shown as mean + individual concentrations. No statistically significant difference between groups.

Comparing global metabolomic analysis by Metabolon[®] with targeted analysis at QIB did not reveal statistically significant correlations in sulphate quantification in either the adipose or prostate tissues (Figure 4-6). While this may represent metabolic differences between separate tissue biopsy samples, this would be especially unlikely in adipose tissue as it is known to be highly homogenous [130]. Another possibility could be the standard method of metabolite extraction and processing in global metabolomics, which is not optimised for highly water-soluble metabolites such as inorganic sulphate. Tissue extraction was carried out in 80% methanol/20% water, and a further substantial increase in the requirement for organic solvents is encountered with other methods on the Metabolon[®] platform, such as HILIC. Furthermore, the most likely limitation is that the reverse phase LC-MS Negative platform used by Metabolon[®] for sulphate analysis is too polar for accurate detection of inorganic anions, a problem that was overcome at QIB by using the Hypercarb column.

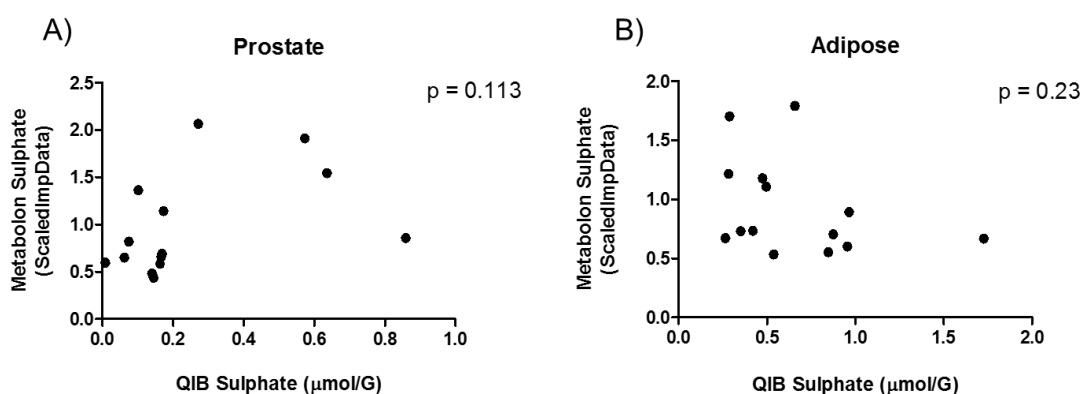


Figure 4-6 Graphs showing quantification of inorganic sulphate on a global metabolomics platform compared to targeted analysis at QIB in human prostate (A) and adipose (B) tissue samples collected from the SAP study. No significant correlation seen in either prostate or adipose tissue by Pearson correlation coefficient analysis.

4.4.3 A Broccoli-Enriched Diet Led to a Significant Accumulation of SMCSO in Urine and Prostate

Results obtained by carrying out a targeted analysis of SMCSO in samples collected from the SAP study revealed significantly higher levels of SMCSO following the broccoli intervention (Figure 4-7) in accordance with global metabolomics data reported in 0. The results positively correlate with strong statistical significance ($p < 0.001$, $r^2 = 0.536$), validating the method of SMCSO quantification in urine used by Metabolon[®] (Figure 4-8).

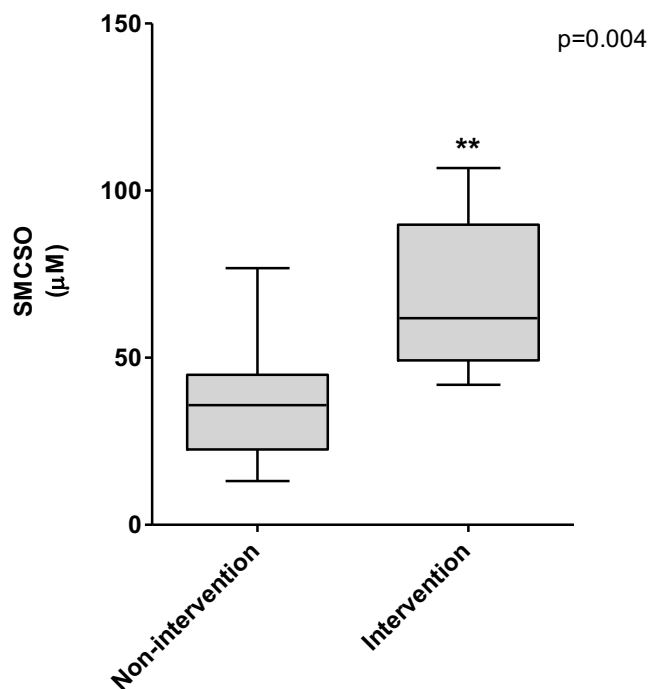


Figure 4-7 Graph of SMCSO quantification in the urine of SAP study participants by targeted LC-MS/MS analysis. The line in the middle of the box-and-whiskers plots is plotted at the median. Significantly higher concentrations by unpaired Student's t-test in the intervention group confirm previous findings from global metabolomics analysis reported in [Figure 3-3](#).

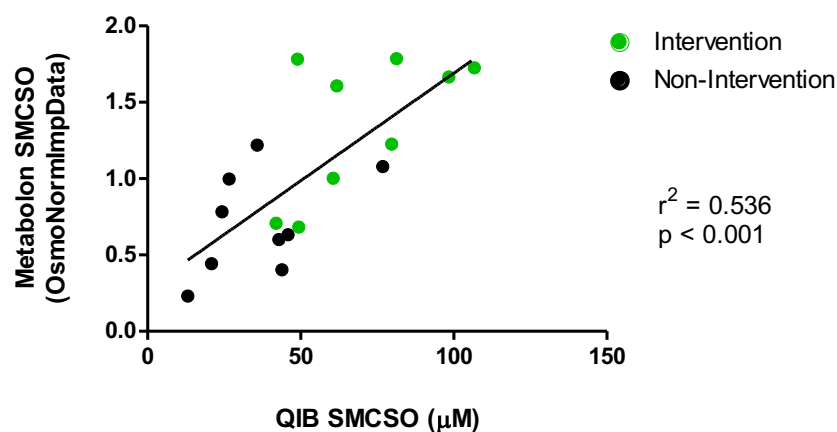


Figure 4-8 Graph showing strongly significant positive correlation between SMCSO detected in SAP study urine by global metabolomics and targeted analysis at QIB by Pearson correlation coefficient analysis.

Whereas SMCSO had been below the limit of detection for global metabolomics in prostate and adipose tissue, the aqueous extracts from both tissue types were found to have detectable levels by targeted LC-MS/MS analysis. The concentrations were higher in adipose tissue than prostate, and Grubbs' test was used again to determine outliers in the adipose data set; two in the non-intervention and one in the

intervention group. Both tissue types appeared to contain higher concentrations of SMCSO in the intervention group, although statistical significance was only reached in prostate tissue ($p = 0.005$, Figure 4-9). This was possibly due to a reduction in statistical power once the outliers were removed from the adipose data set.

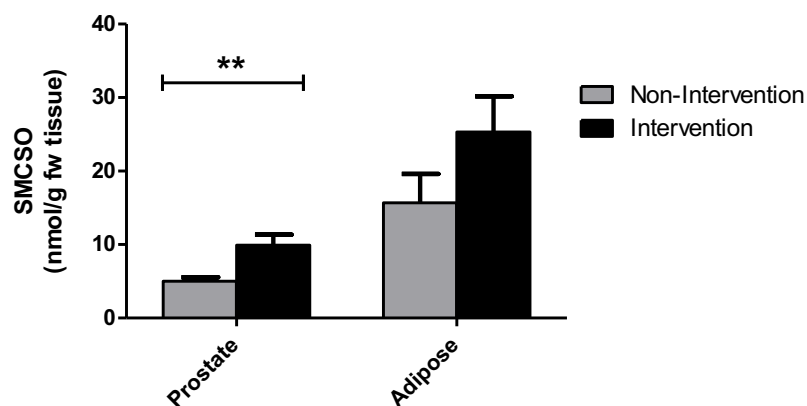


Figure 4-9 Graph of SMCSO concentrations in prostate and adipose tissue of SAP study participants. Data shown as means \pm SD. Significantly higher levels by unpaired Student's t-test were seen in the prostate of men randomised to the intervention arm (** = $p < 0.01$). The increase in adipose tissue did not reach statistical significance, possibly due to a reduction in power following removal of 3 outliers.

4.4.4 Isothiocyanates Were Not Detectable in Adipose or Prostate Tissue

Using the LC-MS/MS method described for quantification of urinary ITCs (Section 2.3.8), the aqueous extracts from both prostate and adipose tissue were also analysed. No detectable peaks were identified for sulforaphane, SF-Cys, SF-NAC or ER-NAC. This may reflect either an absence of accumulation in the tissue, or a transient increase after broccoli consumption that had cleared by the time the samples were collected.

4.4.5 A Broccoli-Enriched Diet Did Not Reduce ATP levels in the Prostate

Developing a novel method for quantifying ATP, ADP and AMP in prostate tissue required optimisation at several stages of the analysis. A range of extraction solvents have previously been reported for quantifying ATP in both plant and animal tissues, including water, TCA, perchloric acid, ethylene glycol, 50% methanol and Phenol-TE [126, 131, 132]. As the highest recovery rates of ATP in human and animal tissues have been with methanol and Phenol-TE, these were compared both with each other and against a simple aqueous extract (data not shown). Consistent with findings in visceral tissues obtained from mice, Phenol-TE yielded the highest and most reproducible results [126].

ATP supplies are rapidly depleted in ischaemic tissue, so comparison was made between samples that had been obtained by TPB *in vivo* and immediately snap frozen, and those that were obtained from *ex vivo* prostatectomy specimens (Figure 4-10). The total levels of ATP did not reach a statistically significant difference between groups and were low relative to both ADP and AMP throughout.

ADP and AMP were both significantly higher ($p < 0.01$ and $p < 0.05$ respectively) in the *ex vivo* prostatectomy tissue, as was the AMP:ATP ratio ($p < 0.01$), confirming the effects of ischaemia on energy balance.

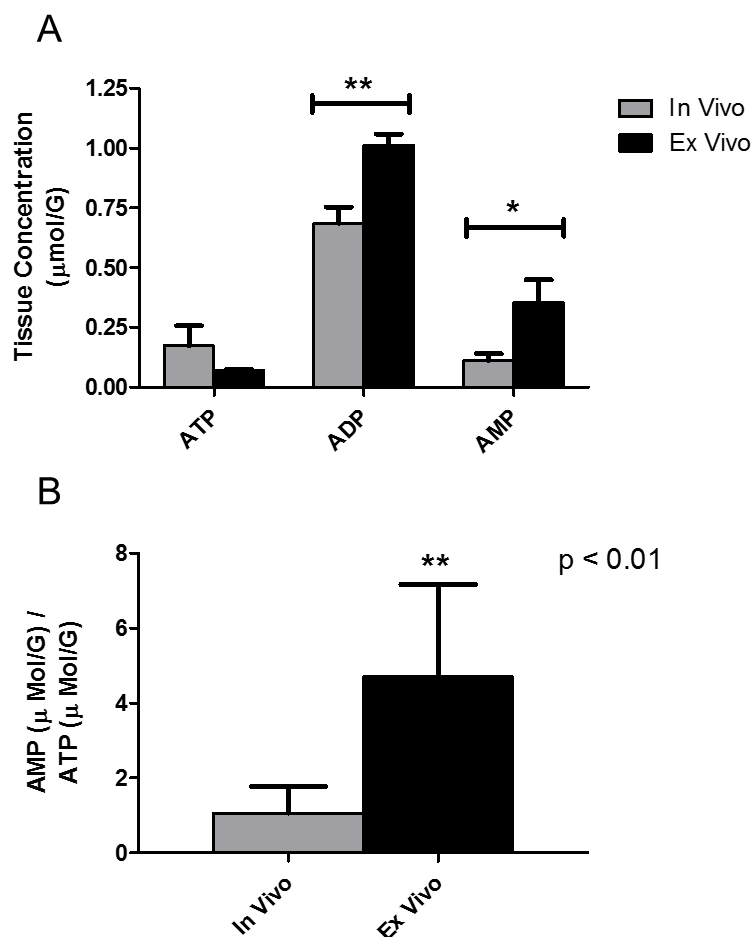


Figure 4-10 Graphs showing (A) concentrations of ATP, ADP and AMP ($\mu\text{mol/g}$) in 8 perfused '*in vivo*' biopsy tissue samples and 3 ischaemic '*ex vivo*' prostatectomy tissue samples and (B) the proportion of AMP to ATP. Data shown as means \pm SD. Statistical analysis by unpaired Student's t-test (* = $p < 0.05$, ** = $p < 0.01$).

Analysing the prostate tissue from SAP study volunteers revealed no significant difference in levels of ATP, ADP or AMP, or in the AMP:ATP ratio between study arms (Figure 4-11). The results previously obtained for levels of ADP by global metabolomics analysis (Chapter 2) suggested a significant increase ($p = 0.03$) because of the dietary intervention. The results obtained by targeted analysis at QIB did not detect similar results despite having a statistically significant ($p < 0.05$) but not strong correlation ($r^2 = 0.252$) to the data from Metabolon[®] (Figure 4-12). We have shown that ischaemia time has an impact on ADP levels, thus the 24-hour period at room temperature and subsequent drying down of extraction solvent in the Metabolon[®] protocol may account for these conflicting results.

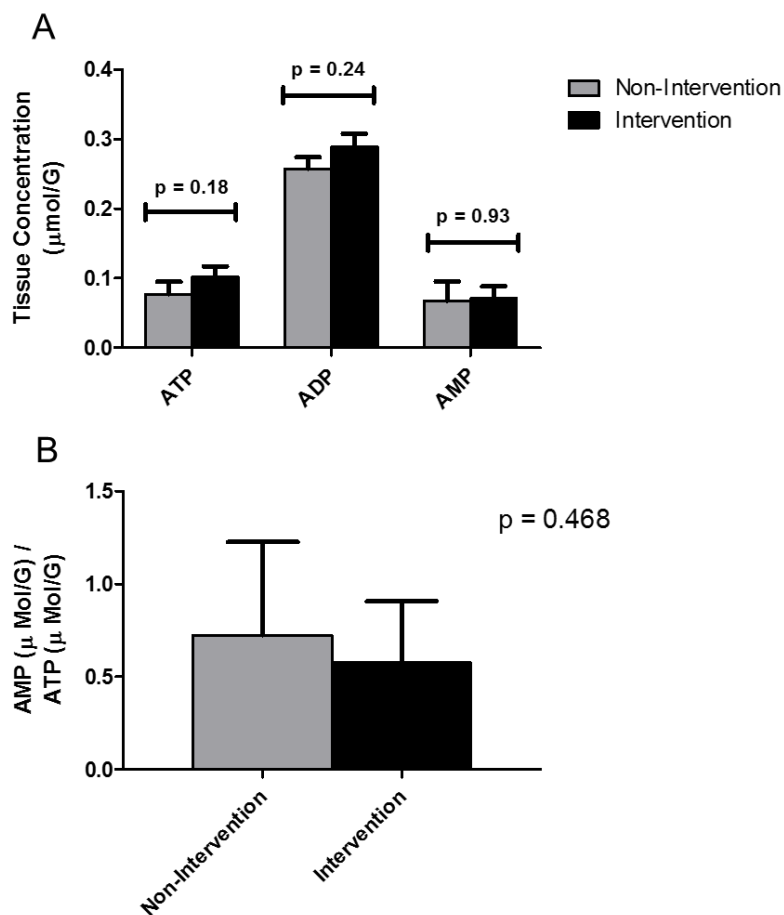


Figure 4-11 Graphs showing (A) concentrations of ATP, ADP and AMP ($\mu\text{mol/g}$) in the prostate tissue of SAP study participants and (B) the proportion of AMP to ATP. Data shown as means \pm SD. No statistically significant differences detected by unpaired Student's t-tests.

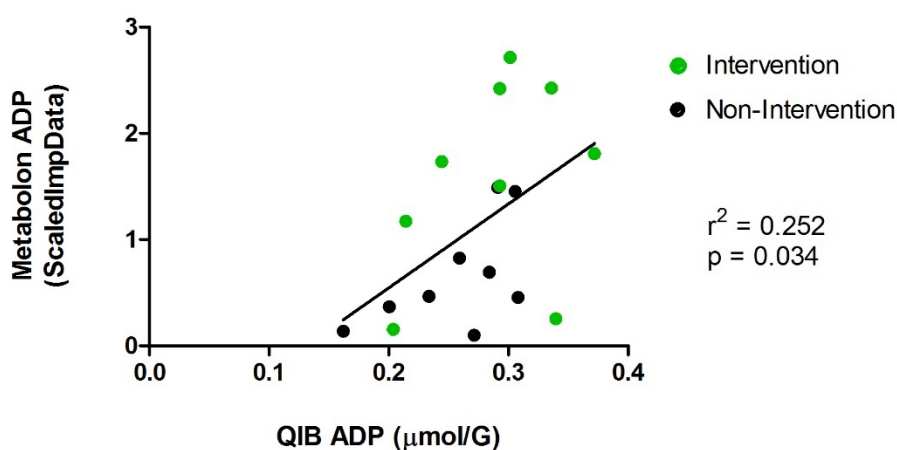


Figure 4-12 Graph showing correlation between ADP detected in SAP study prostate tissue by global metabolomics carried out by Metabolon[®] and targeted analysis at QIB. Weak but statistically significant positive correlation detected by Pearson correlation coefficient analysis ($r^2 = 0.252$, $p < 0.05$).

4.4.6 Sulphate, SMCSO and ATP Levels in the Prostate Were Not Influenced by Other Explanatory Variables in the SAP Study

The baseline characteristics of the SAP study participants were evaluated as explanatory variables in the results of targeted metabolite quantification. Multi-linear regression analysis was performed with sulphate, SMCSO and ATP as the dependent variables and study arm, GSTM1 genotype, cruciferous vegetable intake and histological findings as the explanatory variables. The only variable to account for a significant metabolite change was the intervention arm of the SAP study and higher levels of SMCSO in both urine and prostate.

4.5 Discussion

Despite the wealth of information provided by global metabolite profiling across a range of biological samples, independent analysis of target metabolites is crucial to confirm specific findings.

The method for quantifying inorganic sulphate described in this chapter utilises an extraction protocol that has been optimised for biological samples of human origin (prostate/adipose tissue). The LC-MS/MS method used also offers a robust method for retention of highly polarised metabolites. Not only is inorganic sulphate widely abundant, posing risks for sample contamination, but the content in purified water is also variable [110]. The importance of highly-controlled experimental conditions and same-day analysis were highlighted by the differences in batched samples during the earlier analysis of prostatectomy specimens. Findings obtained from the method validation process will be extremely valuable for future studies. The results of targeted sulphate analysis provided no evidence to support the SAP study hypothesis that a broccoli-enriched diet leads to accumulation of sulphate in the prostate. The apparent bimodal separation of sulphate levels in the prostate of men in the intervention arm (Figure 4-4) suggests that a few individuals may have characteristics that make them susceptible to an effect. However, these were not attributable to any of the explanatory variables accounted for in the study.

SMCSO represents an under-investigated phytochemical with potential biological activity supporting anti-cancer, anti-diabetic and cardiovascular health benefits [57]. A wide range of analytical techniques have been applied for the analysis of SMCSO in plant materials [133-135], but few reports exist for analysis of samples of human origin [136, 137]. Results obtained from optimising a LC-MS/MS method in urine and different tissue types (prostate and adipose) will help further work not only for assessing SMCSO bioavailability and biodistribution *in vivo* but also for investigating its biological activity in *ex vivo* models. Targeted quantification of SMCSO in this chapter independently confirms the higher levels seen in the urine of men in the intervention arm of the SAP study, with close correlation to the previous global metabolomic results (Figure 4-8). It also reveals for the first time an accumulation of intact SMCSO in adipose and prostate tissues, with a significant increase in the prostate after just 4 weeks of intervention with a broccoli-enriched diet. The degradation of SMCSO, which is known to ultimately yield a high proportion of sulphate, includes a number of reactive intermediate products [123]. In cell and animal models, used at higher levels than those of SMCSO detected in prostate and

adipose tissue, these products have been proven to induce apoptosis in cancerous cell lines, including prostate cancers, and protect against a number of pro carcinogens [55]. Both caspase-dependent and -independent mechanisms have been suggested, leaving extensive scope for further investigation [52].

The emergence of therapeutic ATP depletion to target lung and breast cancers, and the recent finding that ATP synthase is overexpressed in aggressive prostate cancer, mark the need for accurate methods to quantify ATP in tissue and assess response to treatment [138, 139]. The novel method of quantification described in this Chapter surpasses commercially-available fluorescence-based assay kits by simultaneously measuring ATP, ADP and AMP against labelled internal standards. It also offers preparation and extraction techniques specific to small-volume prostate tissue biopsies, which have not previously been described. As the changes in tissue after a period of ischaemia were also quantified, the method was validated against a positive control for detection of a significant change in energy state.

No significant differences were detected in ATP or its dephosphorylation products in the prostate tissue of SAP study participants. The broccoli-enriched diet at the dose administered (3 x 300g portions per week) did not, therefore affect ATP levels in the tissue. As the results of global metabolomic profiling had initially suggested an increase in ADP, further *in vitro* studies were carried out to investigate the effects of sulforaphane and SMCSO on mitochondrial energy metabolism; ATP production rather than the hypothesised mechanism for depletion. Results obtained from *in vitro* models are reported in Chapter 4.

4.6 Conclusion

Highly-sensitive LC MS/MS techniques have been developed to quantify sulphate, SMCSO and ATP in biological samples. These methods represent valuable analytical tools for carrying out targeted analyses in future dietary-intervention studies. The broccoli-enriched diet of the SAP study intervention led to one consistent and significant metabolic change; an accumulation of SMCSO in urine and prostate tissue that was unrelated to any of the other explanatory variables. The mechanisms of biological and potential anti-cancer activity of SMCSO require further investigation, but do not appear to be linked to either an accumulation of sulphate or a resultant depletion of ATP. The absence of detectable ITCs in prostate tissue, despite their presence in urine, may signify greater importance to the finding of SMCSO accumulation in the prostate.

5 *In Vitro* Effects of Sulforaphane and SMCSO on Prostate Mitochondrial Energy Metabolism

Summary

Dysregulation of mitochondrial metabolism is characteristic of cancer in the prostate and other body sites. The role for regulating or reversing these changes by diet is increasing, as is our understanding of the mechanisms that link specific phytochemicals to cancer prevention. In Chapter 3 a global metabolomics approach was taken to analyse the effects of broccoli consumption on prostate tissue *in vivo*. A key finding was a significant increase in ADP, which was suggestive of ATP depletion because of the study diet. Targeted LC MS/MS analysis revealed no significant difference in net ATP levels (Chapter 4), so an *in vitro* approach was taken to test the dynamic effects of broccoli-derived sulforaphane and SMCSO on real-time prostate bioenergetics.

5.1 Introduction

The role of dietary ITCs, including sulforaphane, has yielded promising results in both *in vitro* and animal models of prostate cancer [43, 44]. Multiple chemopreventive mechanisms have been suggested that involve pathways targeting inflammation, anti-oxidant defence, phase I enzyme modulation, and induction of phase II enzymes and apoptosis [140]. The overall effects are, therefore, likely to interact with mitochondrial metabolism in numerous directions. Recent studies in breast cancer have revealed additional roles for diet-derived ITCs in restricting mitochondrial bioenergetics [141, 142]. ATP depletion is also a common finding following a range of *in vivo* interventions with natural compounds that inhibit prostate cancer growth, such as ursolic acid, curcumin and resveratrol [85]. At high doses, sulforaphane induces reactive oxygen species (ROS) production in the cancerous PC3 prostate cell line, inducing apoptosis by interaction with the mitochondrial electron transport chain (ETC) [143]. Although not tested in prostate cells, high-dose sulforaphane is a known inhibitor of complex III of the ETC, with similar properties to antimycin (discussed below) [144, 145]. However, Nrf-2 activation by sulforaphane maintains mitochondrial homeostasis, preventing oxidative stress from affecting rate-limiting enzymes [146, 147]. Thus, metabolic reprogramming, which is inherent in prostate cancer, represents another potential target among the multi-modal effects of broccoli-derived bioactive compounds.

Global metabolomic analysis of prostate tissue from men enrolled in two broccoli-enriched dietary intervention studies undertaken at QIB have independently revealed an increase in ADP. In the ESCAPE study (REC 13/EE/0110; NCT01950143), paired samples were taken before and after 12 months of broccoli soup, investigating the effects of increasing glucosinolate content compared to a standard broccoli control. Participants in the SAP study (Chapter 2) were randomised to either a non-intervention arm or a high-dose broccoli-enriched diet for four weeks prior to prostate biopsy. The significant increase in ADP suggested a reduction of ATP, either through increased utilisation or reduced production. No difference in ADP levels was seen between intervention arms of the ESCAPE study (unpublished doctoral thesis), and increasing concentrations of SMCSO (another broccoli-derived metabolite) in urine of SAP study participants was the most significant metabolite change (Table 3-3). The biological activity on energy

metabolism of both sulforaphane and SMCSO therefore required separate investigation.

The Seahorse XFp Bioanalyser (Agilent®) was selected to simultaneously measure oxygen consumption rate (OCR) and extra-cellular acidification rate (ECAR) in cultured cells, indicating the rates of oxidative phosphorylation and glycolysis respectively [148]. Assays are run in real time with the capacity for acute injections of up to four test compounds. The dynamic response of cells to stressed conditions elicits their capacity to adapt various parameters of mitochondrial function. However, individual cell lines and assay conditions require careful optimisation before tightly-controlled and reproducible results can be obtained. In this chapter the application of the Agilent Mitochondrial (Mito) Stress Test, the Glycolysis Stress Test and the Mito Fuel Flex Fatty Acid Oxidation Capacity Test will be fully described [149-151].

The substrates of respiration, NADH and FADH₂, donate electrons to the mitochondrial ETC [152]. NADH donates directly to complex I, initiating a series of redox reactions that release energy and pump protons out of the matrix and into the intermembrane space [152]. FADH₂ releases electrons less readily and is dependent on complex II, which does not span the membrane or transport protons [152]. Electrons from both complex I and II are transported by the mobile electron carrier ubiquinone (Q) to complex III, at which point more protons are pumped out and the electrons continue via another carrier (cytochrome C) to complex IV. In addition to pumping out further protons, complex IV is the site of oxygen consumption; O₂ dissociates and then binds with protons from the matrix to form water. The flow of protons back down their electrochemical gradient via ATP synthase provides the energy for ATP production from ADP and phosphate [153]. The Mito Stress Test allows quantification of ATP production, maximal respiration and non-mitochondrial respiration by manipulation of the mitochondrial electron transport chain. Figure 5-1 shows the sites of action of the compounds injected during the assay. OCR is measured before and after sequential injections of oligomycin (ATP synthase inhibitor), carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP, mitochondrial membrane uncoupler) and rotenone/antimycin A (complex I and III inhibitors). The OCR response at each stage can be used to determine the separate characteristics of mitochondrial function (Figure 5-2A).

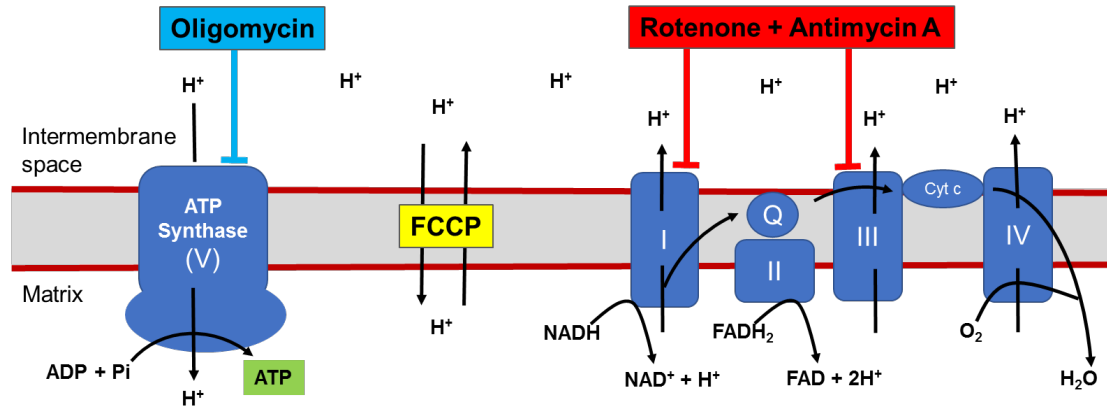


Figure 5-1 The mitochondrial electron transport chain and the sites of action of the compounds injected during the Mito Stress Test assay.

OCR can also be used to determine the capacity and dependency on specific substrates fuelling oxidative phosphorylation. Inhibition of individual pathways relative to combined inhibition of others can be applied to glucose, glutamine and fatty acids with the respective injections of a mitochondrial pyruvate transporter inhibitor (UK5099), a glutaminase inhibitor (Bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulphide, BPTES) and an irreversible O-carnitine palmitoyltransferase-1 (CPT-1) inhibitor (etomoxir).

The Glycolysis Stress Test allows quantification of glycolysis and glycolytic capacity by measuring ECAR (Figure 5-2B). Pyruvate produced by metabolism of glucose in glycolysis can either enter the mitochondrial TCA cycle or be converted to lactate in the cytoplasm [154]. The result of lactate production is extrusion of protons into the extracellular space, which in cultured cells results in acidification of the media [155]. Measuring ECAR under glucose-starved conditions and after a saturating dose of glucose determines the rate of glycolysis under basal conditions. Adding oligomycin blocks mitochondrial ATP synthesis, forcing cells to their maximal glycolytic capacity to maintain ATP. A final injection of 2-deoxyglucose (2-DG) completely inhibits glycolysis, confirming the basal levels of ECAR that were unrelated to glycolysis.

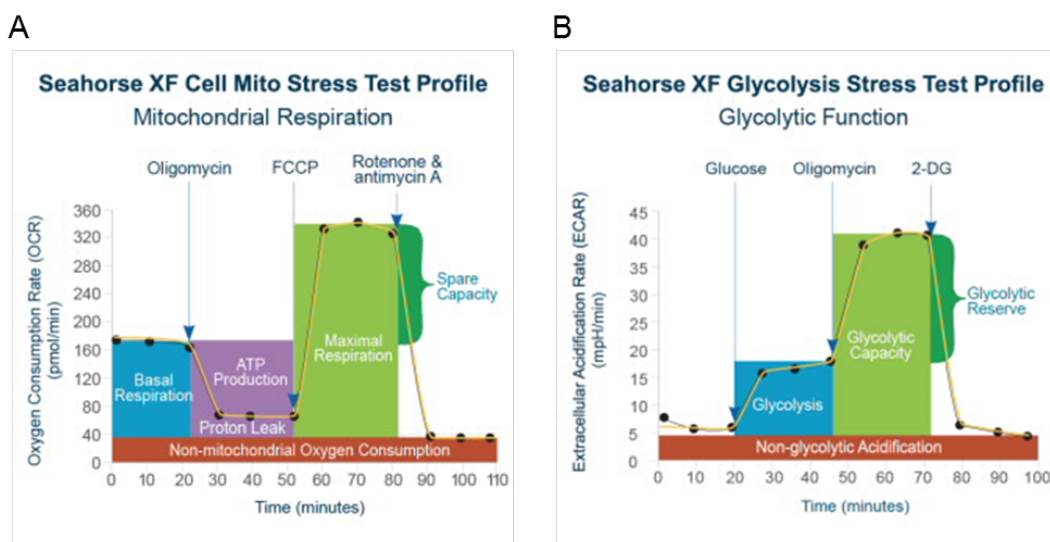


Figure 5-2 Examples of stress test parameters (source: Agilent®). (A) Parameters of mitochondrial function derived from injections of Mito Stress Test compounds. (B) Parameters of glycolytic function derived from Glycolysis Stress Test injections after basal measurements under glucose-starved conditions. *Oligomycin* (ATP synthase inhibitor), *FCCP*: carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (mitochondrial membrane uncoupler); *Rotenone/antimycin A* (complex I and III inhibitors). *2-DG*: 2-deoxyglucose (inhibitor of glucose-6-phosphate production).

5.2 Aims

- To characterise normal and cancerous prostate cell lines of human origin and optimise assay conditions for use with the Seahorse XFp Bioanalyser
- To investigate the effects of sulforaphane and SMCSO on mitochondrial function in normal and cancerous prostate cells
- To perform preliminary analyses on other metabolic pathways such as glycolysis and fatty acid oxidation
- To test whether sulforaphane protects against ROS-induced mitochondrial dysfunction

5.3 Materials and Methods

5.3.1 Cell Culture

All *in vitro* work was carried out with immortalised human Caucasian prostate cell lines. Human normal prostate epithelial cells (PNT1A) (Cat. 95012614) were purchased from the European Collection of Authenticated Cell Cultures (ECACC) and routinely cultured in RPMI-1640 (Sigma-Aldrich®) medium. Cancerous cell lines of human prostate adenocarcinoma, VCaP (Cat. CRL-2876) and DU145 (Cat. HTB-81), were purchased from the American Type Culture Collection (ATCC®). VCaP and DU145 were cultured in DMEM and EMEM media respectively. For all three cell lines, the media was supplemented with 10% fetal calf serum (FCS) (Sigma®) and 1% penicillin/streptomycin (Gibco®). Cells were grown as adherent monolayers

(semi-adherent for VCaP) at 37° C, in humidified conditions supplemented with 5% CO₂.

5.3.2 Cell Seeding and Sensor Cartridge Hydration

Optimisation of assay conditions (characterisation) was performed on each cell line as per the manufacturer's protocol for the Seahorse XFp Bioanalyser (Agilent®). Cells were grown to 70% confluent and seeded in a total volume of 180µl in the central 6 wells of an XFp cell culture miniplate (2.5 to 6 x 10⁴ cells/well) (Figure 5-3). The outer wells were filled with culture media and the surrounding moats filled with 400µl of sterile phosphate-buffered saline (PBS). An additional step was required for immobilisation of VCaP cells, which are semi-adherent; Cell-Tak™ (Corning®) was applied with 1M sodium hydroxide and 0.1M sodium bicarbonate to wells A-H in accordance with the manufacturer's Basic Adsorption Protocol. Following application, excess Cell-Tak™ solution was removed and the wells were washed twice with 200µl of sterile water before cells were seeded. All cells were seeded and relevant pre-treatments applied in the usual growth media for that cell line.

48 hours prior to the assay run an XFp Sensor Cartridge was hydrated with 200µl XF Calibrant solution per well and 400µl in each of the surrounding moats. The cartridge was placed in a 37° C non-CO₂ incubator until required for the assay.

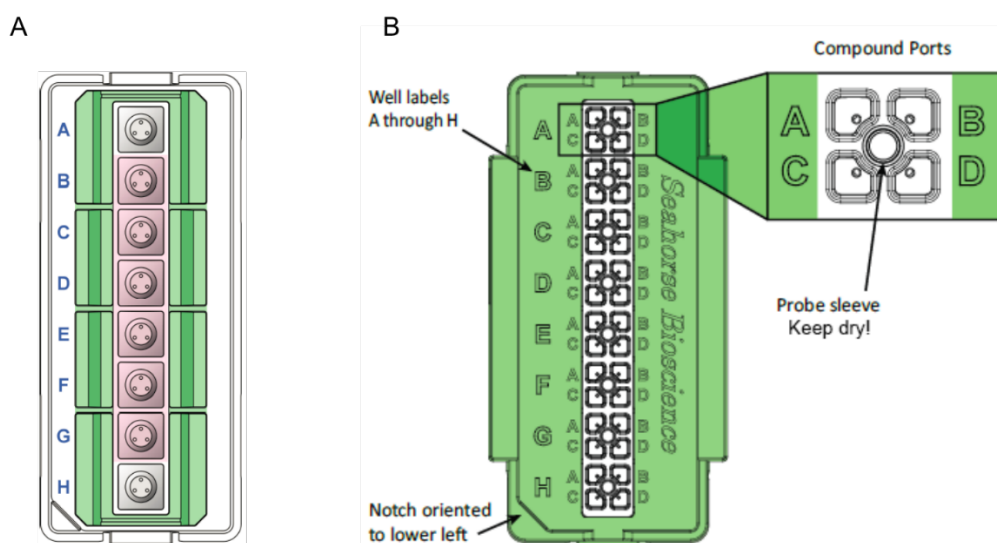


Figure 5-3 XFP cell culture miniplate (A) and sensor cartridge with compound injection ports (B). The inner 6 wells (B-G) are seeded with cells in normal growth medium. The outer wells (A and H) contain sterile media without cells and the moats are filled with sterile PBS. Source: Agilent®.

5.3.3 Assay Optimisation

A target basal OCR is specified by Agilent® at 50–200 pmol/min. Cells were seeded in triplicate at multiple densities (from 2.5 to 6 x 10⁴ cells/well). On the day of the assay, cells were washed twice and incubated in 180µl of warmed assay medium (Agilent® XF base DMEM, pH 7.4, supplemented with L-glutamine 2mM, pyruvate 2mM and glucose 2g/L) for 1 hour in a 37° C non-CO₂ incubator. Blank assay conditions were specified on the Seahorse XFp and optimal cell numbers determined by microscope appearance (viability and confluence) and OCR.

FCCP optimisation was carried out using components of the Mito Stress Test Kit (Agilent®) on the pre-determined cell number. Injection port A was loaded with 20µl of 10mM oligomycin A for a final well concentration of 1.0mM. PNT1A were known to require low dose FCCP, so injection ports B were loaded in duplicate with 22µl of 1.25, 2.5 or 5µM FCCP for a 10-fold dilution into the well (0.125, 0.25 and 0.5 µM) as part of a complete mitochondrial stress test (e.g. Figure 5-2 A). Serial FCCP dose titration was carried out for both DU145 and VCaP (Table 5-1). The concentration that achieved the highest peak OCR was considered optimal.

Table 5-1 Template for loading injection ports of sensor cartridge for FCCP optimisation.

Port Designation	Volume per port	Port Conc. Wells A-D (Low dose)	Port Conc. Wells E-H (High dose)	Final Well Conc. Wells A-D (Low dose)	Final Well Conc. Wells E-H (High dose)
A (oligomycin)	20µL	10µM	10µM	1µM	1µM
B (FCCP)	22µL	1.25µM	5µM	0.125µM	0.5µM
C (FCCP)	28µL	1.25µM	5µM	0.25µM	1.0µM
D (FCCP)	30µL	2.5µM	10µM	0.5µM	2.0µM

5.3.4 Treatment with Sulforaphane or SMCSO

24 hours after seeding into the cell culture miniplate, an appropriate treatment or vehicle control was applied in triplicate in growth medium for a further 24-hour incubation. R, S-Sulforaphane ((4-(methylsulfinyl) butyl isothiocyanate) (CAS 4478-93-7) (purity > 98%) was purchased from LKT Laboratories (St. Paul, USA) and used in parallel with a dimethyl sulfoxide (DMSO) vehicle control. S-methyl L-cysteine sulfoxide (CAS 6858-87-8) (purity >98%) was also purchased from LKT and used in parallel with a water control.

5.3.5 Mitochondrial Stress Test

Assays were initially carried out with components of the Mito Stress Test Kit (Agilent®). Once the procedural technique was established, oligomycin A, FCCP, rotenone and antimycin A were purchased separately from Sigma® and diluted in DMSO to generate 10mM stock solutions, which were stored at -20° C. On the day of the assay working solutions were generated by dilution in warm assay media. FCCP concentration was determined by cell type, while concentrations of oligomycin and combined rotenone/antimycin A remained constant (Table 5-2).

Table 5-2 Template for loading injection ports of sensor cartridge for mitochondrial stress test.

Port Designation	Volume per Port	Port Conc.	Final Well Conc.
A- Oligomycin	20µl	10µM	1.0µM
B - FCCP	22µl	5.0µM (PNT1A, DU145) 20µM (VCaP)	0.5µM 2.0µM
C – Rotenone/ Antimycin A	25µl	5.0µM	0.5µM

5.3.6 Glycolysis Stress Test

Growth medium was removed from cells and glucose starvation medium (XF base DMEM + 2mM L-glutamine) applied for 1 hour in a 37° C non-CO₂ incubator prior to the assay. Working injection-port solutions of 100mM glucose (Fisher[®]) and 10µM oligomycin A (Sigma[®]) were diluted in warm glucose starvation (assay) media, and 2-DG (Sigma[®]) was loaded direct from a 500mM stock solution.

Table 5-3 Template for loading injection ports of sensor cartridge for glycolysis stress test.

Port Designation	Volume per Port	Port Conc.	Final Well Conc.
A- Oligomycin	20µl	10µM	1.0µM
B - Glucose	22µl	100mM	10mM
C – 2-DG	25µl	500mM	50mM

5.3.7 Exposure to Hydrogen Peroxide

Hydrogen peroxide (H₂O₂) was purchased from Sigma[®] (Cat. 16911) and diluted in sterile Milli-Q[®] water to generate a stock solution of 20mM on the day of use. H₂O₂ solutions were protected from light. Working port solutions were generated by dilution in warm assay medium to concentrations of 1.25mM, 2.5mM and 5.0mM. Injection ports A and B were loaded with 1.25mM, port C with 2.5mM and port D with 5.0mM H₂O₂, achieving serial 2-fold increases in final well concentrations of H₂O₂ from 125µM to 1.0mM (Table 5-4).

Table 5-4 Template for loading injection ports with hydrogen peroxide at increasing concentrations.

Port Designation	Volume per Port	Port Conc. Of H ₂ O ₂	Cumulative Well Conc.
Port A	20µl	1.25mM	125µM
Port B	22µl	1.25mM	250µM
Port C	25µl	2.5mM	500µM
Port D	28µl	5.0mM	1.0mM

5.3.8 Mito Fuel Flex Fatty Acid Oxidation Capacity Test

Growth medium was removed from cells and flex test assay medium (XF base DMEM, pH 7.4, + 1mM pyruvate + 2mM L-glutamine + 10mM glucose) was applied for 1 hour in a 37° C non-CO₂ incubator prior to the assay. Injection port A was loaded with UK5099 (20µM) and BPTES (30µM) from the Mito Fuel Flex Test Kit (Agilent[®]) and port B was loaded with etomoxir (40µM). Fatty acid oxidation capacity was calculated by the following formula:

$$1 - \left[\left(\frac{(\text{Baseline OCR} - \text{OCR after injection of UK5099} + \text{BPTES})}{(\text{Baseline OCR} - \text{OCR after injection of all 3 inhibitors})} \right) \right] \times 100$$

Equation 1. The formula for calculating fatty acid oxidation capacity of cells as part of the Agilent[®] Mito Fuel Flex Test [151].

5.4 Results

5.4.1 Oxygen Consumption was Higher in Cancerous VCaP and DU145 than Benign PNT1A Cells

Cells were seeded 48 hours before the Seahorse assay, allowing 24 hours for normal adherence and growth and a further 24 hours for application of pre-treatment. The optimal seeding density was determined by appearance and OCR at the end of this period. To achieve a basal OCR of around 100 pmol/min, PNT1A required a seeding density of 6×10^4 cells per well. At the time of the assay, cells were 100% confluent but appeared viable and yielded a more desirable OCR than lower cell numbers (Figure 5-4 A). Both VCaP and DU145 achieved basal OCR levels of around 120 pmol/min with 2.5×10^4 cells per well, less than half the number for PNT1A. Increasing the cell numbers further resulted in either an OCR outside the target range (VCaP) or significant over confluence affecting cell viability (DU145) (Figure 5-4 B, C).

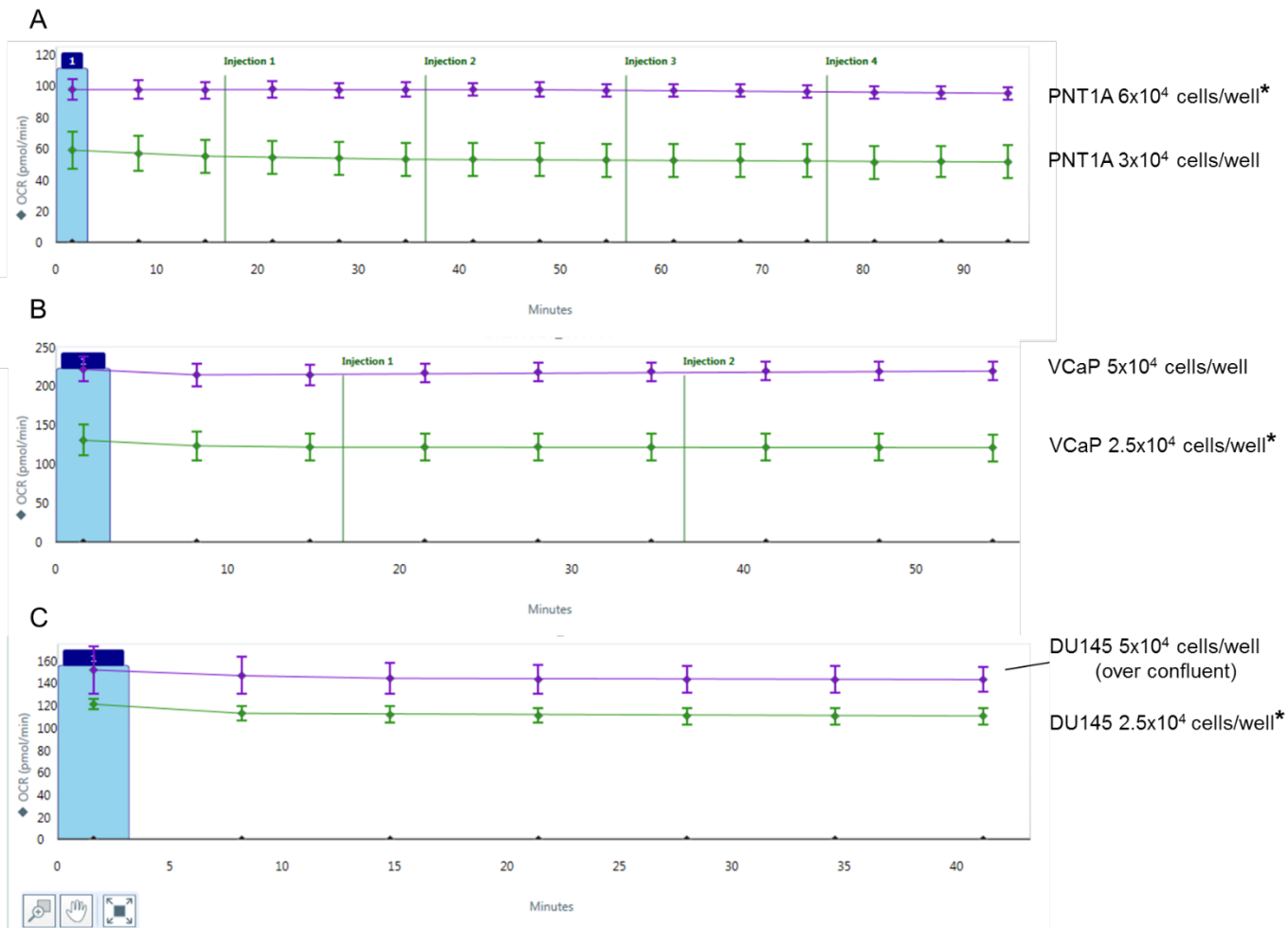


Figure 5-4 Basal oxygen consumption rates (OCR, pmol/min) for prostate cell lines at varying seeding densities. Optimal seeding density (*) determined for (A) PNT1A, (B) VCaP and (C) DU145 by satisfactory confluence and OCR in the target range of 50-200 pmol/minute. Data are expressed as mean \pm SD of wells in triplicate

5.4.2 VCaP Cells Required Higher Concentrations of FCCP than PNT1A and DU145 for Maximal Respiration

FCCP dose optimisation was performed after 48 hours incubation and at the seeding densities described above. Maximal OCR response was seen for both PNT1A (Figure 5-5) and DU145 (Figure 5-6 B) at 0.5 μ M FCCP, consistent with other studies on mitochondrial function in the prostate [156]. VCaP cells demonstrated a dose-dependent increase in OCR up to the maximal concentration of FCCP (2.0 μ M), with no evidence of toxicity (Figure 5-6 A). No previous studies on VCaP were available for comparison on the Agilent Seahorse Cell Reference Database [157].

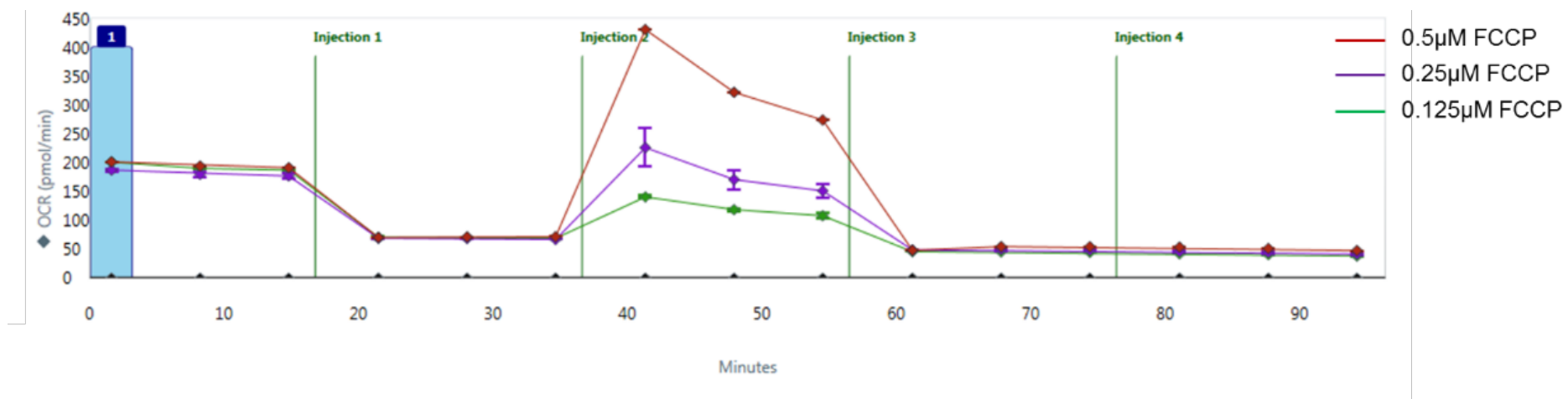
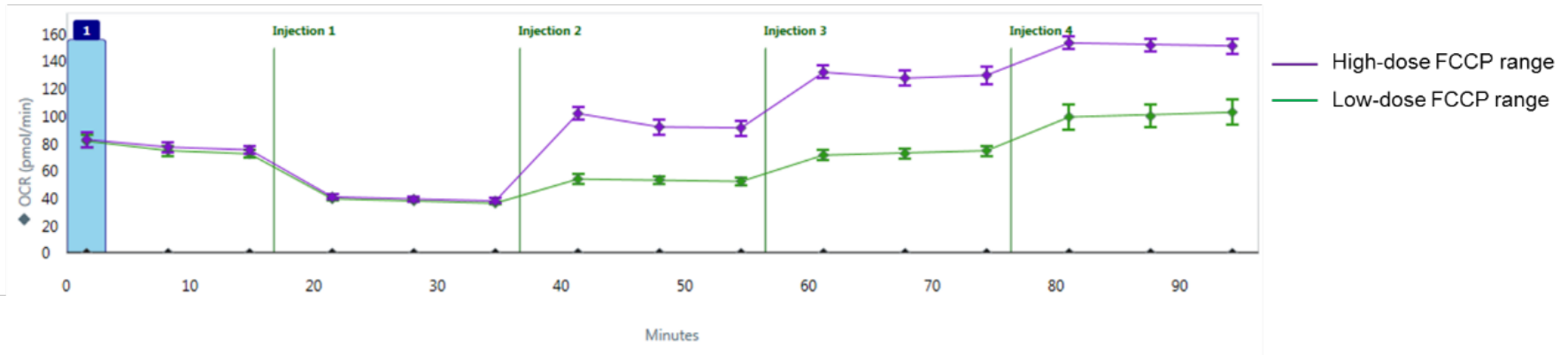


Figure 5-5 FCCP dose optimisation for PNT1A (6×10^4 cells/well). Mitochondrial stress test with no pre-treatment and a range of FCCP (0.125, 0.25 and 0.5 μ M) at injection 2. Greatest response seen at 0.5 μ M. Data are expressed as mean \pm SEM of wells in duplicate.

A



B

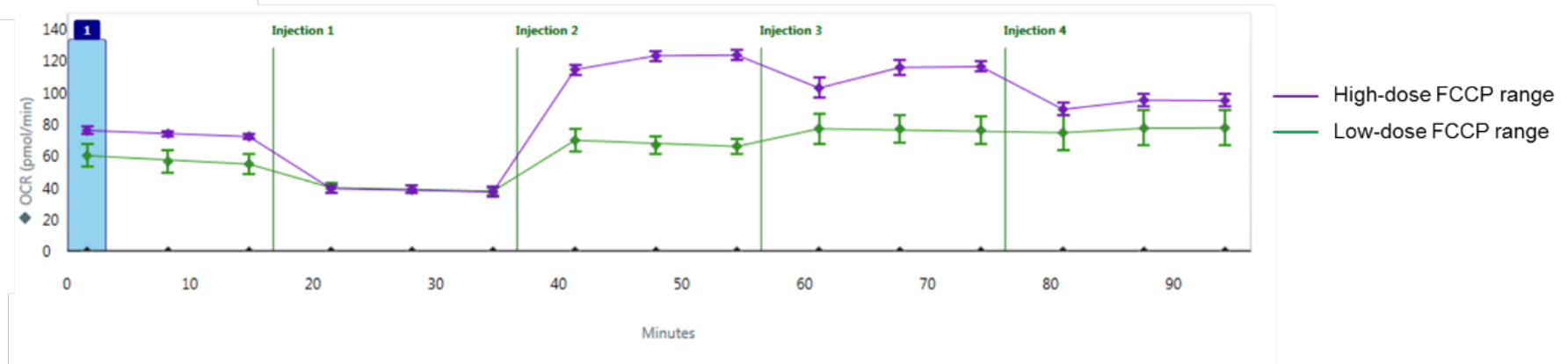


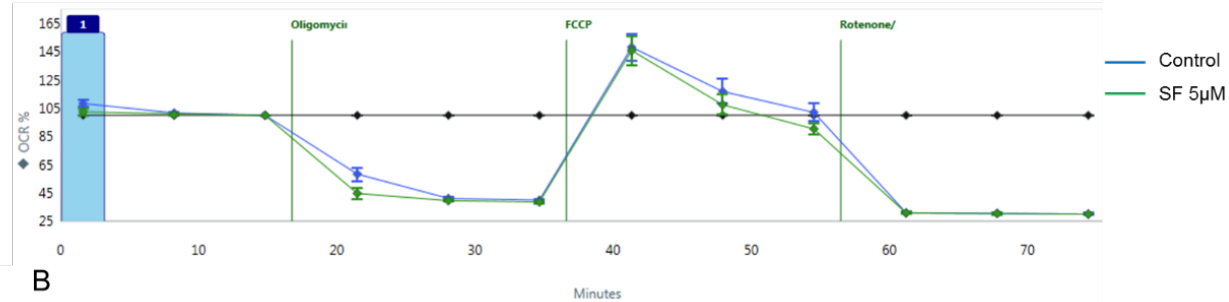
Figure 5-6 FCCP dose optimisation for (A) VCaP and (B) DU145, both at seeding densities of 2.5×10^4 cells/well. $1.0 \mu\text{M}$ oligomycin given at injection 1, followed by serial injections of FCCP to achieve both low (0.125 , 0.25 and $0.5 \mu\text{M}$) and high (0.5 , 1.0 and $2.0 \mu\text{M}$) dose ranges. Maximal response in VCaP seen at $2.0 \mu\text{M}$. Maximal response in DU145 seen at $0.5 \mu\text{M}$, with toxicity at $1.0 \mu\text{M}$ and above. Data are expressed as mean \pm SEM of wells in triplicate.

5.4.3 Sulforaphane Exposure Did Not Alter Mitochondrial Metabolism in Normal or Cancerous Prostate Cells

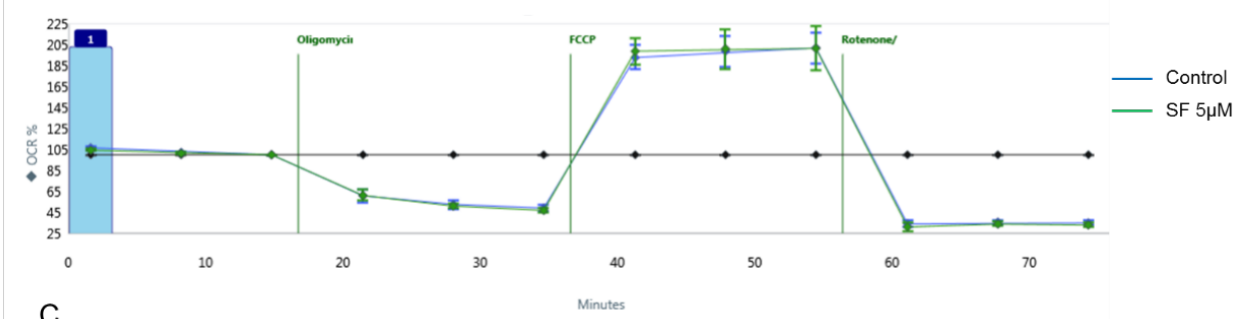
PNT1A, VCaP and DU145 were all incubated for 24 hours with 5 μ M sulforaphane prior to mitochondrial stress test under optimised conditions.

Experiments were duplicated for each cell line and data extracted from the assay of highest technical quality; a representative experiment for each cell line is presented in Figure 5-7. Compared to baseline, the spare respiratory capacity of VCaP cells was significantly higher than both PNT1A and DU145 ($p < 0.0001$). However, no significant differences were detected in either basal ATP production or spare respiratory capacity as a result of sulforaphane treatment versus controls (Figure 5-8).

A



B



C

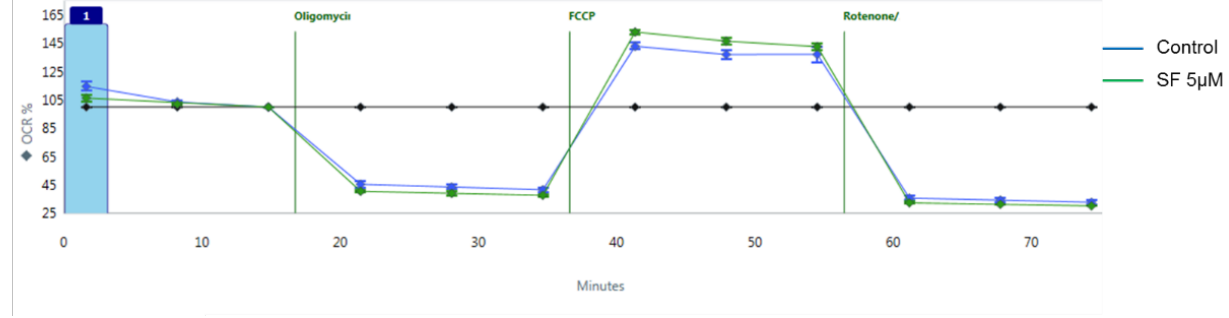


Figure 5-7 Mitochondrial stress test graphs performed under optimised conditions, after a 24-hour pre-treatment with 5µM sulforaphane (SF) in normal and cancerous prostate cell lines; (A) PNT1A, (B) VCaP and (C) DU145. Data shown as mean % of third basal OCR measurement ± SEM for each time point.

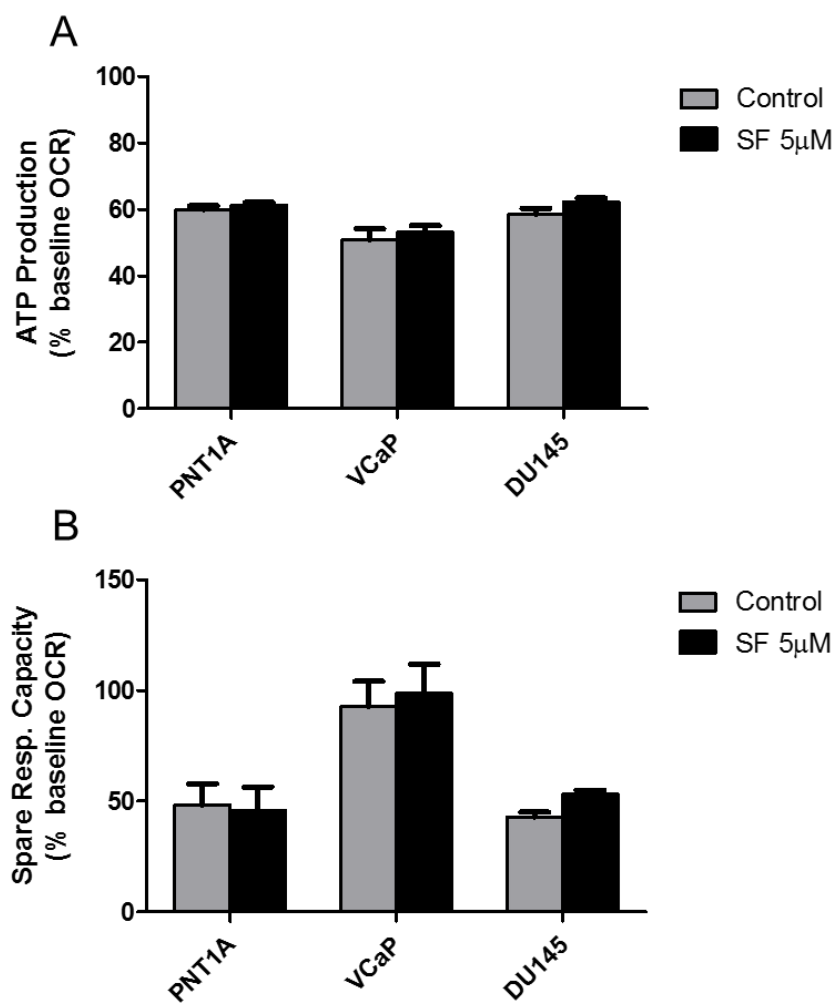


Figure 5-8 Graphs showing (A) ATP production and (B) spare respiratory capacity as percentages of basal OCR in prostate cells in response to sulforaphane (SF). Results derived from mitochondrial stress tests under optimised conditions for three prostate cell lines (PNT1A, VCaP and DU145) after 24 hour pre-treatments with 5µM sulforaphane. Data are shown as means \pm SD. No significant differences by unpaired Student's t-test were detected due to sulforaphane exposure.

5.4.4 SMCSO Exposure Did Not Alter Mitochondrial Metabolism in Normal or Cancerous Prostate Cells

Mitochondrial stress tests were repeated under the same optimised conditions for the three cell lines (PNT1A, VCaP and DU145) after incubation for 24 hours with 200 μ M SMCSO. Similar to sulforaphane, no statistically significant differences were detected in either basal ATP production or spare respiratory capacity after SMCSO treatment versus control (Figure 5-9).

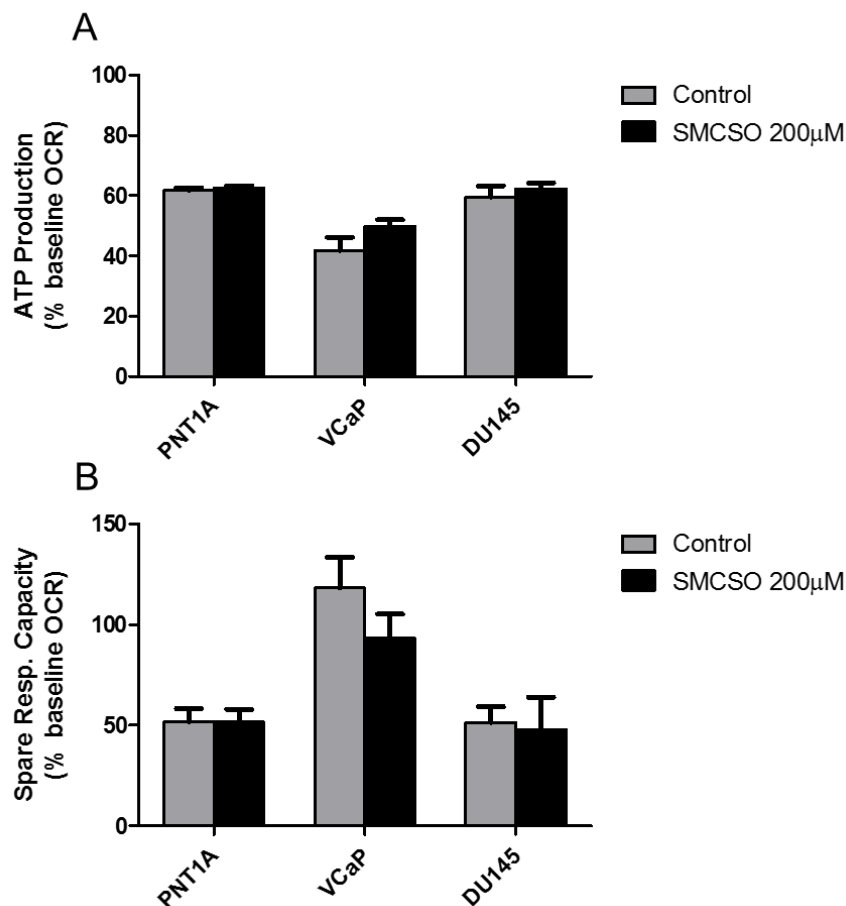


Figure 5-9 Graphs showing (A) ATP production and (B) spare respiratory capacity as percentages of basal OCR in prostate cells. Results derived from mitochondrial stress tests under optimised conditions for three prostate cell lines (PNT1A, VCaP and DU145) after 24-hour pre-treatment with 200 μ M SMCSO. Data are shown as means \pm SD. No significant differences by unpaired Student's t-test ($p < 0.05$) due to SMCSO exposure were detected.

5.4.5 Sulforaphane Treatment Did Not Affect the Outcome of Glycolysis Stress Testing in PNT1A Cells

PNT1A cells were treated for 24 hours with either 5 μ M or 10 μ M sulforaphane before glycolysis stress test assay (Figure 5-10). No statistically significant differences were detected at either treatment concentration for either basal glycolysis or forced glycolytic capacity (Figure 5-12). This was consistent with ECAR results from previous mitochondrial stress tests, which had shown no significant differences either before or after oligomycin injection (Figure 5-11). Expansion of the glycolysis stress test to other cell lines and treatment applications was not, therefore, considered relevant.

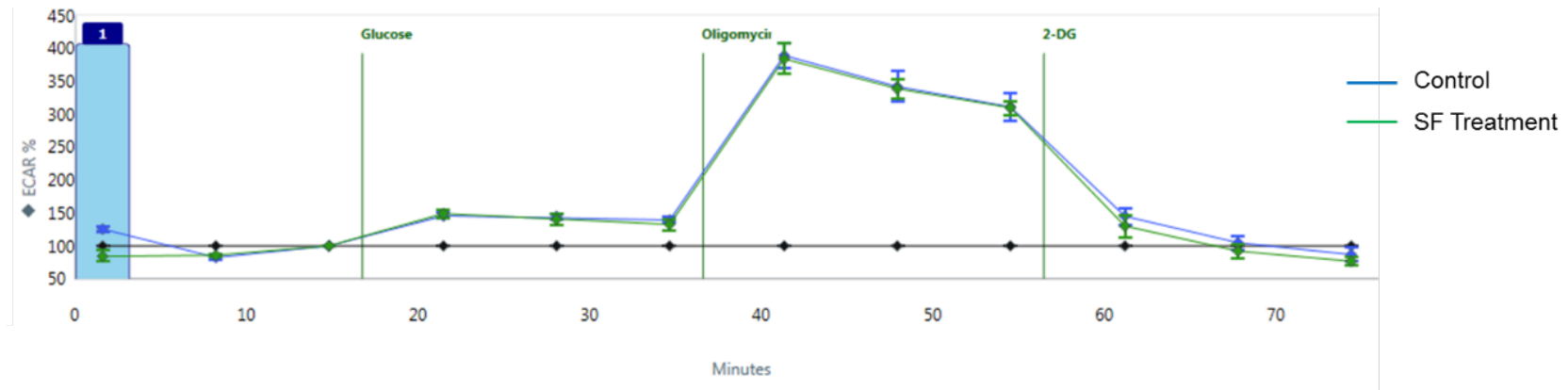


Figure 5-10 Graph of typical glycolysis stress test assay with PNT1A cells after a 24-hour pre-treatment with sulforaphane (SF). Data shown as mean \pm SEM at each time point. No significant difference seen by unpaired Student's t-test between treatment conditions at any stage or sulforaphane dose.

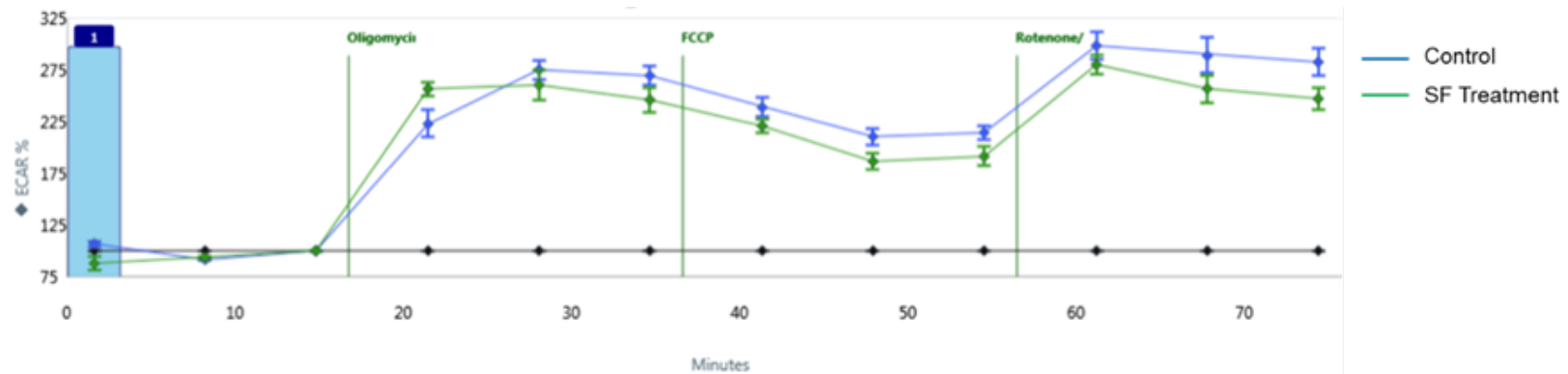


Figure 5-11 Graph of ECAR (mpH/min) during Mito Stress Test of PNT1A treated for 24 hours with sulforaphane (SF) (Figure 5-7A). Data shown as mean \pm SEM as percentage of basal ECAR. No significant differences detected by unpaired Student's t-test after sulforaphane exposure.

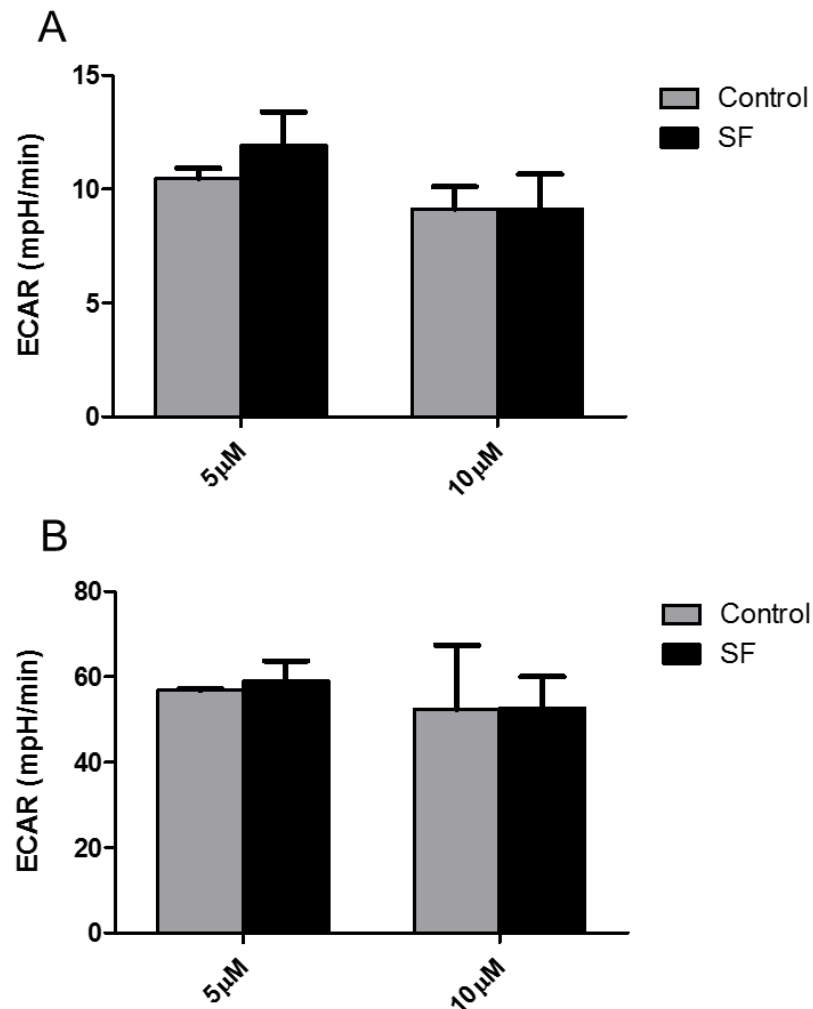


Figure 5-12 Graphs of glycolytic function expressed as ECAR (mpH/min) in PNT1A cells treated for 24hours with 5µM or 10µM sulforaphane (SF) before glycolysis stress testing: (A) glycolysis and (B) forced glycolytic capacity after oligomycin injection. Data are expressed as mean \pm SD. No significant differences detected by unpaired Student's t-test following sulforaphane exposure.

5.4.6 Combined Treatment with Sulforaphane and SMCSO Did Not Protect Mitochondrial Metabolism Against Oxidative Stress in PNT1A

PNT1A cells were treated with a combination of sulforaphane (5µM) and SMCSO (200µM) for 24 hours before assay with progressive two-fold higher injections of H₂O₂ (0-1mM). The OCR declined compared to baseline in response to each injection of H₂O₂, with the most significant impact seen above 500µM. The interaction of ROS is known to cause inhibition of TCA cycle enzymes, including aconitase and α -ketoglutarate dehydrogenase, which accounts for the change in OCR [158, 159]. Treatment with sulforaphane and SMCSO for 24 hours did not induce a sufficient change in antioxidant defence to protect PNT1A cells against these effects (Figure 5-13).

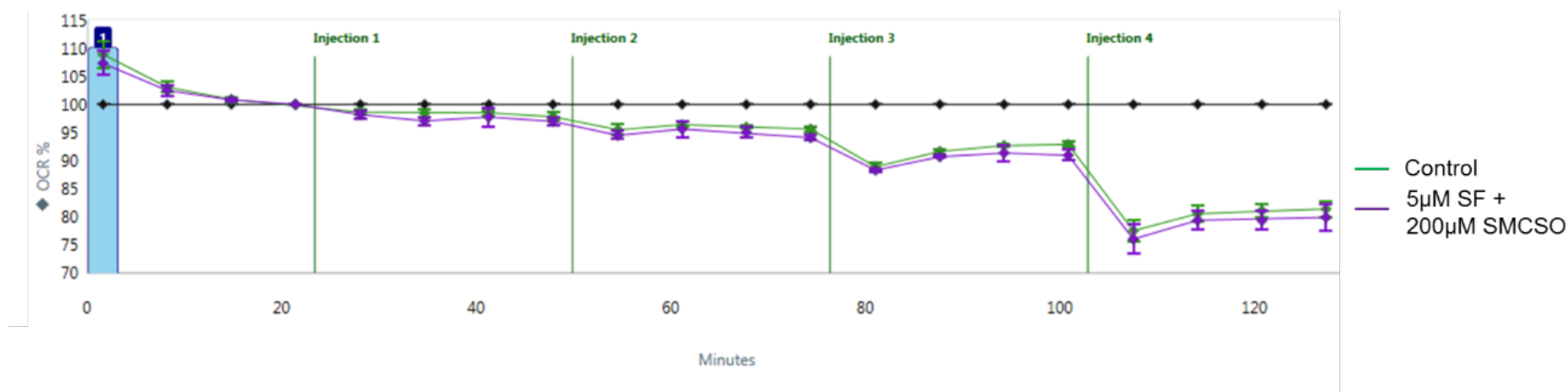


Figure 5-13 Graph of effect on OCR (% baseline) with increasing concentrations of H₂O₂. PNT1A cells pre-treated with combination of sulforaphane (5µM) and SMCSO (200µM) for 24 hours. Two-fold increases in H₂O₂ concentration in well after each injection; 125µM (injection 1), 250µM (injection 2), 500µM (injection 3) and 1mM (injection 4). Data shown as mean ± SEM percentage of baseline OCR at each time point. No significant differences by unpaired Student's t-test or protective effects seen after treatment.

5.4.7 DU145 Cells Had Greater Capacity for Fatty Acid Oxidation than PNT1A

Blockade of pathways involved in the metabolism of glucose and glutamine (UK5099 and BPTES, respectively) increases the dependency of cells on fatty acid oxidation. Establishing the level of OCR before and after subsequent blockade of fatty acid oxidation with etomoxir reveals the contribution of this pathway to oxidative phosphorylation via acetyl CoA production. Fatty acid metabolism, both in synthesis and beta oxidation, is known to be upregulated in prostate cancer [30, 160]. These assays revealed a fatty acid beta oxidation capacity of 44.6% in benign PNT1A cells. The cancerous cell line, DU145, had a significantly higher capacity of 68.4%. However, no effect was seen in either cell line after 24-hour treatment with sulforaphane (Figure 5-14).

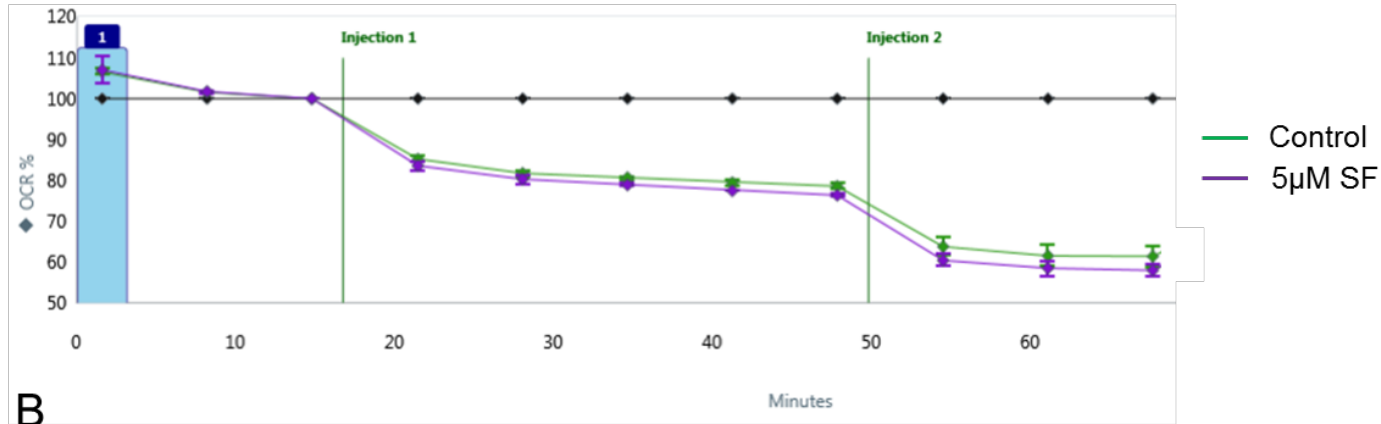
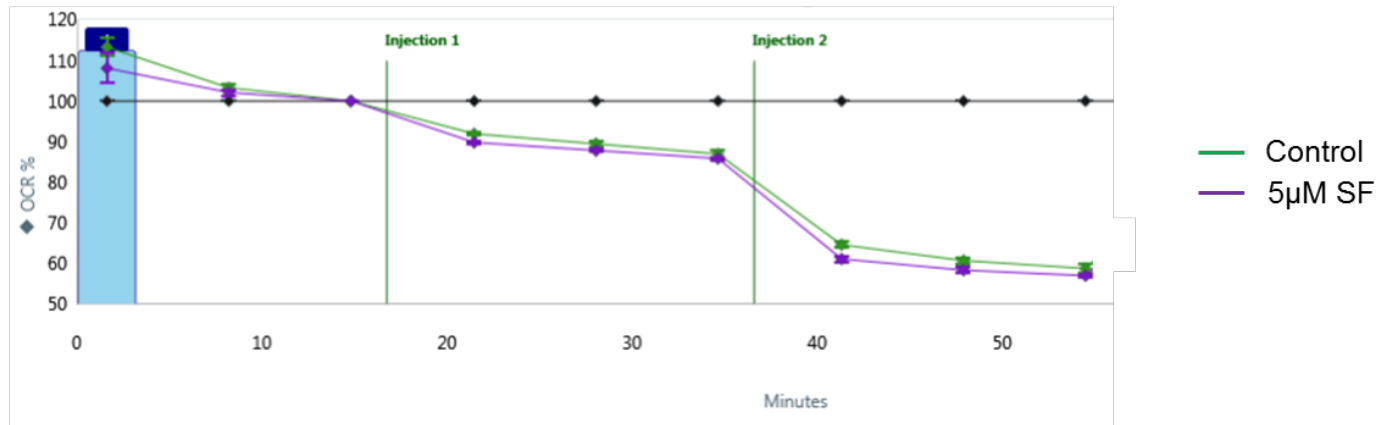
A**B**

Figure 5-14 Graphs of mito fuel flex fatty acid oxidation capacity tests. (A) PNT1A and (B) DU145 were pretreated with sulforaphane for 24 hours before acute injection of BPTES+UK5099 (injection 1) and etomoxir (injection 2). Data shown as means \pm SEM of third basal OCR (%) measurement. No significant differences detected by unpaired student's t-test due to sulforaphane treatment.

5.5 Discussion

The Seahorse Bioanalyser provides a useful tool for studying the interaction between dietary components and real-time metabolism in prostate cells. As ATP regulation becomes an increasingly popular target for therapeutics, this will also be a useful adjunct to the novel LC MS/MS method of ATP quantification (developed in Chapter 4) for comparison of *in vitro* and *in vivo* outcomes. A major limitation of the Seahorse XFp is the limited number of wells in the culture miniplate, which allows for comparison of only one treatment concentration and controls in triplicate.

The significant findings of this chapter are primarily limited to fundamental differences between prostate cell lines. Normal PNT1A cells required a seeding density more than two-fold higher than both cancerous cell lines (VCaP and DU145) to achieve comparable OCR at 48 hours. The results are similar to those of other studies defined by OCR, supporting the higher energy turnover of cancerous cells [156]. Although growth rate will ultimately influence OCR, this does not account for the lower levels in PNT1A; population doubling time for VCaP for example is known to be especially slow [161]. During mitochondrial stress testing, VCaP cells were found to have a significantly higher spare respiratory capacity than both PNT1A and DU145 ($p < 0.0001$) (Figure 5-8). This may be due to either a dose-dependent response to FCCP (2.0 μ M vs 0.5 μ M), to which VCaP were less susceptible to toxicity, or reflect the metabolic adaptations of VCaP as an aggressive, malignant and androgen-insensitive cell line [162]. Another metabolic trait of prostate cancer cells was confirmed by the differences in fatty acid oxidation capacity between PNT1A and DU145 (Figure 5-14).

Existing research on the biological activity of SMCSO and energy metabolism has only been concerned with antidiabetic and hypolipidaemic effects in rats, and not specific to the prostate or prostate cancer [163, 164]. The hypothesis of the SAP study described in Chapter 4 was that degradation of SMCSO to sulphate was depleting ATP as both substrate and energy source for synthesis of PAPS. It was therefore unknown whether ATP synthesis would also be affected. The 200 μ M dose of SMCSO was selected according to levels in SAP study urine over 48 hours post broccoli consumption, and peaks in the first 24 hours of the Bioavailability of Broccoli Soup (BOBS) study undertaken at QIB, but had no significant effect on oxidative phosphorylation (Figure 5-9).

Sulforaphane, among other ITCs, has received more focused research in the context of prostate cancer metabolism. A recent, high-throughput study screened levels of ATP in a mouse cell line after exposure to naturally-occurring compounds, including sulforaphane, known to be efficacious against prostate cancer [85]. Treatment with a wide range of sulforaphane doses (0-20 μ M) did not exert any effect on ATP levels. For this reason, no further investigation was carried out in samples obtained from the dietary intervention studies described in this thesis. In studies with human prostate cancer cell lines (PC3 and DU145), mitochondrial dysfunction due to ROS production was only achieved at non-physiological doses of sulforaphane (up to 40 μ M) [143]. Most recently, phenethyl isothiocyanate (PEITC), which is also present in broccoli, was shown to induce down-regulation of a range of fatty-acid metabolism enzymes and reduce serum ATP levels in the transgenic adenocarcinoma of the mouse prostate (TRAMP) model. Physiological

concentrations of PEITC (2.5-5 μ M) were equally effective in an androgen dependent human prostate cancer cell line (LnCaP) [165]. A physiologically relevant concentration of sulforaphane (5 μ M) was selected for use throughout the experiments carried out using the Seahorse XFp [166]. No effect was seen on any of the parameters of oxidative phosphorylation, glycolysis or fatty acid oxidation capacity in the cell lines tested. The Nrf-2 regulated induction of anti-oxidant defence by sulforaphane will be discussed in greater detail in Chapter 6. In the presence of an exogenous source of ROS (H₂O₂), no protective effects on oxidative phosphorylation were seen in PNT1A cells after sulforaphane and SMCSO treatments.

Interestingly, data obtained from investigating broccoli-derived compounds in the *in vitro* models of the human prostate described in this chapter confirmed the findings of the broccoli-enriched dietary-intervention study (SAP). The initial global metabolomics results from the SAP study detected a significant increase in ADP, suggesting reduced tissue levels of ATP (Chapter 3). However, targeted quantification by LC-MS/MS later revealed no significant difference in the net ATP levels within prostate tissue (Chapter 4). Accordingly, exposure to physiological concentrations of sulforaphane and SMCSO during the Seahorse experiments has not demonstrated any evidence of a dynamic shift in metabolism in prostate cells.

Future work with the Seahorse Bioanalyser could extend to a range of higher treatment concentrations, ranging from those achievable by diet to those that may require purified compound supplements in humans. The bioenergetics of individual prostate cell lines can be clearly characterised on the Seahorse platform, so ongoing investigation is required to test compounds with the potential to prevent or reverse the malignant metabolism of prostate cancer. The observed accumulation of SMCSO in the prostate following dietary intervention (Chapter 4) brings into question whether it is intact SMCSO or its degradation products that have the highest potential to influence metabolism. Testing these compounds either in isolation or combination, as they would naturally be delivered in food, will provide useful information on their potential effects and dose thresholds.

5.6 Conclusion

Three human prostate cell lines of both benign and cancerous origin have been optimised for analysis on the Seahorse XFp Bioanalyser. Whilst individual cell lines demonstrated significant heterogeneity in mitochondrial metabolism, sulforaphane (5 μ M) and SMCSO (200 μ M) did not disrupt their homeostasis after a 24-hour treatment. Further work is required to investigate whether these dietary bioactives are able to protect cells from exogenous stressors known to influence cellular metabolism. These compounds exert their anti-cancer potential via a number of recognised mechanisms, though the effects of combinatorial treatment, rising concentrations and degradation to more reactive intermediates on bioenergetics are yet to be investigated.

6 Effect of Sulforaphane and SMCSO on Redox Status of a Key Tumour Suppressor Protein in the Prostate

Summary

The PTEN gene is a tumour suppressor responsible for inhibition of the PI3K pathway, which drives signalling cascades via AKT, required to promote the rapid growth and proliferation of cancerous cells. Although PTEN allelic deletions and mutations are common in prostate cancer, PTEN protein is also susceptible to post-translational modification, such as inactivation by oxidative stress. This chapter describes the use of laboratory techniques to quantify both reduced and oxidised forms of PTEN following exposure to an exogenous inducer of oxidative stress. To investigate potential links between diet-induced, anti-oxidant defence and PTEN redox status, the effects of both sulforaphane, a potent inducer of Nrf-2, and SMCSO from broccoli were investigated.

6.1 Introduction

The PTEN gene, also known as MMAC1 (mutated in multiple advanced cancers), is an important tumour suppressor that belongs to the family of protein tyrosine phosphatases [167]. PTEN acts as the only known inhibitor of the phosphoinositide 3-kinase (PI3K) pathway, one of the most studied cancer promoting pathways [168]. In response to extracellular stimuli such as insulin, growth factors and chemokines, PI3Ks are recruited to the membrane by G-protein coupled receptors or activated receptor tyrosine kinases (RTKs). Here they catalyse the phosphorylation of phosphatidylinositol-4,5 bisphosphate (PIP2) to the active second messenger phosphatidylinositol-3,4,5 trisphosphate (PIP3). In cancer it is class IA PI3Ks that have the most central role, responding to upregulated growth-factor activation of RTKs [169]. The potent lipid phosphatase activity of PTEN antagonises this action by dephosphorylating PIP3 to PIP2 [87, 170]. If unregulated, PIP3 recruits a range of proteins to the membrane, including AKT and 3-phosphoinositide dependent kinase 1 (PDK1). AKT is activated upon phosphorylation by PDK1, influencing multiple pathways related to growth, proliferation and apoptosis, including loss of downstream inhibition of the mammalian target of rapamycin complex 1 (mTORC1) (Figure 6-1) [171].

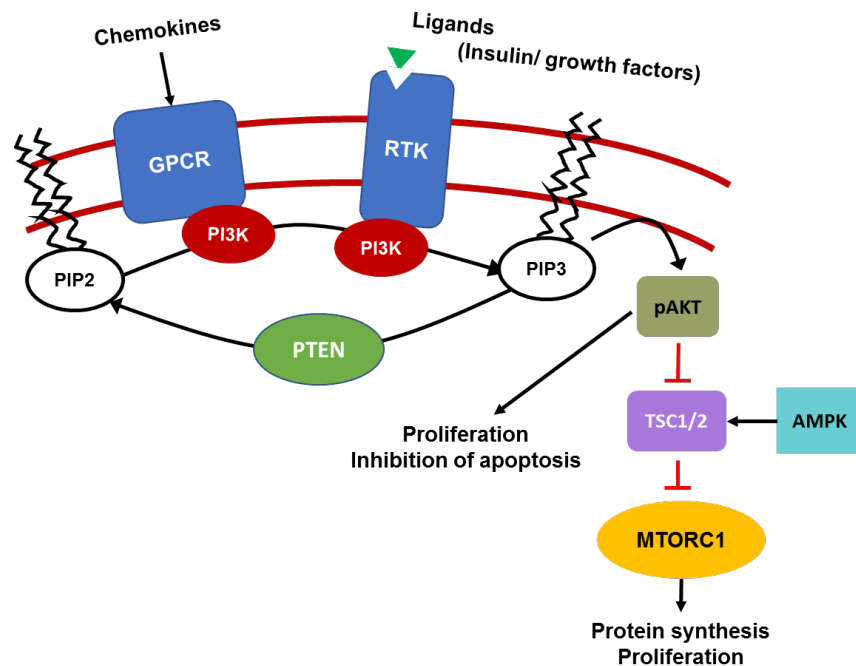


Figure 6-1 The PI3K/AKT signalling pathway and inhibition by PTEN. Abbreviations: GPCR, G-protein coupled receptor; RTK, receptor tyrosine kinase; PI3K, phosphoinositide 3-kinase; PIP2, phosphatidylinositol-4,5 bisphosphate; PIP3, phosphatidylinositol-3,4,5 trisphosphate; pAKT, phosphorylated AKT; TSC1/2, tuberous sclerosis complex 1/2; AMPK, AMP-activated protein kinase; mTORC1, mammalian target of rapamycin complex 1.

Abnormalities in the PTEN gene, either as mutations or deletions, occur in both prostate adenocarcinoma and the presumed pre-malignant condition known as high-grade prostatic intra-epithelial neoplasia (HG-PIN) [10, 172, 173]. Tissue from primary human prostate cancer lesions have identified a frequency of bi-allelic homozygous deletions occurring in 10-15% and monoallelic deletions in up to 70% [88, 89]. However, the reported loss of PTEN activity at the protein level is greater than expected from the latter loss of heterozygosity, suggesting other non-genomic influences on overall function [174]. The implication of PTEN deletion is an association with worse clinical outcomes from prostate cancer; integrated genomics analyses of metastatic prostate cancer deposits revealed an incidence of bi-allelic deletions up to 45% [173]. Decreasing PTEN protein expression in primary tumours is also associated with a higher Gleason score (grade) and stage of tumour at the time of diagnosis, and a significant increase in early biochemical (PSA) recurrence and progression to metastatic disease after radical prostatectomy [175, 176].

As well as other post-transcriptional (mi-RNA) and post-translational (phosphorylation and ubiquitination) regulatory mechanisms, PTEN exhibits similar sensitivity to oxidative stress as other phosphatases [177]. The formation of disulphide bonds between cysteines Cys71 and Cys124 occurs with exposure to ROS and renders the PTEN protein inactive (Figure 6-2). This method of oxidation of PTEN has been shown in an *in vitro* model, but it is unclear whether this occurs in prostate tissue of men with localised prostate cancer [90]. If demonstrated, it may well represent a post-translational mode of modification in prostate carcinogenesis.

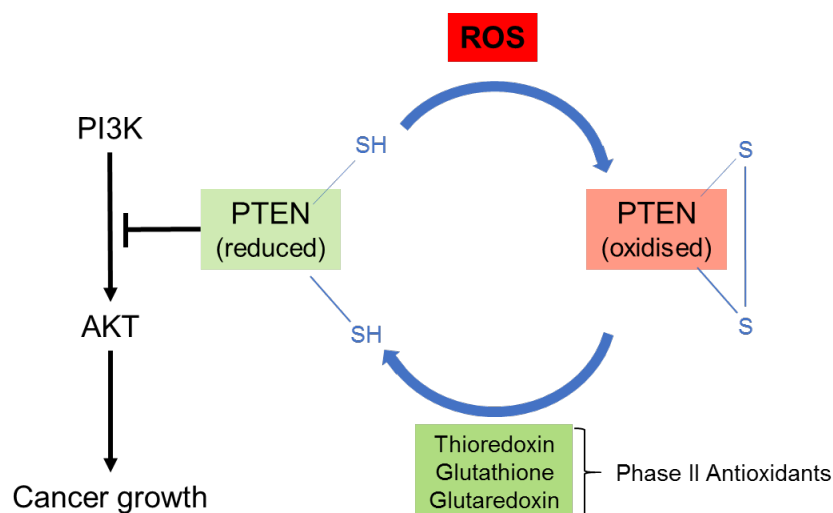


Figure 6-2 Activity of PTEN protein dependent on reversible redox status. Phase II antioxidants function both to detoxify ROS, preventing oxidation of PTEN, and reduce PTEN to its active form.

Research into the negative epidemiological association between cruciferous vegetable intake and prostate cancer risk has focused on the effects of dietary ITCs and antioxidant defence [178]. Sulforaphane, which is derived from glucoraphanin, is the most intensively studied bioactive compound to be delivered by broccoli consumption. Sulforaphane exerts its influence on cellular redox status in a dose-dependent manner, ranging from protective to cytotoxic effects [179]. At low (physiological) concentrations, exposure to sulforaphane prevents KEAP-1 ubiquitination of Nrf-2, increasing its localisation to the nucleus and binding to the antioxidant response element (ARE) [180]. Downstream induction occurs for a range of genes responsible for phase II detoxification and antioxidant defence [181]. However, at higher (pharmacological) concentrations, conjugation with sulforaphane depletes stores of the antioxidant glutathione, and direct inhibition of complex III of the mitochondrial electron transport chain generates higher levels of ROS [144, 182]. Increased sensitivity and production of ROS following exposure to sulforaphane are thought to induce apoptosis in cancerous cells, while normal cells benefit from an increase in Nrf-2-regulated antioxidant defence [145].

SMCSO, another bioactive compound in broccoli, has also been shown to demonstrate antioxidant properties in animal studies by induction of both superoxide dismutase and catalase [163]. Although the mechanism for this is less well understood, SMCSO protects against pro-carcinogens and readily accumulates in human prostate tissue after broccoli consumption (Chapter 4) [183].

Considering the key role of dietary agents in preventing cancer development and modulation of cellular redox homeostasis, this chapter describes the development of highly-sensitive techniques to monitor the redox status of non-cancerous prostate cells in response to exogenous factors. The redox status of PTEN protein could represent a quantifiable and highly-relevant biological outcome to test the capacity for sulforaphane and SMCSO to induce antioxidant defence and protect against prostate carcinogenesis.

6.2 Aim

- To investigate the influence of sulforaphane and SMCSO on the redox status of PTEN after H₂O₂ exposure
- To investigate the ability of sulforaphane and SMCSO to protect prostate cells against H₂O₂ and generation of ROS

6.3 Methods

6.3.1 Cell culture

PNT1A (95012614), a benign epithelial prostate cell line, was purchased from ECACC and cultured in RPMI-1640 (Sigma-Aldrich) medium supplemented with 10% FCS (Sigma[®]) and 1% penicillin/streptomycin (Gibco[®]). Cells were grown as an adherent monolayer at 37° C in humidified conditions, supplemented with 5% CO₂.

6.3.2 Treatment with Sulforaphane, SMCSO and H₂O₂

PNT1A cells were incubated at 70% confluence for 24 hours with an appropriate treatment or vehicle control. R, S-Sulforaphane ((4-(methylsulfinyl) butyl isothiocyanate) (CAS 4478-93-7) (purity > 98%) was purchased from LKT Laboratories (St. Paul, USA) and used in parallel to a DMSO control. SMCSO (CAS 6858-87-8) (purity >98%) was also purchased from LKT and used in parallel with a water control.

H₂O₂ was purchased from Sigma (Cat. 16911) and diluted in sterile Milli-Q[®] water to generate a stock solution of 20mM on the day of use. Prior to protein extraction, appropriate concentrations of H₂O₂ were achieved by dilution in growth medium and applied to cells for a 10-minute incubation period. For fluorescence assays, H₂O₂ stock solution was diluted in Hank's balanced salt solution (HBSS) supplemented with 10% FCS.

6.3.3 Protein Extraction and Quantification

All reagents for protein extraction and subsequent quantification were purchased from Sigma[®]. After treatment with H₂O₂, cells were treated with catalase 1µg/ml and washed using ice cold PBS. Cells were then incubated for 5 minutes on ice with lysis buffer (20mM Tris pH 7.4, 150mM NaCl, 5% glycerol, 0.1% NP40, 100mM phenylmethanesulfonyl fluoride (PMSF), 50mM indole acetic acid (IAA) and 0.1% sodium dodecyl sulfate, scraped into Eppendorf tubes with 1µl benzonase added to each tube. Samples were sonicated in a cold-water bath for 30 seconds and cell debris spun off by centrifugation at 14,000g, 4° C for 10 minutes. The supernatants were transferred to new Eppendorf tubes and protein quantification performed by bicinchoninic acid (BCA) assay as per manufacturer's protocol. Fluorescence was measured from 96-well plates using a FLUOstar-Optima plate reader (BMG technologies) at 565nm.

6.3.4 Western Blot for PTEN Redox Status

The application of western blot analysis to PTEN redox status in PNT1A was a continuation of previous method development work carried out by Dr Karen Chambers (QIB research scientist). Briefly, protein lysates were run by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% Bis-Tris gels with NuPAGE[®] MOPS running buffer (Invitrogen[®]) under non-reducing conditions. Semi-dry transfer of proteins was carried out using the i-Blot 2[®] system and polyvinyl difluoride (PVDF) membrane transfer stacks (Invitrogen[®]). Membranes were initially blocked by 1-hour incubation at room temperature with 5% bovine serum albumin (BSA), and incubated overnight in 5% BSA at 4° C with addition of primary antibody; anti-PTEN rabbit monoclonal antibody (9188L, Cell Signaling Technology) at a concentration of 1:1000. After washing steps, membranes were incubated for a further 1 hour at room temperature with secondary antibody in 5% BSA; HRP-linked anti-rabbit IgG (7074S, Cell Signalling Technology) at a concentration of 1:5000. Following further washing, membranes were developed by chemiluminescence with the Clarity™ ECL Western Blotting Substrate kit (Bio-Rad[®]) and fluorescence intensity measured using Bio-Rad Fluor-STM Multimager Quantity One software.

6.3.5 Developing a Highly-Sensitive Quantitative Method for PTEN Redox Status

For the purpose of this study, a novel LC-MS/MS method to quantify the redox status of PTEN was under development by Dr Shikha Saha (Senior Analytical Scientist, QIB). PTEN peptides were identified with the same Agilent[®] system described in Chapter 3. Samples were injected at 2 µl and eluted at a flow rate of 0.4 ml/min on an Aeris 1.7 µm Peptide (100 x 2.1mm) XB-C18 column. Separation was carried out using 0.1% formic acid in Milli-Q[®] water (mobile phase A) and 0.1 % formic acid in methanol (mobile phase B). The gradient started at 5% mobile phase B increasing over 3.9 min to 35% mobile phase B and finally re-equilibrated to 5% mobile phase B for 2.4 min. The column temperature was set at 40 °C. The LC eluent flow was sprayed into the mass spectrometer interface without splitting. PTEN peptide ions were monitored using mass spectrometry in MRM mode in positive polarity with ESI source. The source parameters were: gas temperature was 200 °C with a gas flow of 16 l/minute; sheath gas temperature was 400 °C with a sheath gas flow of 11 l/minute; nebuliser pressure was 50 psi and capillary voltage was 4000 °C. Identification was achieved based on retention time and product ions. The retention time and fragments were determined by comparison with synthetic peptide standards, which were made and authenticated by JPT in Germany. The Agilent optimiser software was used to obtain fragment ions and collision energy, and a fragments calculator was used to verify the fragment ions.

6.3.6 Quantification of Intra-Cellular Reactive Oxygen Species Levels by Fluorescence Assay

PNT1A cells were seeded into clear-bottom, black, 96-well plates at a density of 25 x 10³ cells per well, and incubated in growth medium for 24 hours. Pre-treatment or appropriate controls were subsequently applied for a further 24 hours. After washing with PBS, cells were incubated with a 20µM concentration of 2',7'-dichlorofluorescein diacetate (DCFH-DA), a cell-permeant reagent that is oxidised by ROS to

fluorescent dichlorofluorescein, for 45 minutes in the dark. Cells were washed and incubated in HBSS with 10% FCS throughout the assay detection period. H_2O_2 was added to specific wells as either a positive control or to assess the protective effects of pre-treatment with sulforaphane or SMCSO. Fluorescence was measured with a FLUO-star Optima plate reader at excitation and emission spectra of 485nm and 530nm respectively.

6.3.7 Statistical Analysis

Analysis of variance (ANOVA) and Bonferroni multiple post-comparison tests were used to analyse ROS assay data.

6.4 Results

6.4.1 Hydrogen Peroxide Caused Oxidation of PTEN in PNT1A Cells

Western blot and LC-MS/MS analysis were used to confirm the effects on PTEN of exposure to extrinsically applied oxidative stress (H_2O_2) in benign prostate epithelial cells (PNT1A). Western blot analysis demonstrated that PTEN protein is maintained entirely in its reduced (active) form in PNT1A control cells. However, in the presence of H_2O_2 at concentrations $\geq 250\mu M$ there is a separate oxidised band for PTEN that becomes increasingly prominent as H_2O_2 concentration increases (Figure 6-3). High-performance LC-MS/MS also confirms that only the reduced peptides of PTEN are detectable in control samples, but that oxidised PTEN peptides become apparent after H_2O_2 exposure. However, the developed LC-MS/MS method requires further optimisation due to poor reproducibility.

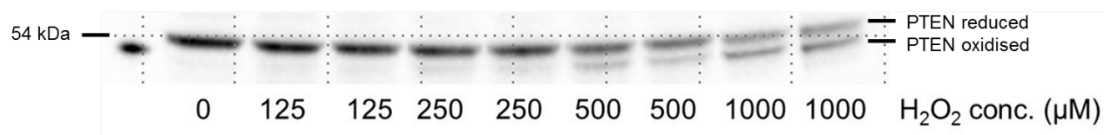


Figure 6-3 Western blot analysis of PTEN under non-reducing conditions in PNT1A cells treated with increasing concentrations of H_2O_2 for 10 minutes. The oxidised (lower) band is first seen at 250 μM and increases in proportion to reduced PTEN (upper band) up to 1000 μM .

6.4.2 Sulforaphane Did Not Protect PTEN Redox Status in PNT1A Against Oxidative Stress

Pre-treating PNT1A cells with physiological concentrations of sulforaphane (0-5 μ M) for 24 hours did not show a protective effect on the redox status of PTEN when incubated for 10 minutes with H₂O₂ (250 μ M) (Figure 6-4).

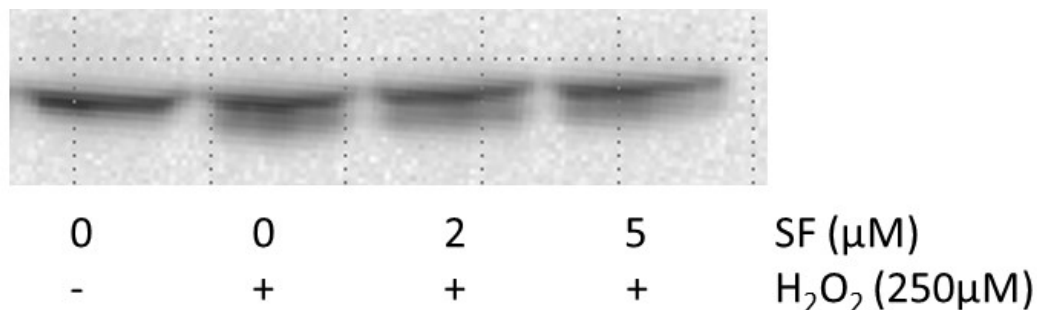


Figure 6-4 Western blot analysis of PTEN under non-reducing conditions in PNT1A cells pre-treated with sulforaphane (SF, 2-5 μ M) or DMSO control before 10-minute exposure to H₂O₂ at the final concentration of 250 μ M. The oxidised (lower) band is present in all cell samples exposed to H₂O₂ with no significant evidence of a protective effect from sulforaphane.

6.4.3 SMCSO Did Not Protect PTEN Redox Status in PNT1A Against Oxidative Stress

Western blot analysis showed that pre-treating PNT1A cells with a range of concentrations of SMCSO (0-100 μ M) for 24 hours did not have a protective effect on the redox status of PTEN when incubated for 10 minutes with H₂O₂ at the final concentration of 250 μ M (Figure 6-5). To further investigate the influence of SMCSO on oxidative stress, a separate experimental approach was taken by performing fluorescence assays for ROS in PNT1A cells (Figure 6-6).

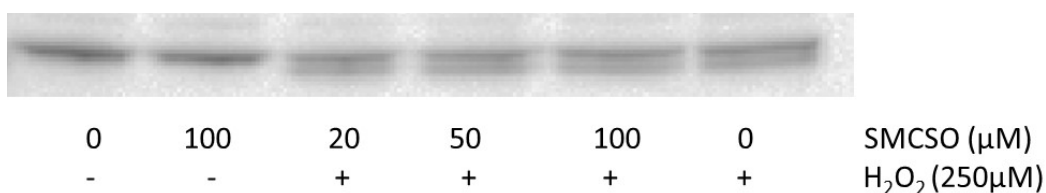


Figure 6-5 Western blot analysis of PTEN under non-reducing conditions for PNT1A cells pre-treated with SMCSO (0 μ M to 100 μ M) or water control before 10-minute exposure to H₂O₂ (250 μ M). The oxidised (lower) band is present in all cell samples exposed to H₂O₂ with no significant evidence of a protective effect from SMCSO.

6.4.4 SMCSO Exposure Did Not Induce Oxidative Stress in PNT1A

Exposing PNT1A cells to varying concentrations of SMCSO (0-100 μ M) did not induce any significant change in mitochondrial ROS production as measured by DCFH-DA fluorescence assay (Figure 6-6). Results obtained were independently confirmed by additional experiments carried out with SMCSO at concentrations as high as 3mM, which did not result in significant generation of ROS or impact on cell viability (unpublished data from Andrew Jones, masters student).

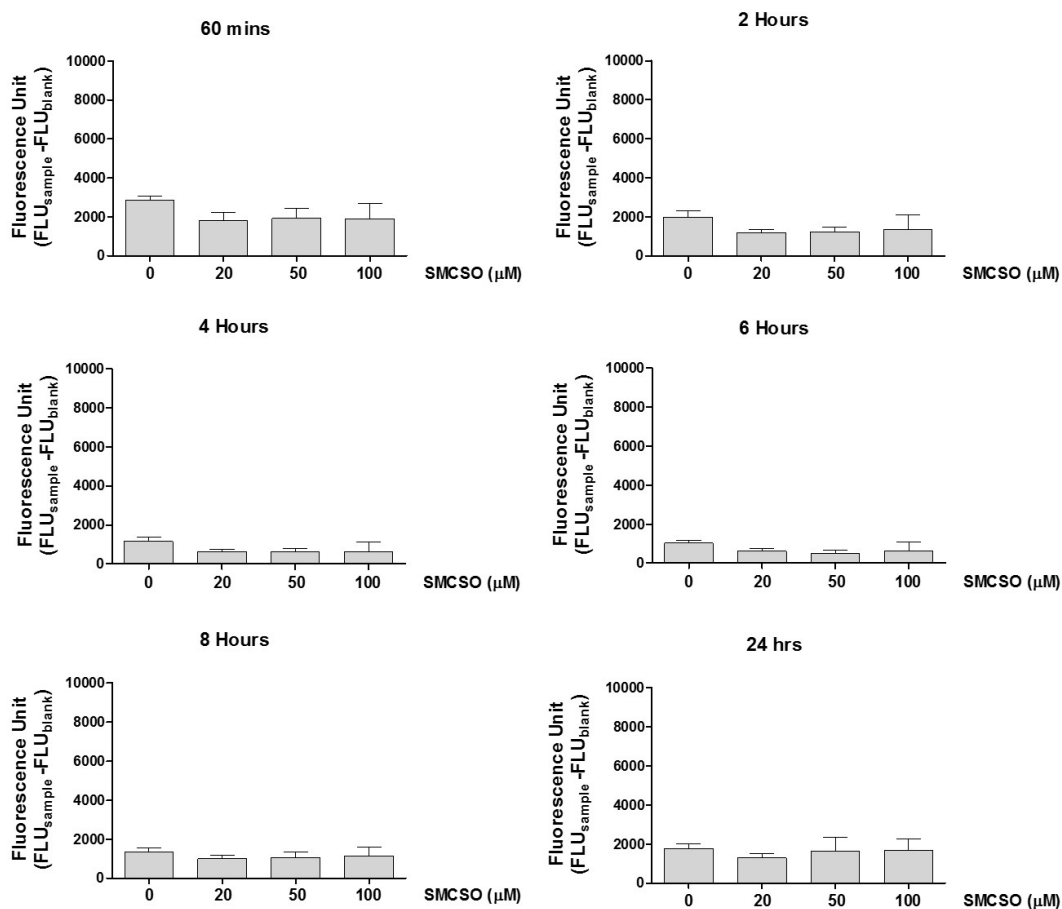


Figure 6-6 ROS levels in PNT1A cells after 24 hour pretreatment with increasing concentrations of SMCSO (0-100 μ M). Data are expressed as means of blank-corrected fluorescence units of 8 biological replicates \pm SD.

6.4.5 Sulforaphane and SMCSO Did Not Reduce Oxidative Stress in H₂O₂-treated PNT1A Cells

Pre-treatment for 24 hours with either sulforaphane (0-5 μ M) or SMCSO (0-100 μ M) did not significantly reduce the levels of ROS measured by DCFH-DA assay when H₂O₂ (125 μ M) was applied to PNT1A cells (Figure 6-7). A significant difference ($p < 0.05$) in detectable levels of ROS occurred at lower concentrations of H₂O₂ by fluorescence assay than Western blot analysis of PTEN redox status (125 μ M vs 250 μ M). Therefore, the lower concentration was used.

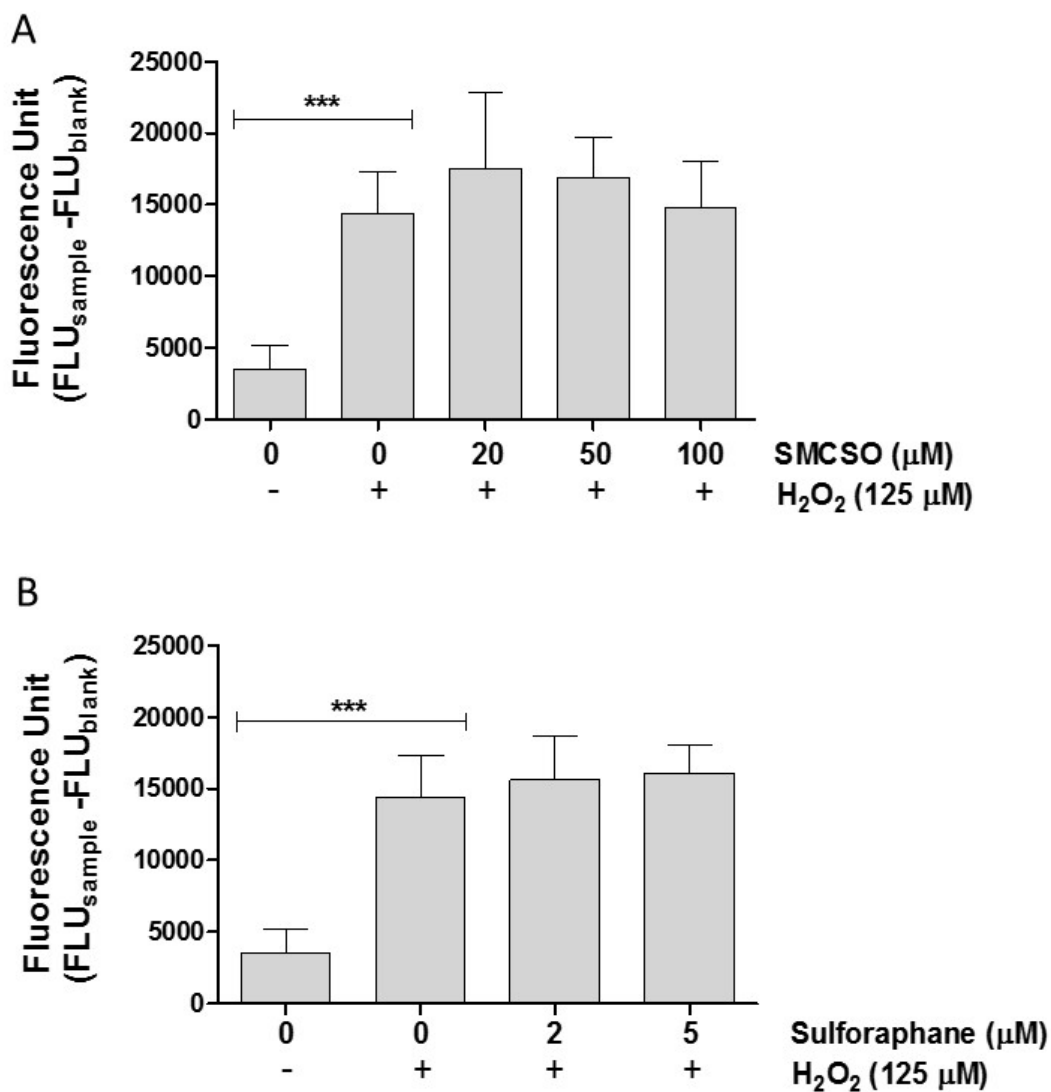


Figure 6-7 ROS level measurements in PNT1A cells after 24-hour pre-treatment with SMCSO (A) or sulforaphane (B) and application of H₂O₂ at start of assay. Data are expressed in blank-corrected fluorescence units from 8 technical replicates as means \pm SD (***) = $p < 0.001$, by Bonferroni post-test).

6.5 Discussion

6.5.1 Role of Oxidative Stress in Prostate Carcinogenesis

ROS are generated during normal mitochondrial metabolism and kept in balance by detoxification systems targeting their removal. Excessive ROS, termed 'oxidative stress,' occurs due to either an increase in ROS production or inadequacy of the cell's antioxidant defence [184]. Oxidative stress is associated with tissue injury and damage to DNA, as well as interaction with signalling pathways responsible for inflammation, proliferation, apoptosis and senescence. These factors have all been implicated in the development of cancers [185]. Prostate cancer is further susceptible because of the link between ROS and androgen receptor activation; higher levels increasing the risk of androgen insensitivity and likelihood of metastatic disease [186].

The potential for cruciferous vegetables to protect against prostate cancer has been largely attributed to their delivery of glucosinolates, which are hydrolysed to biologically active ITCs, including sulforaphane [44]. Central to the multimodal action of sulforaphane is its potent induction of Nrf-2 regulated antioxidant genes, protecting cells against oxidative stress [41]. Reduced levels of Nrf-2 and downstream genes are common findings in both human prostate cancer specimens and TRAMP mice [187, 188]. The latter is reversed *in vivo* by supplementation with sulforaphane [189]. The unique sulphur metabolism of cruciferous vegetables extends beyond glucosinolate content to other specialised compounds, such as SMCSO, which may also play a role in promoting antioxidant defence. However, the bioactivity of SMCSO, especially in terms of antioxidant potential, has not been demonstrated yet. Data obtained from the *in vitro* work described in this chapter clearly indicates that both sulforaphane and SMCSO do not perturb ROS production in normal PNT1A cells. Further work is needed to determine their effects in cancerous cells that are characterised by higher ROS levels.

6.5.2 Monitoring PTEN activity By Measuring Its Oxidation

As expected, western blot analysis detected a progressive rise in oxidised PTEN in PNT1A cells treated for 10 minutes with increasing concentrations of H₂O₂. The increase in oxidised PTEN corresponded to a reduction in PTEN protein that was reduced (Figure 6-3). This is consistent with findings of PTEN redox status reported in a range of cell types, which correspond to loss of PI3K pathway inhibition and an increase in downstream pAKT [90, 190]. Further work is needed to optimise the LC-MS/MS method described in this chapter in order to achieve an accurate quantification of both reduced and oxidised peptides. The use of a validated LC-MS/MS method will help to improve the sensitivity of analysing biological samples collected from *in vivo* studies, such as small-volume prostate biopsy cores.

6.5.3 Effect of Sulforaphane and SMCSO on PTEN Response to Exogenous Oxidative Stress

As Lee et al. have previously shown, the inactivation of PTEN by ROS is reversible *in vitro*, predominantly by upregulation of thioredoxin, but also by other Nrf-2 regulated antioxidant genes, including glutathione and glutaredoxin [90]. Traka and colleagues have also demonstrated that treatment with sulforaphane ameliorated the effects of PTEN deficiency in a prostate-specific PTEN deletion mouse model by

modulating gene expression and alternative splice events [191]. Interestingly, the gene changes significantly correlated with changes seen in human prostate tissue after consumption of both high-broccoli and pea-rich diets, suggesting that additional dietary components within these vegetables may influence cancer risk via the PTEN/PI3K pathway [49].

To test the hypothesis that either sulforaphane or SMCSO would independently protect PNT1A cells from oxidative stress, they were each applied to cultured cells for 24 hours. H₂O₂ was applied at a concentration of 250µM for 10 minutes; the minimum level that gave a reproducible oxidised band for PTEN on detection by Western blot analysis. Neither the use of sulforaphane nor SMCSO at physiological concentrations conferred a protective effect on PTEN redox status. This may be related to the use of PNT1A, a non-cancerous prostate cell line, which inherently generates lower levels of ROS than cancerous cell lines [192]. The highest levels of ROS are generated by prostate cancer cells with the most aggressive phenotype, a characteristic that is often shared with PTEN genomic deletions [193]. The concentration of H₂O₂ used was also above the physiological range for normal human tissues, which are typically up to 30µM in circulating plasma and 117µM in urine [194]. Any potential change to antioxidant defence may, therefore, have been insufficient to remove the high-dose exogenous H₂O₂.

6.5.4 Effect of Sulforaphane and SMCSO in Removing ROS from PNT1A Cells

To test the hypothesis that sulforaphane and SMCSO would induce an antioxidant response capable of removing exogenous H₂O₂ via an alternative experimental approach, ROS fluorescence assays were performed. As previously described, sulforaphane exposure has the potential to increase mitochondrial ROS production at high concentrations [143, 195]. SMCSO was applied at a range of concentrations to PNT1A cells to investigate whether shared biological activity could be established. No significant change in ROS was detected when compared to control cells, providing *in vitro* evidence that levels of SMCSO detected in the urine of SAP study volunteers would not perturb the homeostasis of benign prostate cells (Chapter 4).

A lower concentration of H₂O₂ (125µM) was used for fluorescence analysis, which still gave a strongly significant rise in detectable ROS compared to negative controls (p<0.001). After 24-hour pre-treatment with either sulforaphane or SMCSO, no difference was seen in the levels of ROS in H₂O₂-treated PNT1A cells. This confirms that the benign PNT1A cells' response to exogenous H₂O₂ is unchanged in terms of both ROS detoxification and the redox status of PTEN protein following sulforaphane/SMCSO exposure.

6.5.5 Exogenous Versus Endogenous ROS Production

A limitation of using exogenous H₂O₂ in cultured cells is that it is unlikely to replicate the endogenous ROS generated in the *in vivo* prostate microenvironment [196, 197]. Future work will rely on the development of a more sensitive analytical technique (LC-MS/MS) to detect low levels of PTEN oxidation in prostate tissue. Samples taken following dietary intervention, including those from SAP study

(Chapter 2), could subsequently provide useful information on redox status after a broccoli-enriched diet.

6.6 Conclusion

This chapter has explored a multi-disciplinary approach with three laboratory-based techniques for measuring redox status in response to pro- and anti-oxidant agents in an *in vitro* model of the human prostate. Despite the reported antioxidant effects of sulforaphane and SMCSO, a direct biological mechanism could not be established for prostate cancer prevention by protection of reduced (active) PTEN protein against oxidative stress. Future work should combine: (i) the use of a wider range of treatment conditions with normal and cancerous prostate cell lines; and (ii) the analysis of tissue samples with a more sensitive, quantitative analytical method.

7 General Discussion

7.1 General Discussion

The work conducted within this thesis has made a significant contribution to the current knowledge in the field of cancer and nutrition by providing further insight into the biological activity of broccoli-derived metabolites and their mechanistic links to prostate cancer prevention. As the fifth most common cause of cancer-related death in men worldwide, prostate cancer remains a global health concern [8]. Incidence rates continue to rise, prompting the need for therapeutic strategies with low toxicity to intercept the development of prostate cancer. The increasing role of active surveillance in managing men diagnosed with low-risk prostate cancer also creates an opportunity for lifestyle intervention to reduce the risk of progression to more aggressive disease [198].

The World Cancer Research Fund estimates that 20% of all cancers diagnosed in the US could be prevented with modifications to lifestyle. Not including exposure to tobacco smoke, these measures include weight loss, increasing physical activity and a diet rich in fruit and vegetables [199]. Studying the biological activity of dietary components has led to the identification of a long list of candidate compounds with protective properties against prostate cancer [200-202]. *In vitro* and *in vivo* animal models of prostate cancer have confirmed the beneficial effects of phytochemicals with a broad spectrum of activity across multiple metabolic and signalling pathways. However, the use of isolated compounds in clinical trials against prostate cancer has not yet yielded a preventive or therapeutic benefit. The largest trial to date, known as Selenium and Vitamin E Cancer Prevention Trial (SELECT), recruited over 35,000 healthy men and randomised them to placebo or supplementation with individual or combined treatments. No effect on cancer prevention was detected and the trial was stopped due to non-significant increases in both prostate cancer and type II diabetes incidence in the vitamin E group [203].

Observational studies strongly support the cancer-preventive effects of regular consumption of cruciferous vegetables [40]. However, their protective effects against multiple cancers, including prostate, may not be isolated to the biological activity of ITCs (Figure 1-5). At QIB, preliminary analyses of broccoli-enriched, dietary-intervention studies have shown fundamental changes in prostate metabolism (unpublished data). The use of balanced food products and a comprehensive approach to studying metabolic outcomes provides a framework to identify both sources of biological activity and mechanisms of action relevant to cancer prevention.

This chapter discusses the novel findings obtained from the *in vivo* and *in vitro* work performed to meet the objectives of this thesis as follows:

- Carry out a randomised, parallel-unblinded, high-dose broccoli intervention study in men awaiting TPB
- Investigate untargeted global metabolomic differences in tissue samples of men enrolled in a high-dose broccoli intervention study
- Develop and apply targeted analytical methods to quantify sulphate, ATP and SMCSO in human samples

- Investigate the effects of sulforaphane and SMCSO on real-time bioenergetics in prostate cell lines
- Test the effects of sulforaphane and SMCSO in protecting the PTEN tumour suppressor against oxidative stress.

7.2 The Window-of-Opportunity Study Design for Dietary Intervention

The SAP study was conducted in compliance with local and national regulatory policies, including registration with a publicly-available database as required by the HRA to promote transparency in health research. QIB acted as study sponsor and full ethical approval was received just three months after first registration of the project on the Integrated Research Application System (IRAS), allowing recruitment to begin immediately after receipt of NHS permission.

Unlike the ESCAPE study, SAP recruited participants who were on a clinical pathway for investigation of suspected prostate cancer, rather than following a confirmed diagnosis. The waiting list for TPB provided an ideal opportunity for a short-term, high-dose intervention. Thus, additional experimental evidence was sought for the biological effects of consuming a single broccoli soup each week for 12 months during the ESCAPE study. The high demand for TPB at NNUH generated a large potential study cohort (23 patients/month during the study), which was required to meet the inclusion criteria and recruitment targets. Recruitment was highly successful, reflecting both the enthusiasm of the study team and the willingness of patients to participate in the research. Recruitment to dietary intervention studies is often challenging, reflecting the long-term commitment required by participants [204, 205]. The short intervention and negligible risk of toxicity from the diet were factors contributing to the participant uptake in the SAP study. These observations will be extremely helpful for future dietary-intervention studies, highlighting the pre-biopsy window-of-opportunity trial as an optimal study design for addressing specific research questions in the field of food and health.

Block randomisation was chosen to ensure an equal distribution of recruited patients to the study arms and to initiate intervention in a time-effective manner. Covariates such as GSTM1 genotype, cruciferous vegetable intake and histology were assessed during the study, so their effects on metabolism were accounted for later by multi-linear regression analysis [206]. The distribution of participants between study arms was equivalent by all baseline parameters assessed, allowing comparison of similarly heterogenous groups.

The main limitations of the study were the potential for unaccounted differences in baseline prostate metabolism between individuals, and lack of paired samples. Window-of-opportunity studies in breast cancer overcome this problem by recruiting women with a confirmed cancer diagnosis, performing a study-specific biopsy to obtain tissue before intervention and then analysing the resected tumour specimen following curative surgery [97, 207, 208]. Aside from the risks of complication from additional biopsies, a potential hazard of the window-of-opportunity design is that it could cause a delay to treatment. The intervention is, therefore, often made over

just 2 weeks [209]. In the SAP study, the aim was to further investigate the effects observed by consuming a broccoli-enriched diet for 12 months during the ESCAPE study, so a longer intervention period (≥ 4 weeks) was required. The risk of complications from additional prostate biopsies, especially infection, if not clinically necessary is an important consideration from both ethical and recruitment perspectives [210]. The impact of ischaemia time from different methods of tissue collection would have also had direct implications on levels of labile metabolites such as ATP, an outcome measure of the SAP study. However, the results of the SAP study will be complemented by those obtained after completion of the ESCAPE study, which offers TPB before and after 12 months, providing both samples for research and thorough histological assessment of participants' prostate cancer status as part of active surveillance.

7.3 Metabolomics to Study Dietary Effects on Prostate Metabolism

The application of global metabolomics to the study of prostate and prostate cancer metabolism has increased our understanding of the relationships between diet and numerous metabolic pathways [113, 211]. Analysis of SAP study samples by Metabolon[®] allowed not only comparison of metabolic differences between study groups, but comparison of different tissue types and body fluids (Chapter 3).

The extensive list of metabolites reported by Metabolon[®] in the biological samples (Table 3-1) were ranked by p-value from unpaired t-tests between study arms (Table 3-3). Relevant to the aims of the study were higher levels of SMCSO in urine and ADP in the prostates of men in the intervention arm, which both required further investigation. Multiple correction analysis determined that none of the individual metabolites reached overall statistical significance ($p < 0.05$). However, this approach assumes that the metabolites are independent variables, which explains the need for statistical methods capable of integrating individual metabolite levels into established pathways [212, 213].

The main findings from the pathway analyses of men in the SAP study were significant differences between study arms in both caffeine metabolism and steroid hormone biosynthesis in the prostate (Figure 3-5). Metabolites in both pathways were lower in the intervention group, suggesting either differences between baseline characteristics in both groups, or an increased capacity for androgen and caffeine detoxification with the study diet [119, 120]. Differences in caffeine metabolism were probably caused by higher coffee intake in the non-intervention group, as levels of other coffee metabolites (trigonelline and quinate) were also higher and their metabolism is unrelated to the caffeine pathway. Steroid hormone levels have little evidence of a relationship with constituents of the study diet. A single mouse study found induction of 3α hydroxysteroid dehydrogenases and reduced circulating levels of dihydrotestosterone with a high-dose sulforaphane diet (10mg/kg body weight), and there is some limited evidence of *in vitro* interaction between sulforaphane and the androgen receptor [214, 215]. Alternatively, caffeine may induce an increase in androgenic hormones by stimulation of the stress hormone cortisol [114, 115]. In the SAP study samples, however, there was no difference in cortisol levels between intervention groups. This could reflect either equivalence between groups or especially high levels in all participants on the study day due to the stress response to surgery [216].

Comparing profiles of urine, prostate and adipose tissue revealed similar differences between groups in dietary metabolites trigonelline, quinate and tryptophan betaine (Figure 3-2). As differences in coffee metabolite levels and the caffeine metabolism pathway were marked by greater statistical significance in prostate than adipose, the results do not preclude a role for urinary reflux as a route of metabolite exposure [35]. These metabolites have clearly been widely distributed throughout the body by systemic circulation, as evidenced by their presence in adipose tissue. Whether subsequent glomerular filtration and excretion into urine delivers the metabolites to the prostate for a second time via urinary reflux remains a distinct possibility, and could explain the greater impact of diet on metabolism in the prostate.

7.4 Characterisation of Sulphate, ATP and SMCSO Levels in *In Vivo* and *Ex Vivo* models

Targeted quantification of specific metabolites was achieved by optimisation of extraction techniques and parameters for high-performance LC-MS/MS. There was some discordance between the results of tissue analysis by Metabolon[®] and targeted analysis at QIB for both sulphate and ADP. Although the individual results did not correlate (Figure 4-6), the conclusion was the same for comparison of tissue levels of sulphate between SAP study intervention arms; no significant difference occurred due to the study diet (Figure 4-4). However, four individuals in the intervention group had sulphate levels that appeared distinctly higher than other participants in the group. Variation in dietary intake of sulphate from other sources may have caused a potential difference in levels, as could differences in sulphate metabolism locally in the prostate and by sulphate-reducing bacteria within the gut microbiota [217].

The method developed for quantification of ATP, ADP and AMP was validated against the known effects of ischaemia on cellular energy balance (Figure 4-10) [218]. The highly-sensitive analytical technique was then applied to examine the effects of the SAP study intervention on net energy metabolism. Ischaemic time may also explain the difference in energy status suggested by ADP levels determined by Metabolon[®] and the QIB analysis. The latter technique included meticulous tissue extraction at cold temperatures ($\leq 4^{\circ}\text{C}$) to prevent dephosphorylation of ATP, whereas tissue for Metabolon[®] was incubated at room temperature for 24 hours and subsequently dried down. Ultimately, the significant difference in ADP levels detected by global metabolomics analysis did not represent an overall change in net levels of ATP after independent quantification at QIB. Further information on dynamic energy metabolism was sought with *in vitro* studies on the Seahorse Bioanalyser.

SMCSO was detected in the urine, prostate and adipose tissue samples from the SAP study participants. It was the most significant metabolite difference between study arms in the Metabolon[®] analysis of urine ($p = 0.004$), strongly correlated with independent LC-MS/MS analysis, and accumulated significantly in prostate tissue after just 4 weeks of dietary intervention ($p < 0.01$). Interestingly, the levels of SMCSO were two to three-fold higher in adipose than in prostate tissue (Figure 4-9). Accumulation in adipose is a common route for redistribution of lipophilic drugs [219]. However, SMCSO is water soluble and highly hydrophilic, which means accumulation in adipose would be dependent on alternative methods of uptake

[220]. The lower levels in the prostate could be attributable to lower SMCSO binding capacity of the tissue, diffusion into seminal fluid or urine, or greater localised metabolism of SMCSO. Currently, there is no evidence to explain the metabolic fate of SMCSO within the prostate gland. However, if SMCSO is metabolised locally, the potential biological activity of its degradation products may be exerted directly within the prostate. Further investigation is required to gain new insights into SMCSO metabolism, not only at the systemic level, but also in target organs. This is of particular interest as none of the ITCs quantified in the SAP study were detectable in prostate or adipose tissue.

7.5 Real-time Prostate Bioenergetics in Response to Sulforaphane and SMCSO

The changes that occur to mitochondrial function in prostate cancer are designed to direct metabolism towards production of both energy (ATP) and the building blocks for cell growth and proliferation [22, 29, 30]. Comparing the dynamic oxygen consumption rates of human cancerous prostate cell lines (DU145 and VCaP) with normal PNT1A on the Seahorse XFp Bioanalyser confirmed some of these characteristics. The basal metabolic rate was higher in the cancerous cell lines, VCaP had significantly higher spare respiratory capacity than both DU145 and PNT1A, and DU145 had a significantly higher capacity for fatty acid beta oxidation than PNT1A (Figure 5-8 and Figure 5-14 respectively).

Diet-derived phytochemicals have previously been shown to reduce levels of ATP in both *in vitro* and *in vivo* models of prostate cancer [85]. There is also experimental evidence potentially linking the biological activity of sulforaphane to both maintenance and disruption of ATP production, which is again likely to be dose-dependent. Oxidative stress may inhibit the activity of rate-limiting enzymes in the TCA cycle and mobile electron carriers in the mitochondrial ETC [158]. By inducing Nrf-2-regulated anti-oxidant defence, sulforaphane has the capacity to counteract these effects and preserve ATP production [147]. Increased levels of NADH have been reported after exposure to sulforaphane at physiological concentrations (4 μM), signalling effective reduction of NAD^+ by the TCA cycle [221]. A trend towards higher levels of NADH was also seen in the intervention arm of the SAP study (Table 3-3), though this did not reach statistical significance ($p = 0.056$). In some cell lines, sulforaphane can also exhibit properties similar to antimycin A, an inhibitor of complex III in the ETC [144, 145]. Combining an increase in TCA cycle function with inhibition of oxidative phosphorylation could result in higher levels of NADH. Degradation of SMCSO to dimethyl disulphide may also contribute to these effects by further inhibition of the ETC [222].

Under the experimental conditions described in chapter 5, no changes to mitochondrial or glycolytic function were demonstrated in prostate cells after 24-hour exposure to either sulforaphane (5 μM) or SMCSO (200 μM). These findings were consistent with those of net ATP, ADP and AMP levels in human prostate samples collected for the SAP study. To elicit the potential biological activity of sulforaphane and SMCSO on prostate energy metabolism, a wider range of treatment concentrations will need to be applied. Experimental evidence of SMCSO degradation in the prostate will also be required to establish the presence of its metabolic intermediates and their potential bioactivity.

7.6 The Capacity for Sulforaphane and SMCSO to Preserve PTEN Function by Induction of Antioxidant Defence

Clinical studies support the link between prostate cancer and increasing levels of oxidative stress [184, 223]. ROS production is not only sensitive to androgenic stimulation, resulting in increased levels within prostate tissue, but also interacts with the androgen receptor [224]. Among the numerous damaging effects of ROS to cells, many of which are associated with cancer promotion, is development of androgen insensitivity and progression to lethal prostate cancer [225, 226]. Reversible inactivation of proteins, such as the tumour suppressor PTEN, by oxidative stress could lead to uncontrolled growth and proliferation, as well as increasing the production of ROS [87, 90].

The effects of exogenously applied ROS were investigated separately by Western blot for PTEN redox status and by DCFH-DA fluorescence assay for ROS production in normal PNT1A cells. H₂O₂ applied for 10 minutes was sufficient to oxidise PTEN and to cause a detectable increase in fluorescence for ROS. The capacity for sulforaphane to alleviate these effects by induction of phase II detoxification enzymes and antioxidant defence was therefore tested by both experimental techniques [227]. SMCSO was also evaluated to see if there was any impact on redox status, either by independent generation of ROS or induction of antioxidant defence. However, under the treatment conditions described in chapter 6, no significant protective effects were detected from either compound. The application of lower, more physiological concentrations of exogenous ROS (H₂O₂) and analytical techniques (LC-MS/MS) with greater sensitivity will be required for future analysis with a wider range of treatment concentrations, cell lines and *ex vivo* tissue.

7.7 Conclusions and Future Work

The SAP study proved the success of recruiting to a short-term dietary-intervention study in the waiting list period for TPB. The study design will, therefore, be applicable to future clinical trials undertaken at QIB as we continue to investigate the relationship between diet and prostate biology.

The SAP study revealed a significant accumulation of SMCSO in those receiving the broccoli-enriched diet, which was consistent across both global and targeted analytical methods of quantification. Not only is SMCSO abundant in cruciferous vegetables, but it is also a secondary metabolite of other plant species. This study provides the first evidence of tissue distribution of a dietary sulphur-containing compound from both Alliaceae and Cruciferae families in humans. The detectable presence of this putative anti-cancer metabolite in both adipose and prostate tissue may help to further unravel the health-beneficial effects of cruciferous vegetables. Consumption of phytochemicals, such as sulforaphane and SMCSO, in combination, as they would normally be consumed in the diet, may also confer synergistic biological effects.

In the *in vitro* models of prostate metabolism described in this thesis, the biological activity of sulphur-containing compounds from broccoli has not been linked to either

restriction of ATP production or regulation of PTEN redox status. There is, however, still further information to be derived from the next-generation RNA sequencing of prostate tissue samples from the SAP study. Post-translational differences between study arms may guide future work to focus on specific downstream biological outcomes.

Another important area for future work will be to investigate the tissue-specific metabolism of SMCSO to its intermediate products (Figure 1-5). To date, extensive research has been carried out on ITCs from cruciferous vegetables, which were not detectable in prostate tissue in the SAP study. However, mechanisms of action for SMCSO are relatively poorly understood and the majority of biological activity is reported from its breakdown products [57]. Potential routes of metabolism include both thermal degradation and enzymatic breakdown by cysteine lyase. Although cysteine lyase activity has not previously been confirmed in the prostate, it could either be present endogenously or relate to the bacterial composition of the prostate microbiome, for which there is emerging evidence [228, 229]. Thus, specific bacterial isolates may affect prostate cancer risk by their interaction with local metabolites. Investigating the metabolic fate of SMCSO in prostate tissue could support future clinical and laboratory-based research to elicit its potential role in prostate cancer prevention.

Annex 1



**A pre-biopsy window of
opportunity trial to measure
sulphate levels in human prostate
after broccoli consumption**

Short title: Sulphate Accumulation in Prostate (SAP)

PROTOCOL:

Version 2

Chief Investigator:

Professor Richard Mithen

IFR Investigators:

Mr Jack Coode-Bate

Dr Antonietta Melchini

NHS Investigator:

Mr Robert Mills



PROTOCOL SIGNATURE PAGE

Title: Sulphate Accumulation in Prostate (SAP)

Annex 1; Version 2; 13th January 2016

Sponsor's Approval:

This protocol has been approved by The Institute of Food Research's Human Research Governance Committee (HRGC)

Signature _____ Name _____

Role _____ Date _____

I have fully discussed the objectives of this trial and the contents of this protocol with the Sponsor's representative. I understand that the information in this protocol is confidential and should not be disclosed other than to those directly involved in the execution or ethical review of the trial.

I agree to conduct this trial according to this protocol and to comply with its requirements, subject to ethical and safety considerations and guidelines, and to conduct the trial in accordance with International Conference on Harmonisation (ICH) guidelines on Good Clinical Practices (GCP) and with the applicable regulatory requirements.

Chief Investigator name and address:

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Norwich Research Park

Colney

Norwich NR4 7UA

UK

Signature:

Date:

Executive Summary

Abstract

Epidemiological studies provide evidence for a negative association between prostate cancer and intake of cruciferous vegetables such as broccoli. Preliminary data from on-going intervention trials carried out at the Institute of Food Research suggest that diets rich in broccoli can directly influence the metabolic profile of prostate tissue. The most significant result observed to date is sulphate accumulation within the prostate. The additional sulphate is likely to drive synthesis of 3'-phosphoadenosine-5'-phosphosulfate, a universal sulphate donor, a process which would consume energy from adenosine triphosphate (ATP) and increase adenosine diphosphate (ADP) levels. A significant rise in levels of both ADP and phosphate has also been observed in prostate tissue from these studies, supporting the potential depletion of cellular ATP. Interestingly, our data indicate that the extent of ADP accumulation is negatively correlated with prostate cancer progression.

We now propose to undertake a two arm parallel un-blinded study to test the hypothesis that a short-term intervention with an enhanced dose of broccoli soups will lead to the accumulation of sulphate and ADP in prostate tissue. We also plan to investigate whether these metabolic differences can influence the redox status and the activity of specific genes involved in tumour suppression, such as PTEN. Furthermore, we aim to determine whether the accumulation of sulphate and ADP is specific to the prostate gland through the metabolic analyses of both prostate and non-prostate tissues.

The study will recruit men who have been diagnosed or are under investigation for prostate cancer and require a transperineal template biopsy as part of their standard clinical care. The proposed study does not require the participants to have any additional clinical procedures except that during routine sampling separate tissue cores will be taken from the prostate and adjacent adipose tissue. Participants in one arm will consume three portions of broccoli and stilton soup per week for at least four weeks prior to their transperineal template biopsy, whereas participants randomised to the other arm will not have any diet intervention prior to biopsy. The intervention will be carried out while the patients are awaiting their operation date, with no delay to their clinical care or investigations. Participants in both arms will consent for tissue biopsies (prostate and adipose tissues), blood and urine samples to be taken for analysis.

Primary Aim

- a) To determine whether a broccoli intervention (≥ 4 weeks) will result in a difference in tissue sulphate levels in men scheduled for prostate biopsies.

Secondary aims

- a) To determine whether a broccoli intervention (≥ 4 weeks) will result in a difference in tissue ADP levels.
- b) To determine whether a broccoli intervention (≥ 4 weeks) will affect redox status of phosphatase proteins such as PTEN in prostate tissue.
- c) To determine whether the diet-induced differences observed in tissue are specific to the prostate gland.
- d) To determine whether the diet-induced differences observed in tissue are correlated with differences in urine.
- e) To determine whether the metabolite differences induced by diet are modulated by glutathione S-transferase Mu 1 (GSTM1) genotype or other relevant genotypes.

Study population

This study will recruit men (n=18) aged 18-80 years with a BMI between 19.5 and 35 kg/m² with a referral for transperineal template biopsy at the Norfolk and Norwich University Hospital (NNUH).

Outline of study design

The study outline is presented in **Figure 0-1**. Patients recruited onto this study will be randomly allocated to one of two arms in which they will be required to consume **three portions** of broccoli and stilton soup per week for at least four weeks prior to their biopsy (i) or to follow their normal diet until the operation date (ii). The broccoli and stilton soups will contain glucoraphanin-enriched broccoli named Beneforte®. The study will not be blinded and will involve tissue, blood and urine collection on the same day that patients are scheduled for their diagnostic biopsy at the NNUH. Their habitual intake of cruciferous vegetables and dietary supplements will be assessed.

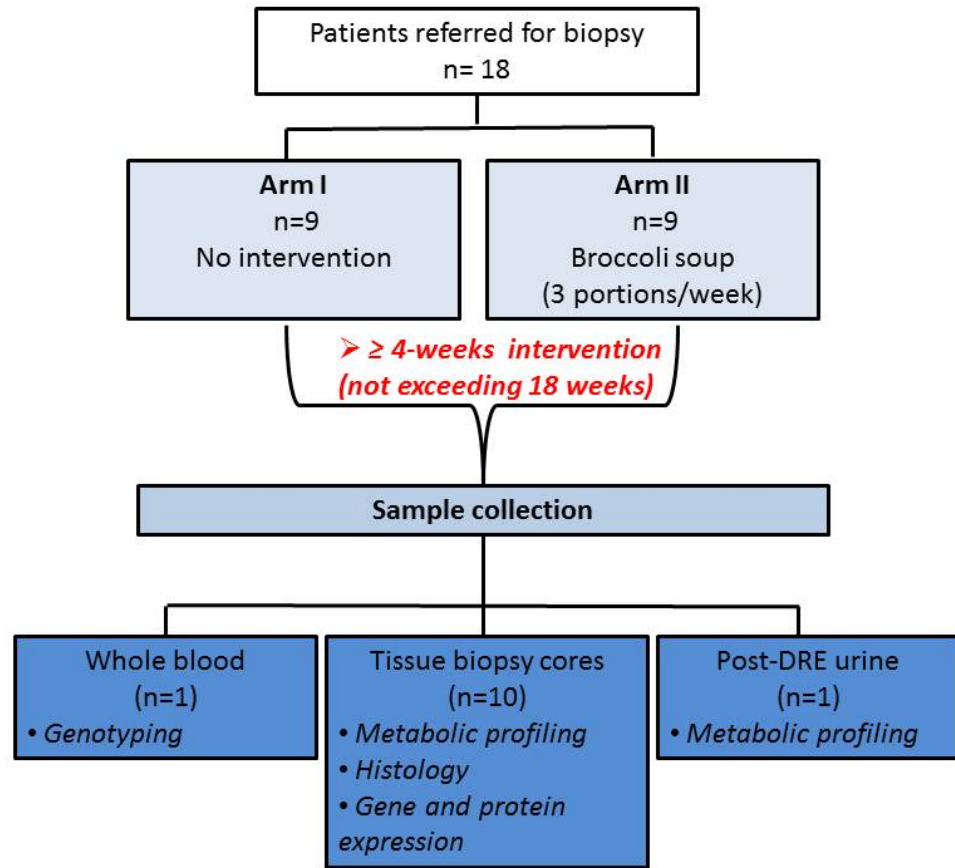


Figure 0-1. Study outline

1 Scientific background

1.1 Bioactives from cruciferous vegetables and prostate cancer

Prostate cancer is the most common form of cancer in men, accounting for over 25% of all new male cancer diagnoses [230]. The aetiology of prostate cancer is not understood. Frequently, several apparently independent cancer foci occur within the prostate. This multifocal nature implies an important 'field effect' in which changes in the gene expression and metabolism of apparently healthy tissue may facilitate the emergence and proliferation of cancerous clones [10]. Diet may be able to modify the metabolism of the prostate and inhibit the emergence of new cancer clones as well preventing the proliferation of existing clones, possibly resulting in their elimination.

Meta-analysis of case-control and prospective epidemiological studies provide evidence for a negative association between prostate cancer and intake of cruciferous vegetables such as broccoli [40]. This association is consistent with experimental studies with cell and animal models that provide evidence that the active compounds within these vegetables may be degradation products of glucosinolates, although other bioactive compounds within these vegetables may also be of importance [231].

We have recently obtained evidence of changes in the metabolite profile of prostate tissue due to a 12-month dietary intervention with a broccoli soup as part of the ESCAPE study (REC 13/EE/0110; NCT01950143). We found that the dietary intervention resulted in metabolic changes in non-cancerous prostate tissue, the most significant of which was an accumulation of sulphate. The impact of this change in metabolism requires further investigation but increasing levels of sulphate are likely to drive the production of 3'-phosphoadenosine-5'-phosphosulfate, a universal sulphate donor. Utilisation of cellular ATP in this process would correspond to an increase in its products ADP and phosphate, which also increased in participants of the ESCAPE study (**Figure 0-2**). Furthermore, the extent of ADP accumulation was negatively correlated with cancer progression (**Figure 0-3**).

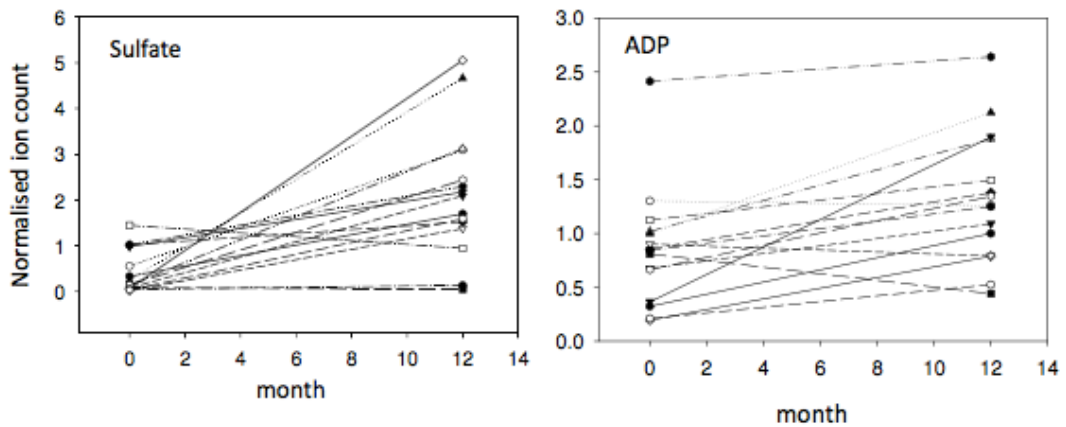


Figure 0-2. Accumulation of sulphate and ADP in prostate tissue of men on active surveillance following a 12-month dietary intervention with broccoli and stilton soups

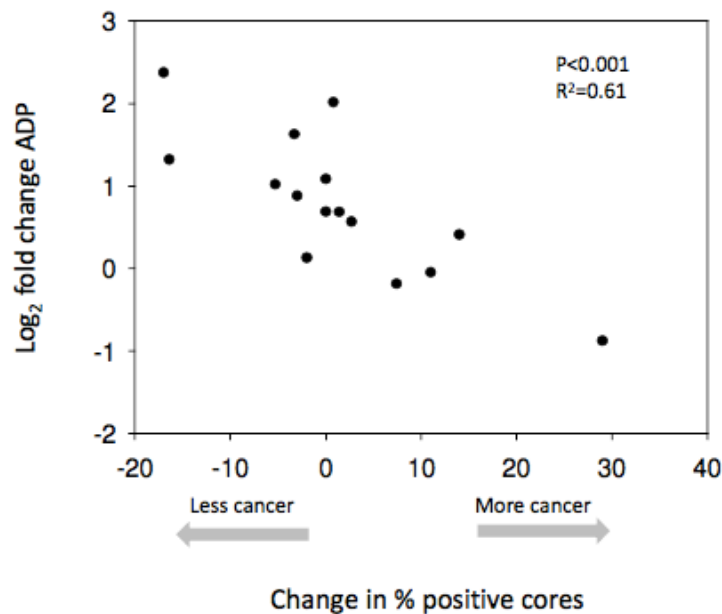


Figure 0-3. Scatter diagram of changes in ADP concentrations with changes in extent of prostate cancer in men who consumed broccoli and stilton soups for 12-months

In an associated study (REC 14/EE/1121; NCT02300324), we have shown that consuming the broccoli and stilton soup results in urinary excretion of the broccoli-derived, sulphur-containing metabolites glucoraphanin and S-methyl cysteine sulfoxide. It is possible that the accumulation of sulphate in the prostate is a result of

urinary reflux of these sulphur-containing metabolites directly into the prostate where they are metabolised to sulphate [123, 232]. This metabolism depletes the tissue of ATP, leading to the observed increase in ADP and phosphate. The result is death of cancer clones, which are more sensitive to ATP depletion than normal tissues, as they cannot adjust their metabolism [233].

These preliminary results provide, for the first time, a mechanistic basis for the association between reduction in prostate cancer progression and a diet rich in cruciferous vegetables in men, and reveal biological processes that have not been apparent from rodent studies. Moreover, while ATP depletion has been theoretically proposed as a therapeutic approach to kill cancer clones [234], it is the first time that ATP depletion has been shown to be induced through either diet or drug intervention in human tissues containing cancerous clones, and represents a promising approach to reduce or prevent the progression of prostate cancer.

We propose to undertake a human intervention study to extend these preliminary findings by exploring whether a short-term intervention with a higher consumption of broccoli will induce accumulation of sulphate and ADP in prostate tissue. This would provide further information concerning how it may be possible to deplete prostate tissue of ATP as a means to reduce risk of cancer progression.

1.2 Redox status of an oncogenic protein phosphatase (PTEN) in prostate cancer

The importance of a phosphatase and tensin homologue known as PTEN in prostate cancer progression is now well established. PTEN is a tumour suppressor gene that inhibits the phosphoinositide 3-kinase (PI3K)/Akt pathway, which would otherwise promote cell growth and proliferation [87]. It exists as two alleles that are subject to mutation or deletion in prostate cancer, corresponding to worse prognosis in terms of disease recurrence and progression after treatment [88, 89]. However, the activity of expressed PTEN protein is further reduced if it is exposed to oxidative stress in which a disulphide bond forms between Cys71 and Cys124, rendering the protein inactive (**Figure 0-4**) [90].

Preliminary analysis carried out in prostate tissue collected from men with early prostate cancer before and after a 12-month broccoli-rich diet (ESCAPE study) has shown changes in glutathione metabolites in addition to sulphate and ADP accumulation described in section 1.1. Changes in glutathione metabolism within the prostate tissue could be indicative of modification of tissue redox status. This may have consequences on the redox status of proteins that have important regulatory roles in metabolism, such as PTEN. The change in glutathione metabolism observed in the biopsies from the ESCAPE study may suggest that PTEN is maintained in a largely reduced and therefore active state.

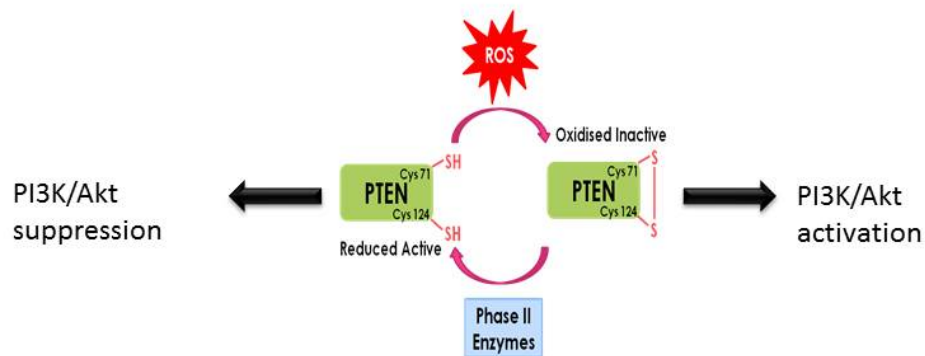


Figure 0-4. PTEN activity as result of its redox status

Thus, a secondary endpoint of the proposed study will be to determine the effect of the dietary intervention on the redox status of PTEN in prostate tissue.

1.3 Diagnostic prostate biopsy as an opportunity for a short dietary intervention

Conventional trans-rectal ultrasound scan (TRUS) and prostate biopsy is indicated for investigation of men with a raised prostate-specific antigen (PSA) level and/or digital rectal examination (DRE) suspicious for prostate cancer. Current guidelines from the National Institute of Clinical Excellence (NICE) recommend the use of transperineal template biopsies (TPB) of the prostate for those patients in whom cancer is still suspected but who have had a negative or inconclusive TRUS biopsy [235, 236]. It is also considered in men on active surveillance, to map the prostate prior to focal therapy or as an adjunct to novel imaging modalities.

The Norfolk and Norwich Hospital (NNUH) is a tertiary referral centre for prostate cancer, and template biopsies are regularly performed under general anaesthetic. We propose to undertake a pre-biopsy, window-of-opportunity dietary intervention study in men who have decided in clinic to proceed to template prostate biopsy. This trial differs from standard window-of-opportunity (phase 0) trials because it will be carried out before a diagnostic procedure rather than surgery. The study design will also avoid any delay in the patients receiving their investigation for a potential prostate cancer. A similar trial design has been recently published by Atwell and colleagues in Cancer Prevention Research [237].

2. Hypothesis, primary and secondary aims

2.1 Hypothesis

In men awaiting a routine template biopsy of the prostate, a broccoli intervention will lead to a higher sulphate concentration within the prostate compared to a non-intervention control.

2.2 Primary aim

To determine whether a diet rich in broccoli will result in a difference in sulphate levels in tissue samples obtained from men scheduled for prostate biopsy.

2.3 Secondary aims

- To determine whether a diet rich in broccoli will result in metabolic differences such as increased ADP levels in prostate tissue of men scheduled for prostate biopsy.
- To determine whether differences in sulphate and ADP levels induced by diet are specific to prostate tissue.

- To demonstrate if the metabolite differences induced by diet will affect the redox status of phosphatase proteins such as PTEN in prostate tissue.
- To determine whether the differences in metabolites induced by diet in prostate tissue are associated with metabolite differences in urine.
- To demonstrate if the differences in metabolites induced by diet are modulated by glutathione S-transferase Mu 1 (GSTM1) genotype or other relevant genotypes.

3. Objectives

We propose to undertake a two arm parallel un-blinded dietary intervention study. This study has been designed in order to provide information about differences in sulphate levels within prostate tissue in response to a broccoli-rich diet in men scheduled for diagnostic prostate biopsies. Volunteers recruited into the study will be randomly allocated to two groups, which will receive either a broccoli-rich diet or no intervention prior to a TPB procedure. Participants randomised to the dietary intervention arm will consume three portions of a soup containing high-glucoraphanin broccoli each week for at least four weeks prior to their biopsy. Secondary endpoints of the study will be to evaluate ADP accumulation, ATP depletion and changes to PTEN redox status as a consequence of dietary intervention. Rather than investigate the effect of the broccoli diet on these individual metabolites within prostate tissue, we intend to take a global approach using various techniques described in section 11. Experimental methods. We also intend to analyse urine samples to obtain additional information on the systemic effects of the dietary intervention. Furthermore, it will be determined whether any observed differences associated with the dietary intervention may be related to key genetic polymorphisms including but not limited to glutathione S-transferase Mu 1 (GSTM1). Several studies have reported that the influence of GSTM1 genotypes on the absorption of bioactives from broccoli and so we intend to gain information on participants' specific genotypes [238].

Finally, we intend to assess whether the accumulation of sulphate and ADP is specific to prostate tissue through the metabolic analyses of biopsies from prostate and non-prostate tissue. The metabolite profiling of prostate tissue obtained from participants not receiving the broccoli diet and separate core biopsies from pelvic adipose tissue will establish whether diet-induced metabolic differences are specific to the prostate or occur in other tissue compartments. This study design does not require the

participants to have any additional clinical procedures; during the TPB procedure, separate cores will be taken from the prostate and adjacent adipose tissue.

4. Study design

The study will be led by Prof Richard Mithen. Dr Antonietta Melchini and Mr Jack Coode-Bate will act as IFR investigators. Mr Jack Coode-Bate is an NHS doctor and part of the Urology team at the NNUH. He is also a MD student with the University of East Anglia (UEA) and his research activities will be based at the Institute of Food Research (IFR). This project is an integral part of his research degree.

The study will be carried out in collaboration with Mr Robert Mills and Professor Richard Ball at the Urology and Histopathology Departments of the Norfolk and Norwich University Hospital (NNUH) NHS Trust. Urologists at the NNUH will specifically help in identifying volunteers who are being scheduled for TPB. The 'window of opportunity' for dietary intervention will be the waiting list time between decision to proceed with TPB and the actual operation date. All TPB procedures will be carried out by trained urology consultants, and would otherwise form part of the volunteers' routine clinical care for potential diagnosis and staging of prostate cancer.

The study team will recruit 9 volunteers to each arm of the study.

Volunteers will be randomised to 1 of 2 arms:

Arm 1- **No dietary intervention**

Arm 2- **Three portions** (300g each) per week of a soup containing glucoraphanin-enriched broccoli (Beneforte®)

The randomisation will be undertaken by a third party with the use of an electronic randomisation generator (www.randomization.com). This uses a method called "Block randomisation" whereby volunteers are equally distributed to both study arms.

We will request volunteers' consent for additional cores of prostate tissue to be taken during their TPB that would otherwise form part of their routine clinical care. We will also seek consent for 2 cores of adipose tissue to be taken through the template grid

used for the transperineal biopsy, but outside of the prostate. The collection of adipose tissue does not require any additional clinical procedures.

In addition to prostate and adipose tissue, we will collect blood and urine samples at the time of TPB. All study samples will be used for global and targeted metabolite analyses. A specific dietary questionnaire will be carried out at the end of the intervention to establish their habitual intake of cruciferous vegetables.

5. Recruitment policy

A fixed 6-month period from the start of the study will be dedicated in order to allow recruitment of 18 volunteers and their follow up to the time of biopsy. Men aged 18 to 80 years and with a BMI of 19.5 to 35 kg/m² who have agreed to undergo a template biopsy of the prostate will be identified and recruited by Mr Robert Mills and other consultant urologists at the NNUH. The standard waiting list time between a referral for TPB and the actual procedure is currently 6-8 weeks at NNUH, allowing a dietary intervention of 4-6 weeks from time of recruitment.

After deciding to proceed to template prostate biopsy, patients will be provided with an information pack either directly from the urology clinic or by arrangement to send it out by post. The urologist will provide the information packs and offer patients who would like more information on the study an opportunity to either return the response letter or contact one of the study team directly with the information provided. Members of the study team will construct information packs in advance of the clinics and hand them to the relevant collaborator. The study scientist will ensure that the clinics have an ample supply of information packs. The information packs will consist of a letter of invitation (**Annex 2**) and participant information sheet (PIS) (**Annex 3**). It is important to stress that non-NHS members of the IFR study team will not have access to any patient files. All searches of patient files will be carried out by the NHS staff outlined below or their NHS colleagues. Identified patients will be contacted by telephone by the NHS members of the study team at least one week after the packs have been distributed to ensure that they have been received. Potential volunteers will be asked to complete a response slip that includes their contact details and preferred time of contact (**Annex 3**). They will have the option to submit these directly to the urologist in clinic or return them to the study team in the pre-paid envelope provided. The information packs will also provide contact details of the IFR study scientists (Dr Antonietta Melchini and Mr Jack Coode-Bate).

If the patient agrees, he will be contacted by telephone to discuss the study in detail and establish interest in ongoing participation. IFR study scientists will encourage the volunteers to ask questions in order to ensure their full understanding of the study protocol prior to obtaining written informed consent (**Annex 4**). Volunteers are given at least 3 days to decide whether they would like to take part or not, and during this consideration period the volunteers will not be contacted. After this consideration period volunteers will attend the first visit at the Human Nutrition Unit (HNU) to give written informed consent and receive the broccoli and stilton soups if they are randomised to the intervention arm (Visit 1). Volunteers who are randomised to the no intervention arm will give written, informed consent before undergoing the clinical procedure (Visit 2).

Recruitment will continue until 18 volunteers have completed the study. Information Services at NNUH have confirmed that 190 TPBs were performed in 2015 and this number is likely to continue to increase. It is, therefore, expected that recruitment of sufficient numbers will be possible within 6 months.

6. Screening criteria

6.1.1 Basic inclusion criteria

- Males
- Scheduled for TPB as part of routine investigation or staging for prostate cancer
- Aged 18-80 years
- BMI between 19.5 and 35 kg/m²
- Smokers and non-smokers

6.1.2 Basic exclusion criteria

- Those regularly taking 5 α -reductase inhibitors or testosterone replacement medicines
- Those on warfarin treatment
- Those diagnosed with diabetes

- Those diagnosed with or suspected to be high-risk for human immunodeficiency virus (HIV) and/or hepatitis
- Those allergic to any of the ingredients of the broccoli and stilton soups
- Those taking dietary supplements or herbal remedies which may affect the study outcome. Please note that some supplements may not affect the study and this will be assessed on an individual basis
- Those that are unable to understand English or give informed consent
- Parallel participation in another research project that involves dietary intervention
- Any person related to or living with any member of the study team

7. Study procedure

A flow chart of the overall study is presented in Figure 0-5

. This study will involve 18 volunteers aged between 18-80 years who will undertake the study as described below.

7.1 Information exchange

Volunteers who respond positively to the study invitation letter (**Annex 2**) will be contacted by telephone by a member of the study team and all aspects of the study will be discussed. The study scientist will go through the PIS (**Annex 3**) and answer any questions. Volunteers will be given at least 3 days to decide and are advised that they may withdraw from the study at any time without affecting their clinical care.

7.2 Visit 1 at the HNU

Volunteers who verbally agree to proceed after the consideration period will be randomised to one of the two arms. If allocated to the dietary intervention arm, volunteers will be invited to the Human Nutrition Unit (HNU) at the IFR to obtain written consent (**Annex 4-5**) and receive the first batch of frozen soups. In the non-intervention arm, volunteers will not be required to attend this visit at IFR but will sign consent forms on the day of their prostate biopsy (Visit 2). All volunteers willing to participate in the study will have the opportunity to discuss the study and tissue bank consent forms (**Annexes 4-5**) with a study scientist in person (*Visit 1, intervention arm; Visit 2, no intervention arm*). Consent for the clinical procedure (TPB) will be

carried out by NNUH urologists using a standard NHS consent form on the day of biopsy (Visit 2) (**Annex 6**). In total, volunteers will be asked to sign three consent forms (**Annexes 4-6**). Copies of the consents will be kept in the study records, the patient hospital notes and a copy given to the volunteer to keep. GPs will be informed by letter of their patients' participation in the study (**Annex 7**).

Volunteers will be assigned a code and only the study scientists will be able to link codes to volunteers. All personal information will be kept confidential and known only to the Chief and Principle Investigators and NNUH collaborators.

7.3 Visit 2 at the NNUH

Volunteers will be provided with a validated Cruciferous Vegetable Food Frequency Questionnaire (CVFFQ) to be completed after the biopsy and returned to the study team using a pre-paid envelope (**Annex 8**). We wish to measure the habitual consumption of cruciferous vegetables in each group as participants may already consume high levels of these vegetables as part of their normal diet. CVFFQ is a 6-page, 79-item questionnaire that has been designed and validated by the University of Arizona Health Sciences and assesses cruciferous vegetable intake over the previous 12-month period [92]. Volunteers will be asked to use the CVFFQ to review the time period between the biopsy referral and the actual procedure using a pencil provided by the study team.

On the day of the biopsy, volunteers will also be asked to complete a health questionnaire detailing their past and current medical history and record all regular prescribed and over-the-counter medications (**Annex 9**).

With informed, signed and written tissue banking consent (**Annex 5**) from all volunteers as detailed above, study samples (blood, urine and tissue biopsies) will be collected at the NNUH and transported to IFR to be stored until analysis. At the end of the study, the remaining blood and urine samples will be transferred to the local Human tissue bank to help future research studies. Participants will still be eligible to take part in the study even if they do not want their remaining samples to be transferred to the Norwich Biorepository.

Tissue biopsy samples

TPB of the prostate is a useful diagnostic tool in diagnosing and staging prostate cancer. It is performed under general anaesthetic with intra-vascular aminoglycoside

antibiotic prophylaxis. The patient is positioned flat on their back (supine) with their legs supported in stirrups and the skin cleaned with an appropriate antiseptic solution. An ultrasound probe is inserted into the rectum and allows direct visualisation of the prostate throughout the procedure for accurate targeting. A specially designed grid is attached to the ultrasound probe and placed flat against the skin of the perineum. Holes in the grid are spaced at 5mm intervals and allow systematic sampling of all zones of the prostate, while taking care to avoid the urethra. An average of 25 tissue cores is taken, but may vary depending on prostate gland volume.

We propose to take an additional 8 needle cores of prostate through the template grid for study purposes. One core will be transferred immediately to methanol for metabolomic analysis, 5 cores will be snap frozen in dry ice and stored at -80°C for target metabolomic, oncogene and protein expression analyses, and 2 cores will be placed in RNAlater for RNA extraction.

At the time of template biopsy we also propose to take 2 cores of adipose tissue from ischio-rectal (pelvic) fat to act as a control sample. These will be taken through the template grid in order to avoid a separate operation site. Both cores will be taken at the start of the procedure and using trans-rectal ultrasound to ensure no prostate tissue is involved in the core, before being appropriately processed for subsequent analyses.

It is important to stress that the collection of additional tissue biopsy cores for this research project has been discussed with the consultant urologist (Mr Mills, NHS principal investigator) and will not affect patients' clinical care. It is also important to take into consideration that the proposed sample collection is not expected to increase the risks of any associated complications. The prostate biopsies will be taken from the same zones as routinely sampled during TPB and the peri-prostatic adipose tissue samples avoid the need for a separate operation site. Intravenous antibiotic prophylaxis will be given as standard to reduce the risk of wound infection.

Blood sample

During routine venous cannulation prior to administration of general anaesthetic (standard procedure for template biopsy), a 5ml blood sample will be collected for genotyping. The blood will be mixed with an anti-coagulant (EDTA), divided into

aliquots and placed on dry ice until they can be safely transported back to the IFR to be stored at -80°C until required.

Urine sample

Prior to the prostate biopsy a first-pass urine sample (20-30ml) will be collected following digital rectal examination (DRE) for metabolomic analysis. The sample will be divided into aliquots, placed and transported on dry ice and stored at IFR at -80°C until required.

At the end of the study, the remaining blood and urine samples will be transferred to the NNUH Human tissue bank (The Norwich Biorepository) to help future research studies (participants will give written consent for tissue banking, Annex 5). It is important to stress that any further analysis will be carried out in full compliance with ethical requirements.

8. Post-Intervention Follow Up

Volunteers will not be required to attend any further clinical follow up as part of the study. Their histology results, including both standard and study cores, will be reviewed by the clinician responsible and discussed in a multi-disciplinary team meeting if cancer is detected. All ongoing care will be determined as per standard treatment in the NNUH Urology Department.

Volunteers in both arms of the study will be offered a 3 month supply of Beneforte® broccoli and stilton soup for their own personal consumption if desired but this will not form part of the study. They will have the option to collect the soups from the HNU or to be delivered directly to their home. In case of collection from HNU, the study team will not cover travel expenses.

9. Study diet

Volunteers will be randomly allocated to either no intervention or consumption of 3 portions of Beneforte® (glucoraphanin-enriched) broccoli and stilton soup per week as part of their normal diet for a minimum of 4 weeks prior to their prostate biopsy. As

a day-case, elective procedure the expected waiting time between referral and the actual TPB procedure is currently 6-8 weeks at the NNUH. Volunteers will be asked to continue the soup intervention until the day of the biopsy in case the clinical procedure is delayed. The maximum waiting list duration set by the UK government is 18 weeks, but as an investigation for potential cancer TPBs are prioritised by the NNUH [239].

Beneforte® broccoli variety has been developed by conventional breeding and has the same appearance and flavour as standard broccoli. The broccoli and stilton soups will be prepared by Bakkavor®, a leading international producer of freshly prepared foods. Ingredient declarations, nutritional information and allergy statements for the soups have been provided from the producer (**Annex 10**). Bakkavor will make frozen soups and then arrange to deliver them to IFR (**Figure 0-5**). Once delivered to IFR, soups will be stored in a dedicated freezer (IFR West Side A). IFR study team members who have successfully completed “level 2” training in food safety will be responsible for providing the frozen broccoli and stilton soups either directly to volunteers attending IFR or delivering them to volunteers’ homes. Volunteers will be informed on how to consume the soups by reading the participant information sheet (**Annex 3**) and a specific soup information sheet (**Annex 10**).



Figure 0-5. Broccoli and stilton soups provided by Bakkavor® will be packed in a carton designed to freeze soups in individual portions

9.1 Monitoring of diet and compliance

Volunteers randomised to the intervention arm will be asked to fill in a soup record sheet during the pre-biopsy intervention period, recording each time they eat their soups (**Annex 11**). This is intended to aid compliance with the dietary interventions and also to be used in conjunction with the Arizona cruciferous vegetable intake questionnaire (**Annex 8**). Volunteers will also be asked to return the record sheets with the lids of empty soup containers, which will be counted by members of the study team to monitor compliance.

We appreciate that volunteers may wish to go on holiday during the intervention period. Volunteers will be allowed to go on holiday during the course of the intervention provided they are able to transport and store the soups appropriately and continue to incorporate them into their diet. We will ask the volunteers to inform us of any planned holiday dates so that we can keep a record and review each case on an individual basis.

10. Withdrawal of a participant from the study

Volunteers may withdraw from the study at any time, without giving a reason, without affecting their clinical care at the NNUH Urology Department or their participation to other research studies. A withdrawal letter will be sent to the participant by the study team (**Annex 13**) and his withdrawal will be appropriately documented in the study withdrawal/termination log.

11. Experimental methods

11.1 Prostate biopsy sampling and processing

We intend to collect 8 prostate biopsies and 2 adipose tissue biopsies from each volunteer through a TPB procedure:

- Two cores will be used for global metabolomics:

Biopsy samples (1 prostate core, 1 adipose core) will be deposited individually into pre-labelled sample vials containing room temperature extraction solvent (methanol). Following incubation at room temperature (up to 48 hours), biopsy samples will be removed from the extraction solvent. The removed prostate core will be used for histological analysis; instead the adipose core will be disposed by incineration.

Histological analysis of prostate biopsies will be carried out by Professor Richard Ball, consultant histopathologist at the NNUH, as part of normal diagnostic procedure for patients under investigation for prostate cancer.

The vials containing extracted metabolites in methanol will be stored at IFR at -80°C until required for analysis.

- Six cores will be used for target analyses

Biopsy cores (5 prostate cores, 1 adipose core) will be placed individually into pre-labelled vials and transported in dry ice until they can be safely transported back to the IFR to be stored at -80°C until required for analysis.

- Two cores will be used for gene expression analyses

Two biopsy cores will be immersed in RNAlater solution at the point of collection, safely transported to IFR and subsequently snap frozen in liquid nitrogen before being stored at -80°C.

11.2 Blood sampling and processing

A single blood sample (5ml) will be collected by a clinician in the operating theatre before starting the biopsy procedure. This will be divided into aliquots, transported on dry ice to IFR and stored at -80°C until required for genotyping.

11.3 Urine sampling and processing

A first-pass urine sample will be collected following prostate massage immediately before the patient undergoes the prostate biopsy procedure. This will be divided into aliquots, transported on dry ice and stored at -80°C until required for global and targeted metabolomic analysis.

11.4 Genotype determination

The genomic DNA will be extracted from the blood sample using the QIAamp DNA Mini kit protocol (Qiagen Inc.) prior to genotyping for selected genotypes considered relevant for the purposes of this study.

11.5 Gene expression analysis

We will undertake both global and targeted gene expression analysis from prostate tissue biopsies. Total RNA will be extracted following snap freezing of samples in RNAlater™ (Ambion). This product ‘freezes’ gene transcription and stabilises the RNA present so that it can be extracted at a later stage. Total RNA isolation will be performed using RNeasy kits (Qiagen) according to the manufacturer’s instructions; these are used routinely for the extraction and purification of RNA. Preliminary investigation has demonstrated that sufficient RNA of high quality can be obtained from a single needle biopsy sample [63]. RNA extracted from the biopsy samples will be concentrated and the quality assessed using Agilent technologies lab-on-a-chip RNA 6000 nano assay. RNA of sufficient quality will be analysed by using new technologies such as Next generation sequencing. In addition, a selection of genes, including established prognostic biomarkers and interesting candidates identified through the global approach, will be confirmed by target analysis if considered relevant for the purposes of this project.

11.6 Metabolite profiling

The metabolomic analysis of tissue and urine samples will be carried out by an US company called Metabolon®. Metabolon® is a service and diagnostic products company with the ability to identify and produce a profile of up to 350 known metabolites using standard metabolomics techniques including liquid chromatography/mass spectrometry (LC/MS) and gas chromatography/mass spectrometry (GC/MS) [104].

Tissue and urine samples will be sent for analysis at:

Metabolon Sample Acceptance,

800 Capitola Drive, Suite 1

Durham, NC 27713, USA.

Briefly, the sample preparation process will be carried out using the automated MicroLab STAR® system from Hamilton Company. Standards will be added prior to the first step in the extraction process for quality control purposes. Samples will be prepared using a series of organic and aqueous extractions to remove the protein fraction while allowing maximum recovery of small molecules. The resulting extract will then be divided into two fractions; one for analysis by LC and one for analysis by GC. Samples will be placed briefly on a TurboVap® (Zymark) to remove the organic solvent. Each sample will then be frozen and dried under vacuum and thus prepared for the appropriate instrument, either LC/MS or GC/MS.

Metabolon® will provide detailed reports on the difference in levels of sulphate, ADP and phosphate as well as a much wider range of metabolites following dietary intervention. The Metabolon® platform can identify sulphate with very high confidence levels, even without an internal standard. Sulphate has a unique mass defect and isotope pattern which are highly characteristic of the sulphur atom, ensuring fragmentation is accurate.

Sulphate and other relevant metabolites will also be measured in tissue and urine samples by chromatography methods following IFR standard operating procedures. Sulphate quantification will be performed by liquid chromatography/mass spectrometry using an external standard calibration curve. IFR scientists have established a standard operating procedure for sulphate quantification that will be followed for the purposes of this study.

Target analyses to determine ADP/ATP levels in tissue samples will be carried out using commercially available assay kits.

11.7 Protein expression analysis

The redox status of PTEN and associated proteins will be evaluated from snap frozen biopsy cores. Semi-quantitative analysis of PTEN oxidation will be performed using SDS-Page protein electrophoresis and Western blot analysis. A chromatography method based on liquid chromatography tandem mass spectrometry (LC/MS-MS) for detecting the reduced and oxidised PTEN (disulphide bond between Cys71 and Cys124) will be used for further quantitative analysis.

12. Confidentiality

Volunteers who are successfully recruited onto the study will be assigned a unique code number which will be kept in a secure file. A lockable filing cabinet or cupboard will be used to keep paper documents that include the file linking the volunteer to the code and personal information. All electronic data will be stored on a password protected shared data file. Confidential data will be accessed only by the study scientists. The samples sent to the other laboratory facilities will be known only by their code number. All data collected will also be identified by code only.

Volunteers' personal data will be held in a locked cabinet or password protected electronic file in the IFR. Only the study scientists will have access to these data. Data will be stored for at least 15 years after completion or discontinuation of the study. The data will be stored in the IFR human studies archive. Access to archived data will be limited to the study scientist and chief investigators (CI) of the study or the CI's successor. The quality assurance auditors may also be allowed access with the permission, and in the presence, of the CI. The main computer storage will be on one main IFR computer, but as part of a password protected shared network. All IFR computers are individually password protected and the shared network access is limited to those working within the research area. Only the study scientists will have access to the file linking personal data to the volunteers' unique code. Manual files/folders will consist of separate named and numbered files for each participant. No data with the volunteers' name will be filed in the numbered file and vice versa.

13. Statistical Analysis

13.1 Sample size calculation

There are no data (from previous literature) reporting the effect of a diet rich in broccoli on sulphate levels within prostate and at the systemic level. The sample size for the proposed study has been estimated taking into consideration the preliminary data obtained from the metabolomic analysis of samples collected as part of the ESCAPE study (REC 13/EE/0110; NCT01950143). The metabolite profiling of prostate tissue from patients (n=15) randomised to a 12-month broccoli intervention has shown a significant accumulation of sulphate in prostate tissue after the intervention period compared to baseline.

To detect a difference of 1.735 (normalised ion count) at a 5% significance level with 90% power and assuming a within group standard deviation of 1.056 (normalised ion count) requires a sample size of 9 individuals in each group (total 18). These sample sizes were calculated for a two-group study design (broccoli intervention vs no intervention) assuming a two-sided comparison (i.e. to detect a difference rather than a higher level).

13.2 Data analysis

The statistical analysis for data generated from this study will be performed by using different methods.

To determine if the dietary intervention affects sulphate levels, a Welch's t-test (or unequal variance test) will be used for data obtained from the two groups (broccoli intervention vs no intervention).

One of the secondary aims of this study is to determine whether metabolic differences occurring in response to diet within prostate tissue are correlated with metabolite levels in urine. The association between the metabolic differences in prostate and those measured in urine will be assessed using an appropriate correlation test.

The analysis will use ANCOVA to test for the effect of the diet whilst taking account of the glutathione S-transferase Mu 1 (GSTM1) genotype or other relevant genotypes. Genotype will be included as an explanatory variable in this analysis.

14. Ethical considerations

Food safety: IFR has standard operating procedures for the storage and delivery of food (broccoli and stilton soups) to study participants. Those procedures will be adhered to when processing and handling our food items, in accordance to Environmental Health Guidelines.

Toxicity: There is no evidence from animal or human studies that broccoli is harmful. The level of glucoraphanin that will be consumed in the soup will be similar to that being consumed in a current intervention study carried out at IFR (*Effect of Sulforaphane on Prostate CAncer PrEvention; REC ref: 13/EE/0110*).

Risk of complications: The additional prostate and adipose tissue biopsies are not expected to increase the risk of potential complications. Antibiotic prophylaxis will be offered as standard and the operation site will not extend beyond the template grid. To date the incidence of complications in participants undergoing TPB, including 8 additional prostate tissue cores, as part of the ESCAPE study has been lower than reported in the British Association of Urological Surgeons' information sheet.

The use of study samples for future research: Volunteers will be asked to sign a consent form agreeing to the storage of their samples in the tissue bank for research purposes. It is envisaged that the banking of the remaining blood and urine samples at the end of the study at The Norwich Biorepository will be valuable for obtaining additional information for this study and for designing further studies.

Travel expenses: Volunteers will be reimbursed travel expenses to and from the HNU. This will be reimbursed at the IFR's current mileage rate or by reimbursing public transport costs on production of a ticket or receipt. If volunteers require transport by taxi, this will be arranged by prepayment.

Use of documents previously approved by RECs: Volunteers will be provided with detailed information on how to consume and store the study diet (Annexes 10 and 12). They will be also asked to complete some paperwork for the purpose of this study (Annexes 9 and 11). The annexes stated above are currently used in other dietary intervention studies; they have been reviewed and approved by the NRES Committee East of England- Cambridge South as part of two separate ethical applications (*REC ref: 13/EE/0110; REC ref: 14/EE/1149*).

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Annex 2

Annex 2–Invitation letter NNUH

Version 2-SAP

13th January 2016

Norfolk and Norwich University Hospital 
NHS Trust

Date

Dear Sir

I am writing to tell you about a research study that is being organised jointly between the Institute of Food Research in Norwich and the Urology department of the Norfolk and Norwich University Hospital. The study is called:

Sulphate Accumulation in Prostate (SAP)

We are recruiting a group of men scheduled for transperineal template biopsy of the prostate at the Norfolk and Norwich University Hospital (NNUH). This study aims to investigate the effect of a broccoli-rich diet on the levels of certain compounds, derived from these vegetables, within the prostate gland. You have received this letter because a member of the medical staff from the department of Urology at the NNUH has suggested that you may fit the criteria for this study.

If you are interested in receiving more information, please contact one of the study scientists; Dr Antonietta Melchini on 01603 255030 (email antonietta.melchini@ifr.ac.uk) or Mr Jack Coode-Bate on 01603 255030 (email jack.coodebate@ifr.ac.uk).

If you are interested in taking part in the current study, please complete the response form included in the participant information sheet (PIS) and return it to the Institute of Food Research (IFR) using the freepost envelope provided. The study scientist will then contact you as soon as possible.

Participation in the study is completely voluntary and not taking part will not affect the standard of care that you receive in any way. You are free to withdraw from the study at any time and do not have to give a reason.

Thank you in advance for your time.

Yours Faithfully,
Mr Robert Mills (MB ChB, FRCS),
Consultant Urologist



Annex 3



Institute of Food Research

PARTICIPANT INFORMATION SHEET

Sulphate Accumulation in Prostate (SAP)

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve for you. Please take time to read the following information carefully. Talk to others about the study if you wish.

- Part 1 tells you the purpose of this study and what will happen if you take part
- Part 2 gives you more detailed information about how the study will be conducted

Please ask us if there is anything that is not clear or if you would like more information.

Take time to decide whether or not you wish to take part.

This information sheet is yours to keep.

Thank you for reading this.

.....

Study scientist contact details:

Dr Antonietta Melchini

Mr Jack Coode-Bate

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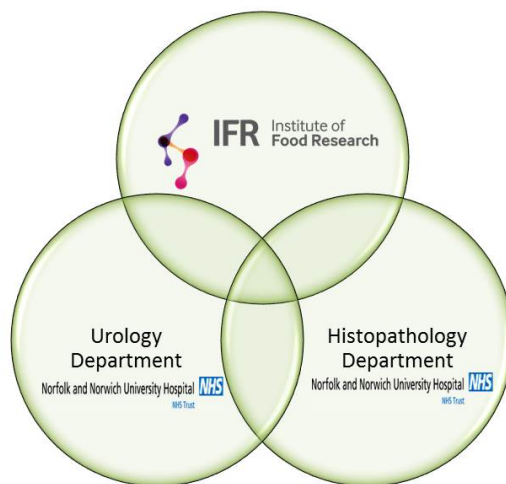
richard.mithen@ifr.ac.uk

Norfolk and Norwich University Hospital 
NHS Trust

PART 1 of the information sheet

What is the purpose of the study?

This study is part of a research project currently running at the Institute of Food Research (IFR) in collaboration with the Norfolk and Norwich University Hospital (NNUH) (Urology and Histopathology departments).



It is also part of a research project for Mr Jack Coode-Bate, a Specialty training Registrar (StR) in Urology in the East of England deanery, and MD student at the University of East Anglia (UEA) and IFR.

Current research suggests that diets rich in cruciferous vegetables (like broccoli) are beneficial to our health and may reduce the risk of some cancers, including prostate cancer.

We have designed this study to investigate the relationship between diets rich in broccoli and prostate

metabolism. We hope to expand our understanding of how eating these vegetables will influence prostate metabolism and so, ultimately, we can find ways to improve prostate health.

The SAP study will explore the effects of consuming three portions of broccoli and stilton soup per week for at least four weeks prior to a template biopsy of the prostate (biopsies taken through a specially designed grid placed on the skin of the perineum). The broccoli used for production of the soups is a specially cultivated, glucoraphanin-enriched variety known as Beneforte®.



Once processed by the body this delivers high concentrations of sulforaphane – the naturally occurring chemical thought to be responsible for the beneficial effect of these vegetables. However, Beneforte® broccoli also retains the same compounds as normal broccoli, which could also be important.

Previous studies carried out at IFR have identified specific changes in prostate metabolism, and particularly sulphate levels, as a result of a 12 month broccoli-enriched diet in men with prostate cancer.

We now aim to measure sulphate levels and expand our understanding

of key genetic changes and products of metabolism in the prostate and urine after consuming Beneforte® broccoli and stilton soups.

Why have I been invited to take part?

We are contacting men who are under investigation for a potential, or previously diagnosed, prostate cancer and have been scheduled for a template biopsy of the prostate at the NNUH.

We aim to recruit 18 men aged 18-80 to take part in our study.

You will not be able to take part if:

- Your Body Mass Index (BMI) is lower than 19.5 kg/m² or greater than 35 kg/m²
- You are being prescribed 5 α -reductase inhibitors (e.g. finasteride or dutasteride)
- You are receiving testosterone replacement medicines
- You are receiving warfarin treatment
- You have been diagnosed with diabetes
- You have been diagnosed with human- immunodeficiency virus (HIV) or hepatitis
- You have a food allergy to the ingredients of the study diet

- You are taking dietary supplements or herbal remedies, which may affect the study outcome. (Please note that some supplements may not affect the study and this will be assessed on an individual basis)
- You are unable to understand English or give informed consent to take part.
- You are participating in another research project involving dietary intervention.
- You are related to or living with a member of the study team.

Do I have to take part?

It is entirely up to you to decide whether or not to take part. If you do decide to take part you will be asked to sign the relevant consent forms. You are free to withdraw from the study at any time without giving a reason. A decision to withdraw or not to take part will not affect the standard of care you receive.

An expression of interest does not mean you are committed to participating in the study.

After reading this information sheet if you are interested in taking part in the study you should contact the study scientists named on the first page, or complete the attached response form (page 20) and return

it either directly to your urologist in clinic or using the pre-paid envelope enclosed.

Please feel free to say no by not returning the response letter we have provided.

A member of the study team will contact you by telephone after one week to ensure you have received the information pack but will not attempt to persuade you to join the study.

What do I have to do?

If you decide to take part your involvement in the study will last until the day of your biopsy at the NNUH. This will be at least four weeks from the point of decision to participate, but will continue until the biopsy has been performed (depending on hospital waiting list times). We do not expect your participation in the study to exceed 6 weeks.

Your participation in the study will not delay your biopsy for any reason.

During this period you can contact the study team at any time to ask

any questions that might arise. We will request details of any holiday arrangements in case this may affect the study.

What will happen to me if I take part?

You will be contacted by a member of the study team by telephone at a mutually convenient time. At this point the details of the study will be discussed and any initial questions answered.

If you decide to participate you will be randomly allocated into one of two groups (arms) of the study.

Arm I: You will consume 3 portions of broccoli and stilton soup per week until your biopsy day.

Volunteers in this group will be asked to visit the Human Nutrition Unit (HNU) at the IFR on **one occasion** to sign consent and receive the first batch of frozen soups. All travel expenses will be reimbursed and if transporting the frozen soups is not convenient we will arrange for them to be delivered directly to your home.

Arm II: You will continue your normal diet until the biopsy day.

Written consent from volunteers in this group will be taken at the hospital on the day of their biopsy, so will not require any additional study visits.

On the day of the biopsy, all volunteers will be asked to complete a health questionnaire and a food frequency questionnaire to measure your normal intake of cruciferous vegetables.

While under anaesthetic for the template biopsy of the prostate, *additional samples for the study will be taken of both prostate tissue and the fat surrounding the prostate through the template grid.*

You will not be asked to undergo any additional surgical procedures outside of your routine clinical care. The collection of tissue samples for the purpose of this research will not affect the clinical procedure and will not increase your risk of any potential complications. All usual precautions surrounding the biopsy will be maintained and these are discussed in greater detail later.

Single blood and urine samples will also be collected on the day of your prostate biopsy.

Study talk over the phone

If you have replied to tell us you are interested in participating, a study scientist will contact you by telephone. The details of the study will be discussed, as well as the content of this participant information sheet.

This will be a good opportunity to ask any questions you may have.

At the end of the discussion you will be given a 3-day consideration period to decide whether you would like to participate. We will not contact you during this period in order to allow you time to think about the study and discuss it with friends and family if you wish.

If you decide to participate, you will be asked to contact one of the study scientists and he/she will arrange for you to be randomly allocated into either arm of the study.

Depending on whether or not you will be receiving soups we will then arrange to meet with you either at

the IFR HNU (**Arm I**) or at the hospital on the day of your biopsy (**Arm II**).

Health and diet questionnaires

On the day of your biopsy you will be asked to complete two questionnaires: **Health questionnaire** and **Arizona Food Frequency Questionnaire**.

Health questionnaire

The health questionnaire will be carried out by a member of the study team at the NNUH and all your information will be considered strictly confidential.

This will include a list of prescribed and over-the-counter medications as these can occasionally influence your metabolism.

This questionnaire will inform us about your general health, your past and current medical history.

Arizona Food Frequency Questionnaire

We will ask you to complete a dietary questionnaire called the Arizona Cruciferous Vegetable Food Frequency Questionnaire. This has been specifically

developed by the University of Arizona to assess cruciferous vegetable intake over the time period you have been asked to review. In your case this will be between your biopsy referral and the actual procedure.

It is a 6-page questionnaire that will ask you questions related to the frequency and portion size (small/medium/large) of the range of individual cruciferous vegetables in your normal diet. It must be completed using the pencil provided by the study team.

Questions are asked regarding both raw and cooked vegetables including typical methods of cooking. There is also a section to assess intake of oriental mixed dishes and condiments such as mustard that contain compounds of a similar nature to those delivered by eating cruciferous vegetables.

The questionnaire should take approximately 20 minutes for you to complete. You have the option to return the completed questionnaire to the study team on the day of your biopsy or to send it by post using a pre-paid envelope.

This questionnaire will help us to calculate what is normal for you and

which additional nutrients are likely to have come from the soups.

Prostate biopsy procedure

You have been invited to take part in this study because you have been scheduled for a template prostate biopsy (TPB) at the NNUH. This means that your urology consultant has already discussed the type of procedure, its intended benefits and serious or frequently occurring risks. Consent for this procedure will be carried out by NNUH urologists using a standard NHS form on the day of the biopsy. A copy of this consent is retained in your hospital notes.

You already know that TPB is a surgical method for taking a larger number of prostate biopsies through the perineum (skin between scrotum and anus). TPB is performed under general or spinal anaesthetic with you flat on your back and legs supported in stirrups. The prostate is examined and an ultrasound probe inserted via the back passage (anus) to view and measure the prostate directly. The template grid is then placed flat

against the skin of the perineum, which accurately guides the biopsy needle to sample regions throughout the whole prostate. The total number of biopsies taken may be as high as 50 depending on the size of your prostate, and allows access to regions of the prostate that would not otherwise be possible. A dressing is applied at the end of the procedure and most patients go home on the same day.

For this study we will ask your permission to take an additional 8 prostate biopsies and 2 fat biopsies during a TPB that would otherwise form part of your routine clinical care.

It is important to stress that these samples will be collected only if you have given written informed consent for the study before undergoing TPB.

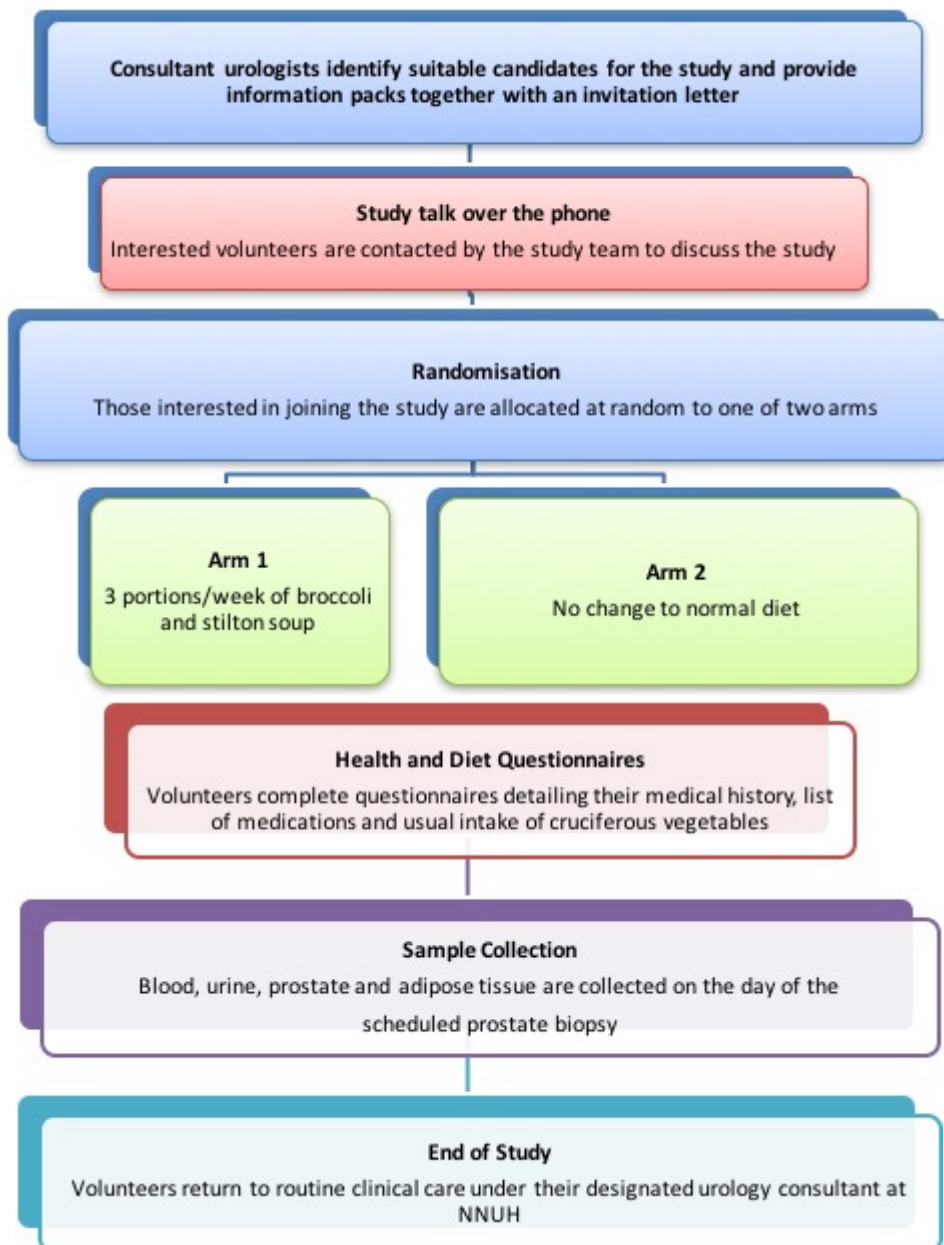
These will not affect the histology or sampling of the prostate and all research samples will be independently reviewed by a consultant histopathologist.

The biopsies taken from the fat (adipose) surrounding the prostate can also be directed through the

template grid and will therefore avoid the need for a separate operation site.

The analysis of these fat biopsies will provide very useful information to assess metabolic changes elsewhere in the body (outside the prostate) as a result of eating the soups.

We do not expect an increase in the risk of complications as a result of taking additional samples for the study.



Flow chart detailing the different stages throughout the SAP study

When do I give consent for this study?

If you agree to participate you will be asked to **sign three separate consent** forms confirming your enrolment (study consent), your consent to undergo the biopsy procedure (NHS standard consent) and permission to store your samples in the tissue bank for research purposes (The Norwich Biorepository consent). You will still be eligible to take part in the study even if you don't want to store your samples in the tissue bank. These forms will also be signed by the study scientists and/or your urologist and you will be given a copy to keep.

According to the study arm to which you have been allocated, you will either be asked to attend the HNU at IFR where we can go through the consent forms with you (**Arm I**), or meet us for the first time face-to-face on the day of your prostate biopsy where all three consent forms will be signed (**Arm II**).

After signing the consent form you are still free to withdraw at any time without giving a reason. This means that additional samples for

this research will not be collected during your biopsy procedure.

Broccoli and stilton soup provision

Broccoli and stilton soups will be prepared by Bakkavor®, one of the world's leading fresh prepared food providers in the UK (Spalding, Lincolnshire).

Bakkavor® make the soups using fresh



Beneforte® broccoli.

You will not be able to choose which arm of the study you are allocated to. The random allocation will be performed by a computer-based programme (equivalent to a coin toss or roll of a dice), and so members of the study team could not influence this choice.

If you are allocated to the arm receiving the diet intervention, you will be asked to eat **three portions** (300g) of this soup a week for the duration of the study (at least 4 weeks prior your biopsy). Volunteers will be offered their soups in frozen 300g portions directly from the HNU. Depending on the number that is convenient for you, we can arrange for any further

batches of soup to be delivered directly to your home.

The delivery of soups will be tailored to each participant taking into account their availability and storage space.

The soups are provided frozen, so you will be asked to follow the cooking instructions (described separately in this information pack). We will contact you by telephone every fortnight to check that everything is going OK with the trial.

What happens on the day that my biopsy has been scheduled at the NNUH?

It is important for you to know that your biopsy procedure scheduled at the NNUH will not be delayed for any reason related to your participation in this study.

Questionnaires, blood and urine sample collection

On arrival at the NNUH you will be met by your urologist and the study scientists. Once all consent forms have been signed, we will help you to complete the brief health questionnaire and the diet questionnaire.

After a digital rectal examination (DRE) of your prostate by an

urologist, we will ask you to provide a urine sample of 25-30ml. This will be divided into portions and will be stored at the IFR until analysis. The remaining samples will then be transferred to the Norwich Biorepository (tissue bank) for future research.

A 5ml (1 teaspoon) blood sample will be collected when the venflon (fine plastic drip tube) is inserted into a vein on your hand or arm by a urology or anaesthetic doctor. The venflon will remain in place as it will later be used during your anaesthetic. The blood sample will be transported to the IFR and stored until analysis. The remaining samples will then be transferred to the Norwich Biorepository (tissue bank) for future research.

Prostate and adipose tissue biopsy

The study samples will be collected first, targeting adipose (fat, 2 biopsies) and prostate (8 biopsies) with the ultrasound and template grid. The method of TPB will not alter in terms of anaesthetic type or approach to the biopsy. A full, standard set of prostate biopsies will then be taken and sent for histology to meet the needs of your routine clinical care.

Results of the prostate biopsies

Analysis of tissue needs to be done carefully; it usually takes 2-3 weeks to complete. After obtaining the results of your biopsy procedure your doctor may decide that you need further investigations or treatment.

The study will have no effect on your ongoing treatment options, and subsequent decisions will be made between you and your urologist.

Your routine follow up visits at the hospital

Your participation in this study will not change your hospital appointments to see your urologist.

It is important to stress that you will not be required to attend any additional urology outpatient appointments before or after your biopsy for the purposes of this study.

What will happen to my samples?

The samples will be collected from the hospital and taken to IFR for analysis. Some of your samples will be sent for

analysis in the USA. These specimens will not carry any personal information. Instead they will be labelled with an identification code. Transport of such material will be arranged with a specialist courier service. With your consent the remaining samples will be stored at the Norwich Biorepository (tissue bank). Otherwise they will be destroyed.

Blood samples will be used for genetic tests. It is important to find out if the genetic characteristics of each individual may influence the effects of the diet. This study will involve genetic analysis that will help us understand how the volunteers respond to the intake of broccoli. In the future, it may be possible to recommend specific diets to people with certain genetic characteristics.

It is important to stress that genetic analysis has no clinical relevance to you, your relatives or the treatment you receive; therefore participants will not receive results of this test.

Are there any benefits of taking part?

The information we obtain from this study may help in the prevention and treatment of prostate cancer in the future. We also hope the dietary modifications used in this study may be

of benefit to you. However, this cannot be guaranteed.

Will my GP be informed?

Yes, it is routine practice for us to inform your GP that you are participating in a study at IFR. This is one of the things you are agreeing to when you sign the consent form for taking part in the study.

Are there any risks or side effects from participating in this study?

There can be a small amount of discomfort associated with taking blood. This generally only occurs on insertion of the needle. You should not experience pain during the procedure or afterwards. You may develop a small bruise at the site of the blood sample but this will fade like any bruise.

The surgical procedure of template biopsy carries a risk of certain complications. There is a small risk of infection. In order to reduce this risk, you will be given some intravenous antibiotics with your anaesthetic. This is normal practice for everyone having a prostate biopsy.

A small proportion of men will suffer from urinary retention (less than 1 in 10) as a result of the procedure, requiring a short-term catheter to be inserted into the bladder. This would later be removed once the swelling from surgery had subsided.

It is normal to see blood in your urine or with bowel motions for a few days after the procedure. Blood can also be present in the semen and may persist for several months.

Major complications from general anaesthesia are related to your health and whether you suffer from other medical conditions. The anaesthetist you meet will discuss these with you and advise you on the best anaesthetic technique. It is important to inform the pre-operative assessment nurse or the anaesthetist if you have other medical conditions or if there is family history of serious reactions to anaesthetics.

Do I get paid for doing this?

Participating in this study is voluntary. However, we do recognise that being involved in the study can cause you some inconvenience and there are travel costs associated with visiting us. Therefore, travelling expenses will be reimbursed for journeys to and from the HNU where parking is provided

free of charge. Expenses will be reimbursed on production of a receipt/ ticket for buses or trains or at the current mileage rate (45p per mile). If you require transport to and from the HNU, please let us know. We will arrange and pay for a taxi.

This completes Part 1 of the information sheet.

If the information in **Part 1** has interested you and you are considering taking part, it is important that you read the additional information in **Part 2** before making any decision

PART 2 of the information sheet

Access to your personal information

Any information relating to you will be held in strictest confidence. Once recruited, you will be issued with a volunteer code number. This number will be used on all your samples so that no one outside the study group will be able to link them to you.

Access to your records is restricted to scientists who are running the study, research nurses, hospital medical staff and your GP.

Will my taking part be kept confidential?

Yes. We follow Ethics and Research Governance and Good Clinical Practice (GCP) requirements.

All information collected about you during the course of this study will be kept strictly confidential. Information will be stored in locked filing cabinets at the IFR.

Your volunteer code number will be used to protect your personal

information and samples so no one will be able to link them to you. These codes are kept in a separate filing cabinet at IFR that is also kept locked. Access to your personal records is restricted to the study team, study nurses, NHS medical staff and your GP. Your data will be kept for up to 15 years after the end of the study in a secure archive at the IFR.

All research is subject to inspection and audit and although your records may be accessed for this purpose any personal information remains confidential.

Please note that IFR has CCTV cameras in use for security purposes. However, provision has been made so that volunteers attending the HNU will not be identified.

What if relevant new information becomes available or changes to the study are made?

If this happens, we will tell you. If changes to the study have to be made, you may be asked to sign another consent form.

What happens when the research study stops?

Findings of this study will be reported in the scientific literature. No individual data will be identified.

Research Governance Committee (HRGC) at the IFR – Dr Linda Harvey.

Will I be told the results of the study?

The results of the study will be published in scientific journals and presented at national and international scientific meetings. We are unable to tell you any of your individual results since it is only possible to draw conclusions from the group as a whole.

At the end of the study, we will try to provide feedback of what we have found as a result of your help and what it may mean for future research.

What if there is a problem?

If you have a concern about any aspect of the study, you should ask to speak to the study scientists who will do their best to answer your questions (Dr Antonietta Melchini, 01603 255030; Mr Jack Coode-Bate, 01603 255030). If you remain unhappy and wish to complain formally, you can do this through the chairperson of the Human

What if something happens to me while I am on the study?

If you are harmed by taking part in this research project there are no special compensation arrangements. If you are harmed due to someone's negligence, you may have grounds for legal action, but you may have to pay for it. Regardless of this, if you wish to complain or have any concerns about the way you have been treated whilst taking part in this study at the NNUH clinics, there will be a local hospital complaints procedure that you can follow. If you wish to complain you should contact the Patient Advice and Liaison Service (PALS) on 01603 289036 (email: pals@nnuh.nhs.uk). Their offices are located next to Kimberley Ward, East Block Level 2 or please ask at the main reception desks at the Inpatient and Outpatient hospital entrances. The office has an answerphone which is available 24 hours a day and messages will be responded to as quickly as possible. IFR accepts responsibility for carrying out trials and as such will give consideration to claims from participants for any harm suffered by them as a result of participating in the

trial, with the exception of those claims arising out of negligence by the participant. IFR has liability insurance in respect of research work involving human volunteers. Please note that the Institute will not fund any legal costs arising from any action unless awarded by a court.

What will happen at the end of the study?

After the prostate biopsy you will be offered a 3 month supply of the Beneforte® broccoli and stilton soups for your own personal consumption if desired. You will have the option to collect the soups from the HNU or to be delivered directly to your home. In case of collection from the HNU, we will not cover travel expenses. This will not form part of the clinical trial, which will finish once we have collected all samples and questionnaires.

You will not be asked to attend for any further clinics, tests or procedures as part of the trial. Instead, you will continue with routine clinical care at the NNUH.

Who has reviewed this study?

To protect your safety, rights, wellbeing and dignity the study was reviewed by the Institute of Food Research Human Research Governance Committee (HRGC) and an independent group called the National Research Ethics Service (NRES) Research Ethics Committee (REC) (insert REC name once approval issued).

What should I do now?

If you are interested in taking part in the study then please complete the response slip in your invitation letter and return it to your urologist directly, or in the FREEPOST envelope. If you are not interested in taking part you need not do anything. No one will contact you about the study.

Taking part in the research is entirely voluntary! You are free to withdraw from the study at any time without giving a reason.

For further information or to arrange a study appointment, please contact study scientists:

➤ **Dr Antonietta Melchini**

+44 (0)1603 255 5030

antonietta.melchini@ifr.ac.uk

➤ **Mr Jack Coode-Bate**

+44 (0)1603 255 030

jack.coodebate@ifr.ac.uk

Sulphate Accumulation in Prostate (SAP)

I am interested in taking part and/or finding out more information about this study (please complete the personal details below).

Name:

Address:

.....

Daytime telephone no.:

.....

Evening telephone no.:

.....

Mobile no.:

I am happy for a message to be left via my daytime/evening number:

YES/NO

**please circle as applicable*

Preferred time to call:

.....

E-mail address:

.....

Please return this form in the **FREEPOST** envelope provided to:

Dr Antonietta Melchini/Mr Jack Coode-Bate

Institute of Food Research

FREEPOST NC 252

Norwich Research Park

Conley


Norwich

NR4 7BR

Expressing an interest does not commit you to taking part in the study

Annex 4

Annex 4—Consent form	Version 3-SAP	26 th February 2016
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Norfolk and Norwich University Hospital 

NHS Trust

INFORMED CONSENT FORM FOR RESEARCH STUDY

Study Title: **Sulphate Accumulation in Prostate (SAP)**
 Chief investigator: **Prof Richard Mithen**

Patient Identification Number for this Trial: _____

Volunteer please initial each box

I confirm that I have read and understand the information sheet dated; version no. for the above study, and I have had the opportunity to consider the information, discuss the study and ask questions.

I agree that I do not fall within the basic exclusion criteria listed for this research study

I confirm that I have received satisfactory answers to my questions.

I understand that my participation is voluntary, and I am free to withdraw from the study (1) at any time without giving a reason and (2) without my withdrawal affecting future participation in other research studies at IFR and at Norfolk and Norwich University Hospitals NHS Foundation Trust.

With who have you discussed the information for this research study?
 Name: Role:


I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from IFR, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.

I understand that my personal information and data will be held confidentially at IFR and that it will be destroyed after 15 years.

I have been informed about the purpose of the genetic test and that it has no significance for me or my family.

I agree and consent to having a blood sample taken for this test.

1 copy of the signed consent form must be given to the volunteer to keep.
1 copy of the signed consent form must be kept in the study records at IFR and 1 copy for hospital notes.



Page 1 of 2

Annex 4-Consent form

Version 3-SAP

26th February 2016

Norfolk and Norwich University Hospital 
NHS Trust

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

Name and address of your General Practitioner:

I understand that my study data, without my name and contact details, will be transferred to an analytical group in the US for analysis.

I understand that all research is subject to inspection and audit.
NB: although your records may be accessed for this purpose your personal information remains confidential

I agree to take part in the above study.

Signed: Print Name:.....

Date: Date of Birth:

I confirm that the volunteer above has been given a full verbal and written explanation of the study.

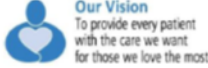

Signed: Print Name:.....

Role: (in BLOCK letters) Date:

1 copy of the signed consent form must be given to the volunteer to keep.
1 copy of the signed consent form must be kept in the study records at IFR and 1 copy for hospital notes.



Annex 5

Annex 5-Tissue Bank Consent Form	Version 2-SAP	13 th January 2016
	Norfolk and Norwich University Hospitals  <small>NHS Foundation Trust</small>	

The Norwich Biorepository

The donation, collection, storage and use of samples of tissue and/or fluids and/or other material from an adult for research

Information sheet for patients - Version 15 (21 February 2014)

Thank you for considering giving a sample for biomedical research. This information sheet provides a brief summary to help you to understand what this means and involves.

There is a consent form after the information sheet. It is important that you complete and sign it, if you decide to give us a sample. Please complete all parts of the consent form.

Doctors and other health professionals often take samples (which may be blood, small biopsies, or something else) from patients to help tell us what is wrong and how best to treat it. Larger pieces of tissue or whole organs may be removed by surgeons from some patients as part of their treatment.

Quite often, some of the test sample or surgical tissue is left over at the end. The left-over sample can be used by other doctors or researchers in special experiments to learn more about illness, how it happens and how to treat it, and sometimes to help make new medicines.

We would like to do some experiments on any left-over sample or surgical tissue, once your tests are finished. To do that, we need your permission and signed consent.

Samples donated (given) to the Norwich Biorepository are not:

- Normally used in animal research. It will be made clear to you if animal research is an integral part of the project for which we are seeking a donation.
- Used in cloning experiments. However, the Biorepository would consider the use of donations in non-reproductive cloning experiments based on their scientific value and in the context of prevailing law and ethical standards. It will be made clear to you if cloning experiments are part of the research project for which we are seeking a donation.

If you want to help us, please sign the form at the end of this document.

Continued.....

<small>Norwich Biorepository – Information Sheet for Patients and Consent Form – Adult; Version 15 (21 February 2014)</small>	<small>Page 1 of 7</small>
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If you give permission for a sample to be taken –

- The Hospital will own the sample.
- The sample may be stored, usually in a deep freezer, until it is used. The freezer is referred to as a tissue bank in the consent form.
- Nobody involved in the research will know where the sample has come from.
- The sample will be used only in experiments that are ethical and to help other people. Please see the section entitled 'Scientific and ethical approval' below to understand what we mean by *ethical*.
- Your donated sample(s) and any genetic material derived from it (them) may be stored for an indefinite amount of time for future research projects, which may include whole genome sequencing. *Whole genome sequencing* means reading your total DNA code (your genetic blueprint) in a single assessment.
- We might give some of or all the sample to other doctors or researchers for their experiments, if they are ethical and to help other people. Some of these people might work in companies in this country or abroad.
- Data derived from your sample(s) may be placed anonymously in an international database to be used in future research. While we will take all possible steps to maintain your anonymity and protect your privacy, there is a very small risk that genetic information produced in the research and stored on databases could lead to your identification by being linked to other stored information.
- We will keep some facts about you on our Biorepository database.
- Although these facts might be given to the research doctors or scientists to help their experiments, we will NOT tell them your name or other details that would let them know who you are.
- Doctors in the Hospital might also read your hospital records to help them understand what the doctors or scientists find out in the experiments. This is possible because your hospital records can be linked to the anonymous research sample without loss of confidentiality as far as the researchers are concerned. If the research results are important for you, it might be possible using this linkage to feed back the information to your doctor, so that any appropriate action can be considered.

Your treatment will not be affected in any way, if you do not feel able to say yes to our request for a research sample.

The next sections give you some more detailed information. If you have any questions, please do not hesitate to ask the person who is seeking your permission.

WHAT WILL HAPPEN

Tissue, blood or other samples taken from you for diagnosis and/or treatment of your condition will be sent to the Pathology Laboratories, where they will be tested to decide exactly what they are and whether any further treatment will be necessary. This is a standard part of treatment. **Only as much tissue or fluid as is needed will be removed.**

Continued.....

MEDICAL RESEARCH AND WHY THIS PROGRAMME IS IMPORTANT

When all the routine tests have been done, if any samples are left over, with your consent, they could be donated (given) for use in medical research. As part of a research programme which now includes the Norfolk and Norwich University Hospitals NHS Foundation Trust, the James Paget University Hospitals NHS Foundation Trust, the University of East Anglia (UEA), and the Institute of Food Research (IFR), some of the sample or material extracted from it will be stored in the Norwich Biorepository (usually in a special deep freezer) for use by ourselves or by researchers from other centres at a later date. Some of this research may involve an assessment of genetic material (DNA and/or RNA) to help us understand the genetic basis of health and disease.

The purpose of this research is to understand more about human health and disease, and to develop new methods of prevention or new treatments for the benefit of future patient care. Some of these research programmes could lead to the development of new products and processes, which may be developed commercially for the improvement of patient care, in which case there would be no financial benefit to you.

Medically qualified doctors or other suitably qualified staff at the hospital may need to review your hospital records, including case notes, as part of this research in order to relate the research findings to the clinical outcome. It is important to be able see how you progress after the tissue or other samples you have donated (given) have been used in the research project(s). They will not give your name to those doing the research.

The research may also involve training doctors and researchers in scientific medicine, and may lead to higher qualifications for them (e.g., PhD or MD degrees). This is important for future research into diseases and for looking for new, more effective, treatments for them.

LINKS WITH OTHER ORGANISATIONS

If you agree, we may send stored material or products derived from it to other approved tissue banks or companies in this country or abroad to support their research programmes or the research programmes of those companies' clients. Such outside organisations will provide financial support for the Norwich Biorepository (our tissue bank), to help it recover its operating costs. We are not, however, allowed to sell tissue or other samples in order to make any financial profit from these commercial links.

SCIENTIFIC AND ETHICAL APPROVAL

The Norwich Biorepository acts as a custodian of the samples it holds. It releases them only to individuals or organisations that have an acceptable scientific background and work to high ethical standards. We require that all such medical research has been approved by a properly constituted Research Governance Committee before it starts. It must also be approved by a Research Ethics Committee or on behalf of the Research Ethics Committee that oversees the work of the Norwich Biorepository under the terms of the Biorepository's own Research Ethics Committee approval. That committee is the Cambridge East Research Ethics Committee. These committees look particularly at the purpose and validity of the research proposal, the welfare of any participants and issues of consent and confidentiality. We will release samples to commercial companies only if they work to appropriate ethical and scientific standards.

Continued.....

DONATING EXTRA SAMPLES FOR RESEARCH

In certain circumstances you may be asked by the doctor treating you (or by a doctor, research nurse or nurse practitioner working with him/her) to consider donating (giving) tissue or other samples in addition to those to be removed as part of your diagnostic investigation or treatment. **Such extra samples will be taken only if you give your consent and if their removal does not cause you any harmful effects now or in the future.**

YOUR RIGHTS

If your samples are stored, information about your case will be kept on a computer in the Norwich Biorepository. This will help us understand what your illness was like and relate what we find in experiments to what happens to patients. Under the Data Protection Act, you are entitled to ask to see what is recorded about you by applying to the Chairman of the Norwich Biorepository Committee, Norfolk & Norwich University Hospital, c/o Dept. of Cellular Pathology, The Cotman Centre, Colney Lane, Norwich, NR4 7UB. No one other than you has the right to see these records and any information needed for research purposes will be made anonymous before it is given to the researcher.

The researchers will not be able to find out your name or any personal details about you from the information that they receive.

You will have the opportunity to discuss with a doctor issues relating to the possible use of your samples for research purposes. He or she will answer any questions you may have.

MAKING A DONATION (GIFT) OF TISSUE AND/OR OTHER MATERIAL FOR RESEARCH

If you decide that you want your tissue, etc., to be stored in the Norwich Biorepository and used for research purposes, you will be asked to sign a special Consent Form confirming your decision and stating that you have read and understood this sheet. When you sign the form you will give the ownership of the tissue or other samples to the Norfolk & Norwich University Hospitals NHS Foundation Trust. The tissue or other samples will then belong to the Trust, which will store it for an indefinite period of time and will be able to decide how it should be used for research. It will also have the right to dispose of unused stored material in an appropriate legal and ethical manner following normal procedures.

If you do not want your tissue to be stored in the Norwich Biorepository, please tell us and do NOT sign the special Consent Form.

If you do not sign this form, the tissue or other samples will still be sent to the laboratory to undergo those tests that are necessary for your care but they will not be used for research purposes. All unused tissue from your procedure will be disposed of using normal hospital methods. We will respect your decision and it will not affect in any way the treatment you receive.

Continued.....



Affix an addressograph label here or complete the following details:

Patient's name.....

Date of birth.....

Hospital no.

The Norwich Biorepository

Consent for the collection, storage and release of human samples for research

I agree (Please initial small box) that the following tissue or other material 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:

List samples for research:

I also agree that (Please initial small boxes, as appropriate):
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

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 case may be kept on the Norwich Biorepository database

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 Medical records, including case notes, as appropriate, for the purposes of research using the donated samples

These samples may be used in ethically approved animal research

These samples may be used in ethically approved cloning research

Continued.....



Affix an addressograph label here or complete the following details:

Patient's name.....
Date of birth.....
Hospital no.

The Norwich Biorepository

Consent for the collection, storage and release of human samples for research

I confirm that:

- 1) I have read and understand the Information Sheet for Patients, Version 15, dated 21 February 2014
- 2) The issues have been explained to me, and that I have had the opportunity to ask questions.

Signed _____ (Patient) Date _____

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

Signed _____ Print name _____
Medical / Nursing Practitioner

Date _____



CONSENT FOR TAKING EXTRA SAMPLES FOR RESEARCH

Please initial the appropriate box for each item:	YES	NO
In addition to the removal of tissue, blood or other fluid samples as a necessary part of my procedure, I also consent to the removal of additional tissue, blood or other fluid samples from the operation site during my procedure PROVIDED THAT SUCH REMOVAL CAUSES ME NO HARM now or in the future, is limited to what I and the doctor treating me (or a research nurse or nurse practitioner delegated by him/her) have discussed and agreed, and which is specified below. * <i>Please initial appropriate box</i>		
*Please list additional samples for research:		

Signed _____ (Patient) Date _____

I have explained the request for the donation (gift) of extra tissue and/or other samples for research purposes and have answered such questions as the patient has asked.

Signed _____ Print name _____
Medical / Nursing Practitioner

Date _____

Annex 6

Annex 6-Example NNUH Consent for clinical procedure Version 2-SAP 13th January 2016 **D**

Norfolk and Norwich University Hospitals **NHS**
NHS Foundation Trust

CONSENT FORM (1)

Addressograph Label

For staff use only

Patient's surname/family name:
Patient's first name(s):
Date of birth:
NHS number (or other identifier):
Male Female

Patient agreement to Investigation or Treatment

Name of Procedure

.....

Please bring this form on each visit to the hospital.
You will be asked to read this form carefully and you and your doctor (or other appropriate health professional) will sign the document confirming your consent to the procedure.

For staff use only: Does the patient have any special requirements?

An Interpreter Other communication method Other

.....

Important note: (tick if applicable) See also advance directive / living will (eg Jehovah's Witness form)

TO BE RETAINED IN PATIENT'S NOTES

UNH 225B

Consent Form - Part A

For staff use only

Patient's surname/family name: _____
 Patient's first name(s): _____
 Date of birth: _____
 NHS number (or other identifier): _____
 Male Female

Name of proposed procedure or course of treatment
 (include brief explanation if medical terms are not clear)

Statement of health professional: (to be filled in by health professional with appropriate knowledge of proposed procedure as specified in consent policy)

I have explained the procedure to the patient. In particular, I have explained:
 The intended benefits _____

Serious or frequently occurring risks : _____

Any extra procedures which may become necessary during the procedure

Blood transfusion Other procedure (please specify) _____

I have also discussed what the procedure is likely to involve, the benefits and risks of any available alternative treatments (including no treatment and any particular concerns of this patient).

The following leaflet/tape has been provided: _____

This procedure will involve:

general and/or regional anaesthesia local anaesthesia sedation

Anaesthetic leaflet received: Yes No (please tick)

Signed: _____ Date: _____

Name (PRINT): _____ Job Title: _____

Complete as appropriate: Female of reproductive age:

During your operation it may be necessary to take an X-ray to assist the surgeon with the procedure. It is important that X-rays should be avoided if there is a possibility of pregnancy.

Date of last menstrual period _____

Is there a possibility of you being pregnant Yes No (if yes can this procedure be deferred or does the clinical urgency override the risk to the pregnancy) Yes No

Signed: _____ Date: _____

Name: (PRINT) _____ Job Title: _____

Contact details (if patient wishes to discuss options later)

Statement of interpreter (where appropriate)

I have interpreted the information above to the patient to the best of my ability and in a way in which I believe she/he can understand.

Signed: _____ Date: _____

Top copy accepted by patient: Yes No (Please tick)

Consent Form - Part B

For staff use only	
Patient's surname/family name:	
Patient's first name(s):	
Date of birth:	
NHS number (or other identifier):	
Male <input type="checkbox"/>	Female <input type="checkbox"/>

Statement of patient

Please read this form carefully

Do ask if you have any further questions, we are here to help you. You have the right to change your mind at any time, including after you have signed this form. You may ask for a relative, or friend or a nurse to be present whilst the procedure is being explained and consent obtained.

Training doctors and other health professionals is essential to the continuation of the Health Service and improving the quality of care. Your treatment may provide an important opportunity for such training, where necessary under the careful supervision of a senior doctor or health care professional. **You may decline to be involved in the formal training of medical and other students. This will not affect your care and treatment.**

Please tick boxes to indicate you have understood and agree to the statements below.

- I agree** to the procedure or course of treatment described on this form.
- I agree** to the use of photography for the purpose of diagnosis and treatment
- I agree** to photographs being used for medical teaching
- I agree** that any samples or tissue taken for testing or examination may be stored and may subsequently be used for research and development purposes under strict legal and ethical guidelines.
- I understand** that you cannot give me a guarantee that a particular person will perform the procedure. The person will, however, have appropriate experience.
- I understand** that I will have the opportunity to discuss the details of anaesthesia with an anaesthetist before the procedure, unless the urgency of my situation prevents this. (This only applies to patients having general or regional anaesthesia.)
- I understand** that any procedure in addition to those described on this form will only be carried out if it is necessary to save my life or to prevent serious harm to my health.
- I have been told** about additional procedures that may become necessary during my treatment. I have listed below any procedures, **which I do not wish to be carried out** without further discussion.

Patient's signature: _____ **Date:** _____

Name: (PRINT) _____

If the patient is unable to sign but has indicated his or her consent, a witness should sign below.

Signature: _____ **Date:** _____

Name: (PRINT) _____

Confirmation of consent

(To be completed by a health professional when the patient is admitted for the procedure, if the patient has signed the form in advance)

On behalf of the team treating the patient, I have confirmed with the patient that she/he has no further questions and wishes the procedure to go ahead.

Signed: _____ Date: _____

Name: (PRINT) _____ **Job Title:** _____

Important Note: (tick if applicable) Patient has withdrawn consent (ask patient to sign / date below)

Signature: _____ Date: _____

Guidance to health professionals (to be read in conjunction with consent policy)**What a consent form is for**

This form documents the patient's agreement to go ahead with the investigation or treatment you have proposed. It is not a legal waiver – if patients, for example, do not receive enough information on which to base their decision, then the consent may not be valid, even though the form has been signed. Patients are also entitled to change their mind after signing the form, if they retain capacity to do so. The form should act as an *aide-memoire* to health professionals and patients, by providing a checklist of the kind of information patients should be offered, and by enabling the patient to have a written record of the main points discussed. In no way, however, should the written information provided for the patient be regarded as a substitute for face-to-face discussions with the patient.

The law on consent

See the Department of Health's *Reference guide to consent for examination or treatment* for a comprehensive summary of the law on consent (also available at www.doh.gov.uk/consent).

Who can give consent

Everyone aged 16 or more is presumed to be competent to give consent for themselves, unless the opposite is demonstrated. If a child under the age of 16 has "sufficient understanding and intelligence to enable him or her to understand fully what is proposed", then he or she will be competent to give consent for himself or herself. Young people aged 16 and 17, and legally 'competent' younger children, may therefore sign this form for themselves, but may like a parent to countersign as well. If the child is not able to give consent for him or herself, some one with parental responsibility may do so on their behalf and a separate form is available for this purpose. Even where a child is able to give consent for him or herself, you should always involve those with parental responsibility in the child's care, unless the child specifically asks you not to do so. If a patient is mentally competent to give consent but is physically unable to sign a form, you should complete this form as usual, and ask an independent witness to confirm that the patient has given consent orally or non-verbally.

When NOT to use this form

If the patient is 18 or over and is not legally competent to give consent, you should use form 4 (form for adults who are unable to consent to investigation or treatment) instead of this form. A patient will not be legally competent to give consent if:

- they are unable to comprehend and retain information material to the decision and/or
- they are unable to weigh and use this information in coming to a decision.

You should always take all reasonable steps (for example involving more specialist colleagues) to support a patient in making their own decision, before concluding that they are unable to do so.

Relatives **cannot** be asked to sign this form on behalf of an adult who is not legally competent to consent for himself or herself.

Information

Information about what the treatment will involve, its benefits and risks (including side-effects and complications) and the alternatives to the particular procedure proposed, is crucial for patients when making up their minds. The courts have stated that patients should be told about 'significant risks which would affect the judgement of a reasonable patient'. 'Significant' has not been legally defined, but the GMC requires doctors to tell patients about 'serious or frequently occurring' risks. In addition if patients make clear they have particular concerns about certain kinds of risk, you should make sure they are informed about these risks, even if they are very small or rare. You should always answer questions honestly. Sometimes, patients may make it clear that they do not want to have any information about the options, but want you to decide on their behalf. In such circumstances, you should do your best to ensure that the patient receives at least very basic information about what is proposed. Where information is refused, you should document this on page 2 of the form or in the patient's notes.

Annex 7

Annex 7–GP letter

Version 2-SAP

13th January 2016

Norfolk and Norwich University Hospital 
NHS Trust

DEPARTMENT OF UROLOGY
Norfolk and Norwich University Hospital NHS Trust
Colney Lane
Norwich
NR4 7UY

DateDear Doctor (*insert name of patient's GP*)**RE:** (*insert name of patient and home address*)

I am writing to inform you that your patient has consented to take part in a collaborative research study organised by the Institute of Food Research in Norwich and the Urology department of the Norfolk and Norwich University Hospital. The study is led by Professor Richard Mithen and is entitled '**Sulphate Accumulation in Prostate**' (SAP). The study has been approved by the Institute of Food Research (IFR) Human Research Governance Committee (HRGC) and the NRES Research Ethics Committee (REC) (*insert REC name once approval is issued*).

Your patient was identified because he has been scheduled for a transperineal template biopsy of the prostate at the NNUH. The study will investigate whether short-term consumption of broccoli results in changes in metabolites within the prostate gland and at the systemic levels.

The study involves collecting tissue biopsies (prostate and adipose tissue), urine and blood samples on the day that your patient undergoes a transperineal template biopsy of the prostate at the NNUH.

Thank you for taking the time to read this letter. If you require any further information about the study then please do not hesitate to contact Professor Richard Mithen at the Institute of Food Research 01603 255259 (email richard.mithen@ifr.ac.uk) or myself at the NNUH robert.mills@nnuh.nhs.uk. You can also contact the study scientists, Dr Antonietta Melchini on 01603 255030 (email antonietta.melchini@ifr.ac.uk) or Mr Jack Coode-Bate on 01603 255030 (email jack.coodebate@ifr.ac.uk).

I have enclosed a copy of the Participant Information Sheet for your reference.

Thank you for taking the time to read this letter.

Yours sincerely
Mr Robert Mills (MB ChB, FRCS)
Consultant Urologist



Annex 8

Annex 8-CVFFQ

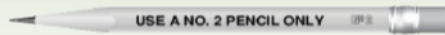
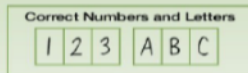
Version 2-SAP

13th January 2016

Arizona Cruciferous Vegetable Food Frequency Questionnaire

DIRECTIONS

- Use a #2 pencil.
- Keep marks within the circles.
- Thoroughly erase as needed.



Demographic Information

Please print the date, your age and your gender in the spaces provided below:

TODAY'S DATE:

--	--	--	--	--	--	--	--	--	--

AGE:

--	--

 years old Male Female

How would you describe your appetite over the past few months or the time period you have been asked to review?

- Normal/usual
- Increased
- Decreased

OFFICE USE ONLY

ID NUMBER:

--	--	--	--	--	--	--	--

CLINIC VISIT NUMBER:

--	--	--	--	--	--	--	--

STUDY NUMBER:

--	--	--	--	--	--	--	--

Interview method:

- Clinic visit
- Telephone
- Mail
- Email
- On line (Web-based)

PLEASE DO NOT WRITE IN THIS AREA



SERIAL #

FOOD INTAKE

The following two sections refer to COOKED vegetables ONLY.

A. How often did you eat the following COOKED cruciferous vegetables?

Please also mark your usual portion (S = small, M = medium, L = large).

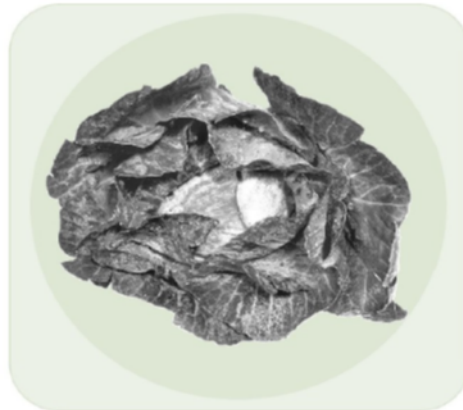
	NEVER	< 1 SERVING/ MONTH	1 to 3 SERVINGS/ MONTH	1 SERVING/ WEEK	2 to 3 SERVINGS/ WEEK	4 to 6 SERVINGS/ WEEK	1 - 2 SERVINGS/ DAY	3+ SERVINGS/ DAY	SERVING SIZE
Bok or Pak choy	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	(S)(M)(L)
Broccoli spears	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	(S)(M)(L)
Broccoli florets	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	(S)(M)(L)
Broccoflower	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	(S)(M)(L)
Broccolini	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	(S)(M)(L)
Brussels sprouts	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	(S)(M)(L)
Cabbage (green)	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	(S)(M)(L)
Cabbage (red / purple)	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	(S)(M)(L)
Cauliflower	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	(S)(M)(L)
Chinese cabbage / Napa	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	(S)(M)(L)
Collard greens	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	(S)(M)(L)
Daikon root	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	(S)(M)(L)
Garden cress / water cress	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	(S)(M)(L)
Kale	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	(S)(M)(L)
Kohlrabi	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	(S)(M)(L)
Mustard greens	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	(S)(M)(L)
Rutabaga	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	(S)(M)(L)
Sauerkraut	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	(S)(M)(L)
Turnip	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	(S)(M)(L)
Turnip greens	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	(S)(M)(L)
On AVERAGE, how often do you eat the above COOKED cruciferous vegetables?	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	(S)(M)(L)

2

The following section refers to RAW vegetables ONLY.

**C. How often did you eat the following RAW (Uncooked) cruciferous vegetables?
Please also mark your usual portion (S = small, M = medium, L = large).**

	NEVER	< 1 SERVING/ MONTH	1 to 3 SERVINGS/ MONTH	1 SERVING/ WEEK	2 to 3 SERVINGS/ WEEK	4 to 6 SERVINGS/ WEEK	1 - 2 SERVINGS/ DAY	3+ SERVINGS/ DAY	SERVING SIZE
Arugula	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	(S)(M)(L)
Bok or Pak choy	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	(S)(M)(L)
Broccoli Spears	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	(S)(M)(L)
Broccoli Florets	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	(S)(M)(L)
Broccoli flower	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	(S)(M)(L)
Broccolini	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	(S)(M)(L)
Broccoli sprouts	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	(S)(M)(L)
Brussels sprouts	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	(S)(M)(L)
Cabbage (green)	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	(S)(M)(L)
Cabbage (red / purple)	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	(S)(M)(L)
Cauliflower	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	(S)(M)(L)

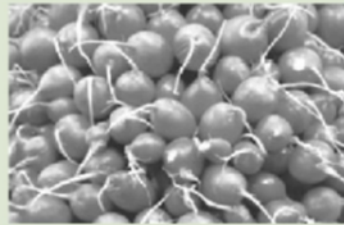


RAW vegetables ONLY (Continued)

	NEVER	< 1 SERVING/ MONTH	1 to 3 SERVINGS/ MONTH	1 SERVING/ WEEK	2 to 3 SERVINGS/ WEEK	4 to 6 SERVINGS/ WEEK	1 - 2 SERVINGS/ DAY	3+ SERVINGS/ DAY	SERVING SIZE
Chinese cabbage/Napa	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	S M L
Coleslaw	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	S M L
Daikon root	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	S M L
Garden cress / water cress	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	S M L
Kale	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	S M L
Kohlrabi	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	S M L
Mustard greens	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	S M L
Radicchio	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	S M L
Radish	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	S M L
On AVERAGE, how often do you eat the above RAW cruciferous vegetables?	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	S M L

3/8 spine part

CONTINUE ON THE NEXT PAGE



E. The following questions relate to the use of certain condiments or seasonings.

On average, how often did you use the following to season food? Please also mark your usual portion S = small (1/2 tsp. to 1 tsp.), M = medium (1 1/2tsp. to 1 tbsp.), L = large (more than 1 tbsp.)

	NEVER	< 1 SERVING/ MONTH	1 to 3 SERVINGS/ MONTH	1 SERVING/ WEEK	2 to 3 SERVINGS/ WEEK	4 to 6 SERVINGS/ WEEK	1 - 2 SERVINGS/ DAY	3+ SERVINGS/ DAY	SERVING SIZE
Horseradish	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	S M L
Chinese mustard	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	S M L
Brown mustard	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	S M L
Yellow mustard	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	S M L
Mustard seed / powder	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	S M L
Wasabi	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	S M L
On Average, how often did you use the above condiments to season food?	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	S M L

38" spine part

Thank You!



OpScan INSIGHT™ forms by Pearson NCS M0250045-1 054321 HC99 Printed in U.S.A.

Developed by the Arizona Diet and Behavioral Assessment Center, Tucson, Arizona, USA
 Azdiet-behavior.azcc.arizona.edu

Annex 9

Annex 9–Health questionnaire

Version 2-SAP

13th January 2016

Norfolk and Norwich University Hospital 
NHS Trust

Sulphate Accumulation in Prostate (SAP)

CONFIDENTIAL

Volunteer Health Questionnaire

Volunteer code

Sex:

D.O.B

Age

Height

Weight

B.P.

Body Mass Index (BMI)

Have you ever had any of the following:

If **yes** give details below each relevant section.

Angina/Heart disease: Y N

.....
.....

Thrombosis: Y N

.....
.....

High blood pressure: Y N

.....
.....

High Cholesterol: Y N

.....
.....

Chest problems: Y N

.....
.....

Diabetes: Y N

.....
.....

Depression or anxiety: Y N

.....
.....

Digestive problems: Y N

.....
.....

Skin Conditions: Y N

.....
.....

Other:

.....
.....



IFR Institute of Food Research

Annex 9–Health questionnaire

Version 2-SAP

13th January 2016

Norfolk and Norwich University Hospital 
NHS Trust

Volunteer Code:.....

Are you currently on any medication: Y N

If **yes** give details:.....

Prescribed
medication:.....
.....
.....
.....
.....
.....

Are you currently taking any supplement: Y N

If **yes** give details:.....

Dietary supplements Y N

If **yes** give details:.....

Herbal remedies: Y N

If **yes** give details:.....

Other Y N

If **yes** give details:.....

Have you had a major physical injury/operation:.....

If **yes** give details:.....
.....
.....

Are you currently suffering from any illness/injuries: Y N

If **yes** give details:.....
.....



IFR Institute of
Food Research

Annex 9–Health questionnaire

Version 2-SAP

13th January 2016

Norfolk and Norwich University Hospital 
NHS Trust

Volunteer Code:.....

Do you/ or have you ever smoked: Y N

When did you stop smoking:.....

If **yes** how many per day:.....

Do you drink alcohol: Y N

How many units per week:.....

Have you any known allergies: Y N

If **yes** give details:

Food:.....

Other:.....

Do you agree to us, informing your General Practitioner of your participation in this study:

If you have answered NO to this question then we are unable to accept you on this study.

What is the name and address of your General Practitioner?

.....
.....
.....

Form completed by:

Print Name

Role: Study scientist

Signature

Date:



IFR Institute of
Food Research

Annex 10

Annex 10-Soup information sheet Version 2- SAP 13th January 2016

Broccoli and stilton soups: ingredients and nutritional value

Broccoli and Stilton Soup

Ingredients Declaration: Water, Beneforte® Broccoli (28%), Fresh Milk, Single Cream, Diced Onion, Potato, Stilton Cheese (4%), Cornflour, Rapeseed Oil, Salt, Black Pepper.

Allergens: Milk, celery

Theoretical Nutrition:

		Energy Kcal	Fat	protein	CHO	Na	Sugar	Sat Fat	Salt	Fibre
Per:	300g portion	175	11.36	8.29	9.91	0.62	3.00	4.53	1.80	2.57
Per:	100g	58	3.78	2.76	3.30	0.21	1.30	1.51	0.53	0.86

Broccoli and stilton soups: storage and cooking guidelines

Storage instructions

Frozen soups delivered to you should be stored in the freezer on receipt. Please **DO NOT** re-freeze soups once they have thawed.



Cooking instructions

Hob: Empty chilled contents into a pan. Heat gently, stirring occasional for 4 minutes. Ensure soup is piping hot.

Microwave: Can be heated from chilled or frozen. Remove lid. Lay lid back on the pot and place pot on microwaveable plate and heat for the times below, stirring halfway through the cooking time. Allow to stand for 2 minutes after heating.

Microwave	B. 650W	D. 750W	E. 850W
Chilled	6 minutes	5 minutes	4 ½ minutes
Frozen	9 minutes	8 ½ minutes	8 ½ minutes

Annex 11

Annex 11–Soup record sheet

Version 2-SAP

13th January 2016

Broccoli Soup Record Sheet

Volunteer code:

Please record a tick ✓ on the day that you eat your vegetable portion.

WEEK COMMENCING (fill in date)	MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	SATURDAY	SUNDAY
dd/mm/yy							

WEEK COMMENCING (fill in date)	MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	SATURDAY	SUNDAY
dd/mm/yy							

WEEK COMMENCING (fill in date)	MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	SATURDAY	SUNDAY
dd/mm/yy							

WEEK COMMENCING (fill in date)	MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	SATURDAY	SUNDAY
dd/mm/yy							

Annex 12

Annex 12-Donated equipment form

Version 2- SAP

13th January 2016



IFR Institute of Food Research

DONATED EQUIPMENT DISCLAIMER

Name of Equipment:Freezer.....

Make and Model of Equipment:

Supplier:

The Institute of Food Research (IFR) is pleased to be able to donate this equipment, but in doing so recommends that the equipment is examined and thoroughly checked by the donee prior to use. IFR makes no guarantee as to the fitness for the purpose for which it is subsequently used by the donee and, to the extent permissible by law, excludes liability for any injury or damage howsoever caused by the installation and use of the equipment.

I agree to take full responsibility for the above donated equipment.

Name of donee:Date:.....

Signature of donee:Date:.....

Annex 13

Annex 13–Participant Withdrawal Letter

Version 2-SAP

13th January 2016

Norfolk and Norwich University Hospital 
NHS Trust

Patient's Address

Date

Dear *(insert name of patient)*

Re: Sulphate Accumulation in Prostate (SAP)

I am writing to confirm your decision to withdraw from the SAP study. This will not affect your ongoing clinical care in any way and you will remain under the Urology Department at the Norfolk and Norwich University Hospital (NNUH). It is important for you to know that this will also not affect your participation in future research studies at the Institute of Food Research or at the NNUH.

You will not be contacted again and will not be asked to provide any further information for this study. However, if we can answer any other questions or you would like to discuss your decision with a member of the study team, please do so with the details provided on your original participant information sheet.

In any case we would like to thank you for your interest and involvement so far.

Yours sincerely,

Dr Antonietta Melchini/ Mr Jack Coode-Bate

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