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The Role of Phytochemicals in Modulating Intrinsic Human Cellular Defence Processes

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Abstract

The plant kingdom contains a large number of unique phytochemicals, some of which are known to be beneficial to the function of human cells. Where the macro- and micronutrients in plant foods were once thought to be the reason for their essentiality in human health, phytochemicals have emerged as being significant non-nutritive contributors. The rapidly-evolving science of nutrigenomics directs greater focus on the intricate signalling pathways that relate dietary phytochemicals and human biomolecules. The effects of phytochemicals in modifying gene expression are becoming increasingly documented.

This thesis investigates the nutrigenomic effects of two unrelated plant-derived materials (SOD/gliadin and sulforaphane) in terms of their clinical potential. The original plan was to conduct two clinical trials using SOD/gliadin only; however, the first study resulted in null findings leading to the decision to investigate a second supplement for the remainder of the thesis.

The thesis is divided into two sections, with each covering one of the compounds. Section 1 contains Chapters 1-3 with Chapter 3 describing a randomised placebo-controlled trial conducted to examine the potential health benefits of SOD/gliadin supplementation. This chapter has been published in the Journal, *Phytomedicine*. The trial examined the effects of 3 months of SOD/gliadin supplementation on perceived fatigue in 40 post-menopausal women. It was hypothesised that the SOD/gliadin would increase the activity of the primary antioxidant enzymes, superoxide dismutase (SOD) glutathione peroxidase (GPx) and Catalase (Cat) and that this would lead to a decrease in aconitase, the rate-limiting enzyme in the synthesis of adenosine triphosphate (ATP), with a view to reducing perceived fatigue in this population. The results showed that the SOD/gliadin supplement had no significant effect on self-perceived fatigue, antioxidants, oxidative stress or hormones in women aged 50-65 years.

Section 2 (Chapters 4-9) sees the focus of the thesis shift to investigating a broccoli sprout-containing product which yields sulforaphane (SFN). Chapters 5 and 6 contain two published review articles (*Nutrition Reviews* and *Oxidative Medicine and Cell Longevity* respectively). Chapter 7 highlights methodological issues associated with using phytochemicals like SFN in clinical trials.

Chapter 8 details the second clinical trial (The EASYGENEX Study). The primary objective was to examine gene expression across two dose levels of encapsulated whole broccoli sprout raw material, optimised for its sulforaphane yield. An open-label dose-escalation study was conducted with 21 young, healthy, physically-active men. Plasma was collected before and after consuming encapsulated whole broccoli sprout supplements over two 7-day continuous periods (53 g SFN/day during Week 1 then 106 g SFN/day in Week 2). Genotypes were identified from buccal swab samples. Liquid chromatography with tandem mass spectrometry was used to measure plasma SFN and its metabolites. Gene expression analysis was conducted using Ingenuity Pathway Analysis with peripheral blood mononuclear cells (PBMCs) on a microarray platform.

Both upregulated and downregulated significantly differentially-expressed genes were identified over the 14-day period of the study, with downregulated genes predominating. The major network identified by Ingenuity Pathway Analysis (IPA) centred on NF-kB, a transcription factor associated with pro-inflammatory effects; the study confirmed the known inhibitory effect of SFN on NF-kB. Also identified were 8 differentially-expressed genes not documented elsewhere in association with SFN. Two were upregulated, *DDC* and *ACSM2A*, with the remainder downregulated: *HERC6, PDIA4, ZBTB2, IGF2B2, DDX3X* and *GK5*. All the significantly differentially-expressed genes could be grouped by function into the six overlapping categories: immune modulation (anti-inflammatory), metabolism (adipogenesis, glucose metabolism, insulin sensitivity), neurotransmitter synthesis, cytoprotection, cardioprotection and redox modulation. Some are known to exhibit multiple effects.

Development of the EASYGENEX Study highlighted a number of significant methodological issues inherent in using phytochemicals like SFN as intervention materials. Chapter 7 identifies these issues, describes their potential for flawed outcomes and suggests ways to mitigate against such design flaws. In so doing, it highlights the background to the key genes discussed in Chapter 8, exploring relevant aspects of their mechanisms in the function of human cells.

The summation and concluding remarks for this thesis form the final chapter, Chapter 9.

Declaration by Author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, financial support and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my higher degree by research candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Publications during candidature

Peer-reviewed publications

Houghton CA, Fassett RG, Coombes JS. *Sulforaphane and other nutrigenomic Nrf2 Activators: can the Clinician's Expectation be Matched by the Reality?* Oxid Med Cell Longev. 2016;2016:7857186.

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Publications included in the thesis

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Contributor	**Statement of contribution**
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Robert Fassett	Conception and Design (0%) Analysis and Interpretation (0%) Drafting and Production (10%)
Jeff Coombes	Conception and Design (10%) Analysis and Interpretation (0%) Drafting and Production (15%)

Contributions by Others to the Thesis

Professor Jeff S Coombes

Professor Coombes, as my principal adviser, assisted with all aspects of the development of this thesis, providing advice on the designs and implementation of the trials, as well as the preparation and editing of the manuscripts. His department also provided funding support for the trials.

Professor Robert Fassett

Professor Fassett assisted in grant application to a funding source and personal presentation to the Ethics Committee of that funding body. In addition, he provided editorial assistance with manuscripts prior to submission for publication.

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Dr Briskey assisted in development, modification and implementation of assay protocols required for the clinical trial detailed in Chapter 8. He also assisted in editing Chapters 7and 8 and in providing advice associated with thesis preparation.

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Dr Archard assisted in the implementation and preliminary data analysis of the EASYGENEX Study.

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Statement of parts of the thesis submitted to qualify for the award of another degree

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Research Involving Human or Animal Subjects

The University of Queensland's Human Ethics Unit provided ethics approval for the two intervention studies forming this thesis:

Clinical Trial #1 (The Fatigue Study), appearing here as Chapter 3.
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Sulforaphane, Redox, Bioavailability, Dose-Response, Antioxidant, Cellular Defence, Nutrigenomics, Gene Expression.

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FoR code: GROUP 0604, GENETICS **20%**

THE THESIS

The Role of Phytochemicals in Modulating Intrinsic Human Cellular Defence Processes

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ABBREVIATIONS

8-OHdG	8-hydroxy deoxyguanosine
ACE	Angiotensin Converting Enzyme
ACLY	ATP citrate lyase
ACSM	Acyl-CoA synthetase medium-chain
AGE	Advanced glycation end
AhR	Aryl hydrocarbon receptor
AIP	Atherogenic Index of Plasma
AMPK	AMP-activated protein kinase
ANZSRC	Australian and New Zealand Standard Research Classifications
ARE	Antioxidant Response Element
ATP	Adenosine Triphosphate
AUC	Area Under Curve
Bax	Apoptosis regulator Bax, also known as bcl-2-like protein 4
BSE	Broccoli sprout extract
CSC	Cancer Stem Cells
CAT	Catalase
CEBPB	CCAAT/enhancer-binding protein
CFS	Chronic fatigue syndrome
COMT	Catechol-O-methyl transferase
COPD	Chronic obstructive pulmonary disease
COX-2	Cyclo-oxygenase 2
CTG	Centre for Translational Genomics
CYP450 2E1	Cytochrome P450 2E1
DDC	Dopa decarboxlyase
DEP	Diesel exhaust particles
DHEA	Dehydroepiandrosterone
DIM	Di-indolyl methane (a dimer of I-3-C)
DSHEA	Dietary Supplement and Health Education Act
DTC	Dithiocarbamate
EAhy926	Transformed permanent human umbilical vein endothelial cells
EGF	Epidermal Growth Factor

EL	Endothelial Lipases
EpRE	Electrophile-Responsive Element
ER	Endoplasmic reticulum
ERα	Estrogen Receptor-alpha
ERA	Excellence in Research for Australia
ERK	Extracellular signal-Regulated kinase
ERN	Erucin
ESP	Epithiospecifier Protein
GCL	Glutamate-cysteine ligase
GLUT-1	Glucose Transporter 1
GLUT-2	Glucose Transporter 2
GLUT-4	Glucose Transporter 4
GPx	Glutathione peroxidase
GPX1	Glutathione peroxidase gene
GRN	Glucoraphanin
GSH	Glutathione (reduced form)
GSR	Glutathione reductase
GSSG	Glutathione (oxidised form)
GSN	Glucosinolate
GST	Glutathione-S-transferase
GST	Glutathione-S-transferase
HAEC	Human arterial endothelial cells
HDAC	Histone Deacetylase
HDL	High density lipoprotein
HMEC-1	Human microvascular endothelial cells
HO-1	Haemoxygenase-1
HPLC	High performance liquid chromatography
I-3-C	Indole-3-Carbinol
ICD-10	International Classification of Diseases – 10th edition
IFN	Interferon
IkB	Inhibitor of kappa B
IKK	IkB kinase
IL-6	Interleukin-6

IL-8	Interleukin-8
IL-10	Interleukin-10
iNOS	Inducible Nitric Oxide Synthase isoform
INR	International Normalised Ratio
IPA	Ingenuity Pathway Analysis
ITC	Isothiocyanate
LADME	Liberation Absorption Distribution Metabolism Excretion
LPS	Lipopolysaccharide
LTR	Long terminal repeats
MAP	Mitogen-Activated Protein Kinase
MAPK	MAP kinases
MCT	Medium-chain triglycerides
MCT	Medium-chain triglycerides
MDA	Malondialdehyde
ME/CFS	Myalgic encephalomyelitis/ Chronic Fatigue Syndrome
MetMIN	Metabolic Minutes
MFI	Multidimensional Fatigue Inventory
MnSOD	Manganese Superoxide dismutase
MYR	Myrosinase
NAC	N-acetyl cysteine
NADH	Nicotinamide Adenine Dinucleotide (coenzyme)
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (coenzyme)
NAFLD	Non-alcoholic fatty liver disease
NAPQI	N-acetyl-p-benzoquinone imine
NF-κb	Nuclear Factor-kappa b
NHBEC	Normal Bronchial Epithelial Cells
NOS	Nitric Oxide Synthase
NQO1	NAD(P)H: Quinone reductase
Nrf2	Nuclear factor erythroid 2-related factor 2
p-53	p53 Tumour Suppressor Protein
PAH	Polycyclic aromatic hydrocarbon
PBMC	Peripheral blood mononuclear cells
PCA	Principal Component Analysis

PEITC	Phenyl-ethyl isothiocyanate
PHQ-9	Depression scale questionnaire
PPAR-γ	Peroxisome proliferator-activated receptor gamma
PrEC	Prostate Epithelial Cells
PSA	Prostate Specific Antigen
QR	Quinone reductase
RAGE	Receptor for Advanced Glycation End Products
RDI	Recommended Dietary Intake
RELB	RelB gene
RIN	RNA Integrity Number
RMA	Robust Multi-Array Average
RNS	Reactive Nitrogen Species
ROS	Reactive oxygen species
RT-qPCR	Real-time quantitative polymerase chain reaction
SERM	Selective estrogen receptor modulator
SFN	Sulforaphane
SNP	Single nucleotide polymorphism
SOD	Superoxide dismutase
T2DM	Type 2 Diabetes
TAC	Total antioxidant capacity
TAS	Total antioxidant status
Th1	Type 1 T Helper Cell
Th2	Type 2 T Helper Cell
TNBC	Triple Negative Breast Cancer
Trx	Thioredoxin
TrxR	Thioredoxin reductase
UGT	UDP-glucuronosyl transferase
VCAM-1	Vascular Cell Adhesion Molecule 1
VDR	Vitamin D receptor
VEGF	Vascular endothelial growth factor
WHO	World Health Organisation

CHAPTER ONE

Introduction

1.0 General Principles

We live in an era where modern medicine is strongly focused on relief of symptoms, the pharmaceutical industry providing many solutions to address this demand. It is becoming increasingly apparent however, that the diseases which cause most distress at the individual level and are the most expensive at the public health level are typically managed by pharmaceuticals which may provide only short-lived symptomatic relief. Few if any modern pharmaceuticals modulate fundamental etiological disease processes. As a consequence, there is a groundswell of interest in therapies which address the fundamental *upstream* causes of disease as opposed to symptom-relief alone. Strategies to address these fundamental causes of disease may help elucidate the pathophysiology of both chronic diseases and acute self-limiting conditions. Such findings may inform development of new therapeutic solutions.

1.0.1 Searching for Upstream Factors

A search for *upstream* factors in the etiological processes of disease is the focus of considerable global research, with such research closely investigating signalling pathways within cells and organelles. Typical of the current trajectory for chronic disease as a whole is the increasing global prevalence of cardiovascular disease and type 2 diabetes. It is emerging[3] that the primary upstream factor which links endothelial dysfunction with cardiovascular disease and type 2 diabetes is closely related to oxidative stress.[4] Attempts to intervene with the classical antioxidant vitamins to enhance endothelial function and related glucose regulation have largely resulted in no response in some studies and adverse effects in others.[5-9]

1.0.2 Phytochemicals as Inducers of Endogenous Defences

A possible alternative approach to the modulation of the oxidative stress underpinning such cellular dysfunction involves the application of phytochemicals with nutrigenomic potential.[10] By definition, a phytochemical is a plant-derived

chemical substance that is biologically active but typically non-nutritive[11]; nutrigenomics describes the way in which phytochemicals may affect gene expression. As such, the application of nutrigenomic principles may allow effective dietary intervention strategies to recover normal homeostasis and to prevent or even treat disease.[12]

This thesis focuses on the modulation of gene expression that may occur using plant-derived compounds with desirable and demonstrable nutrigenomic properties. One of the intended effects of this strategy is to increase the production of endogenous antioxidant compounds, including the antioxidant enzymes. Whilst some phytochemicals may upregulate cellular endogenous defences, others may downregulate pathways associated with undesirable effects, including prolonged inflammation.

Although such plant-derived compounds may exhibit direct antioxidant activity, it is their *indirect* antioxidant effect which is attracting growing interest. At the *upstream* cellular level, the effect of the antioxidant enzymes in quenching Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) is catalytic, compared with non-enzyme antioxidants which exhibit only a one-for-one stoichiometric effect. There is considerable evidence to show that induction of such cytoprotective compounds has multiple beneficial effects.[13,14,15,16]

The two compounds selected for investigation in this thesis are derived respectively from the fruit flesh of a particular strain of melon (*Cucumis melo)* and the young germinated seeds of broccoli (*Brassica oleracea var. italica).* An extract from the melon, combined with wheat-derived gliadin polymers has been shown to contain a nutrigenomically-active SOD[17]; the germinated broccoli yields a similarly nutrigenomically-active isothiocyanate, sulforaphane (SFN).

1.1 SOD/Gliadin as an Intervention Compound

The melon-derived nutrigenomic compound (SOD/gliadin)[17] has been shown to be capable of inducing the endogenous synthesis of the three antioxidant enzymes, SOD, glutathione peroxidase (GPx) and catalase (Cat).[18] Each capsule claims to provide 250 U of SOD activity in an orally-bioavailable form, protected from digestive degradation by biopolymers of gliadin. In one study[19] of this compound, it was

shown that treatment with SOD/gliadin (but not vitamins C and E)[20,21] was able to prevent the severe DNA damage associated with hyperbaric oxygen exposure.

It could be hypothesised that treatment with SOD/gliadin may have benefit in any condition where oxidative stress is a contributor to redox dysregulation. Two contrasting cellular events are relevant to this hypothesis: 1) endogenous synthesis of antioxidant and other protective enzymes is known to decline with age and illness. 2) production of the superoxide radical increases with age, illness and various environmental conditions.[22]

Fatigue is one of the most common complaints of patients seen by all health practitioners. In many cases, no obvious or specific cause is found. Anecdotal evidence supplied by the patent-holder suggests that treatment with SOD/gliadin can decrease fatigue in those who use it. The first study described in this thesis and forming Section 1 is the *Fatigue Study*[2] which was designed as a randomised placebo-controlled trial to investigate the effect of SOD/gliadin capsules on unexplained fatigue in otherwise healthy post-menopausal women. However, compared with placebo, this investigation did not find a significant change in fatigue in the subjects taking the intervention capsules. It was subsequently decided to discontinue further investigation of this compound, having become aware of the more extensive and growing database of publications on bioactive compounds derived from cruciferous plants.

1.2 Sulforaphane as an Intervention Compound

1.2.1 Properties and Relevance

Sulforaphane is a plant-derived biomolecule shown to demonstrate nutrigenomic potential. Sulforaphane is naturally derived from certain species of the Brassica vegetable family[23] and most notably from broccoli. These vegetables are also known as *cruciferous vegetables,*[a] well-known for their disease-preventive effects.[24,25] Broccoli has been shown to be the most significant dietary source[26] of the precursor compound, glucoraphanin (GRN) which on ingestion in the presence of the *myrosinase (*MYR) enzyme, is metabolised to SFN. Very young sprouted broccoli

[a] Cruciferous Vegetables include broccoli, cabbage, cauliflower, Brussels sprouts, rocket, arugula, bok choy and others

seeds in the order of 3-7 days have been shown to contain the highest levels of GRN.[27]

There is evidence to suggest that SFN's nutrigenomic effects contribute to the enhancement of the cell's antioxidant capacity.[28] Of the endogenous antioxidants induced by SFN, the better-known include glutathione (GSH), Thioredoxin (Trx), Thioredoxin reductase (TrxR) and Haemoxygenase-1 (HO-1). In addition, a significant role in endogenous cellular defence processes is emerging for the Phase 2 detoxification enzyme, NAD(P)H: quinone oxidase reductase1 (NQO1) which is also substantially upregulated by SFN.[27]

It has now been firmly established that NQO1 provides major antioxidant functions by virtue of its obligatory two-electron reduction mechanism which diverts quinones from participating in oxidative cycling and generation of reactive oxygen intermediates. The finding that the gene coding for NQO1 is highly-inducible and that increased induction protected animals and their cells against oxidative stress, provided a major new perspective on the functional importance of this enzyme.[29-31] SFN is considered to be one of most potent phytochemical inducers of *NQO1*.[32,33]

Given that classical antioxidant supplements have failed to demonstrate significant chemoprotection or preventive benefits against cancer, cardiovascular disease and type 2 diabetes,[5,34-36] it could be hypothesised that intervention with SFN may provide an alternative but effective strategy. A growing number of studies use broccoli sprouts to enhance cellular defences and have been described as showing promising results.[37,38,39,40,41,42] However, these studies fail to provide consistent clinical responses that correlate with the concentration of SFN yielded from its plant source. The issue of dose-response is discussed in greater detail in Chapter 7.

The second more comprehensive study in this thesis and forming Chapter 8 was designed to investigate the potential for SFN to modulate gene expression in young healthy men, using a broccoli sprout ingredient which specifies its level of the key bioactive, glucoraphanin as well as the sulforaphane it yielded via a MYR-dependent enzymatic reaction. The SFN-yielding ingredient was provided to participants at practical doses obtainable from taking broccoli sprout capsules. Chapter 5 provides tabulated data of the available clinical trials for which a dose-response for a

quantifiable amount of SFN can be correlated with clinical outcomes in a variety of human illnesses and abnormalities.

SECTION ONE

CHAPTER 2

Review of Literature – SOD/Gliadin

2.0 Introduction

This review of the literature provides the scientific foundation associated with the mechanisms and clinical application of a patented nutrigenomically-active melon/gliadin used in a clinical trial conducted by our group and published in *Phytomedicine*.[2]

2.1 Background - the History of Superoxide Dismutase Research

The antioxidant enzyme, superoxide dismutase (SOD) was discovered by McCord and Fridovich in 1968. Following recognition of its role in cellular defence mechanisms,[43,44] its potential in clinical medicine was considered appropriate for translation into therapeutic applications. Injectable forms of bovine SOD appeared as pharmaceuticals and were used in a range of conditions[45,46,47,48,49,50,51] for some years until the onset of Creutzfeldt-Jakob disease (*'mad cow'* disease) which necessitated their removal from the market.

In the late 1990's, plant alternatives to bovine SOD were sought. Because SOD as an enzyme is degraded by proteolytic gastric, duodenal and pancreatic enzymes in humans, it was necessary that the SOD be protected against such proteolysis. A French company identified a source of SOD in a particular strain of cantaloupe, or melon (*Cucumis melo LC)* and in 2000, a U.S. patent for the SOD/gliadin combination was granted.[52] The material is known commercially as GliSODin (www.glisodin.org).

The resulting melon-based product was a unique compound bound with gliadin (8.3mg per 250 U enzyme dose), the resulting gliadin biopolymer protecting the SOD from proteolysis.[17] In addition to protection against degradation by gastrointestinal secretions, ingestion of the SOD/gliadin combination was shown in rats to result in significant increases in the three primary antioxidant enzymes, SOD, Glutathione peroxidase (GPx) and Catalase (Cat).[17,18]

These two SOD/gliadin proof-of-concept studies by Vouldoukis et al.[17,18] showed that neither the melon nor the gliadin extract alone was capable of inducing endogenous synthesis of the antioxidant enzymes; it was the combination which demonstrated increases in all three antioxidant enzymes together with an increase in the anti-inflammatory cytokine, interleukin-10 (IL-10).

2.1.1 The SOD/Gliadin Research Timeline

Figure 2.1 shows the evolution of SOD/gliadin research since patent issue in 2000, together with the nature of the clinical trials using SOD/gliadin as the intervention material. The studies undertaken include *in vitro* mechanistic and pharmacokinetic studies, animal studies and human clinical trials. The latter studies focus on conditions known to be associated with cellular redox imbalance, investigating reduction of atheromatous plaque in adults with metabolic syndrome[19] as well as investigations into SOD/gliadin's protective effect against UV radiation-induced fibrosis,[53] hyperbaric oxygen[19] and surgical stress (as ischaemia-reperfusion).[54]

The first intervention study undertaken as part of this thesis was to clinically test the hypothesis that the SOD/gliadin phytochemical combination could increase endogenous synthesis of SOD, Glutathione peroxidase (GPx) and Catalase (Cat) and in so doing might be capable of reducing fatigue in otherwise healthy older women.

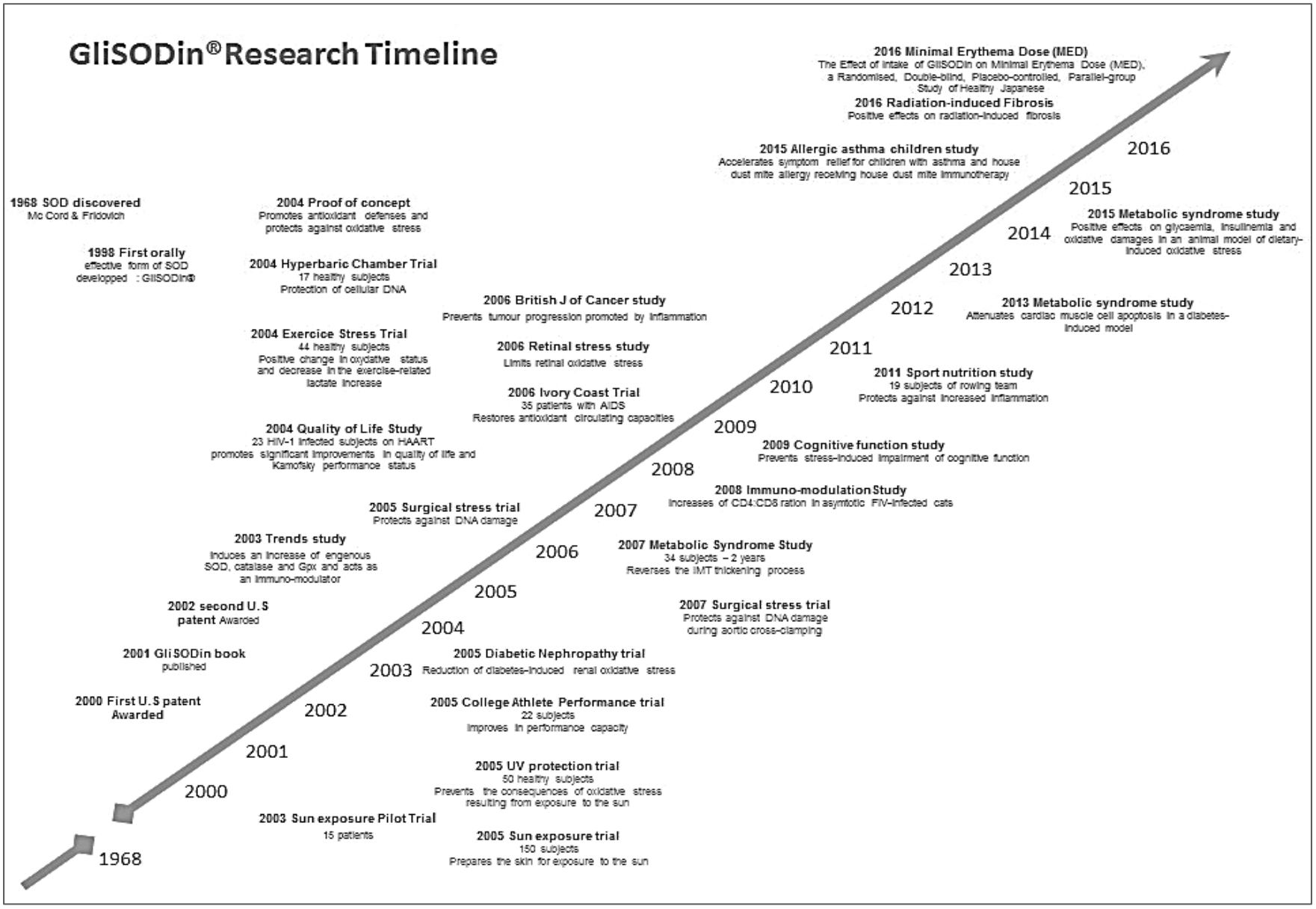


Figure 2.1 Evolution of SOD/gliadin research 2000-2016.

Source www.glisodin.org

2.2 More Recent SOD/Gliadin Research Investigation

Since our study was published, there have been four publications focused on the SOD/gliadin compound. In the first of these[55] and using chloroplasts from the same cantaloupe strain, the patent-holders developed a stable recombinant Cu-Zn SOD fused to a gliadin peptide. Unlike the SOD/gliadin used in our study which has 1 U SOD activity per mg, the recombinant form demonstrated ~ 5,000 U SOD activity per mg. The authors concluded that although the fundamental action of the recombinant form was similar to the lower potency SOD/gliadin, it was also able to switch the gliadin-induced pro-inflammatory cytokine production to the anti-inflammatory pathway.

The second of these studies investigated the effect of 500 mg daily of the same SOD/gliadin used in our trial on 19 male rowers exercising to exhaustion.[56] SOD was shown to be higher in the supplemented group and C-reactive protein lower, although there was no effect on oxidative damage in muscle tissue. A third publication[57] is a review paper summarising the suggested mechanisms of action of the SOD/gliadin combination in addition to tabulating recent studies considered to be significant. The last of these studies[53] examined the effect of SOD/gliadin in mice exposed to ionising radiation on Day 0 and then to the supplement over an 8-day period at age 6 months. Excised tissue in those administered the supplement showed significantly reduced dermal thickness. The value of this animal study may be of limited comparative value in that the doses were much greater than those administered in the reported human clinical trials. However, the development by Intes et al. of the more potent recombinant SOD/gliadin form in their group's study[55] may enable such findings to be replicated in humans.

2.3 The Role of Endogenous Antioxidant Enzymes

SOD is the primary human endogenously-synthesised antioxidant enzyme quenching the ROS, superoxide anion. It is well understood that free radical injury plays a role in all types of cellular damage and is implicated in many diseases[58,59-62] including cardiovascular disease, diabetes and cancer. SOD exists in three main forms, a cytosolic form which uses copper and zinc at its active site, a mitochondrial form which uses manganese at its active site and an extracellular form which also uses copper and zinc as its cofactors.

Dietary antioxidant supplements have been used as intervention tools in a variety of clinical expressions of oxidative stress[63]; the results are conflicting, with some studies showing benefit and others showing no benefit.[5,64] Yet other studies[65] have shown that in some circumstances, there may be reason to avoid the use of isolated antioxidant supplements in supraphysiological doses.

It is known that endogenous synthesis of the three primary antioxidant enzymes, SOD, GPx and Cat declines with increasing age and with illness.[22] It is also known that production of the superoxide radical increases with age, illness and various environmental situations.[66,67]

2.3.1 Biochemical Considerations Associating Elevated ROS with Cellular Dysfunction

In testing the hypothesis that elevated ROS may contribute to unexplained fatigue in humans, the following biochemical factors may be relevant:

- The cell produces energy in the form of Adenosine Triphosphate (ATP), mostly via the mitochondrial electron transport system
- Ageing typically results in a reduction of the endogenous synthesis of antioxidant enzymes, including mitochondrial manganese-dependent SOD (MnSOD). The potential for a greater oxidative burden exists as individuals age.[68]
- Superoxide radical in the presence of nitric oxide generates a toxic ROS, peroxynitrite.[69]
- SOD is deactivated by peroxynitrite.[70]
- A range of stressors can initiate stimulation of inducible nitric oxide synthase (iNOS) activity and its oxidant product, peroxynitrite (in the presence of superoxide).[71]
- The ageing process is associated with pro-inflammatory NF-kappa B (NF-kB) activation. Certain redox-modulating phytochemicals are capable of ameliorating such age-related alterations in signal transduction, *in vitro*.[72]
- The transcription factor NF-kB stimulates iNOS activity, so that increased levels of nitric oxide are produced. This initiates a feed-forward loop where an

initiation of inflammatory activity continues to produce more nitric oxide which in turn produces more peroxynitrite.[73]

- Peroxynitrite has several effects which deplete ATP, including its effect on the mitochondrial electron transport system. Nitric oxide inhibits one of the component enzymes of this system, *cytochrome oxidase*.[74] Both of these factors produce increased superoxide, potentially providing further positive feedback
- Peroxynitrite is formed during sepsis, inflammation, excito-toxicity and ischaemia-reperfusion of tissues, conditions under which cellular production of both superoxide and nitric oxide increase; such elevated sustained peroxynitrite can cause chronic fatigue syndrome.[70]
- Multiple mechanisms have been found to produce increases in both superoxide and nitric oxide, leading to increased levels of the peroxynitrite free radical.[75] Because peroxynitrite inhibits mitochondrial energy production, it is plausible to consider that interventions directed at favourably regulating the production of superoxide and/or nitric oxide could be expected to impact on energy production. It is on this hypothesis that this study is based
- The cell's redox status can have a modulating effect on aconitase in intact mitochondria. As such, superoxide can inhibit the rate-limiting enzyme in the ATP-generating Kreb's Cycle.[76]

In considering these interactions which provide a plausible mechanism linking oxidative stress to reduced ATP synthesis and the possibility of subjective fatigue, the first study in this thesis investigates whether the SOD/gliadin combination has an effect on these processes.

The challenge in such nutrigenomic investigations is to identify oral therapeutics with the ability to enhance activity of the cell's endogenous antioxidant enzymes, since the findings for the therapeutic application of exogenous antioxidant vitamins are equivocal.

2.4 The Intervention Compound

The SOD/gliadin compound has been designed to deliver SOD in an oral delivery system, typically a 2-piece hard vegetable capsule. Its mode of action[18] in inducing the primary antioxidant enzymes makes it different from other exogenous compounds such as the SOD-mimetics which are drugs seeking to mimic the effect of endogenous superoxide dismutase enzyme in human cells. SOD/gliadin is not an SOD-mimetic. The SOD/melon extract is specified for its SOD enzyme activity and has been shown to induce other enzymes apart from SOD.[18] In this capacity, it could be classified as a nutrigenomically-active compound.

As a complex of melon-derived SOD bound to a gliadin biopolymer, SOD/gliadin is structurally different from the SOD-mimetics which in turn are structurally different from native SOD. Native SOD is a homo-tetramer, a polymer consisting of four identical monomers,[77] with an individual subunit molecular weight of about 23,000 Daltons and containing around 205 amino acids per monomer. Although all three compounds have been shown to exhibit superoxide-quenching activity, each is structurally different.

2.4.1 Studies Utilising SOD/Gliadin as the Intervention

The timeline in Figure 2.1 lists the studies performed using SOD/gliadin. The manufacturer of the SOD/gliadin used in this study, (IsoCell NUTRA, Paris, France) has conducted research into the therapeutic properties of the compound. One of these studies demonstrated that in humans, treatment with SOD/gliadin was able to prevent the severe DNA damage associated with hyperbaric oxygen exposure in deep sea divers.[19] By contrast, two similar studies[21,20] using vitamins C and E as intervention tools failed to show any protection by these vitamins against hyperbaric oxygen. Thus, it is hypothesised that treatment with SOD/gliadin is likely to have benefit in conditions wherein marked oxidative stress occurs and that this benefit may occur in conditions where no benefit results from supraphysiological dosages traditional antioxidant vitamin supplements.

2.5 The Rationale for Investigating Fatigue

Fatigue is a common presenting complaint to primary care clinicians.[78] It can be defined as a pervasive sense of tiredness or lack of energy that is not related exclusively to exertion.[79] In many cases, no obvious or specific cause is found. It has been estimated that there is an overall prevalence of fatigue of 27% in ambulatory Australians 60 years of age and over.[80] Studies[81,82,83] in other populations report prevalence from 10-25%, with marked cross-cultural variability.[84] Fatigue syndromes lie along a continuum of severity, from ubiquitous transient and mild states to the more severe and prolonged fatigue disorders, including chronic fatigue syndrome (CFS). If fatigue is prolonged beyond six months, is disabling, and is accompanied by other characteristic constitutional and neuropsychiatric symptoms, then a diagnosis of CFS should be considered.[79] Because the measurement of fatigue is usually based on subjective assessment, there can be considerable confusion in the minds of both patients and practitioners about how to define a particular patient's condition even when it has been longstanding. Is it simple fatigue caused by a medical condition, is it chronic fatigue which has been present over an extended period or is it the more complex CFS?

Chronic Fatigue and *CFS* can be difficult to distinguish from neuropsychiatric syndromes and as such may confound studies on fatigue.[85] Somatoform disorders wherein no physical cause can be found for a symptom, anxiety disorders and major depression can all contribute to fatigue. Fatigue is often found in association with sleep apnea[86] and with a muscle-related condition, fibromyalgia.[87] A 2007 Canadian Consensus document[88] reveals the complexity of the diagnostic process required to classify an individual with CFS, now more frequently described as ME/CFS to incorporate its *Myalgic Encephalomyelitis* aspects.

The Canadian document classifies ME/CFS as *"an acquired organic, pathophysiological, multi-systemic illness that occurs in both sporadic and epidemic forms* (ICD10 G93.3)[89] and stresses that ME/CFS must not be confused with 'chronic fatigue' because ME/CFS represents pathophysiological exhaustion and is only one of many symptoms. Other symptoms include post-exertional malaise, sleep dysfunction, autonomic, neuroendocrine and immune manifestations. This situation

is made worse because there are no precise or validated diagnostic tests for this condition. CFS is usually made on the basis of a *diagnosis of exclusion.*[90]

The cause of chronic fatigue in apparently healthy individuals may not always be determined, even after established etiological factors such as lifestyle behaviours, anaemia, thyroid abnormalities and other medical conditions are excluded. A high rate of co-morbidity with psychological disorders (most often anxiety and depression) has been noted[91] but there still remains a significant percentage of fatigued individuals for whom there is no known somatic or psychological cause.[92] About one-third of this group could be classified as experiencing *chronic unexplained fatigue* and thereby meeting the formal criteria for classification under WHO's ICD-10 as F48.0 neurasthenia.[93]

2.5.1 The Potential for Oxidative Stress to be Associated with Chronic Fatigue

Oxidative stress has been demonstrated to underpin a range of human illnesses and is the result of an imbalance between exogenously and endogenously-generated free radicals and the body's defences.[94,95,96,97,98] When the body is unable to adequately contain the adverse effects of free radical activity leading to redox imbalance, the result is *oxidative stress.*[99] Not all free radical activity is responsible for adverse effects and indeed much of the body's free radical activity is an essential component of a healthy immune response[100] and with healthy cellular function in general.[101,102] Free radicals are also an integral part of the signal transduction pathways which allow intra and inter-cell communication.[103]

CFS has been associated with oxidative stress but there is little reference to the possible association of oxidative stress with uncomplicated but unexplained chronic fatigue.[104,105,106]

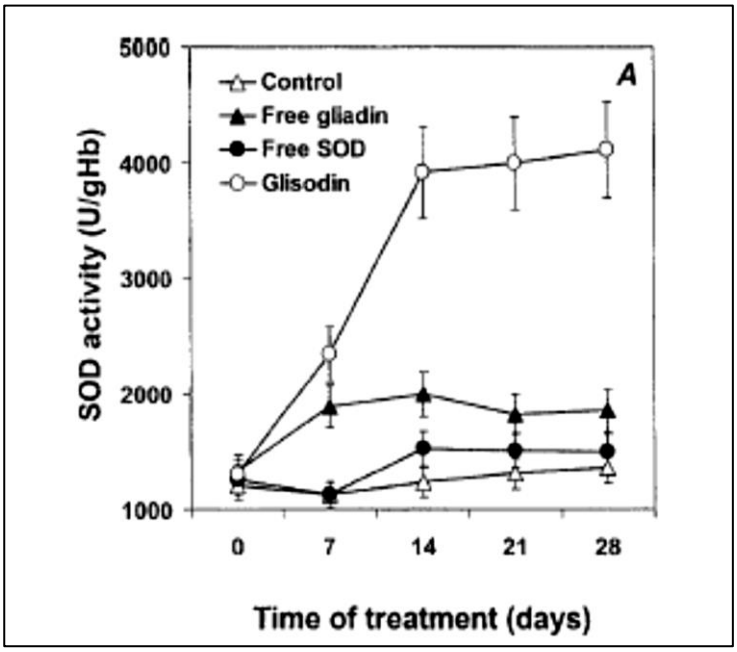
2.5.2 Putative Mechanism - Effect of SOD/Gliadin on Superoxide Dismutase Activity

SOD/gliadin has been developed to deliver SOD as an oral treatment to human cells. The data below is taken from a patent-holder sponsored proof-of-concept study[18] designed to show the effect of SOD/gliadin in mice. The study compared the effects

of free SOD, free gliadin and the combined SOD/gliadin compound, GliSODin®, on blood levels of SOD.

Figure 2.2 Effect of a supplementation with SOD/gliadin (GliSODin™) on circulating SOD activity.

Only the SOD/gliadin compound was shown to increase SOD activity; maximal activity occurred within 14 days with a slower increase thereafter for a total of 28 days.



The same study measured the effects of supplementing the animals with only SOD/gliadin for a 28-day period and measured erythrocyte levels of the endogenous antioxidant enzymes SOD, GPx and Cat. As shown in Figure 10 of the Vouldoukis study below (and as Table 2.1 here), there were increases in Total Antioxidants, SOD, GPx and Cat.

	Supplementation	
	Control	Glisodin®
Antioxidant status (mmol/L)	1.39 ± 0.03	1.98 ± 0.06
SOD (U/g Hb)	1720 ± 125	3250 ± 255
Gpx (U/g Hb)	800 ± 33	1210 ± 89
Catalase (kU/g Hb)	35 ± 5	95 ± 6

Animals were fed every day with control diet or with control diet supplemented with 1 mg/mouse/day of Glisodin® for 28 days. Blood samples were collected and SOD, Gpx and catalase activities were evaluated in erythrocytes. Data represent the mean ± SD of ten animals/group from one representative experiment.

Table 2.1 Effect of SOD/gliadin on erythrocyte changes to antioxidant levels in mice.

2.6 The Role of SOD-mimetic Drugs in Human Pathophysiology

Another avenue in therapeutics has been the exploration of SOD-mimetic drugs. Studies using SOD-mimetics as investigational drugs[107,108,109] are helpful in gaining a better understanding of the role of SOD enzyme in human systems. The use of SOD-mimetics illustrates the diverse application of SOD in a broad range of pathophysiological states. SOD/gliadin differs from the SOD-mimetics in that it is manufactured from a plant extract,[17] whereas the SOD-mimetics are synthetic and are structurally unrelated to those found in nature. Research[107,110] into the SOD-mimetics provides some evidence of the clinical effects of increased levels of SOD in cells. These findings may lend some support to the design of trials in which SOD/gliadin is the intervention compound.

Acting nutrigenomically, SOD/gliadin provides a new class of antioxidant intervention tool for application in conditions related to oxidative stress. By inducing the cell's own antioxidant enzyme synthesis, it has the potential to quench free radicals such as superoxide much earlier in the process and before the cascade of free radical activity is well-established. Where membrane-bound diet-derived lipophilic antioxidants such as vitamin E and β-carotene can act only when free radicals are in proximity to the membrane, antioxidant enzymes exist in several forms located throughout the cell and its organelles. MnSOD is located in the mitochondria and is considered to offer significant protection against the continuously-generated superoxide anion radical. A number of studies[111,112,113,114] related to the secondary complications of diabetes refer to the role of MnSOD overexpression as a positive intervention in controlling the progression of such complications.

In summary, SOD/gliadin is a nutraceutical material derived from *Cucumis melo LC* and bound to gliadin. Each nutrigenomically-active capsule provides 250 U SOD activity in an orally-bioavailable form, protected from digestive degradation by biopolymers of gliadin.

2.7 Hypothetical Construct for the Study

As described in 1.1, it is known that endogenous synthesis of the antioxidant enzymes, *SOD, GPx* and *Cat* declines with increasing age and with illness.[22] It is also known that production of the free radical superoxide increases with age, illness

and various environmental conditions.[115] As an individual ages, these opposing effects contribute to greater likelihood for the development of redox-related conditions.

SOD is the primary human endogenously-synthesised antioxidant enzyme quenching the free radical superoxide anion. It is well understood that free radical injury plays a role in all types of cellular damage and is implicated in many diseases, including cardiovascular disease, type 2 diabetes (T2DM) and cancer. SOD exists in three main forms, a cytosolic form which uses copper and zinc at its active site, a mitochondrial form which uses manganese at its active site (MnSOD) and an extracellular form which also uses copper and zinc for its activity.

It appeared that there existed sufficient biochemical evidence to suggest that the SOD/gliadin intervention may ameliorate subjective feelings of fatigue and change biomarkers indicated by earlier research.

CHAPTER 3

The 'Fatigue' Study

Effects of a Gliadin-combined Plant Superoxide Dismutase Extract on Self-Perceived Fatigue in Women aged 50-65 years.

This chapter was published in *Phytomedicine* (Elsevier) as an original investigation. The abbreviations and referencing style of this document have been altered slightly to more closely reflect the formatting of other chapters and published work in this thesis.

A pdf version of the published manuscript is attached as Appendix A.

Effects of a gliadin-combined plant superoxide dismutase extract on self-perceived fatigue in women aged 50-65 years. Phytomedicine. 2011 Apr 15;18(6):521-6

3.0 Abstract

Fatigue syndromes exist on a continuum of severity from mild and transient to the disabling Chronic Fatigue Syndrome, with oxidative stress linked to its pathogenesis. A thermolabile gliadin-combined plant SOD extract has shown potential in clinical trials as a therapeutic antioxidant. This study investigated the effects of 12 weeks of 500 mg/day of a SOD/gliadin supplement on fatigue. Thirty-eight women aged 50-65 years with self-perceived fatigue entered this randomised, double-blind, placebo-controlled trial. The primary outcome measure was general fatigue determined by the Multidimensional Fatigue Inventory (MFI). Secondary outcome measures included other measures of fatigue from the MFI and blood measures of oxidative stress, antioxidant status and hormones. There were no significant (P>0.05) differences between, or within groups, for decreases in general fatigue (active = 1.6%, placebo = 4.1%). There were no within or between group differences (P>0.05) in other measures of fatigue (physical fatigue, reduced activity, reduced motivation, mental fatigue and total fatigue score). In regard to the biochemical measures, there were non-significant (P>0.05) differences in increases in plasma SOD activity (active=7.1%, placebo=12.2%), plasma GPx activity (active=2.4%, placebo=0.7%), red blood cell GPx activity (active=9.8%, placebo=4.4%). Markers of oxidative stress were decreased but there were no differences (P>0.05) within or between groups; malondialdehyde (active=4.1%, placebo=1.6%), F-2 isoprostanes (active=14.7%, placebo=22.4%). There was a trend (P=0.08) for a decrease in cortisol in the active group (24.6%), however this was not significantly different from the decrease in the placebo participants (4.1%). DHEA differences were not significant (P<0.05) and declined 1.3% in the active group and 14.4% in the placebo group. In summary, the thermolabile SOD/gliadin supplement had no significant effect on self-perceived fatigue, antioxidants, oxidative stress or hormones in women aged 50-65 years.

3.1 Introduction

Fatigue is a complaint commonly presenting to primary care practitioners. It can be defined as a pervasive sense of tiredness or lack of energy that is not related exclusively to exertion. It has been estimated that there is an overall prevalence of fatigue of 27% in ambulatory Australians 60 years of age and over.[80] The cause of fatigue in apparently healthy individuals may not always be determined, even after

established etiological factors are excluded. A high rate of co-morbidity with psychological disorders (most often anxiety and depression) has been noted [91] but there still remains a significant percentage of fatigued individuals for whom there is no known somatic or psychological cause.[92] Fatigue syndromes lie along a continuum of severity, from ubiquitous transient and mild states to the more severe and prolonged fatigue disorders, including CFS characterised in part by disabling fatigue prolonged beyond six months.

Oxidative stress is defined as a disturbance in the balance between the production of reactive species (e.g. free radicals) and antioxidant defences, which may lead to tissue injury.[116] CFS has been associated with oxidative stress[104] but little work has been done on the possible association of oxidative stress with milder forms of uncomplicated but unexplained fatigue. Although classical dietary antioxidants have been trialled extensively as therapeutic agents for fatigue, the results are conflicting.[117,118,119]

Catalytic endogenous enzyme antioxidants such as SOD have a greater capacity to quench free radicals than the classical dietary or supplemental antioxidants.[120, 6,121] An orally-active SOD, derived from a combination of melon (*Cucumis melo LC)* and polymers of gliadin from wheat *(Triticum vulgare)* (SOD/gliadin) has shown promise as a novel means of inducing antioxidant enzyme activity (*SOD, Glutathione peroxidase and Catalase)* in human cells.[122]

The gliadin-combined SOD preparation is a water-dispersible form of a SOD lyophilised extract from melon, spray-dried using maltodextrin as a support (Vouldoukis et al.2004b). The gliadin biopolymer is used to firstly protect the enzyme against gastric proteolysis and secondly it exhibits bio-adhesive properties when in contact with the intestinal mucosa to improve and/or promote the delivery of the active ingredient.[123]

The antioxidant and anti-inflammatory activities of SOD combined with a gliadin polymer have been demonstrated in animal and human trials, producing positive biochemical and clinical outcomes.[17] In healthy volunteers, SOD/gliadin showed protection against DNA strand break induced by hyperbaric oxygen.[19] The same research group later showed that 14 days pre-treatment with SOD/gliadin before aortic cross-clamping to simulate surgery, prevented the oxidative stress

characteristics of ischaemia/reperfusion.[54] A recent study using restraint-stressed mice showed that SOD/gliadin prevented stress-induced impairment of cognitive function, decreased lipid peroxidation and maintained neurogenesis in the hippocampus.[124] Furthermore, a double-blind placebo-controlled study using SOD/gliadin in metabolic syndrome patients following the Lyon Heart Diet showed that intima medial thickening regressed over a 2-year period.[19]

Given that SOD/gliadin has shown positive responses and that oxidative stress may be associated with fatigue as it is with CFS, the aim of this study was to evaluate the effects of the supplement in reducing levels of fatigue in otherwise healthy women aged between 50 and 65 years who self-reported fatigue. It was hypothesised that the encapsulated SOD/gliadin would decrease the perceived fatigue in these individuals.

3.2 Materials and Methods

3.2.1 Study Design

A single-centre, randomised, double-blind, placebo-controlled trial was conducted with approval from the Ethics Committees of The University of Queensland and the Endeavour College of Natural Health.

3.2.2 Participants

The participants were recruited in Brisbane, Queensland, Australia through newspaper advertisements. Respondents underwent an initial telephone screening via non-structured questions to determine eligibility and severity of fatigue. Inclusion criteria were: women aged 50-65 years of age (inclusive) with longstanding (6 months or more) unexplained fatigue. This was defined as fatigue to which a medical cause (e.g. CFS) had not been assigned. Participants needed a history of generally good health and be able to attend fortnightly assessments over a 12-week period. They were ineligible if any of the following exclusion criteria applied: Coeliac Disease or known gluten sensitivity, presence of any serious disease or condition or limited life expectancy of < 1 year due to pre-existing malignancy or other disease, depression according to the PHQ-9 nine item depression scale Questionnaire PHQ-9 Depression Rating Scale during screening,[125] history of alcohol or drug abuse,

primary sleep or movement disorders, recent history of anaemia, history of thyroid dysfunction, currently enrolled in another investigational study, unlikely to comply with study requirements, following a particular dietary pattern considered to be extreme or unbalanced, have had investigational drugs used for diagnosis or evaluation of health status during preceding 30 days, currently supplementing with antioxidants or has supplemented with antioxidants for at least 4 weeks in the previous 3 months or not in a position to obtain adequate sleep (7-8 hours), such as shift workers. Where the women were also taking prescribed medication, they were included as long as the condition for which the medication had been prescribed had stabilised over time.

The trial was conducted at the Clinic of the Endeavour College of Natural Health Clinic in Brisbane, Australia. Two female clinicians experienced in the practice of Nutritional Medicine made all contact with the participants at these premises over seven fortnightly sessions.

At the first meeting, the subjects were given information relating to the nature of the trial and its goals as well as the nature of the supplement they may be randomised to take. Informed consent was obtained from each participant, anthropometric baseline measurements (height and weight) were taken and instructions for baseline collection of blood samples provided. In addition, further information regarding medical history, medications, menstrual status and whether the fatigue appeared to be related to onset of menopause was collected by individual interview. The survey asked participants to rate aspects of their fatigue and its effect on daily life on a Yes/No basis as a means of ensuring that subjects were not experiencing fatigue so debilitating that they were unable to perform normal daily activities. Women were considered to be menopausal if they had finished their last menstruation at least 12 months prior to enrolment in the study.

3.2.3 Intervention

Subjects were randomised to receive either 500mg/day of the SOD/gliadin supplement, or placebo, as 2 x 250 mg capsules per day. The SOD/gliadin was encapsulated in a hard shell 2-piece vegetable cellulose capsule with maltodextrin as the excipient. The placebo capsule appeared identical to the intervention capsule and contained only maltodextrin. At the first group session, the participants were

issued with sufficient capsules for the 12-week trial period plus a small overage allowance (180 capsules supplied, 168 required). Compliance was monitored by counting unused tablets after completion of the trial.

3.2.4 Randomisation and Concealment

Randomisation was achieved using a computer-generated random number sequence at the Launceston General Hospital Pharmacy, Tasmania, Australia. The randomisation sequence and trial codes were stored by the Hospital. Neither the participants nor the personnel implementing the trial had access to any un-blinded data until the code was revealed at the completion of all data collection.

3.3 SOD/Gliadin

Each capsule was specified as containing 3.11mg lyophilised orally-bioavailable and water-dispersible SOD derived from the fruit of *Cucumis melo LC* (melon) standardised to 90 IU/mg combined with a 40% hydro-alcoholic soft gel of gliadin (7.9 mg per capsule). The melon-gliadin combination is spray-dried using maltodextrin as a support and ratio adjusted to obtain a theoretical activity of 1 IU/mg of final dry powder (7.9 mg gliadin).[18] Individuals with gluten intolerance were excluded from the study even though this amount of gliadin is unlikely to trigger a coeliac-like response.[126] The product has been shown to exhibit no acute or chronic toxicity at doses up to 2000 mg/kg in rat studies performed by Laboratoires Lavipharm in Les Oncins, France in January 1999.[127,128] Gliadin, a vegetable prolamine (biopolymer), protects SOD from gastric juices, delays release of the compound in the small intestine, and eases its passage into the bloodstream through the mucosa. The SOD activity of the SOD/gliadin combination had been certified prior to randomisation using a specific enzymatic assay.[129] The capsules (both active and placebo) were manufactured by BTT Synerlab, a division of the Synerlab Group, Erstein 67150, France.

3.4 Outcome Measures

The primary outcome measure was general fatigue determined by the *MFI* that was repeated at 2-week intervals throughout the 12-week period. The developer of the

MFI recommends that for a single measure of non-specific fatigue, the score for *General Fatigue* only should be the main consideration.[130]

Secondary outcome measures included other measures from the MFI; Physical Fatigue, Reduced Activity, Reduced Motivation, Mental Fatigue and Total Fatigue (assessed every two weeks). In addition, blood was collected at approximately 8am at baseline, six and twelve weeks and plasma was analysed for measures of oxidative stress (malondialdehyde; MDA and F_2-isoprostanes), antioxidant status (SOD and glutathione peroxidase (GPx) activity, total antioxidant status) and hormones (dehydroepiandrosterone DHEA and cortisol).

3.4.1 Multidimensional Fatigue Inventory (MFI)

The MFI instrument consists of 20 questions that ask respondents to rate aspects of fatigue on a scale of 1 to 5. The responses are then tallied according to a scale, which classifies the fatigue into 5 categories. It was developed using populations of both healthy and unhealthy people across a range of age groups. The MFI has been validated in more than 1000 individuals, including cancer patients receiving radiotherapy, CFS patients, psychology students, medical students, army recruits and junior physicians.[131]

3.4.2 Oxidative Stress

High performance liquid chromatography (HPLC) was used to determine plasma MDA using a modification of the method of Sim.[132] The principle is that MDA in plasma is derivatised with 2,4-di-nitrophenylhydrazine (DNPH), which forms stable hydrazones that can be easily separated by HPLC and detected with diode array detection (Shimadzu, Kyoto, Japan). F_2-isoprostanes were quantified using stable isotope dilution capillary gas chromatography/electron capture negative ionisation mass spectrometry with slight modifications to the method of Mori et al.[133]

3.4.3 Antioxidants

Plasma SOD activity was quantified by a modified method of Madesh and Balasubramanian[134] that measures the ability of the enzyme to inhibit reduction of a tetrazolium dye. Inhibition is calculated based on the absorbances of two blanks (0 and 100% superoxide production – corresponding to 100 and 0% inhibition

respectively). Glutathione peroxidase (GPx) activity was measured via the oxidation of NADPH to $NADP^{2+}$, according to the method of Wheeler et al.[135] One unit of GPx activity was defined as 1 Kmol NADPH oxidised per minute. The total antioxidant status (TAS) of the plasma was measured using the method of Miller et al.[136] The method uses the radical cation $ABTS^{\cdot+}$ that absorbs at 600 nm. The higher the antioxidant status of the plasma, the greater the quenching of the compound. The concentration of TAS in the plasma was calculated as the mean gradient of the absorbance (relative to trolox standards). All assays were adapted for use on an automated spectrophotometer (Cobas, Mira, Roche Diagnostics, Switzerland) and measured in duplicate. In our laboratory, the coefficients of variation for the SOD, GPx and TAS assays are 7%, 4% and 3% respectively.

3.4.4 Hormones

Serum cortisol was assayed with the ADVIA Centaur test, which is based on a competitive immunoassay using direct chemiluminescent technology. Similarly, serum DHEA-S was measured using the Immulite 2000 solid-phase chemiluminescent enzyme immunoassay. Both tests were supplied by Siemens Healthcare Diagnostics.

3.5 Adverse Events

Adverse events were recorded at each fortnightly meeting with the investigators.

3.6 Determination of Sample Size

To observe a 95% chance of detecting a 2-point difference between the two groups in the mean MFI perceived General Fatigue Score (at the 2-sided 5% level) with an assumed standard deviation of 8 and a loss to follow-up of 20%, 20 women in each group (40 in total) were required.

3.7 Statistical Analysis

Data were first tested for normality using the Shapiro-Wilk normality test. The student's t-test for dependent samples was used to compare baseline data. General linear model two-way (group x time) repeated measures ANOVAs with student t-test

post-hoc tests were used to assess the outcome measures. Two-sided significance tests were used throughout. A *p*-value of ≤ 0.05 was considered significant.

3.8 Results

Participant flow is shown in Figure 3.1. Forty-nine telephone respondents were interviewed. Seven were not eligible (based on inclusion and exclusion criteria), one was withdrawn due to an inability of the phlebotomist to obtain blood on two occasions, one withdrew after the first session and one was subsequently excluded after selection, due to admitting concealing information relating to current treatment for a serious illness. Table 3.1 shows there were no significant (P>0.05) differences between groups in age, body mass, body mass index, menopausal status and number of patients taking anti-hypertensive, anti-depressant or hormone replacement therapy.

Compliance was 89% for the active group and 92% for the placebo group. There were no adverse events reported by any of the women throughout the trial.

There were no significant (P>0.05) changes within or between groups for the primary outcome measure; general fatigue (Figure 3.1). There was a trend for a decrease in the fatigue score in the placebo group from baseline to week 12 (P= 0.06).

Table 3.2 presents secondary outcomes' data. There were no within or between group differences (P>0.05) in other measures of fatigue (physical fatigue, reduced activity, reduced motivation, mental fatigue and total fatigue score). For the biochemical measures, there were no significant (P>0.05) differences in increases in plasma SOD activity, plasma GPx activity, red blood cell GPx activity, or total antioxidant status. Markers of oxidative stress (malondialdehyde, F_2 -isoprostanes) were decreased in both groups but there were no significant (P>0.05) differences within or between groups.

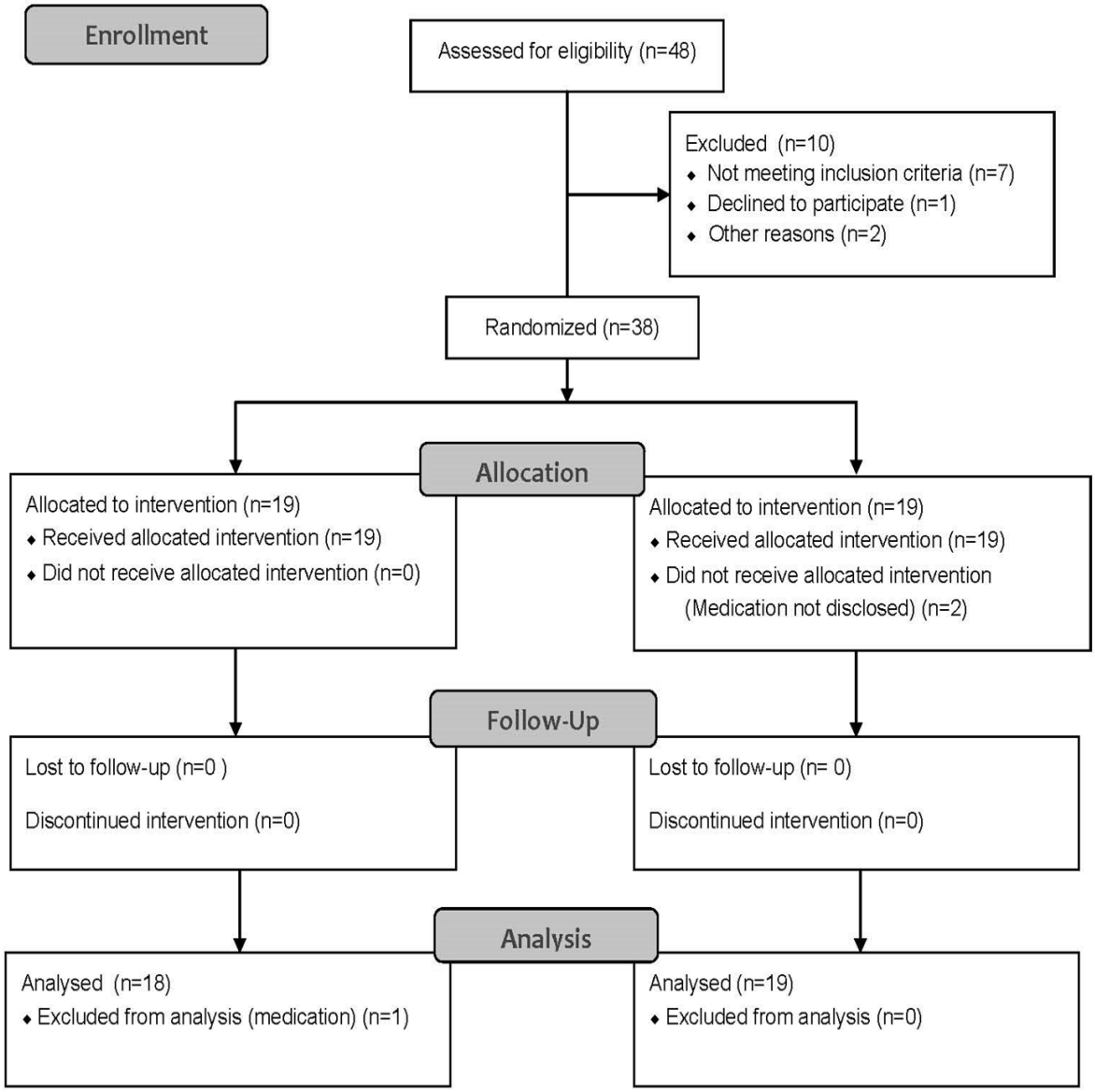


Figure 3.1: Fatigue Study; Participant flow through the study

Table 3.1. Fatigue Study; Participant characteristics. Mean ± SD

	Active	Placebo
	n=19	n=19
Age (years)	56 ± 4.6	56 ± 4.3
Body Mass (kg)	70.9 ± 14.5	71.0 ± 12.5
Body Mass Index	27.0 ± 5.9	27.0 ± 5.4
Pre-Menopausal n (%)	2 (11%)	1 (5%)
Menopausal n (%)	7 (36.8%)	7 (36.8%)
Post-Menopausal n (%)	10 (53%)	11 (58%)
Medication - Anti-hypertensive n (%)	2 (11%)	2 (11%)
Medication - Anti-depressant n (%)	1 (5%)	4 (21%)
Medication - HRT n (%)	4 (21%)	3 (16%)

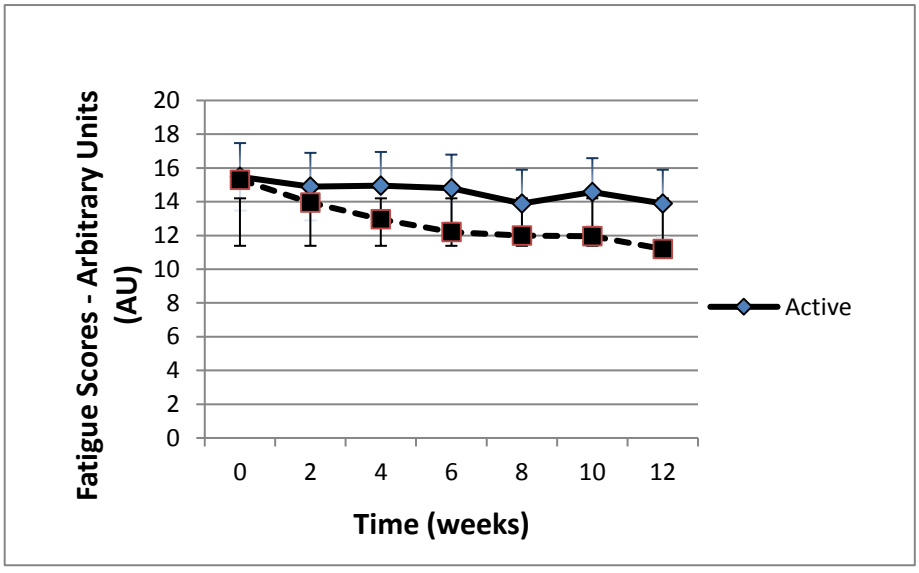


Figure 3.2: Changes in general fatigue in active and placebo groups over the twelve weeks. Mean +/- SD

Table 3.2 Effects of SOD/gliadin supplementation on secondary outcome measures at baseline, and after 6 and 12 weeks of supplementation. Mean ± SD.

	Active Group (n=19)			Placebo Group (n=19)		
	Baseline	6 weeks	12 weeks	Baseline	6 weeks	12 weeks
General Fatigue (AU)	15.5 ± 2.5	14.8 ± 4.3	13.9 ± 4.2	15.3 ± 2.4	12.2 ± 3.8	11.2 ± 3.6
Physical Fatigue (AU)	13.7 ± 13.7	13.3 ± 3.8	12.1 ± 4.0	14.3 ± 3.9	12.2 ± 3.8	11.2 ± 3.5
Reduced Activity (AU)	12.1 ± 4.0	10.1± 3.8	10.3 ± 4.0	11.7 ± 4.6	9.5 ±3.6	8.6 ± 3.3
Reduced Motivation (AU)	10.8 ± 2.7	9.7 ± 3.6	8.7 ± 3.6	9.7 ± 3.0	8.7 ± 3.5	8.1 ± 2.9
Mental Fatigue (AU)	14.2 ± 6.2	10.6 ± 4.2	11.2 ± 3.8	12.8 ± 4.3	10.7 ± 4.2	9.8 ± 3.9
Total Fatigue Score (AU)	66.0 ± 10.7	56.3± 4.4	54.4 ± 16.9	64.1± 13.5	55.2 ± 18.3	50.3 ± 15.2
Plasma SOD activity (U/L)	2.0 ± 0.6	1.7 ± 0.3	2.2 ± 0.4	1.8 ± 0.3	1.9 ± 0.2	2.0 ± 0.3
Plasma GPx activity (U/L)	453.2 ± 53.0	465.3±57.7	464.2±59.9	438.5 ± 47.0	450.5 ± 50.4	441.6 ± 50.0
Red blood cell GPx activity (U/g Hb)	37.7 ± 14.0	40.9 ± 11.8	41.8 ± 13.1	38.9 ± 11.0	40.9 ± 8.9	40.7 ± 8.2
Plasma TAS (mmol/L)	1.8 ± 0.1	1.8 ± 0.1	1.8 ± 0.1	1.8 ± 0.05	1.8 ± 0.1	1.8 ± 0.1
Plasma MDA µmol/L	11.7 ± 1.2	12.9 ± 3.3	11.3 ± 3.5	11.6 ± 1.4	13.5 ± 3.2	11.5 ± 2.8
Plasma F-2 Isoprostanes pg/ml	444.2 ± 107.3	427.7± 92.8	520.4± 122.2	658.5 ± 77.6	436.4 ± 65.1	538.1 ± 111.5
Plasma Cortisol (ng/ml)	454.2 ± 143.3	421.6± 151.1	364.4± 151.1	499.5 ± 148.1	340.5± 128.0	478.3 ± 160.6
Plasma DHEA (ng/ml)	1082.1± 612.1	998.9± 443.4	1068.4± 611.3	782.0 ± 372.3	719.5± 346.2	683.3 ± 268.4
Cortisol/DHEA Ratio	0.42	0.42	0.34	0.64	0.47	0.70

AU = Arbitrary Units; SOD = Superoxide dismutase; MDA = Malondialdehyde; GPx = Glutathione peroxidase; TAS = Total Antioxidant Status; DHEA = Dehydroepiandrosterone.

3.9 Discussion

Twelve weeks' supplementation with a gliadin-combined plant SOD extract had no effect on general fatigue in women aged 50-65 years with longstanding unexplained fatigue. There was a trend (P=0.08) for the supplement to lower cortisol (24.4% decrease). There were no effects on plasma SOD activity or other antioxidants. In addition, the supplement did not significantly lower oxidative stress or change DHEA.

The lack of an effect of the SOD/gliadin supplement is in contrast to previous studies that have shown positive benefits of an oral SOD supplement.[18,19,137] This includes protection against DNA damage following exposure to hyperbaric oxygen,[19] regression of carotid intima medial thickening in metabolic syndrome subjects,[137] prevention of oxidative stress following ischaemia/reperfusion[138] and prevention of stress-induced impairment of cognitive function.[124] The dosage used in these studies was equivalent to that used in our study.

In two of these studies the SOD/gliadin increased tissue activity of SOD, Glutathione peroxidase and Catalase.[19,138] However, we failed to find any changes in either endogenous antioxidant enzymes or oxidative stress. Therefore, a lack of antioxidant effect in our study is a potential explanation for the lack of an impact on general fatigue.

It is possible that the supplement did not have the necessary bioactivity. We did not assay the SOD/gliadin capsules for SOD enzyme activity prior to randomisation. SOD/gliadin contains the temperature-sensitive SOD enzyme which, when exposed to temperatures greater than 40^{O}C can be irreversibly damaged.[139] SOD/gliadin has a shelf life of 2 years when stored between 5 and 25^{O}C.[41] Even though assay of an intervention tool is not customary prior to commencement of a trial, we do not know whether the capsules were exposed to higher temperatures during transit and therefore we cannot be certain that the capsules were bioactive at point of distribution to the participants in the study.

Our findings are in agreement with Zidenberg-Cherr[140] who also failed to find an effect of an oral SOD supplement. However, they used a form of SOD which was degraded by gastric proteolytic enzymes and as such is not likely to increase SOD activity.

Another possible explanation is that fatigue is not responsive to this intervention. Although there were no significant differences in the effects of the SOD/gliadin supplement on general fatigue, there was a substantial non-significant (P=0.06) 4.1% decrease in the placebo group. This is likely explained by a placebo effect although it is surprising that the same was not seen in the active group. It is possible that other biases may have been present and not assessed. For example, some of the participants were being treated for depression (albeit controlled with medication) whilst others reported living in family circumstances that caused them considerable anxiety. These incidental findings were not systematically recorded during the trial. It is known that anxiety/depression and fatigue can be closely correlated and up to three-quarters of patients with fatigue syndromes have co-morbid mood or anxiety disorders.[141] If the underlying and undiagnosed anxiety is contributing to the observed fatigue, rather than oxidative stress, then the supplement would not likely affect this process.

There was a trend for a decrease in cortisol over the twelve weeks in the active group with a non-significant (P<0.08) 24.4% decrease compared to a 4.2% decrease in the placebo group. Elevated cortisol levels are associated with extreme fatigue.[142] An association between CFS and abnormalities of the hypothalamo-pituitary-adrenal axis has also been described.[143] The non-significant lowering of cortisol is interesting but more research, perhaps over a longer period, would need to further investigate this.

In summary, supplementation with the thermolabile SOD/gliadin had no effect on general fatigue in women aged 50-65 years experiencing fatigue. No adverse effects towards the intervention were recorded. The failure of the supplement to affect plasma SOD activity or lower oxidative stress implies that there was either a lack of antioxidant ability or that fatigue in this population was not related to oxidative stress.

SECTION TWO

CHAPTER 4

Sulforaphane Literature Review

INTRODUCTION

The introductory material for this section is supported by two published review papers on clinical aspects of SFN. These publications are separately entitled, *Sulforaphane: translational research from laboratory bench to clinic*[144] and *Sulforaphane and Other Nutrigenomic Nrf2 Activators: Can the Clinician's Expectation be Matched by the Reality?*[145]

These reviews follow this chapter's background discussion as Chapters 5 and 6.

4.0 OVERVIEW

It is 25 years since the identification and isolation of the transcription factor, Nrf2 (coded by the gene NF-E2-related factor 2) was first described in the scientific literature.[146] In the ensuing years, Nrf2 has become a focus of active research on mechanisms of defence in mammalian cells.[147,148,149] The role of Nrf2 in human cells is very relevant to the subject matter of this thesis because SFN significantly activates Nrf2 and as such has the potential to modulate the expression of genes associated with cellular defence. Figure 4.1 illustrates the upward trend in SFN research over the period.

Nrf2 has been variously described by several researchers as an *'activator of cellular defence mechanisms,'*[150] *'the master redox switch'*[151] and *'a guardian of health span and gatekeeper of species longevity*'.[152] As a mediator for amplification of the mammalian defence system against various stressors, Nrf2 sits at the interface between our prior understanding of oxidative stress and the endogenous mechanisms cells use to deal with it.[153]

What is emerging is that diseases known to be underpinned by oxidative stress are proving to be more responsive to such amplification of cellular defences via

Nrf2 activation than by administration of direct-acting antioxidant supplements.[154,35]

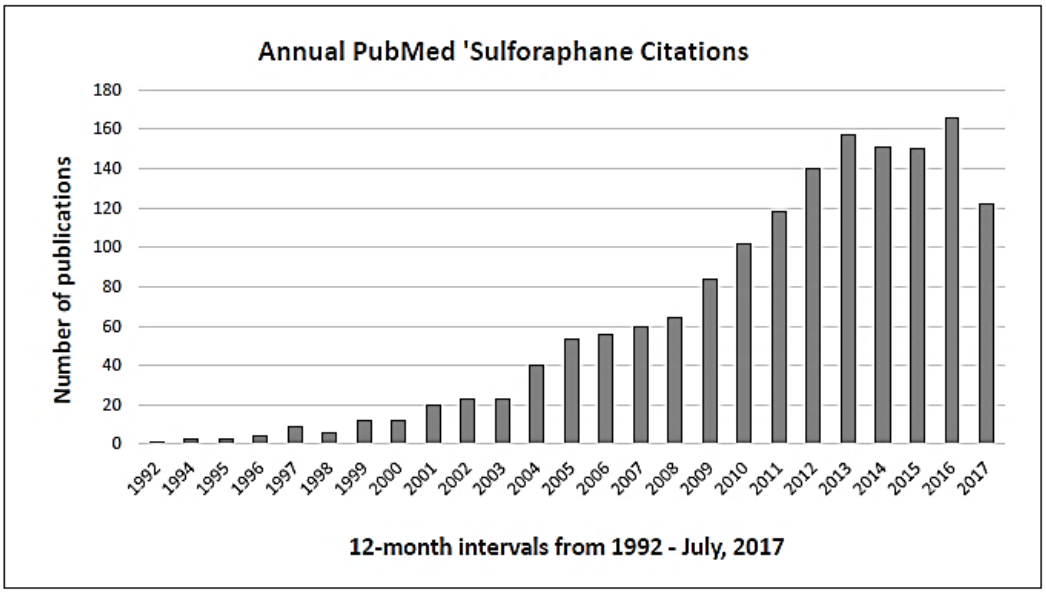


Figure 4.1 Sulforaphane Research Timeline.

Sulforaphane research appears to be accelerating, showing that by mid-2017, the number of published studies is already ~75% of that for the same period in the previous year.

4.1 Phytochemicals on the Drug Discovery Path?

For a phytochemical to be considered as a therapeutic agent, it must be evaluated using many of the same tools used in pharmaceutical product development. Whereas a pharmaceutical is typically a single molecule, plants are complex multicomponent mixtures, the phytochemical composition of which is not constant due to factors which include inherent agricultural and environmental variability.[155] Of the published SFN research to date, the intervention materials are non-standard, with some studies using the pure chemical SFN as the intervention material where others use broccoli vegetable or fresh, dried or homogenised broccoli sprouts; therefore comparison of clinical trial outcomes becomes more difficult.

Nevertheless, when working with isolated bioactive phytochemicals and whole foods as a source of the same bioactive, the biopharmaceutical processes typically used in pharmaceutical development should equally apply. The LADME principles (liberation, absorption, distribution, metabolism, and excretion) described in connection with the pharmacokinetics of pharmaceuticals are

equally relevant to phytochemicals.[156] However, such data is seldom available for the more popular phytochemicals used preventively or medicinally.[155] A comprehensive review on this subject by Pferschy-Wenzig and Bauer[155] highlights the many issues that can be under-appreciated by consumers who self-medicate on the basis of limited safety and efficacy data.

The literature for SFN indicates that many researchers have addressed the various LADME principles, thereby producing a more extensive database that is useful for interpreting the dose-response.

4.2 Pleiotropic Effects of SFN

Although SFN is most often considered for its Nrf2-dependent effects, largely associated with the induction of antioxidant and Phase 2 detoxification enzymes, other less well-characterised mechanisms are associated with this lipophilic, low molecular weight pleiotropic phytochemical molecule. The Nrf2-*in*dependent mechanisms include but are not limited to the induction of apoptotic pathways, suppression of cell cycle progression, inhibition of angiogenesis and anti-inflammatory activity and inhibition of metastasis, primarily relevant to cancer.[157]

One such effect is its action as a Histone Deacetylase Inhibitor (HDAC)[158,159] and there is a growing focus on the role of SFN and other phytochemicals on such epigenetic effects[160,161] and more recently on the role of SFN as an inhibitor of microRNAs.[162] Epigenetic effects are of particular clinical interest in that such changes are potentially reversible and thereby may provide an opportunity for intervention in earlier stages of the cancer process.[163] Tumour suppressor genes may be epigenetically inhibited so that therapies aimed at removing such suppression are attractive options, especially if they can be available through dietary means.

No discussion of SFN and Nrf2 would be complete without reference to the fact that both Nrf2 activators and Nrf2 inhibitors can be utilised in cancer therapy. A very recent paper[164] highlights this dual role and its implications for Nrf2 activation. It suggests that because Nrf2 can modulate the detoxification pathways, its effect on anti-cancer drugs may lead to chemoresistance and that the switch between a beneficial and a detrimental role for Nrf2 in cancer cells

depends on a number of factors which include the tight control of its activity. This poses an obvious dilemma which is already under active discussion and investigation[165-167]; SFN and other phytochemicals capable of modulating Nrf2 form part of such investigation.[164]

4.3 History and Evolution of Sulforaphane Research

Interest in SFN as a food-derived compound with significant clinical potential began in 1992 when a group at Johns Hopkins University published its findings.[27] It appeared that SFN was capable of activating a cytoplasmic transcription factor, Nrf2 which in turn translocated to the nucleus to activate the Antioxidant Response Element (ARE) in the promoter region of several hundred identified genes,[13,168,169,170] many of which are related to cellular defence processes. Interestingly, SFN was identified here as a potent activator of cellular defence mechanisms approximately two years before the isolation of Nrf2.[27,146]

Of the earlier studies, the potential for SFN to intervene in the prevention and/or treatment of cancer through several relevant mechanism gained most attention.[151,171] As SFN research has continued and laboratory techniques have become more sophisticated and widely-available, it has become apparent that this molecule exerts its actions at the level of several of the most fundamental disease-causing biochemical processes.[151] Its mechanism would appear to have clinical implications for chronic disease in general and in this context, SFN has been shown to inhibit endothelial dysfunction[172,173,174] and other aspects of cardiovascular and endocrine[174] function. As endothelial dysfunction compromises normal function of the circulatory system, it has the potential to contribute to chronic disease in general.

4.3.1 Development of Sulforaphane Analogues

Initially, the research focused on SFN itself but as this evolved, SFN analogues[175,176,177] were being developed and investigated for their biochemical and clinical effects. Interestingly however, attempts to develop synthetic analogues have failed to produce compounds with any greater potency and pleiotropy than SFN itself.[178] Some analogues have demonstrated greater

effects in particular aspects under investigation but none has been shown to replicate all the properties of plant-derived SFN.

Nevertheless, pharmaceutical research has resulted in molecules capable of more potently mimicking some of the actions of SFN. A pharmaceutical, *Bardoxolone methyl*,[179] discussed in context in Chapters 6 and 7 is capable of activating the same transcription factor, Nrf2 as SFN but is many times more potent in this action.

4.3.2 Broccoli Sprout vs Vegetable

In addition to their findings that SFN is a powerful inducer of endogenous antioxidant enzymes and Phase 2 detoxification enzymes,[28] the Johns Hopkins group found that the 3-day germinated broccoli seed contained 10-100 times more of the bioactive precursor glucoraphanin than the mature broccoli vegetable.[169] It was this finding that enabled the design of trials which could achieve clinically-relevant SFN effects with small practical doses of dried broccoli sprouts.

Of all vegetables, the cruciferous vegetables are the ones for which most evidence exists for a cancer-preventive effect.[180] Early research on SFN focused on its potential in prevention and to a lesser extent as therapy against cancer. Several of the effects of SFN are directly related to the initiation and progression of the cancer process but as described above, Nrf2 activation is only one aspect of SFN's pleiotropic role in this context.[157]

4.4 Sulforaphane – Structure, Properties and Mechanism of Action

The structure of this small lipophilic molecule (M.W. 177.29), confers upon SFN some unique advantages not afforded other phytochemicals such as the polyphenols which are structurally large and essentially hydrophilic.[181] One of the major advantages for SFN is its higher bioavailability which is a consequence of its structure (Figure 4.2)

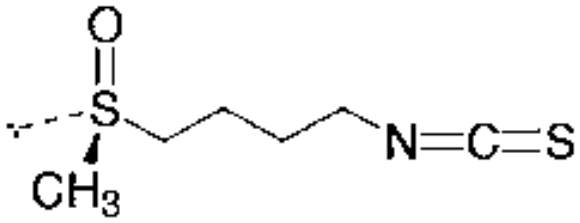


Figure 4.2 Sulforaphane ($C_6H_{11}NOS_2$) – molecular structure of sulforaphane (4-methylsulfinylbutyl isothiocyanate)

4.4.1 Bioavailability

SFN has been demonstrated as having an absolute bioavailability of around 80%[182] and has been shown to peak in the bloodstream around 1 hour following ingestion.[183] By comparison, the polyphenols typically exhibit bioavailability[184] at around 1-8%. SFN can therefore be considered as having at least one of the key properties necessary to be considered for development as a nutraceutical compound.

4.5 The SFN-Nrf2 Relationship

The essence of a very complex biochemical process[147] is that in its basal state, Nrf2 is sequestered to Kelch-like ECH-associated protein 1 (Keap-1) and associated with cytosolic actin filaments; however, when Keap-1 detects a stressor which may threaten the cell's integrity, activation of the complex leads to a dissociation of Nrf2 from Keap-1.[185] Hereafter, it translocates to the nucleus where it may induce expression of its many target genes, aligning with the ARE in the promoter region of these genes. The ARE is a *cis*-acting enhancer sequence that is upstream of many Phase 2 detoxification and antioxidant genes.[33] (Figure 4.3)

Loss of the Nrf2-ARE function in mice has been shown to increase susceptibility[186] to acute toxicity, inflammation and carcinogenesis due to the inability to mount adaptive responses. The elucidation of this process showed that activation of Nrf2-ARE induces a large battery of cytoprotective enzymes.[187]

Cellular Nrf2 levels are under strict control by multiple mechanisms but the best-characterised is the one which is mediated by interaction with (Keap-1.)[188] Keap-1 not only binds Nrf2 to actin filaments in the basal state but it also acts a sensor, especially of subtle redox changes in the cell. Very recently, it has been reported that the regulation of Keap-1/Nrf2/ARE is more complex than previously thought, with other mechanisms including epigenetic regulation of Nrf2 now known to be involved.[149]

The chemistry of sulfur plays an integral role in Nrf2 activation and subsequent modulation of gene expression. All Nrf2 activators react with thiol groups. Keap-1 is rich in sulfur-rich cysteine residues[187] and is under oxidation-reduction (and alkylation) control via its highly reactive thiol groups.

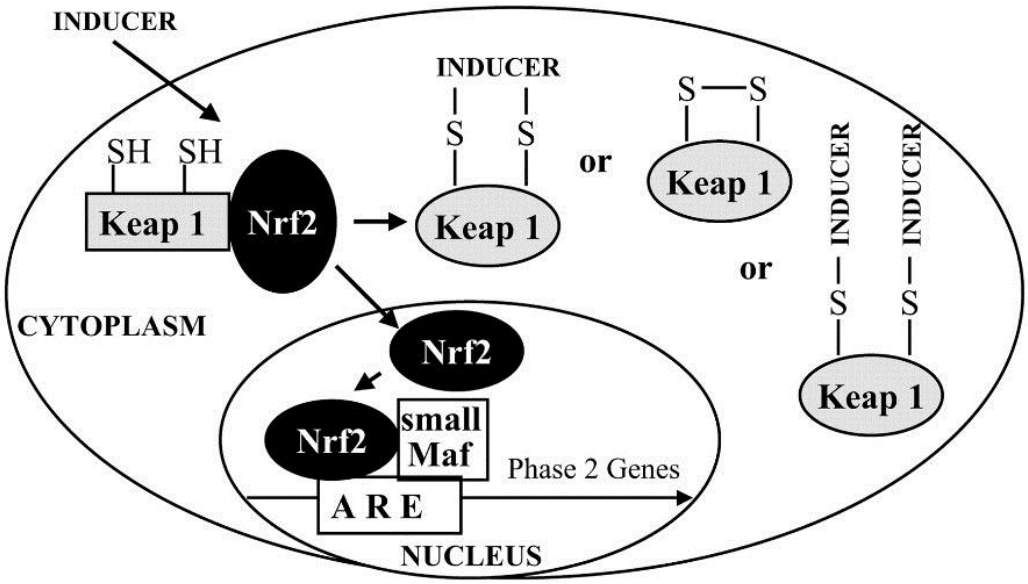


Figure 4.3 Mechanism by which an inducer affects expression of Phase 2 detoxification genes

An inducer such as SFN activates the Nrf2-Keap-1 complex, with sulfur chemistry playing an important role. The presence of sulfur in Keap-1 and Nrf2 inducers is illustrated.[187]

4.5.1 The Significance of the Nrf2-SFN Relationship

Nrf2 is ubiquitously expressed with the highest concentrations (in descending order) in the kidney, muscle, lung, heart, liver, and brain.[146] Because SFN is readily-bioavailable, such universal Nrf2 tissue distribution enhances SFN's potential to modulate systemic gene expression. In this context Nrf2[189] has been

described as a *'master switch'* involved in the induction of cytoprotective genes by some chemopreventive phytochemicals.

4.5.2 Multiple Gene Targets and the Nrf2 /ARE Pathway

It has been suggested that in excess of 500 genes have been identified as being activated by sulforaphane via the Nrf2/ARE pathway[190,191,192] and it is likely that this under-estimates the number as others are being discovered.

The large battery of upregulated cytoprotective genes includes those coding for the endogenous enzyme and non-enzyme antioxidants as well as Phase 2 detoxification enzymes.[168] Nrf2 plays a crucial role in the coordinated induction of those genes encoding many stress-responsive and cytoprotective enzymes and related proteins.[193] These include NAD(P)H:quinone reductase-1 (*NQO1*), haemoxygenase-1 (*HO-1*), glutamate-cysteine ligase (*GCL*), glutathione-S-transferase (*GST*), glutathione peroxidase (*GPX1*), thioredoxin (*TXN*), thioredoxin reductase (*TXNRD1*)[189] and PPAR-γ (*PPARG*).[194]

When Zhang and colleagues[27] of the Johns Hopkins group had been investigating chemoprevention in the early 1990s, they had been working on cytoprotective genes including those coding for the Phase 2 detoxification enzymes *NQO1* and the *GST* families; the discovery that these genes were significantly induced by broccoli sprout-derived SFN provided the foundation for the rapid interest in research in this field.

Of the available SFN clinical trials associated with genes induced via Nrf2 activation, many demonstrate a linear dose-response (Table 4.1). More recently, it has become apparent that SFN can behave hormetically[195] with different effects responsive to different doses and this is in addition to its varying effects on different cell types as outlined in *4.6*. and tabulated in Table 4.2

4.6 Published Clinical Trials

At time of writing[b] there are 1592 published papers which appear in a PubMed search using the term, 'sulforaphane'. However, a limited number of these are for clinical trials utilising either fresh or processed broccoli sprouts (Table 5.1 from

[b] August, 2017

Chapter 5 and Table 4.1 here). Not all trials quantitatively specify the bioactive content of the intervention material. As a result, it is difficult to interpret their findings in a clinical context. In considering SFN as a therapeutic intervention, some important questions to be asked are; *'What quantity of starting material is needed to achieve a micromolar concentration which generates a significant clinical outcome?'* '*How can a broccoli sprout raw material be produced which will be consistent in its composition?'* and *'Is it possible to produce a broccoli sprout raw material that is a practical solution to consumer needs for a SFN-yielding supplement?'*

4.7 The Clinician's Dilemma in Applying Clinical Trial Data

Unlike most of the products categorised by U.S. law as *'dietary supplements'*, the sub-groups of products claiming to be *'nutraceutical supplements'* are typically standardised for their bioactivity; this may require that one or more bioactives is specified with each batch produced. Of the various available supplements which include a dried broccoli sprout ingredient, the label disclosure is inconsistent; this is discussed in some detail later in this chapter and again in Chapters 5 and 6. A consumer or a clinician intending to select an available SFN-yielding supplement on the basis of its dose compared with those used in the peer-reviewed published clinical trials will have great difficulty in doing so.

This thesis explores the issues associated with the process of standardising label disclosure with a view to clarifying a dose-response relationship that can be compared with the currently available SFN clinical trial data. In evaluating the available dose-response evidence, it may be concluded that it is simply too early in the process for clinicians to confidently make such recommendations. Issues associated with dose-response considerations are addressed in some depth in Chapter 7.

4.8 Valuable Contributions from Available Clinical Trial Data.

Table 4.1 lists 33 of the 42 published clinical trials using SFN in a range of forms from pure chemical SFN to fresh sprouts, homogenised fresh sprouts and dried

sprout powder/capsules.[c] These studies have been selected as each contributes significantly to the enhancement of our understanding of this phytochemical's potential in human health and disease. Although dose forms, study populations and endpoints are different across the selected trials, a pattern is emerging to show that clinical outcomes are achievable in conditions such as asthma[196] with daily SFN doses of around 18 mg daily and from 27 - 40 mg in type 2 diabetes.[197,198] Positive outcomes were achieved at a lower SFN dose of around 9-14 mg daily for the autism study by Singh et al.[199] Reduction of Prostate Specific Antigen (PSA) doubling time after radical prostatectomy may require a higher 60 mg daily dose.[200] Chapter 7 explores and analyses the dose-responses when considering an appropriate SFN dose for the gene expression study detailed in Chapter 8.

[c] PubMed accessed 22nd July, 2017

Table 4.1 Selected Clinical Studies Investigating a Dose-Response for Sulforaphane, listed chronologically (2013 – 2017)

The selected trials utilise various forms of intervention to provide a dose of SFN designed to positively affect the chosen endpoint(s). These studies have been published subsequent to our Review paper (Chapter 5) which includes a more comprehensive list of published trials.

Lead Author & Year of Publication	Subject Number	Intervention	Endpoint	Finding	Dosage or Clinical Relevance
Poulton et al. (2013)[201]	24	450 µmols SFN (~ 80 mg) in broccoli sprout extract	CYP3A4 induction in relation to drug adverse effects via the pregnane and xenobiotic receptor (PXR)	SFN did not reduce CYP3A4 induction alone or as co-treatment in co-administration with drugs.	A dose of 80 mg is significantly higher than available from foods or from practical intake from supplements.
Singh; 2014[199]	44	9 - 14 mg SFN based on body weight of a stabilised sulforaphane supplement.	Validated questionnaire-based disease markers for autism	Positive responses in Aberrant Behaviour and Social Responsiveness. Reduced markers of redox imbalance and inflammation.	Favourable effects at 18 weeks gradually trended towards baseline for further 4 weeks. Effect may not have plateaued at 18 weeks.

Lead Author & Year of Publication	Subject Number	Intervention	Endpoint	Finding	Dosage or Clinical Relevance
Egner et al.; 2014[202]	267	262 mg GRN + 7 mg SFN with MYR from daikon in juice	Detoxification of Atmospheric pollutants via urinary excretion of the mercapturic acids of the pollutants, benzene, acrolein, and crotonaldehyde.	Increased levels of excreted glutathione-derived conjugates of toxins.	Benzene-derived mercapturic acid was higher in participants who were GSTT1-positive than in the null genotype. Implications for polymorphism (SNP) screening in individuals.
Heber et al.; 2014[203]	29	18 mg SFN from GRN extract + daikon MYR in mango juice	Nasal Allergic Response. White cell count (WBC) after exposure to diesel exhaust particles (DEP).	85% increased WBC in nasal cells 24 hours after DEP exposure.	Potential preventive therapy for those exposed to environmental inhaled toxins/particles.

Lead Author & Year of Publication	Subject Number	Intervention	Endpoint	Finding	Dosage or Clinical Relevance
Baier et al; 2014[204]	8	Claimed to be 18 mg SFN per gram fresh broccoli sprouts [d] at doses of 34, 68 and 102 g.	Activation of long terminal repeats (LTRs) as off target effects of SFN	SFN increased LTRs dose-dependently, showing peak 10-fold increase at higher dose.	May need to use caution with higher SFN doses.
Brown et al.; 2015[203]	45	18 mg SFN as GRN extract + daikon MYR in mango juice	Reduction of symptoms in moderate asthma; redox and inflammation biomarkers	SFN reduced airway resistance and reduced inflammatory markers.	Potential benefit for asthmatics.

[d] The SFN yield claimed by Baier et al is in stark disagreement with those stated by Myzak et al (2006) using the same fresh sprouts (Broccosprouts®). Refer Chapter 7 for related discussion.

Lead Author & Year of Publication	Subject Number	Intervention	Endpoint	Finding	Dosage or Clinical Relevance
Cipolla et al.; 2015[200]	78	60 mg SFN as stabilised SFN from seed extract	PSA Doubling Time in mean with biochemical recurrence after radical prostatectomy	SFN effects prominent after 3 months. PSA slopes were consistently lower in SFN group.	Potential therapy following prostatectomy where PSA gradually increases.
Atwell et al.; 2015	54	SFN not specified. GRN supplement (180 mg daily).	Breast Tissue Biopsy – presence of SFN and its mercapturic acid metabolites	No change on breast tissue tumour biomarkers. SFN metabolites present.	Confirmation of bioavailability in tissue of women with abnormal mammograms but query dose and SFN Yield.
Doss et al.;2016[205]	16	SFN not specified. Broccoli sprout homogenate (50, 100, 150 g).	Sickle Cell Disease – Nrf2 activation	Dose-dependent increase in Nrf2 target genes, *NQO1, HBG1* and *HMOX1.*	Nrf2 is not maximally activated in sickle cell disease, thereby suggesting SFN as a therapeutic option.

Lead Author & Year of Publication	Subject Number	Intervention	Endpoint	Finding	Dosage or Clinical Relevance
Wise et al; 2016[206,207]	89	Daily SFN at 4.4 mg and 26.6 mg. Whole broccoli sprout powder, described as a broccoli sprout extract (BSE).	COPD – effects on two types of respiratory cells via Nrf2	No change in Nrf2 target genes at either dose.	In diseases where Nrf2 is expressing maximally as a compensatory effect of the disease, Nrf2 activators may be ineffective.
Axellson et al.;2017 [198](Supplementary material)	97	Daily SFN of 27 mg in a 5-gram powder dose. Whole broccoli sprout powder, described as a broccoli sprout extract (BSE).	Type 2 diabetes biomarkers.	Lowered fasting glucose, Inhibition of hepatic gluconeogenesis and lowered fatty liver index. No effect in well-regulated diabetics. No effect on insulin resistance.	Greater effect in patients with elevated HOMA-IR and in obese patients. Potential treatment for those with poor glucose control.

4.9 Diversity of Effect of SFN in Various Cell Types.

Part of the difficulty in the process of establishing an appropriate dose is that *in vitro* evidence clearly shows that effects are tissue-specific and more so, specific to different cell lines of the same tissue. Table 4.2 illustrates this diversity and is also included in our published Review paper included in this thesis as Chapter 5. For example, Ritz et al.[208] showed that bronchial cells exposed to diesel extract in two bronchial cell lines responded differently to the same SFN concentration when measuring Nrf2-induced targets.

Brooks et al.[209] used six different prostate cell lines to show the broad diversity in effect of SFN. Here, normal cells produced 1.35-fold induction of NQO1 at 0.1 µM SFN and 2.46 at 1 – 3 µM but in a prostate cancer cell line, 10 µM was required to produce 4.6-fold induction. Interestingly, the group showed that simultaneous incubation with just 10 nM N-acetyl cysteine (NAC), a direct-acting antioxidant, abrogated all effects of SFN. This may be significant because NAC is available as a dietary supplement and has recently been very actively promoted to non-conventional clinicians who recommend it for a variety of off-label conditions associated with mental health.[210-216]

An animal *in vitro* study investigating tissue- and organ-specific effects with SFN exposure suggested that there may be a need for prioritizing organs when considering the chemopreventive study of SFN.[217]

These large variations are more clearly illustrated in Table 4.4 which shows the lowest effective SFN dose across a range of cell types and a brief summary of the effects.

As *in vitro* studies, these data are not directly relevant to the *in vivo* environment. However, the data linking the intracellular SFN concentrations to a measurable effect is an important step moving such dose responses closer to prediction of clinical responses. These *in vitro* findings are also essential in elucidating the mechanisms that underpin the observed effects.

Table 4.2. Summary of effects of increasing sensitivity to SFN in different tissues or cell types (ascending order)

Tissue or Cell Type	Brief Summary of Effect	Lowest Effective SFN Dose (μM)
Prostate cells, normal and cancer cell lines.[209]	SFN increased activity of GSTP1, silenced in prostate cancer cells. Also increased other Phase 2 enzymes in normal cells	0.1
Neural cortical cells.[218]	SFN protects against dopamine toxicity	0.1
Aortic cells.[219]	Significant dose-dependent induction of Nrf2 target cytoprotective genes	0.25
Bronchial epithelial cells.[208]	Upregulates Phase 2 enzymes and downregulates inflammatory cytokines	0.3
Brain – Substantia nigra cells.[220]	Significant increases in GSH and NQO1 at both doses	0 .5
Bronchial epithelial cells.[221]	Normal cells → significant increases in Nrf2 targets; no effects on cancerous cells	0.5
Retinal epithelial cells.[222]	Linear cytoprotective effects with increasing SFN dose	1.25
Pancreatic β-cells.[223]	Protection against cytokine-induced cell death	2.5

Endothelial cells - arteries and veins.[224]	SFN protective against endothelial lipase at lower dose	2.5 µM (arterial) 10 µM (venous)
Cardiomyocytes.[225]	Greatest effects with SFN exposure up to 48 hours	5 µM
Prostate cancer cells.[226]	Strong Cell Cycle Arrest in Prostate Cancer	10 µM. (No effect at <3 µM)

4.10 Major Actions of SFN at the Cellular Level

The major documented cellular actions SFN are listed in the non-exhaustive summary shown in Table 4.3 along with commentary on their clinical implications. These *upstream* processes have significant *downstream* effects and are associated with the observed effects in clinical trials using SFN or a dietary source of SFN.

Table 4.3 Summary of Clinically-Relevant Actions of SFN

	ACTION	CLINICAL IMPLICATIONS
1.	Increases synthesis of Glutathione.[227]	This has implications for oxidative stress and detoxification as glutathione is the substrate for both pathways. Glutathione is also an antioxidant in its own right.
2.	Inhibits some Phase 1 detoxification enzymes that activate chemical carcinogens.[228]	This reduces the level of toxic intermediates with carcinogenic potential. It also allows Phase 2 to 'keep pace' with Phase 1 processing.
3.	Increases activity of Phase 2 detoxification enzymes. Sulforaphane is considered the most potent of the Phase 2 inducing substances.[27]	As a *mono*functional inducer, sulforaphane is considered to be a significant component of the anticarcinogenic action of broccoli.
4.	Provides significant antioxidant activity, largely due to its ability to induce glutathione synthesis.	Glutathione is a critical factor in protecting organisms against toxicity and disease.[229] The ability of sulforaphane to upregulate glutathione synthesis is highly significant.
5.	Acts as a Histone Deacetylase inhibitor, providing DNA protection.[230, 231, 232.]	Development of Histone Deacetylase inhibitors is a key avenue for cancer drug research.

6.	Induces apoptosis, inhibits MMP-2 (metastasis), inhibits angiogenesis, cell cycle arrest[40,158,233, 234] (interacts at several levels).	Therapeutic interventions which exhibit several related actions targeting the same underlying defect are considered highly desirable.
7.	Limits pro-inflammatory effects of diesel chemicals by upregulation of Phase 2 enzymes.[208]	Environmental pollutants are known to contribute to various lung diseases. Removal of the toxins reduces tendency to disease.
8.	Induces Thioredoxin (Trx) as part of the ARE.	Thioredoxin is implicated in cardioprotection by triggering several *survival* proteins.[235] Sulforaphane may have beneficial effects in cardiovascular disease.
9.	Bactericidal against *Helicobacter pylori* and also blocks gastric tumour formation in animals.[236]	Helicobacter is known to contribute to development of stomach cancer. Elimination of the organism without the use of typical antimicrobial *Triple Therapy* could protect the colonic microflora.
10.	Protects dopaminergic cells from cytotoxicity and subsequent neuronal death (cell culture).[237]	Dopaminergic neurones are associated with Parkinson's' Disease. Pharmaceuticals to treat Parkinsonism are not without risk and the disease is not usually detected until more than 50% of the neurones have been lost. A chemoprotective tool could prevent premature loss.

11.	Increases p-53 (associated with tumour suppression) and bax protein expression, thereby enhancing cellular protection against cancer.[238]	Sulforaphane is an attractive chemotherapeutic agent for tumours with a p53 mutation.[157]
12.	Limits effect of Aflatoxin on Liver Cells.[38]	Interventions which can offer significant protection against environmental and food-borne pollutants could prevent the consequences of these factors. Appropriate doses of Sulforaphane-yielding substances are yet to be determined.
13.	Enhances Natural Killer Cell activity and other markers of enhanced immune function.[227]	The immune system is a critical part of the body's defences against inflammatory as well as infectious diseases. Most diseases benefit from enhancement to immune function.
14.	Suppresses NF-kB, a key regulator of inflammation.[227] NF-kB expression is downregulated by sulforaphane and as such downregulates inducible pro-inflammatory enzymes such as Cyclo-oxygenase (COX-2) and NO synthase (iNOS).	As an inhibitor of NF-kB as well as an activator of Nrf2, SF modulates many cancer-related events, including susceptibility to carcinogens, cell death, cell cycle, angiogenesis, invasion and metastasis.[227]
15.	Sulforaphane is not directly *anti*-oxidant. Instead it exhibits a weak *pro-oxidant* effect.[239]	Because sulforaphane is not directly antioxidant but exerts its antioxidant effect primarily by induction of glutathione and other antioxidant compounds, it is considered to exhibit an *indirect* antioxidant effect.

16.	Potent inducer of HO-1, Haemoxygenase-1.	Haemoxygenase-1 plays an important role in modulating the effects of oxidants in the lungs.[240]

4.11 Cruciferous Vegetables, Composition and Bioactivity

Cruciferous vegetables are known to contain high but variable levels of glucosinolates.[241] Glucosinolates (GSN) are not inherently bioactive but become so when enzymatically converted to their individual isothiocyanates (ITCs) in a hydrolysis reaction catalysed by the enzyme, MYR.[242] GSNs found in the *Brassica* species exhibit their own characteristic properties; the broccoli-derived GRN is the best-known precursor to the bioactive compound, SFN but not the only one.[26] Glucoerucin found in Rocket *(Eruca sativa Mill)* sometimes called 'arugula' is also a source of SFN.[243] SFN is the ITC derived from GRN via a MYR-catalysed hydrolysis reaction. Minimal quantities of GRN are found in several other *Brassica* species.[26]

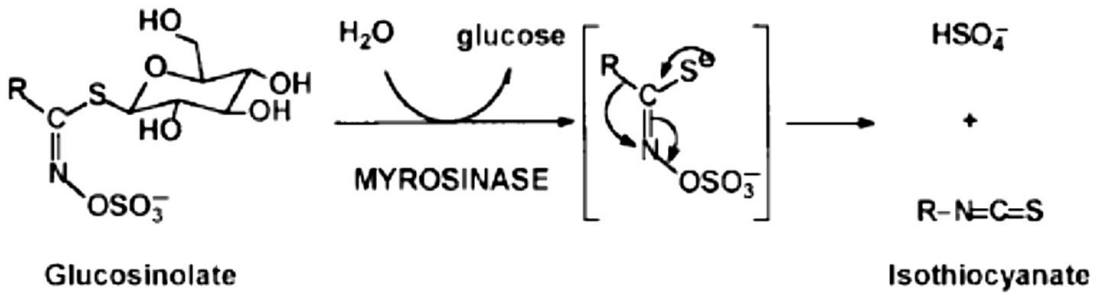


Figure 4.4 ***Conversion of Glucosinolates to Isothiocyanates in the presence of the enzyme, MYR.[242]***

In a hydrolysis reaction, glucose is cleaved from the glucosinolate and after a rearrangement, the ITC is produced. GRN is the GSN from which SFN is derived.

4.11.1 Stability of Bioactives and Effects of Cooking

GSNs are compartmentalised in vacuoles within the plant cell and separated from the MYR which catalyses its hydrolysis. In this way, the GSN remains inactive until the cell structure is disrupted.[242] When the vegetable is cut, chewed or macerated in any way, the enzyme is released from its protective vacuole and immediately reacts with the GSN.

The resulting product is the bioactive ITC shown in Figure 4.4. Once produced, the ITC is relatively unstable with a half-life of around 1.8 hours in humans.[244]

Cooking and especially prolonged cooking denatures the enzyme.[245,246] It becomes clear that for cruciferous vegetables to retain their bioactivity and maintain their efficacy, they must be handled carefully and with thorough understanding of the factors associated with their degradation.[247,248]

MYR activity from broccoli vegetable has been reported to be thermolabile, with loss of approximately 90% activity after only 2 minutes of microwave cooking.[246] Boiling and microwaving cause an initial loss of the MYR-inhibiting SFN nitrile, with a concomitant increase in SFN, followed by loss of SFN, all within 1 minute.[249] By contrast, steaming enhanced SFN Yield between 1.0 and 3.0 min in some broccoli cultivars.[249]

In contrast, it appears that MYR in broccoli sprouts and seeds is much less thermolabile than that of the mature vegetable[250]; nevertheless, the subtle differences in vegetable cooking method and times makes it virtually impossible for a consumer to be assured that MYR activity has been preserved.

4.11.2 Effect of a MYR Inhibitor

To compound the issue of variation in SFN Yield, broccoli seeds and the plants derived from them contain a thermolabile MYR inhibitor, Epithiospecifier protein (ESP)[251,1] which converts a proportion of the GRN to a less active ITC, SFN nitrile (Figure 4.5).

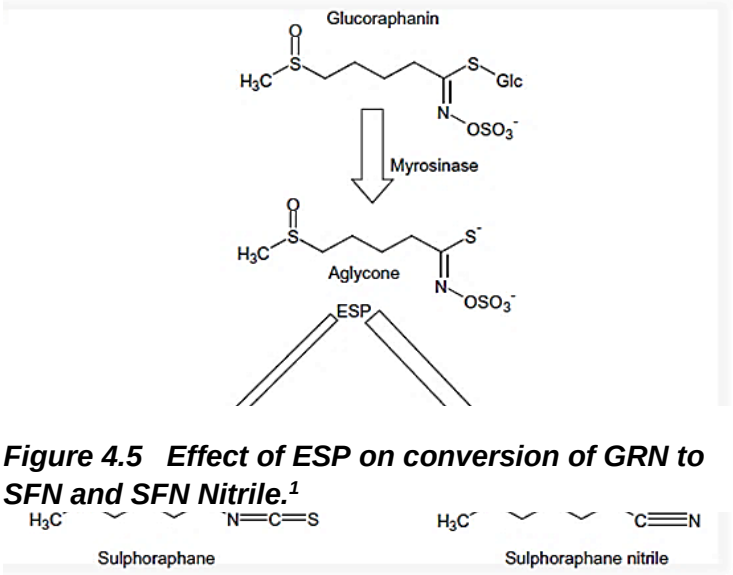


Figure 4.5 Effect of ESP on conversion of GRN to SFN and SFN Nitrile.[1]

Measuring only GRN may lead to erroneous assumptions regarding the expected SFN Yield.

A detailed knowledge of these processes is essential in the production of an enzyme-active broccoli sprout raw material to be used in clinical trials.

In studies which use fresh or dried broccoli sprouts as the intervention, it is clearly essential to characterise the product for its SFN Yield and not just its GRN

content. Failure to do so will impede the ability to interpret clinical findings; these issues are detailed in Chapter 5.

4.11.3 Comparative Glucosinolate Quantity of Mature Broccoli vs the Sprout

SFN is the most potent naturally-occurring monofunctional inducer of Phase 2 enzymes[209] and has been estimated to be around 10 times greater in its inducer activity than other GSNs.[252] The highest source of SFN Potential has been found in the young germinated seedling of certain cultivars of *Brassica* oleracea *italica* (broccoli), with as much as 10 to100-fold the quantity of GRN per gram than found in the mature vegetable.[169]

As the seedling matures, its levels of GRN proportionately diminish within the increasing plant volume,[253] leading to substantial research interest in the young broccoli sprout. A separate line of research has focused on the development of broccoli vegetable with higher GRN content; this has been developed by hybridisation and the vegetable has been subsequently commercialised.[254] Broccoli sprouts as young as 3 days have been used to supply GRN in a number of studies and more recently MYR-inert, GRN-containing extracts of broccoli seeds and sprouts have become commercially-available; the clinical relevance of the MYR-active and MYR-inactive forms is discussed in Chapter 5 and 6.

4.11.4 Variation in Composition of Glucosinolates in Mature Broccoli vs the Sprout

GSNs can be classified into a number of chemical classes on the basis of their structural similarities. The most extensively studied GSNs are the aliphatic, ω-methylthioalkyl, aromatic and heterocyclic (e.g. indole) forms, typical of those found in the Brassica vegetables.[255] Individual *Brassica* plant species contain mixtures of GSN types and these vary significantly between and within species.[255] Not all GSNs yield isothiocyanates with health-promoting properties.[256]

The mature broccoli vegetable contains higher levels of a GSN, glucobrassicin which generates indole-3-carbinol (I-3-C). I-3-C as a bifunctional inducer, upregulates Phase 1 and to a very limited extent Phase 2 detoxification enzymes, whereas SFN as a monofunctional inducer, more significantly induces Phase 2 enzymes.[257] The sprouted

broccoli seed contains high levels of GRN but little to no glucobrassicin, whereas the mature plant contains comparatively much less of the SFN precursor, GRN.[253]

Significantly, the indoles are virtually absent in the broccoli sprout compared with their abundance in the mature vegetable. More specifically, seeds or young sprouts of broccoli (Brassica oleracea var. italica) can contain 70±100 mmol total GSN per gram fresh wt., with <1% contributed by indole glucosinolates and the balance consisting almost entirely of the aliphatic GSNs, GRN, glucoerucin and glucoiberin.[255]

4.11.5 Variability in Glucosinolate and Isothiocyanate Content and Effect

Broccoli oleracea varieties are known to vary widely in their content of bioactives and inhibitor.[256] The most commonly-consumed cruciferous vegetable is broccoli, *Brassica oleracea* var. *italica*.[258] Within this classification are further sub-groups known as cultivars. Analysis of the GRN and SFN Yield varies significantly between the cultivars.[256,259]

A 2003 report showed that only 20% of the GRN in the Marathon cultivar is converted to SFN with 80% converting to the inactive sulforaphane nitrile.[260] In other cultivars, conversion to the inactive SFN nitrile in each case was greater than 50%.[251] At supermarket level, it is not possible for a consumer (or even the merchant) to make an informed choice without knowing what variety or cultivar is being offered.

Some species contain high levels of other GSNs including the potentially thyrotoxic progoitrin which prevents the incorporation of iodine into thyroxine.[261] Indole compounds such as indole-3-carbinol and 3–acetonitrile are formed from the hydrolysis of indole glucosinolates and these have the potential to form mutagenic N-nitroso compounds.[262] However, others suggest that the combined effects of the total GSN composition of a whole crucifer may be quite different from that of an isolated compound and that synergy has been demonstrated when assessing the effects of such combinations.[253]

In considering cruciferous plants as suitable sources of medicinal compounds with clinical efficacy, these factors are among the more important to be considered.

4.12 SFN – Its Potential Clinical Relevance

4.12.1 Nrf2 and Endogenous Cellular Defence Mechanisms

The non-enzyme antioxidant GSH is a major contributor to cellular redox status and the rate-limiting enzyme for its synthesis, glutamate-cysteine ligase (coded by the gene *GCL*) can be induced by SFN.[263] Antioxidants in general and glutathione in particular can be depleted rapidly under conditions of oxidative stress and this can signal inflammatory pathways associated with NF-kB.[264] Nrf2 has been found to be the primary factor inducing the cell's survival system under GSH depletion.[265] Also of interest is the finding that Nrf2 transcriptional activity declines with age,[266] leading to age-related GSH loss among other losses associated with Nrf2-activated genes. This effect has implications too for decline in vascular function with age.[267]

Some of the age-related decline in function can be restored with Nrf2 activation by SFN.[268] Studies in aged mice showed that age-related changes in Th1 immunity could be restored using SFN as an intervention. This finding is compatible with the growing recognition of the importance of the Nrf2 pathway in innate immunity and has implications for human health.[269] As Nrf2 targets, other cytoprotective enzymes such as HO-1 and Trx are also supportive of cellular redox modulation.

4.12.2 Phase 1 vs Phase 2 Detoxification Pathways

As long ago as 1993,[171] it was determined that the ideal chemoprotective compounds are monofunctional inducers of Phase 2 detoxification enzymes. Monofunctional inducers function by upregulating Phase 2 detoxification pathways to metabolise the oxidative and carcinogen-activating products of the Phase 1 enzymes, without having any significant effect on Phase 1 activity itself. Toxins presented to the Phase 1 enzymes produce intermediate compounds which are sometimes more toxic to cells than the initial toxin.[270] It is therefore important that Phase 2 is sufficiently active that the intermediate products cannot accumulate in the cellular environment. Figure 4.6 illustrates the Phase 1 and Phase 2 detoxification pathways.[271] The majority of chemical carcinogens require metabolic activation before they can initiate cancer.[272]

As a monofunctional inducer, SFN has been described as an ideal detoxifier as its effect on Phase 1 is minimal compared with its significant activity on Phase 2.[273] By comparison, many of the most potent of the synthetic SFN analogues[274] are bifunctional inducers and not the monofunctional inducers having the most chemopreventive effect. Several synthetic compounds[275] have been investigated for their chemopreventive

potential against lung cancer in smokers.[175] Human cells are adapted to naturally-occurring L-sulforaphane; they are not necessarily adapted to synthetic analogues. SFN's role as the most potent inducer of the Phase 2 enzyme NADPH: oxido-reductase (NQO1) is discussed in detail in Chapter 6.

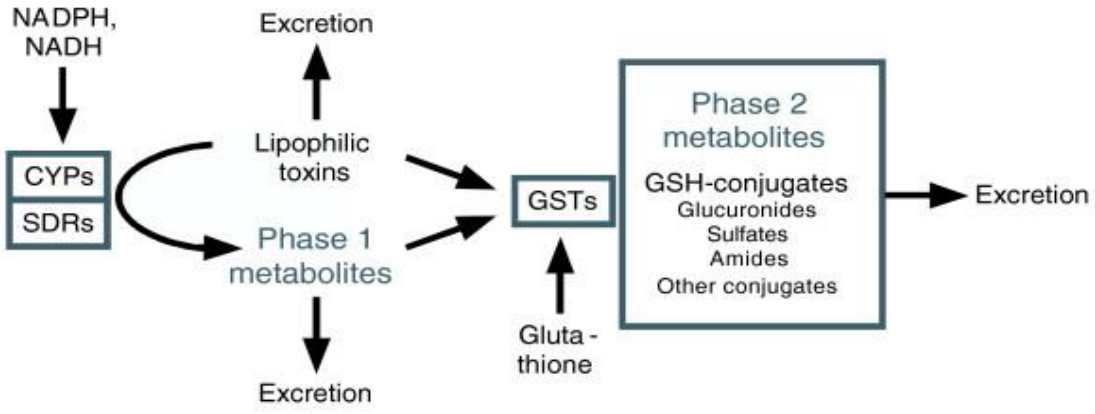


Figure 4.6 Interaction of Phase 1 and Phase 2 metabolites in detoxification

The process of cellular detoxification of both exogenous and endogenous factors entails two phases: Phase 1 (oxidative activation reactions), and Phase 2 (conjugative reactions), effected by several large and diverse gene families. [276]

4.12.3 Significance of Induction of Phase 1 and Phase 2 Detoxification Enzymes

Not all Brassica-derived compounds are monofunctional inducers. Indole-3-carbinol (I-3-C) derived from the mature broccoli vegetable is a bifunctional inducer and as such may lead to the generation of highly toxic intermediate compounds which may overwhelm the capacity of the localised antioxidants to quench them or the Phase 2 processes to detoxify them.[277]

By contrast, SFN selectively upregulates Phase 2 detoxification enzymes, minimising the risk of generating excessive amounts of reactive intermediates (Figure 4.6).[276] As a consequence, although some I-3-C animal studies show an anticarcinogenic effect, other studies using I-3-C show it to have carcinogenic potential where comparable studies using SFN do not.[278,279,280] It should be noted that the comparatively small quantity of I-3-C generated from the GSNs in broccoli vegetable is unlikely to replicate the effects of isolated I-3-C concentrations used in cell culture studies.[26]

4.13 Exploring SFN's Clinical Potential in Relation to *Upstream* Mechanisms

With the knowledge that Nrf2 has broad *upstream* effects on endogenous cellular defence processes, it is to be expected that SFN's clinical effects will be applicable to a broad range of conditions. Endothelial dysfunction adversely affects circulation as described earlier and is known to be a major pathophysiological factor in type 2 diabetes (T2DM). For this reason, T2DM has been selected to model the potential for SFN in chronic disease states underpinned by endothelial dysfunction.

*4.**13**.1 Type 2 Diabetes as a Model to Explore Putative Mechanisms*

The first clinical trial using quantified SFN as the intervention material in T2DM was published in 2011.[197] An earlier 2004 study by another group examined markers of metabolic syndrome but did not quantify SFN in a fresh broccoli sprout intervention.[37] A dose of 10 g of a 0.4% SFN-yielding broccoli sprout powder demonstrated beneficial effects in several cardio-metabolic biomarkers. The trial did not investigate the mechanism to explain the effects but other researchers have hypothesised on the *upstream* etiological factors that closely link T2DM and cardiovascular disease (CVD).[4,6,281]

Uncontrolled T2DM typically results in microvascular complications that significantly contribute morbidity and mortality, with one-third of patients with end-stage kidney disease being diabetic.[282]

4.13.2 Effectiveness of Current Therapy

Although the mainstay of diabetic treatment is in glucose-lowering, there is evidence to suggest that intensive glucose-lowering therapy has no significant effect on the rates of major cardiovascular events, death, or microvascular complications in T2DM patients with longstanding disease.[283, 284] Intensive glucose-lowering may potentially-harmful.[285] Intensive lifestyle modification as an intervention strategy has been shown to be at least comparable[286] in its effect to the drug, metformin, typically the first pharmaceutical a patient will be prescribed. Both metformin therapy and intensive lifestyle intervention reduced the risk of developing diabetes (by 31% and 58%, respectively, in comparison with placebo), showing that it may be possible to use a lifestyle-only approach to reduce the risk of T2DM.

4.13.3 Searching for an Upstream Etiological Factor

An evolving paradigm in the putative etiology of T2DM relates to oxidative stress as being the primary *upstream factor*[4,6,3,287] from which all other manifestations of the disease ensue. Furthermore, it has been stated[288] that oxidative stress as the "root cause" underlies all aspects of the condition: i.e. the development of insulin resistance, beta-cell dysfunction, impaired glucose tolerance and T2DM itself (Figure 4.7).

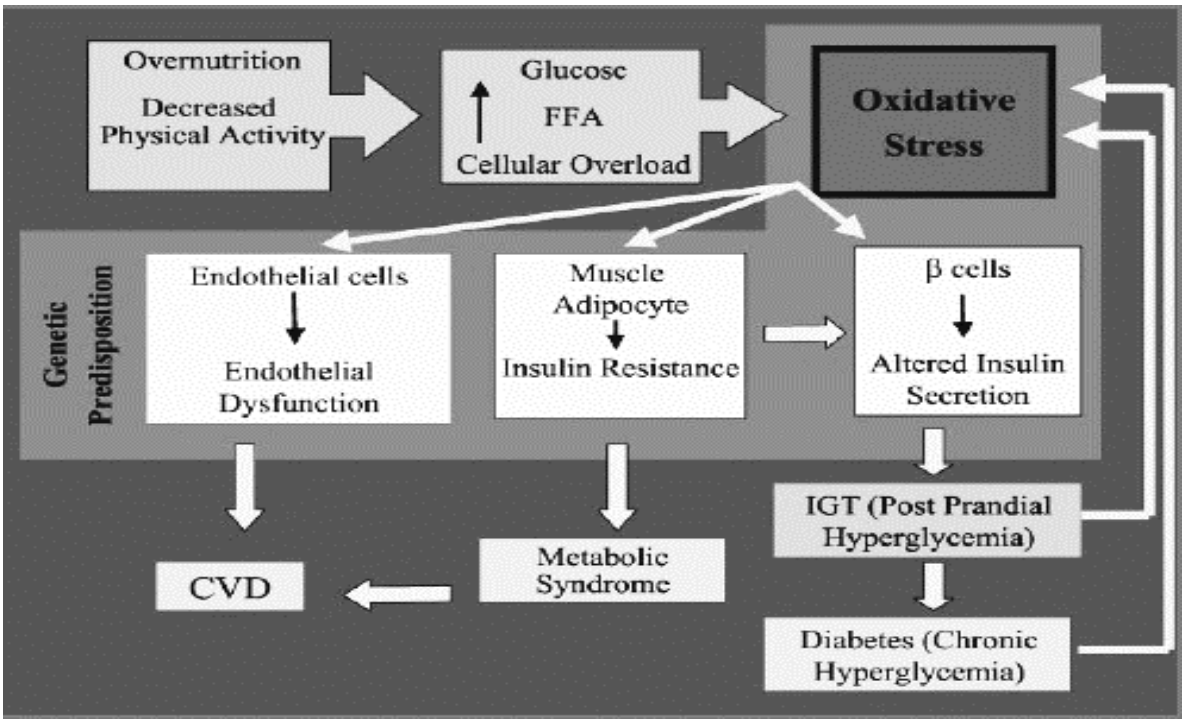


Figure 4.7 Oxidative Stress as a putative upstream cause of T2DM and its complications[4].

The primary causes, overnutrition and decreased physical activity create an oxidative environment which affects endothelial cells, muscle cells and adipocytes and pancreatic β-cells, each contributing to the disease presentation.

4.13.4 Vulnerability of Endothelium and Pancreas

At least six biochemical pathways contribute to oxidative stress in the pancreas and endothelia.[289,290] Pancreatic beta-cells are equipped with about 50% of the SOD activity of liver cells. GPx and Cat activity are even lower at 1%, making beta-cells highly susceptible to the effects of ROS.

Glucose is transported[291] between bloodstream and cells of both endothelium and pancreatic beta-cells via a concentration gradient and facilitated by insulin-independent transcription factors; GLUT-1 in endothelia and GLUT- 2 in beta-cells. This process

being insulin-*independent*, both endothelia and beta-cells are exposed to high glucose levels during hyperglycaemia. This is unlike the situation in muscles and adipose cells which use an insulin-*dependent* GLUT- 4 transporter and are better protected against the effects of ROS.[292]

4.13.5 Influencing the Gene-Environment Interaction

The completion of the Human Genome Project in 2003 led to a surge of interest in cataloguing genes associated with particular diseases. In 2007, it was thought that there were more than 50 genes known to be associated with T2DM, although these genes proved to be low-penetrance and not the high-penetrance genes that were expected to target the disease.[293] In the last decade, other genes have been identified through gene expression studies and these more recent studies have reaffirmed the common *upstream* genetic associations between diabetic cardiovascular and renal complications.[294] Much of the association is localised in the endothelium, such that endothelial dysfunction is a common *upstream* etiological event.

A study[295] using human microvascular endothelial (HMEC-1) cells showed that in the presence of hyperglycaemia, SFN was able to prevent biochemical dysfunction via multiple pathways. SFN was shown to activate Nrf2 with consequent increases in the cellular enzymes, *transketolase* and *glutathione reductase* after a concentration of 4 µmol/l-sulforaphane was added to the cells in the presence of 5 µmol/l glucose.

4.13.6 Are Antioxidant Vitamin Supplements a Logical Therapy in T2DM Patients?

Given that oxidative stress is emerging as the primary *upstream* factor in T2DM, antioxidants have been considered as likely therapeutic agents. Perhaps surprisingly, these trials have been almost uniformly disappointing in their results. A 2010 analysis[296] of major randomised placebo-controlled trials (98,886 subjects in total – Table 4.5) investigating the effects of antioxidant supplementation on prevention of diabetes or glucose homeostasis showed no effect from vitamin E, vitamin C, beta-carotene, selenium, zinc and combinations of these.

Table 4.5 Major randomised placebo-control trials investigating the effects of antioxidant supplement on prevention of diabetes or glucose homeostasis. (Table 1 from Chang and Chuang; 2010)[296]

Study	Study Population	Duration (Years)	Antioxidants (daily dose)	Endpoint	Results
Women Health Study	38,716 healthy US women	10	Vitamin E (α-tocopherol 600 IU)	Incident diabetes	No effect
Women's Antioxidant Cardiovascular Study	6,574 non-diabetic US women at high risk of cardiovascular disease	9.2	Vitamin E (α-tocopherol 300 IU), vitamin C (500 mg), and beta-carotene (25 mg)	Incident diabetes	No effect
Physician Health Study	22,071 healthy US male physician	12	Beta-carotene (25 mg)	Incident diabetes	No effect
Aalpha-Tocopherol, Beta-Carotene Cancer Prevention Study	27,379 non-diabetic male Finnish smokers	12.5	Vitamin E(α-tocopherol 50 mg) and Beta-carotene (20 mg)	Incident diabetes	No effect
Supplementation with Antioxidant Vitamins and Minerals study	3,146 non-diabetic French	7.5	Vitamin C (120mg),Vitamin E (30mg) Beta-carotene (6mg), Selenium (100µg), and Zn (20mg)	Fasting glucose	No effect

4.14 Can Endogenously-generated Antioxidants Play a Role?

Given the role of SFN in induction of Nrf2-dependent cytoprotective genes, SFN might be a useful candidate for modulation of *upstream* genes associated with the etiology of T2DM. A 2016 review paper reaffirms the rationale for the 'unifying hypothesis' proposed by Brownlee in 2001[281] in which generation of ROS is the key central theme linking the pathogenesis of T2DM and CVD.[294] In further support of this hypothesis, Rask-Madsen and King reinforce the possibility that endogenous protective pathways could protect against vascular complications in T2DM.[297]

The Nrf2-dependent target genes of possible relevance are those encoding synthesis of GSH, Trx, HO-1 and NQO-1. Each has been shown to be induced by SFN in a variety of cell types, including endothelial cells.[298,299] In addition, SFN has been shown to inhibit NF-kB in endothelial cells,[300] an effect which may retard inflammation in these and other cell types. A 2006 study[174] using human aortic cells showed that activation of the Nrf2-ARE pathway may represent a novel therapeutic approach for the treatment of inflammatory diseases such as atherosclerosis.

In support of this approach, a 2009 combined cell culture/animal study[173] showed that shear stress in blood vessels keeps Nrf2 in an activated state and as such protects against endothelial function. Activated by SFN, Nrf2 was shown to prevent endothelial cells from exhibiting a pro-inflammatory state via the suppression of p38–VCAM-1 signalling, providing a novel therapeutic strategy to prevent or reduce atherosclerosis.

In other tissues of the cardiovascular system, Nrf2 has been shown to regulate both basal and inducible ARE-controlled cytoprotective genes in cardiomyocytes.[301] As with endothelia, Nrf2 is required for protection against glucose-induced oxidative stress and cardiomyopathy in the heart.

4.14.1 Glutathione

Disturbances of thiol-related mechanisms have been observed[302] in diabetes, with plasma levels of protein-bound thiols lower in T2DM than in controls. These thiols include GSH and Trx. An animal study[303] illustrates the relationship between depressed GSH and the development of atherosclerosis. In this experiment[4], the rate-limiting enzyme needed to synthesise GSH, (*gamma-glutamyl cysteine synthetase*) was shown to be downregulated *early* in the atherosclerosis process. This effect preceded the appearance of lipid peroxidation products by several months. The antioxidant enzyme, GPx was simultaneously downregulated.

The researchers suggest that "*depressed glutathione generation is a key early event and may lead to oxidative alterations involved in the development of coronary artery disease. This suggests that glutathione depression is a contributory factor in oxidative stress rather than a consequence of increased cellular exposure to oxidant species."*

In a study on spontaneously-hypertensive rats, SFN (0.05±1 µmol/l) induced significant and concentration-dependent increases in cellular GSH levels[304], HO-1 protein content and activities of GSH-reductase and GPx in vascular smooth muscle cells. Whether this SFN concentration would have the same effect in humans is yet to be determined.

Erythrocyte levels of GSH have been shown to change depending on the stage of the diabetic process of the individual.[305] Australian research[306] has shown that compared to controls, pre-diabetic patients exhibit a significant lowering of GSH. As the disease progresses to diabetes and later to diabetes with cardiovascular complications, GSH

levels rise; however, they don't reach the levels of controls. The variability in GSH levels depending on the stage of the disease makes it difficult to use GSH as an effective biomarker to measure change in clinical trials.

An infusion of GSH as an intervention in a clinical trial[307] was shown to reverse endothelial dysfunction by strongly potentiating the effect of acetylcholine-mediated vasodilation via enhanced nitric oxide activity. Because GSH as a tripeptide molecule is degraded by gastric proteolytic enzymes, it is not suitable as an oral therapeutic.[308] If sulforaphane can be shown to induce GSH in endothelial cells, this may provide an alternative means of enhancing GSH levels in endothelial and pancreatic beta-cells with a view to reducing the complications of T2DM.

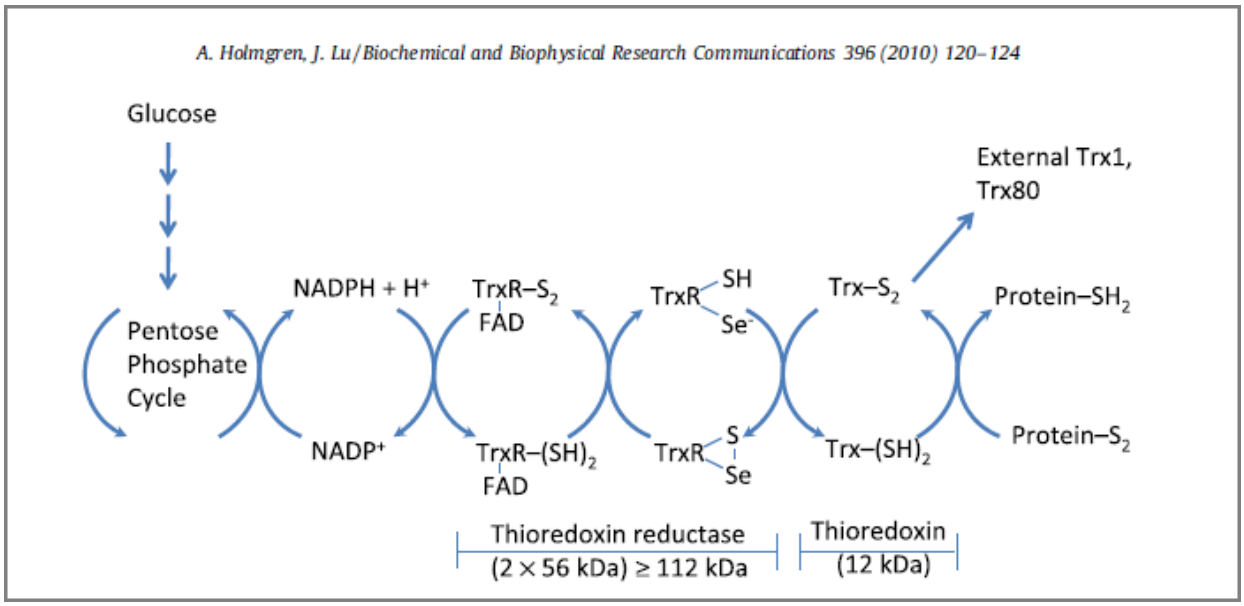
4.14.2 Thioredoxin – Protection from Elevated Blood Glucose

Thioredoxin (Trx) is a potent protein disulfide that participates in many thiol-dependent cellular reductive processes and plays an important role in antioxidant defence, signal transduction and regulation of cell growth and proliferation. As a cellular thiol, Trx has been shown[281] to be associated with the development of diabetic complications. Like GSH, Trx has been shown to protect cells against high ambient glucose.[309] Trx is at its highest levels in metabolically active tissues like the heart and it is critical for normal heart function.

The thioredoxin system (Figure 4.8) consists of thioredoxin, thioredoxin reductase and NAD(P)H. Like GSH, Trx contributes to the cellular thiol pool[310] with the thioredoxin system shown to exhibit *cardio*-protective effects.[311] The pentose phosphate pathway which generates reducing equivalents in the form of nicotinamide adenine dinucleotide phosphate (NADPH) can alleviate much of the oxidative stress created by excess glucose. By upregulating Trx, SFN contributes to cardioprotection by this additional mechanism.

Figure 4.8. The thioredoxin system and its relationship with glucose metabolism in the pentose phosphate cycle. [312]

The pentose phosphate cycle generates reducing equivalents which are transferred along a series of cycling redox reactions. Induction of Trx and Trx reductase by SFN enables glucose to be metabolised as an alternative to the synthesis of superoxide radical, thereby alleviating much of the metabolic stress associated with T2DM.



There are few studies to associate SFN with heart disease but significant cardioprotection was demonstrated in an animal study[235] using fresh broccoli homogenate. Changes included improved post-ischaemic ventricular function, reduced myocardial infarct size and decreased cardiomyocyte apoptosis after the rats were sacrificed. These findings correlated with increased levels of Trx as well as HO-1, with the function of the latter discussed in relation to SFN in 4.14.7.

A 1997 study[313] investigating the role of thioredoxin in vascular biology describes the induction of mitochondrial MnSOD by Trx. The researchers also comment that Trx reduces and protects the function of several classes of proteins during oxidative stress. These include proteins important in cell homeostasis and intermediary metabolism such as glyceraldehyde-3-phosphate dehydrogenase(GADPH),[6] an enzyme which has a 'gatekeeper' function in glucose regulation. In addition, Trx influences hormones such as insulin as well as glucocorticoid receptors and other proteins such as endothelial nitric oxide synthase (NOS) and signalling proteins such as transcription factors. The findings of a Phase 1 clinical trial[37] demonstrated that 100 g of fresh broccoli sprouts over a 7-day period provided cardiovascular benefits which included favourable changes in blood

lipids as well as reduction in biomarkers of oxidative stress. This study however did not assay the broccoli sprouts for their sulforaphane yield, limiting its usefulness.

4.14.3 Quinone Reductase

NQO1, often described simply as Quinone reductase (or QR) is emerging as an Nrf2-induced enzyme with broad cytoprotective properties. A paper[314] published almost two decades ago claims that *an extensive body of evidence supports the conclusion that catalysing obligatory two-electron reductions of quinones to hydroquinones, (NQO1) protects cells against the deleterious effects of redox cycling of quinones and their ability to deplete glutathione.* The same researchers[30] have since published again on this topic discussing what they describe as *a multifunctional antioxidant enzyme and exceptionally versatile cytoprotector.* They suggest too that NQO1 with cytoprotective roles which extend well beyond its catalytic function could be considered as a 'marker cytoprotective enzyme'. Further, they state that *NQO1 is one of the most consistently and robustly inducible genes among members of the cytoprotective proteins.*

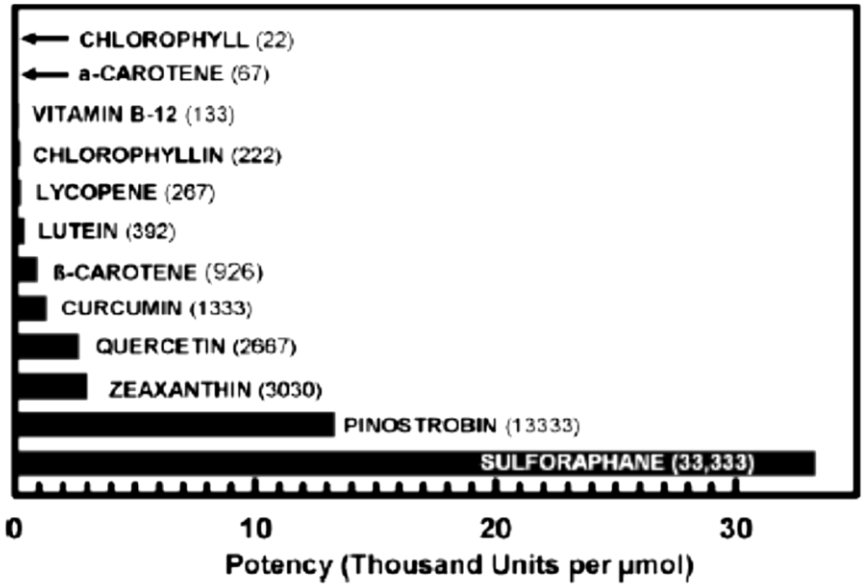


Figure 4.9. Induction of the chemoprotective enzyme NQO1 by phytochemicals in cell culture.

The comparatively much higher induction by SFN against popular plant-derived supplements is evident.[32]

NQO1's antioxidant capacity extends to scavenging superoxide directly,[315] albeit not as efficiently as does SOD. As well as its antioxidant properties, NQO1 is also a key Phase 2 detoxification enzyme, capable of being induced by SFN. It has been claimed here and

elsewhere that sulforaphane is the most potent naturally-occurring inducer of of this enzyme (Figure 4.9).[32,316]

4.14.4 Other Functions of NQO1

NQO1's other functions extend to the maintenance of quinones, Coenzyme Q 10 (Ubiquinone) and vitamin E in their reduced active forms.[30,317] Induction of NQO1 by SFN also co-ordinately induces[168] genes encoding cellular NADPH-regenerating enzymes such as glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and malic enzyme. NADPH in turn assists in maintaining GSH in its reduced state. NQO1, a highly-inducible enzyme provides major antioxidant functions by virtue of its two-electron reduction mechanism; this diverts quinones from participating in oxidative recycling and production of ROS.[30]

4.14.5 Pharmacokinetics of NQO1 Following Induction by SFN

(a) Sulforaphane and its metabolites Women were given a single serve of broccoli sprout homogenate containing 200 μmols SFN one hour before reduction mammoplasty.[183]

Figure 4.10 shows that SFN peaks in the blood at around 1 hour, with a subsequent peak at 12 hours and then declining until 48 hours, in both plasma and tissue. Knowledge of these relationships contributes valuably to the determination of a dose-response for SFN in various tissues and under different conditions.

(Special Note: It would appear that the units on the right *y axis* are incorrect in listing pmol/mg; with respect to the left y axis, the correct units are more likely to be pmol/g.)

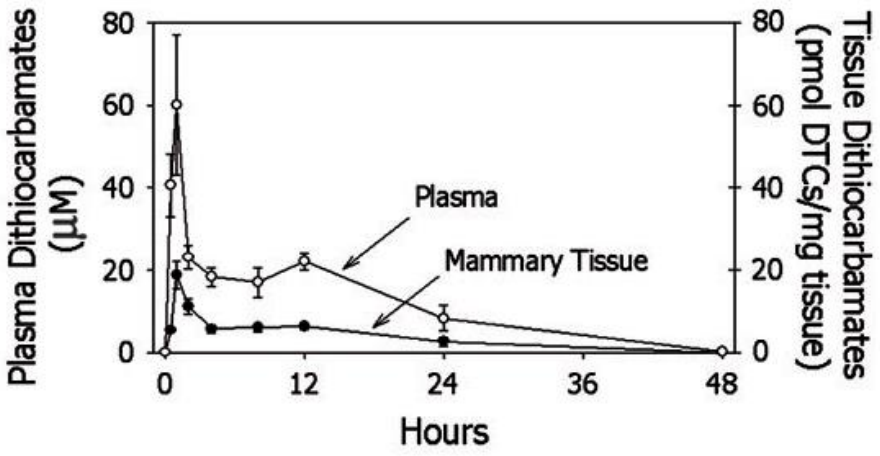


Figure 4.10 Plasma and tissue dithiocarbamate levels over time following sulforaphane ingestion. [183]

The pharmacokinetics of the SFN plasma dithiocarbamate (DTC) levels are shown in μM concentrations on the left vertical axis and the tissue pmol DTC concentrations on the right vertical axis, illustrating the significantly lower concentrations available to cells.

(b) Transcripts of NQO1 and Haemoygenase-1 (HO-1). In the same study, NQO-1 and HO-1 transcripts both peaked at around 12 hours, declining until around 48 hours (Figure 4.11)

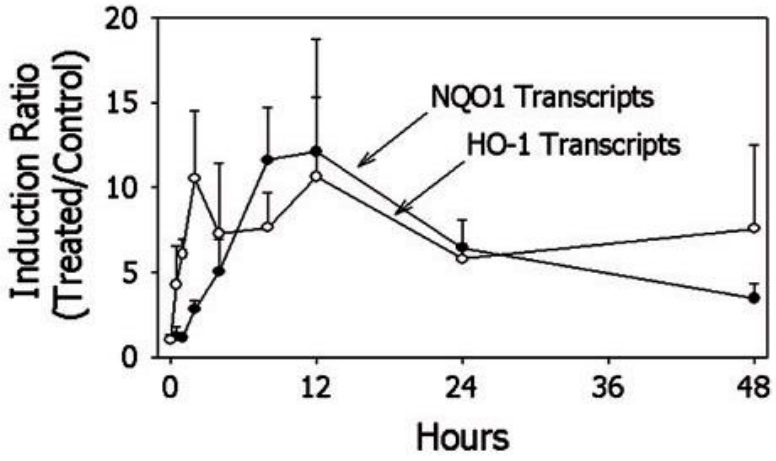


Figure 4.11. Plasma and tissue NQO-1 and HO-1 transcripts over time following SFN ingestion. [318]

Both NQO-1 and HO-1 transcripts reached their maxima at 12 hours. However, HO-1 transcripts remained more elevated than NQO-1 at 12 hours.

(c) NQO1 activity Maximal induction of NQO-1 occurs at around 24 hours, declining over time. This peak represents an approximate 2.8-fold induction (Figure 4.12).

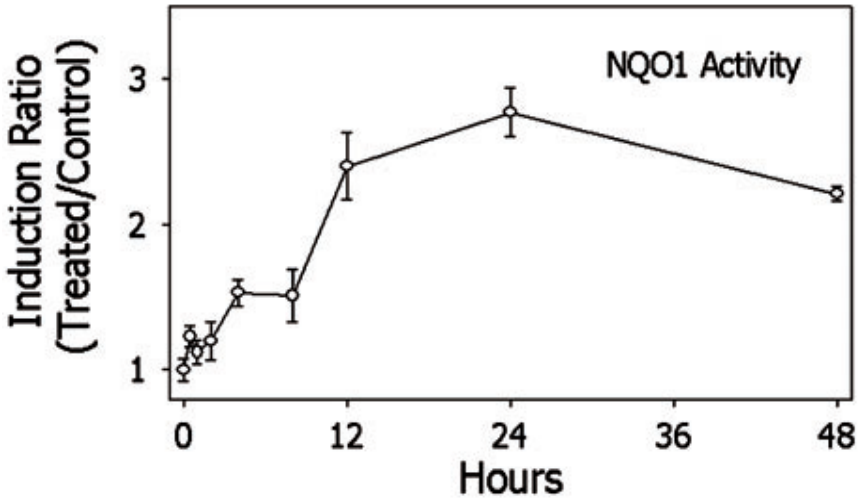


Figure 4.12 NQO-1 activity over time following sulforaphane ingestion.[183]

4.14.6 The Effect of NQO1 Polymorphisms

The relationship between the risk of *NQO1* C609T polymorphisms and carotid artery atherosclerosis in patients with type 2 diabetes has been investigated.[319] The major finding of the study was that the *NQO1* C609T polymorphism is associated with carotid atherosclerosis in patients with T2DM. The gene coding for NQO1 has a genetic polymorphism[320] (C → T) at nucleotide position 609. This polymorphism was shown to reduce NQO1 enzyme activity, thereby diminishing the protection provided by NQO1. Whether diminished NQO1 activity can be influenced nutrigenomically in individuals with one variant allele is not known. Nevertheless, knowledge of the *NQO1* genotypic presentation in clinical trials could be useful, given the consequent variation in protein expression.

In a later chapter, the role of the better-known GST family of polymorphisms in relation to SFN metabolism are explored.

4.14.7 Haemoxygenase-1 (HO-1)

HO-1 is an inducible isoform of the first and rate-controlling enzyme of the degradation of haem into iron, carbon monoxide, and biliverdin, the latter being subsequently converted into bilirubin.[321] HO-1 is considered to have potent cytoprotective effects which include

antioxidant and anti-inflammatory properties in cardiovascular and other tissues. It has been suggested that cytoprotection may be due to bilirubin directly inhibiting NADPH oxidase activity, thereby reducing superoxide generation.[322]

Although the mechanism for the anti-inflammatory effect of HO-1 has not been fully elucidated, there are known associations between HO-1 and a number of cytokines. The 5'-flanking region of the *HO-1* gene contains binding sites for the transcription factors that regulate inflammation, including NF-κB and Activator Protein-1 (AP1).[323] *Leukocyte HO-1* gene expression is significantly lower in patients with and without diabetic microangiopathy compared with control subjects and normalisation of blood glucose results in a reduction in HO-1 antigen in the cytoplasm of mononuclear leukocytes.[324]

Hyperglycaemia is known to increase the formation of advanced glycation end products (AGEs). In endothelial cells, the interaction of the AGE with its receptor, (RAGE) induces generation of ROS, NF-kB translocation and expression of several pro-inflammatory and pro-coagulatory molecules.[325] In normal cells, RAGE is present at low levels but is increased in the endothelia of diabetics.[322]

Both *NQO-1* and *HO-1* are upregulated in response to AGEs.[326] Both are products of Nrf2 induction and may provide an effective endogenous defence mechanism in diabetes and other vascular diseases. Given the effect of SFN on induction of both genes as discussed in 14.4.5, a further mechanism to explain the observed beneficial effects of SFN in T2DM may be associated.

4.14.8 8-OH-2-deoxy-guanosine (8-OHdG)

Because rising levels of oxidative stress play an important role in the pathogenesis of T2DM, it is useful to have a marker which can detect individuals who are at risk. As diabetes progresses from normal through pre-diabetes to diagnosed type 2 diabetes, DNA damage increases. Superoxide anions which are elevated in hyperglycaemic states cause DNA strand breakage with a consequent increase in 8-OHdG. A 2010 study[327] showed a significant and progressive increase in 8-OHdG in prediabetics and diabetics compared to controls. Because markers such as GSH may again increase in later stages of disease following an initial drop,[306] measurement of 8-OHdG may be a more reliable marker for the early detection of diabetic risk and or a tool for monitoring treatment.

4.15 The Role of Selenium in Sulforaphane Chemistry

Although identified 200 years ago, selenium continues to be recognized as an essential element in biology and medicine.[328] Its biochemistry resembles that of sulfur, yet differs from it by virtue of both redox potentials and stabilities of its oxidation states.[329] Selenium can substitute for the more ubiquitous sulfur of cysteine and as such plays an important role in more than a dozen selenoproteins. These proteins include the Nrf2-induced GSH, GPx, Trx and Trx reductase.

4.15.1 Selenium Dietary Requirement

Selenium is an essential trace element with most diet-derived organic forms being highly-bioavailable at > 90%.[330] By contrast, inorganic selenium, typically sodium selenite, is only about 50% bioavailable. Compared with populations in the United States, Australians tend towards selenium deficiency due to the nature of the local soils[331] and soil levels appear to be further declining. Australians average an intake of 57-87µ selenium/day with the Recommended Dietary Intake (RDI) listed as 85 µ per day.[332]

4.15.2 Potential Selenium Toxicity

There is increasing evidence linking selenium status to cancer risk and immune dysfunction but blood levels are poorly correlated to tissue levels.[333,334] The eight-fold gap between the estimated average requirement and the upper limit of safe intake is relatively narrow.[335] For this reason, supplements in Australia are closely regulated to limit the daily intake from supplements to 150 µg.[336] Selenium is representative of a micronutrient for which the dose-response is hormetic; both low and high doses exhibit adverse effects in human cells.[337] The concept of hormesis is very relevant to consideration of the dose-response and is further addressed in later chapters.

4.15.3 Synergy Between Sulforaphane and Selenium

The activation of Nrf2/Keap-1 by SFN results in the induction of several selenoproteins each of which contains an ARE in its promoter region. Translational realisation of the enhanced transcripts depends on adequate selenium supply, which explains the synergism of Nrf2 activators and selenium.[338]

If selenium incorporation at the active site of the enzyme is the rate-limiting step, then less than optimal levels of the enzyme will be synthesised. Synergy between SFN and selenium in the induction of Trx-1 reductase requires both transcriptional and translational modulation.[339] A cell culture study using human hepatocytes[340] investigated the induction of Trx reductase-1 due to a SFN-yielding broccoli sprout material and selenium separately and then combined. The results showed that induction by SFN at 1.6-8 µM ranged from 1.7 to 2.2-fold. When the cells were co-treated with selenium (0.2 – 1 µM), the enzyme activity was high in the range from 3.0 to 3.3-fold, demonstrably greater

Another study[338] using a human endothelial cell line, EAhy926, showed that the combination of selenium and SFN produced an increase in Trx-1 reductase expression that was significantly greater than that achieved when each agent was added alone. The study also showed that SFN increased Trx-1 reductase but not GPx and in doing so conferred protection against oxidative damage in vascular endothelial cells. In contrast to these cell culture findings, a long-term (7.7 years) selenium supplementation study[341] on individuals with T2DM showed that 200 µg daily of selenium does not seem to prevent disease risk and in fact, may increase risk. Further investigation of these findings is warranted, especially in the context of the use of isolated nutrients as intervention materials. Such study protocols may be more suited to a pharmaceutical intervention than a nutrient intervention.

4.16 Implications for Type 2 Diabetes as a Representative Chronic Disease

4.16.1 Oxidative Stress – Is It the Common Upstream Factor Linking CVD and T2DM?

It is widely-accepted that we are in the midst of a global epidemic of T2DM. It has been estimated that up to 70% of patients with acute myocardial infarction have either diabetes or impaired glucose tolerance.[342] Diabetes-specific microvascular disease is a leading cause of blindness, renal failure and nerve damage with diabetes-accelerated atherosclerosis leading to increased risk of myocardial infarction, stroke and limb amputation.[281]

The observation that these seemingly-unrelated conditions are all manifestations of prolonged hyperglycaemia in certain individuals has led to a search for a common

upstream factor to provide an etiological mechanism. It has been proposed[4,343] that oxidative stress is the pathogenic mechanism linking insulin resistance with dysfunction of both beta-cells and endothelium, eventually leading to overt diabetes and cardiovascular disease.

4.16.2 Pancreatic Beta-Cell and Endothelial Cell Vulnerability

The beta-cells of the pancreas and those of the endothelium are particularly susceptible to the effects of oxidative stress.[289] As the first line of protection for the vascular wall and occupying a strategic position between the blood and underlying tissues, endothelial cells appear very susceptible to glucose toxicity.[344] This is especially so when compared with regulation of glucose uptake via glucose transporters by smooth muscle cells.[291]

Several metabolic pathways which link hyperglycaemia in the endothelia to clinical diabetes are associated with oxidative stress.[6,345] Recent studies demonstrate that a hyperglycaemia-induced process of overproduction of superoxide by the mitochondrial electron transport chain seems to be the first and key event in the activation of all other pathways involved in the pathogenesis of diabetic complications.[3]

4.16.3 Antioxidant Vitamins as Therapy?

Although it might appear that classical antioxidants such as vitamins C, E and beta-carotene would play a role in reducing oxidative stress in diabetes, they have been generally unsuccessful[346] and there would seem to be a significant disconnect when comparing the findings of epidemiological and intervention studies.[347] Furthermore, it appears that megadoses of vitamin antioxidants may impair induction of endogenous antioxidant enzymes and other cytoprotective compounds.[348] This may help to explain some of the negative findings of studies using doses of vitamins well in excess of reasonable dietary intake.

4.16.4 Exploring Nutrigenomic Strategies

Investigation into alternative redox-regulating strategies continues. The catalytic effect of antioxidant enzymes such as SOD provides greater potential to reduce oxidative stress[121] than do antioxidants acting stoichiometrically. SOD-mimetics, as introduced in Chapter 2, have been used experimentally to show that quenching excessive superoxide

anion reduces endothelial dysfunction.[349] Since epidemiological studies[350] show that a diet high in fruits and vegetables confers protection against disease, various non-nutrient compounds of plant origin are also under consideration as suitable agents to prevent oxidative stress-induced cell damage.

Polyphenolic compounds from plant foods have significant *in vitro* antioxidant potential but their low bioavailability[351,352] limits their clinical usefulness as systemic antioxidants. On the other hand, SFN derived primarily from broccoli and with absolute bioavailability[182] of around 80%, shows promise as a nutrigenomically-active compound capable of increasing several endogenous antioxidant compounds via the transcription factor, Nrf2.[168]

These cytoprotective compounds[28] which include NQO1 can protect the vascular wall against inflammatory processes.[174] There is evidence[353] to suggest that strategies to increase antioxidant activity of GSH and GPx in particular within the pancreatic islet cell may protect it against glucose toxicity. Substantially reduced levels of erythrocyte GSH occur in the pre-diabetic stage[306] rising with disease progression for 6-10 years after diagnosis. Estimates suggest that 40-50% of individuals with pre-diabetes will develop type 2 diabetes within 10 years[354] and this highlights the value of early detection of glucose dysregulation. As SFN is known to be a potent inducer[182, 355,301] of Nrf2 and has shown promise in clinical trials related to diabetes and its complications, this may indicate that SFN might play a preventive and therapeutic role in this disease epidemic. [295,172 ,173, 356, 357, 198]

To more fully elucidate the Nrf2-dependent and Nrf2-*in*dependent mechanisms that underpin the observed effects of SFN, further investigation will be required. Better understanding of these mechanisms may extend the clinical application for SFN, especially those associated with redox imbalance.

4.17 DIRECTION OF FUTURE SFN RESEARCH

4.17.1 The potential for SFN to be Used in Conjunction with Pharmaceuticals

The diverse properties of Nrf2 are such that it is being increasingly considered as a novel drug target with potential applications across a broad range of conditions. Interestingly, the Nrf2-activating properties of SFN have been experimentally used in conjunction with

pharmaceuticals. As one example, SFN's effect on Nrf2 has been investigated in this context as a means of minimising the nephrotoxicity which typically limits the use of the chemotherapeutic drug, cisplatin.[358]

A further very recent example illustrates the effects of combining members of the chemotherapeutic taxane drug family with SFN.[359] Taxanes are widely-used chemotherapeutic drugs for triple negative breast cancer (TNBC) which is unresponsive to more common receptor-targeted therapies. Taxane therapy typically targets only differentiated cancer cells without exerting any beneficial effect on the disease-promoting cancer stem cells (CSCs). More significantly, taxanes may result in expansion of the breast CSC population.

Because SFN has been shown to eliminate CSCs in some cancer cell types, it was trialled in conjunction with taxane. Where taxane enhanced inflammatory cytokine production in TNBC cell lines, SFN reduced production of IL-6, IL-8, and NF-kB activity to inhibit CSCs. These newer studies wherein SFN is tested in combination with an existing therapy provide numerous avenues for further SFN research.

CHAPTER 5

Sulforaphane: Translational Research from Laboratory Bench to Clinic

This chapter was published in *Nutrition Reviews* as Review – Special Article. The abbreviations, formatting and referencing of this document have been altered slightly to more closely reflect the formatting of other chapters and published work in this thesis. Section 5.11 has been updated to include reference to studies comparing the differing effects of SFN on normal and cancer cells.

A pdf version of the published manuscript is attached as Appendix B.

Houghton CA, Fassett RG, Coombes JS. *Sulforaphane: translational research from laboratory bench to clinic*. *Nutr Rev*. Nov 2013;71(11):709-726.

5.0 Abstract

The chemopreventive benefits of cruciferous vegetables in human health are widely acknowledged but these vegetables are not generally consumed at levels which effect significant change in biomarkers of health. Because consumers have embraced the notion that dietary supplements may prevent disease, this review considers whether an appropriately validated sulforaphane-yielding broccoli sprout supplement may deliver clinical benefit. The crucifer-derived bioactive phytochemical, sulforaphane is a significant inducer of Nrf2 (nuclear factor erythroid 2-related factor 2), the transcription factor which activates the cell's endogenous defences via a battery of cytoprotective genes. For a broccoli sprout supplement to demonstrate *in vivo* bioactivity, it must retain both the sulforaphane-yielding precursor compound, glucoraphanin and the activity of its intrinsic MYR enzyme. Many such supplements are MYR-*in*active but current labelling does not reflect this. For the benefit of clinicians and consumers, this review summarises *in vitro* and clinical trial findings, interpreting these in the context of clinical relevance. Standardisation of sulforaphane nomenclature and assay protocols will be necessary to remove the inconsistency and ambiguity in the labelling of currently available broccoli sprout products.

Key Words sulforaphane, broccoli sprout, myrosinase-active, bioactivity, bioavailability, glucoraphanin, broccoli seed extract

5.1 Introduction

The cruciferous vegetable family includes broccoli, cauliflower, cabbage, kale and others and consumption is associated with reduced cancer risk.[180,360] As foods, crucifers are a significant source of micronutrients including folate and vitamin C but perhaps more significant is their nutrigenomic potential.[361] This potential is due to generation of a family of bioactive compounds, known as isothiocyanates (ITC). Broccoli-derived ITCs for example, are not present in the plant cell but produced when the cell wall is ruptured, resulting in an enzymatic reaction between the MYR enzyme and an inert precursor, glucoraphanin. The well-researched broccoli-derived ITC is sulforaphane (SFN) and it is this compound which appears to be responsible for the larger part of the plant's health-promoting and cancer-preventive properties beyond its nutrient content.[362,363,364] Whereas many plant-derived nutraceutical supplements

contain *extracts* of the major identified bioactive compound(s), the intrinsic instability[365,366] of the naturally-occurring SFN molecule presents a challenge for which a manufacturing solution has yet to be found; SFN *per se* is simply not sufficiently stable to enable production as a supplement. Alternative means of addressing this challenge are discussed herein.

Given the prevalence of diet-related chronic disease and the evidence that many consumers have accepted a role for complementary medicines in their personal health management, there may be a place for a validated bioactive plant-derived SFN-yielding supplement capable of modifying biochemical and physiological risk factors for disease. It is against this background that this review investigates the possibility of delivering such supplements with significant clinical potential.

5.2 The Emergence of Nutrigenomics

Described in 2004[367] as the *'next frontier in the post-genomic era'*, nutritional genomics (more commonly 'nutrigenomics') harnesses multiple disciplines and rests at the interface between the nutritional environment and cellular/genetic processes.[368] Understanding the relationships between phytochemicals and their ability to affect an individual's gene expression underpins the principle of an evolving model of 'personalised nutrition'.[369] Where pharmacogenomics[370] describes the individual effects of pharmaceuticals on a patient's genome, nutrigenomics describes similar responses to bioactive molecules in foods.[371] Plant biomolecules as components of the human diet take on a new significance as the relevance of their roles in intracellular signalling and nutrigenomic modulation of gene expression continue to unfold.[372]

As a weak pro-oxidant, SFN is a phytochemical with demonstrable nutrigenomic potential, activating the expression of a battery of cytoprotective genes via the transcription factor, Nrf2 (nuclear factor erythroid 2-related factor 2).[373] Nrf2 co-ordinates the regulation of over 200 genes in humans and animals,[192,194,219] largely related to mechanisms of endogenous cellular defence and survival.[374] Nrf2 has been variously described as, '*the master redox switch',*[151] an '*activator of cellular defence mechanisms*[150] and '*a guardian of health span and gatekeeper of species longevity'.*[152] As a mediator for amplification of the mammalian defence system against various stressors, Nrf2 sits at the interface between our prior understanding of oxidative stress

and the endogenous mechanisms cells use to deal with it. The knowledge that a weak *pro*-oxidant such as SFN can initiate a sequence of events culminating in increased expression of endogenous *anti*-oxidant molecules such as glutathione, thioredoxin and hemoxygenase suggests that we re-examine the premise that dietary antioxidant supplements are generally beneficial to human health.[34,375]

5.3 Nutrigenetics

Where nutrigenomics investigates the effects of food nutrients and bioactive food-derived compounds on gene expression, nutrigenetics identifies and characterises gene variants associated with differential responses to nutrients, relating this variation to disease states; both aim to unravel diet-genome relationships.[376] Nutritional epidemiology investigates the association between patterns of food consumption and disease risk, with nutrigenetics asserting that the effects of particular foods or food components may vary with genetic differences that affect the metabolic makeup of the individual.[377] Furthermore, nutrigenetics helps to explain why the findings of epidemiologic trials are often inconsistent, since to express their bioactivity, phytochemicals may need to be metabolised by enzymes with known single nucleotide polymorphism (SNP) variants.[378,379]

5.4 Differential Responses Due to Nrf2-Target SNPs

Glutathione S-transferases (GST) constitute a large multigene family of Phase 2 enzymes involved in detoxification of potentially genotoxic chemicals.[378] The bioactive ITCs of cruciferous vegetables are metabolised via the mercapturic acid pathway to form a GSH conjugate, catalysed by GST enzymes.[379] Polymorphisms in these enzymes have a significant nutrigenetic impact on overall ITC metabolism, affecting SFN bioavailability, so that in some cases cruciferous vegetable intake confers greater chemoprevention in those with certain GST polymorphisms; in other studies, the opposite is observed.[380-382] It has been reported that the differing effects of phenotypic expression of *GSTM1* on SFN metabolism is such that *GSTM1* null subjects excreted almost 100% of ingested SFN.[383] In a later study, Gasper et al showed that there were apparent thresholds of intracellular SFN concentration below which gene expression did not occur.[384] This could have implications for dietary recommendation to consume more cruciferous vegetables because it has been shown that some fresh broccoli purchased

from retail stores yielded very low SFN, even consumed raw and even when ingested repeatedly.[385] The same study in humans consuming 300mls of liquidised commercially-available raw broccoli florets achieved peak plasma SFN level of just 0.07 μM. A later study[386] which compared the peripheral SFN exposure in volunteers consuming a single serving of lightly-cooked *fresh* broccoli with lightly-cooked *frozen* broccoli showed an approximately 10-fold difference in plasma concentration, ~ 0.2 μM for the fresh vs ~ 0.02 μM for the frozen. Moreover, the lower physiological concentrations observed after ingestion of the frozen vegetable were described as being below the threshold required to cause a range of responses in cellular models. *GSTM1* on SFN metabolism is such that *GSTM1* null subjects excreted almost 100% of ingested SFN. In a later study, Gasper et al showed that there were apparent thresholds of intracellular SFN concentration below which gene expression did not occur. This could have implications for dietary recommendation to consume more cruciferous vegetables because it has been shown that some fresh broccoli purchased from retail stores yielded very low SFN, even consumed raw and even when ingested repeatedly.

Wide variations exist in glucosinolate content among varieties of the Brassica oleracea seeds[387] from which broccoli sprouts are grown. This would suggest the likelihood of marked variation in the quantities of isothiocyanates derived from hydrolysis of their glucosinolates (GSNs) from which broccoli sprouts are grown. This would suggest the likelihood of marked variation in the quantities of isothiocyanates derived from hydrolysis of their GSNs. A 27-fold difference between the highest and the lowest levels of glucoraphanin has been observed.[256] As a consequence, for a nutraceutical supplement to demonstrate consistent responses, it will be essential for a MYR-active plant powder to contain a standardised content of glucoraphanin, with selection of an appropriate seed cultivar an important consideration.

GST polymorphisms can be prevalent in the population, with up to 50% of people exhibiting the null genotype for the *GSTM1* isoenzyme.[382] Such a genotype may confer an advantage to *GSTM1*-null individuals because their reduced GST activity could result in slower elimination and longer exposure to ITCs after cruciferous vegetable consumption.[388] The GST-null phenotypes have been associated with reduced risk of breast, prostate and colon cancers, adding support to SFN's potential as a chemopreventive and cytoprotective agent[25,389,390,391] but not all studies show that the

null genotype confers such protection.[392] A study in which human hepatocytes were treated with the hepatocarcinogen *Aflatoxin B1* after pre-exposure to SFN at doses from 10-50 μM were investigated for their ability to resist DNA adduct formation; reduction in DNA adduct formation occurred at both doses, with greater reduction at the higher concentration.[393] Analyses of gene expression in the SFN-treated hepatocytes demonstrated that SFN greatly decreased cytochrome P450 (CYP3A4) mRNA but did not induce the expression of *GSTM1*, suggesting that GST induction is but one means by which SFN exerts chemoprevention. The effects of SFN on CYP3A4 are discussed in more depth in a later section.[387]

5.5 Sulforaphane's Diverse Modes of Chemoprotection

A number of cell defects have been observed in cancer cells.[394] Among such defects, mutation of the p53 suppressor gene occurs in about 50% of all cancers.[395] Accumulated and varied defects in the cell's intrinsic cytoprotective mechanisms gradually weaken these defences. The most common molecular genetic change in prostate cancer involves silencing of the expression of the gene which codes for *GSTP1*, a critical enzyme of carcinogen defence.[209] This change occurs early in prostate carcinogenesis because it is found in virtually all cases of intraepithelial neoplasia (PIN) and is a near universal finding in clinical prostate cancers regardless of the grade or stage.[209]

Normal GST function is required to detoxify potential carcinogens; without it, prostate cells are exposed to the damaging effects of endogenous and exogenous toxins. Fimognari[396] showed that SFN is capable of offsetting the effects of a mutated p53 gene by virtue of its various modes of action, providing evidence that SFN confers chemoprotection even when genetic defects are already apparent, a finding of considerable clinical significance. These findings reaffirm the value of cruciferous vegetables in human diets.

Whether it is possible to develop a clinically efficacious supplement based on the nutrigenomic potential and the pharmacokinetic properties of phytochemicals like SFN is a key question posed by this review.[397,398] A close investigation of many of the phytochemical-derived supplements popular with consumers reveals that many of these exhibit poor bioavailability, in contrast to that of SFN.[399,400,41] As a result, it is doubtful

that some of the bioactive compounds which demonstrate impressive effects in highly-publicised *in vitro* studies exist in sufficient systemic concentration to exhibit a clinical effect.[401]

5.6 Developing an Evidence-Based Sulforaphane Supplement

The finding that SFN is both a potent[32] and a bioavailable[41] inducer of Nrf2 places it in a category distinct from most other supplemental phytochemicals, especially the polyphenols.[352] Cell culture studies are a valuable starting point in dose determination and various human cell types have been investigated as substrates for SFN intervention studies.[402] Of particular value are the studies which reveal the biochemical effect of a specific micromolar concentration of SFN.[318,402] These data can then be reviewed in relation to the SFN Yield from a specified oral dose.

Whereas the primary bioactive component of many phytochemicals is extracted and the standardised extract used in the manufacture of a nutraceutical supplement, the intrinsic instability of the SFN molecule prevents this method of delivery.[366] As such, broccoli-derived foods or supplements require the hydrolysis of the glucoraphanin precursor in the presence of the MYR enzyme; typically, this occurs when the fresh plant cell is ruptured or when a dry whole powder becomes moist. In short, SFN must be formed close to the time of ingestion. The challenge for the manufacturer of a SFN-yielding supplement lies in the ability to retain both the glucoraphanin and the MYR enzyme during the processing from fresh whole plant to powder; this aspect is explored in more detail later.

5.7 Sulforaphane Yield vs Sulforaphane Content

Broccoli sprouts yield more SFN than any other known plant and contain10-100 times the concentration of glucoraphanin (the major glucosinolate in broccoli) of the mature broccoli vegetable.[32,169] The broccoli seed therefore contains more glucoraphanin per gram than does the sprout or the vegetable of the same seed. The higher level in broccoli seeds compared to young sprouts is apparently due to the fact that there is no significant net synthesis of the glucosinolates over the few days following germination.[169,403] As the maturing plant becomes established and is exposed to environmental factors including the influence of herbivorous predators, additional

glucosinolates can be synthesised as part of the plant's defence against predators.[404] Nevertheless, the very young broccoli sprout is typically a far more significant source of SFN on a weight basis compared with the vegetable and it is for this reason that the sprout is chosen as a source of sulforaphane-yielding precursors in many clinical trials.

In addition, different cultivars of Brassica oleracea contain varying levels of glucoraphanin, further establishing the wide variation in glucosinolate content between broccoli sprout and the broccoli vegetable.[387] Importantly however, the plant does not *contain* any SFN, instead producing it during the hydrolysis of glucoraphanin in the presence of MYR.[27] As a result, broccoli sprouts are correctly described as As a result, broccoli sprouts are correctly described as *releasing, generating* or *yielding* but not *containing* SFN. The ideal broccoli sprout ingredient contains separately compartmentalised glucoraphanin and MYR which react at, or close to the time of ingestion.[387] The absence of MYR in a broccoli sprout food powder or supplement renders the product unable to produce SFN on ingestion.[398]

5.8 Role of Epithiospecifier Protein (ESP)

The presence of a pH-sensitive modifier protein, ESP affects the amount of SFN produced on hydrolysis.[405] In broccoli, the primary glucosinolate glucoraphanin, can be converted to two principal products, SFN and SFN nitrile, the latter being several orders of magnitude lower in Nrf2-activating potency than SFN.[253] The importance of this finding is that, whereas SFN has been shown to have anticarcinogenic properties, SFN nitrile has not.[406] Because ESP is temperature-sensitive, heating broccoli sprouts to 60 degrees C decreases the formation of SFN nitrile.[251] A challenge for manufacturers of a SFN-yielding supplement lies in deactivating the ESP without simultaneously deactivating the MYR enzyme or degrading the glucoraphanin.

5.9 Presence of Erucic Acid in Broccoli Seed Lipid Fractions

Erucic acid is a lipid found in broccoli seeds as well as in other members of the Brassicaceae family. Its toxic effects in animals consuming rapeseed (another member of the *Brassicaeae* family) are well-known and include myocardial lipidosis, myocardial necrosis, and impaired oxidative phosphorylation.[407] Concerns regarding its possible effects in humans are sufficiently important that erucic acid in rapeseed is

regulated in the U.S.[408] Broccoli seeds contain about 28% lipid but broccoli sprouts contain only about 1%. Therefore, as further described by West et al (2002), one gram of broccoli seed may contain approximately 120mg of erucic acid but 28 g of broccoli sprouts contains 90mg of erucic acid[407] ; this would indicate that the seed contains around 37-fold more than the sprout. They calculate that 35 g per week of broccoli seeds could equal the estimated maximum allowable amount of erucic acid, extrapolating from FDA requirement to limit the content of erucic acid in canola oil to a level not exceeding 2% of the component fatty acids. Although it is unlikely that an individual would consume 35 or more g of broccoli seed per week, it is not impossible; consumption of supplements derived from broccoli seeds is therefore not recommended.[408]

The production of an aqueous broccoli seed extract provides a way of removing the erucic acid; however, this process also activates the enzymatic conversion of glucoraphanin to SFN, which exhibits relatively high reactivity,[387] making SFN unsuitable as a compound for direct fortification of foods or supplements. A number of MYR-inactive broccoli seed extracts are commercially-available; these products are a source of glucoraphanin but are devoid of the MYR necessary to convert it to SFN on ingestion.[397]

5.10 Sulforaphane's Effects on Target Genes

The more than 200 identified Nrf2 target genes can be classified[194] broadly as those which code for a range of cytoprotective proteins, including antioxidants (enzyme and non-enzyme), drug-metabolising enzymes, drug-efflux pumps, heat shock proteins, NADPH regenerative enzymes, growth factors, growth factor receptors, heavy metal binding proteins and various transcription factors including PPAR-γ as well as for Nrf2 itself.[409]

An illustrative study[318] investigated the effect of a single oral dose of 200 µmols of pure SFN in women immediately before undergoing reduction mammoplasty. This dose can be calculated to be equivalent to 35 mg SFN, given that 5.7 µmols SFN is equal to 1mg SFN (M.W. = 177.29). Two important biomarkers in this study were hemoxygenase-1 (HO-1), a cytoprotective enzyme expressed following Nrf2 induction of the target gene[410] and NAD(P)H:Quinone oxido-reductase (NQO1), a Nrf2-inducible

Phase 2 detoxification enzyme[253] exhibiting a range of other cytoprotective properties. The dose selected was based on an earlier study[411] which showed that SFN dithiocarbamate metabolites were detected at 15 minutes in the plasma and peaked at 2.00 ±0.3 µM one hour after ingestion, indicating rapid uptake.

A maximal 12-fold induction of NQO1 transcripts was observed in the mammary gland 12 h after dosing, with significant induction as early as 2 h. A biphasic pattern of HO-1 transcript induction was observed, with an initial peak at 2 h followed by a subsequent peak at 12 h, indicative of the delayed effect of SFN conversion to dithiocarbamates (DTCs). The minimal time to statistically-significant HO-1 induction was 1 h.

Cornblatt's study[183] is significant in providing a guide to the expected micromolar concentration for a given quantity of ingested SFN. A dose of 35mg SFN (200 µmols) appears to be achievable from a MYR-active broccoli sprout powder, provided such a powder is able to yield 1% SFN per gram of powder. As such, a supplement weight of 3.5 g is within a practical human dose range, ingested either as a powder or as several capsules.

5.11 Relevance of *in vitro* Studies

Much of the preclinical research on SFN examines its effect in various cell types and at varying micromolar concentrations. It soon becomes clear that different tissues respond differently to a specified dose. Even different cell types in the same organ can respond differently.[208]

The expression of Nrf2 target genes such as NQO1 occurs at lower SFN concentrations, whereas other effects appear to require much higher intracellular concentrations.[157] SFN exhibits effects beyond its ability to activate Nrf2; these effects include HDAC (Histone Deacetylase) inhibition,[412] apoptosis,[159] anti-angiogenesis[413] and anti-metastasis[414] in cancer cells. Induction of NQO1 in the rat colon is 3-fold higher than it is in the liver for the same dose.[415] Clearly, an effect detected in one tissue cannot be extrapolated to others. This may also suggest that the beneficial effects from both dietary crucifers and supplements may be greater in organs such as the digestive tract and bladder than in organs reached only systemically. A recent study[416] showed that SFN levels measured in the stomach and

bladder are higher than for the other organs measured. It is possible that tissue uptake of SFN is highest in the stomach due to direct exposure but the levels are seen to decline rapidly in the descending gastrointestinal tract, possibly a result of metabolic conversion.[416,417] Paradoxically, induction of GST and NQO1 are relatively modest in the stomach; the authors state that the reason for high gastric uptake of SFN but low induction of GST and NQO1 remains unexplained. An earlier study by the same researchers showed that SFN tissue uptake level in the bladder was second only to that in the stomach. There would appear to be remarkably high exposure of the bladder epithelium to dietary SFN, with a peak at 6 h after dosing and levels 64–5509 times higher than in the plasma.[416] Unlike the relatively low extent of enzyme induction observed in the stomach, significant induction of GST and NQO1 occurred in bladder cells.[416] This may have clinical implications for patients with bladder cancer.[418]

It would seem too that the doses required for disease prevention are far lower than those required to effect change in established disease. A study[419] on men at risk of prostate cancer showed that increasing intake of cruciferous vegetables from one serve per week to more than three serves per week (presumably cooked) was enough to provide 41% decreased apparent risk. Data shown in Table 5.1 review a number of *in vitro* studies[208,209,218-226] which evaluated the effect of SFN in different tissue and cell types. Notably but not surprisingly, the micromolar concentrations to effect change are highly variable across cell types and it has also been shown that the time during which the cells were exposed to SFN markedly altered the response. In cardiomyocytes exposed to hydrogen peroxide (H_2O_2), a 5 μM SFN dose for 6 hours maintained 45% cell viability but when exposed for 24 and 48 hours, cell viability increased to 82% and 92% respectively.[225] How the findings of such cell culture studies translate to the human *in vivo* situation is not yet known.

The examples shown in Table 5.1 are a cross-section of the many published *in vitro* SFN trials of this type. The data show that in some cell types, the required biochemical changes could be readily achievable by supplementation of a MYR-active standardised broccoli sprout supplement. It is equally likely that in some cells and in certain conditions, such supplementation may not provide the required micromolar concentration. For example, inhibition of transcription factor, NF-κB and induction of transcription factor, Nrf2 appear to be achievable at micromolar concentrations much

lower than for the induction of HDAC inhibition, cell cycle arrest or anti-angiogenesis in cancer cell lines.[232,420] As an example, where low SFN doses between 0.1 and 3 μM activate Nrf2-related cellular defences in prostate cells,[209] higher doses of 10 μM for DU145 cells and 30 μM for LNCaP cells are necessary to induce cell cycle arrest and apoptosis.[226]

As a further consideration, when comparing the effects of SFN in normal and cancer cells, SFN has been shown to exhibit different behaviours. A study comparing the effect of SFN on three tumour cell lines and a normal cell line showed differing abilities of SFN to induce apoptosis and arrest the cell cycle for the three tumour cell lines; more significantly, in the normal cell line, SFN was shown to be less toxic than to the tumour cell lines.[421] In a separate study investigating effects on prostate cells, SFN was shown to exhibit a different pattern of chemoprevention-related gene expression when comparing normal and cancer cells.[422] These effects extend beyond comparison of cancer and normal cells, so that in a rheumatoid arthritis model, SFN induces the cytoprotective transcription factor Nrf2 in naïve synoviocytes, whereas it induces apoptosis in inflamed synoviocytes.[423]

Table 5.1 Studies showing the diversity of effects in different cell types when varying µM SFN and incubation time.

Lead Author & Year of Publication	Tissue	SFN µM Conc.	Cell Type & Effect	Finding
Brooks et al. (2001)[209]	Prostate	0.1 – 15 µM	6 prostate cancer cell lines + normal cells. Effect of SFN evaluated – including effect on GSTP1 silencing	Normal cells: SFN after 48 hours→ 1.35-fold NQO1 induction at 0.1 µM and 2.46 at 1 – 3 µM LNCap cells: SFN → 4.6-fold induction at 10 µM Modest induction of GST isoforms to 1.7-fold in all cell lines All effects of SFN completely abrogated by 10 nM NAC
Gao et al. (2004)[222]	Retinal epithelium	1.25 – 5 µM	Human adult retinal pigment epithelial cells (ARPE-19). Challenged with UV light + all-trans retinaldehyde on photo-oxidative damage	Increased cell survival with increasing SFN doses; linear cytoprotective effect with dose Where only 9.4% of cells survived, pre-incubation with SFN raised survival 3-fold to 27.4% at SFN = 5 µM
Cho et al.	Prostate	10 µM	Effect of SFN on prostate	Strong cell cycle arrest (G2/M phase) and apoptosis at SFN = 10 µM. No

Lead Author & Year of Publication	Tissue	SFN µM Conc.	Cell Type & Effect	Finding
(2005)[226]	Cancer		cancer cell line DU145	effect at < 3 µM LNCaP cells required SFN > 30 µM for arrest Effects of SFN completely abrogated by NAC
Ritz et al. (2007)[208]	Bronchial epithelium	0.3 – 10 µM	Effect of SFN on bronchial cells exposed to diesel extract in two cell lines BEAS-2B and NHBEC	All doses induced GST, NQO1 but to different degree in each cell line SFN at 5 µM → 15-fold increase in NQO1 in BEAS-2B cells GST increased 1.9-fold in NHBEC at SFN = 1.25 µM → 1.43-fold increase in BEAS-2B Expression of inflammatory cytokines, IL-8, IL-1β and GM-CSF were all significantly reduced at SFN = 0.625 µM
Zhu et al. (2008)[219]	Aorta	0.25 – 5 µM	Effect of SFN in aortic cell cytoprotection in rat aortic smooth muscle cells (A10 cell line)	Significant dose-dependent induction of cytoprotective Nrf2 target genes at SFN as low as 0.25 µM. SFN at 0.25 µM → 25% increase in GST and 66% increase in NQO1. SFN at 0.5 µM → 33% increase in GSH levels. SFN protected against toxicity of superoxide, H_2O_2, acrolein and peroxynitrite.
Angeloni et al.	Cardiac	5 µM	Effect of SFN on	Greater effects when pre-dosing for 48 hours

Lead Author & Year of Publication	Tissue	SFN µM Conc.	Cell Type & Effect	Finding
(2009)[225]	tissue		cardiomyocyte cell viability in presence of reactive oxygen species (ROS) over 6-48 hours	H_2O_2 reduced cell viability to 40% SFN at 5 µM for 6 hours → 45% viability SFN at 5 µM for 12 hours → 75% viability SFN at 5 µM for 24 hours → 82% viability SFN at 5 µM for 48 hours → 92% viability
Song et al. (2009)[223]	Pancreatic β-cells	0.1 – 10 µM	Effect of SFN in protecting β-cells (RIN cell line) against oxidative damage, restoring insulin secretion and preventing type 1 diabetes in STZ-treated mice	~ Linear response in Nrf2 target genes to induction by 2.5, 5.0 and 10.0 µM SFN. Protection against cytokine-induced β-cell death by SFN. Cytokines reduced cell viability to ~54%. SFN at 10 µM increased viability to ~96%. SFN at 5.0 µM increased viability to ~92%. SFN at 2.5 µM increased viability to ~80%.
Kivela et al. (2010)[224]	Endothelial cells or arteries and veins	Human Umbilical Endothelial Cells (HUVEC)	Effect of SFN on Endothelial Lipases (EL) and TNF-α expression in HUVEC and HAEC cells	SFN at 10 µM inhibits EL in HUVECS venous cells SFN at 2.5 µM inhibits EL in HAEC arterial cells EL activation occurs through classical (canonical) NF- κB pathway

Lead Author & Year of Publication	Tissue	SFN µM Conc.	Cell Type & Effect	Finding
		0 - 10 µM Human arterial endothelial cells (HAEC) 0 - 10 µM		
Mas et al. (2011)[220]	Brain – Substantia nigra	0 .5 - 5 µM	Dopaminergic human neuroblastoma cells (SK-N-SH cells). Potential for SFN to protect against ox stress-induced by anti-psychotics	2.5 µM and 5.0 µM → 60% and 140% increase in GSH. 2.5 µM and 5.0 µM → 175% and 250% increase in NQO1 activity. Doses > 5 µM reduced cell viability. 2.5 µM and 5.0 µM maintained normal lipid peroxidation in the presence of Haloperidol. SFN at these doses protects dopaminergic cells from the oxidative stress-induced by antipsychotic drugs, including Haloperidol at a dose to reduce cell viability by 80%.
Tan et al. (2010)[221]	Bronchial epithelium – normal and	0.5 – 2.0 µM	Normal human bronchial epithelial cells (NHBE)	NHBE cells: SFN → ~ 4-fold increase in NQO1 at 0.5 and 1.0 µM at 24 hours and 11.8-fold at 6 days HBEC cells: SFN → 7.5-fold increase in NQO1 at 2.0 µM, only at 48 hours

Lead Author & Year of Publication	Tissue	SFN µM Conc.	Cell Type & Effect	Finding
	cancerous		& Immortalised human bronchial epithelial cells (HBEC) Malignant lung adenocarcinoma cells (A549 cells)	A549 cells: SFN → no change at the doses tested No toxicity at 10 µM SFN
Vauzour, D et al. (2010)[218]	Neural cortex	0.1 – 1.0 µM	Cortical neurones. Protection against dopamine toxicity	SFN → maximum protection (~ 30%) at 0.1 µM and ~ 24% at 1.0 µM No toxicity at 10 µM SFN.

5.12 Data from Clinical Studies

A summary of twenty-two published clinical studies using a variety of intervention materials is tabulated in Table 5.2.[37,38,41,197,245,318,383-385,397-399,412,424-432] These materials include mature broccoli vegetable (raw and cooked), fresh broccoli sprouts, MYR-inactive broccoli extract beverage, MYR-inactive powdered broccoli sprout extract (BSE), MYR-active broccoli sprout powder and capsules made from MYR-inactive broccoli extract. Several of the studies[38,397,398,430] confirm the necessity for MYR in optimising SFN Yield, citing cooking and extraction as being the primary processes leading to loss of activity. confirm the necessity for MYR in optimising SFN Yield, citing cooking and extraction as being the primary processes leading to loss of activity.[424,245,399] The SFN bioavailability[399] from raw broccoli was shown to be 10-fold higher than for cooked broccoli. Plasma SFN peaked at about 1.6 hours after ingesting raw broccoli but peaked later at around six hours after cooked broccoli. Also apparent in the MYR-inactive materials was that inter-individual variability in SFN concentration was considerably higher in those taking the MYR-inactive material.[430] The SFN bioavailability from raw broccoli was shown to be 10-fold higher than for cooked broccoli. Plasma SFN peaked at about 1.6 hours after ingesting raw broccoli but peaked later at around six hours after cooked broccoli. Also apparent in the MYR-inactive materials was that inter-individual variability in SFN concentration was considerably higher in those taking the MYR-inactive material.

Others[425,428,433,434] demonstrate the bactericidal effect on the Helicobacter pylori organism associated with gastric reflux and cancer. Forty-eight *H pylori*-infected subjects were given 70 g fresh broccoli sprouts daily.[428] Three markers of *H pylori* infection declined within eight weeks to below the diagnostic cut-off point. However, once the intervention had stopped, the levels of *H pylori* returned to baseline levels after 8 weeks.

In evaluating the effect of 25 to 200 g of a 1:4 fresh broccoli sprout homogenate, a dose-escalation study[41] found that there was a safe and effective induction of Phase 2 enzyme expression in the upper airways in a dose-dependent manner. At the highest intake, there was a 200% increase in NQO1 activity. This study is valuable

in that it demonstrates the dose required to double activity of NQO1 in tissues of the human airway; more importantly, it shows that such doses are achievable through diet.

HDAC inhibition is an epigenetic phenomenon associated with chemoprotection. SFN was shown to act as an HDAC inhibitor[412] in a mechanism unrelated to its activation of Nrf2. In this study, a single dose of 68 g of broccoli sprouts (claimed to yield 105mg SFN) "*inhibited HDAC activity significantly in peripheral blood mononuclear cells 3 and 6 hours following consumption".* The effect lasted 24-48 hours.

An important question a clinician might ask relates to the possibility that the beneficial effects bestowed upon normal cells by SFN might also protect cancer cells. To be certain that SFN as a therapeutic intervention is both safe and clinically-relevant, a recent cell culture study compared the effects of SFN on cancer cells and normal cells.[435] Using four cell types in a prostate model, normal (PrEC), cancer (LnCaP and PC3) and benign prostatic hypertrophic (BPH1) cells were subjected to 15 µM SFN for varying time periods. The results showed that SFN selectively targets benign hyperplastic cells and cancerous prostate cells whilst leaving the normal prostate cells unaffected. These findings hold clinical significance, highlighting the potential of SFN as a non-toxic chemopreventive agent readily incorporated into the diet as an uncooked vegetable or taken as a MYR-active supplement.

Table 5.2 Clinical studies investigating Sulforaphane (2000 – 2012)

Lead Author & Year of Publication	Subject Number	Intervention	Endpoint	Finding	Dosage Relevance
Conaway et al. (2000)[424]	12	200g fresh or steamed broccoli vegetable– single dose	Compare metabolic fate of steamed vs fresh broccoli	Bioavailability of isothiocyanates from fresh broccoli is approximately three times greater than that from cooked broccoli.	• Total isothiocyanate plasma metabolites peaked between 1 and 8 hours • Total urinary excretion of total isothiocyanate equivalents (esp. SFN-NAC) occurred at 2-12 hours
Galan et al. (2004)[425]	9	14, 28, 56 g fresh broccoli sprouts twice-daily for 7 days	Can orally consumed broccoli sprouts eradicate Helicobacter pylori infection?	Consumption of oral broccoli sprouts was temporally associated with eradication of *H pylori* infection in three subjects.	No data provided on sprout composition or SFN Yield
Murashima et al. (2004)[37]	12	100 g daily fresh broccoli sprouts for 7 days	Markers of oxidative stress and lipid metabolism in healthy males and females	Improved lipid parameters and reduced oxidative stress markers. HDL increased only in females.	No data provided on sprout composition or SFN Yield

Lead Author & Year of Publication	Subject Number	Intervention	Endpoint	Finding	Dosage Relevance
Kensler et al. (2005)[38]	200	Hot water infusions of 3 day-old broccoli sprouts nightly for 2 weeks	Could MYR-inactive infusion alter the disposition of aflatoxin and phenanthrene, predisposing to hepatic carcinoma	Significant inter-individual differences in bioavailability but no overall difference between intervention arms was observed.	• Each dose contained 400 µmol SFN • Inter-individual variability between 1-45% of dose • DTC (Dithiocarbamate) excretion = 49 µmol/12 hours
Gasper et al. (2005)[383]	16	3-phase crossover dietary trial of standard broccoli vegetable, super broccoli vegetable, and water	Comparison of sulforaphane metabolism in GSTM1-null and GSTM1-positive subjects in different broccoli types	GSTM1 genotypes have a significant effect on the metabolism of sulforaphane derived from standard or high-glucosinolate broccoli - greater protection that GSTM1-positive persons.	• GSTM null subjects excreted ~ 100% of urinary metabolites • GSTM positive subjects excreted ~ 70% SFN
Shapiro et al. (2006)[426]	12 as 3 groups of 4. 3:1 active to	21 doses for 7 days of glucoraphanin or SFN	Evaluation of safety, tolerance and metabolism	No significant or consistent subjective or objective abnormal events (toxicities) associated with any of the sprout extract ingestions were	Non-toxic at well in excess of any practical dose

Lead Author & Year of Publication	Subject Number	Intervention	Endpoint	Finding	Dosage Relevance
	placebo			observed.	
Cornblatt et al. (2007)[318]	8	Single dose oral broccoli sprout delivering 200 µmol SFN ~ 1 hour pre-surgery	Evaluation of plasma DTC concentration at ~ 100 minutes after ingestion	Approx 40-fold increase in urine SFN metabolites and a 90-fold increase in plasma SFN metabolites.	200 µmol oral SFN → 2µM in breast tissue
Rungapamestry et al. (2007)[245]	12	Single meal broccoli vegetable	Comparison of beef meal composition on 150g lightly-cooked or broccoli or broccoli seed with 3 g mustard	SFN Yield 3-fold higher after lightly-cooked than fully-cooked. No effect of meal matrix, only with cooking.	No effect of meal matrix on SFN bioavailability. Complete cooking more destructive than light cooking which is more destructive than raw
Gasper et al. (2007)[384]	16	3-phase crossover dietary trial of standard broccoli, 3-fold higher glucosinolate	Comparison of gene expression in gastric mucosa 6 hours after consuming broccoli	Only one gene upregulated by more than 1.5-fold in standard broccoli. Several genes upregulated in the high-glucosinolate broccoli.	Apparent threshold for doses of SFN influencing gene expression

Lead Author & Year of Publication	Subject Number	Intervention	Endpoint	Finding	Dosage Relevance
		broccoli, and water	vegetable of varying glucosinolate content		
Traka et al. (2008)[427]	22	Broccoli vegetable-rich diet over 6 months (400 g broccoli per week)	Does broccoli alter expression of GSTM1 to perturb oncogenic signalling in prostate?	Broccoli interacts with GSTM1 genotype to result in complex changes to signalling. pathways associated with inflammation and carcinogenesis in the prostate.	Only the broccoli-rich diet influenced expression of genes in the androgen receptor pathway, insulin signalling TGF-β and EGF (Epidermal Growth Factor) receptors
Vermeulen et al. (2008)[399]	8	200 g broccoli vegetable, raw and cooked	Determine the bioavailability and kinetics of sulforaphane	Broccoli eaten raw (bioavailability of 37%) versus cooked (3.4%). SFN absorption delayed when cooked broccoli was consumed (peak plasma time - 6 h) versus raw broccoli (1.6 h).	10% greater bioavailability from raw vs cooked
Myzak et al.	3	68 g broccoli sprouts	Effect of SFN on HDAC	Strong inhibition of HDAC activity 3-6 hours after	• 68 g fresh sprouts → 105mg SFN • Human HDAC activity reduced to

Lead Author & Year of Publication	Subject Number	Intervention	Endpoint	Finding	Dosage Relevance
(2007)[412]		containing approx 105 mg sulforaphane	activity in peripheral blood mononuclear cells	ingestion. Trend towards increased histone acetylation.	~65% after 3 hours - lasted 24-48 hours
Riedl et al. (2009)[41]	65	Dose-escalation from 25 g to 200g fresh broccoli sprouts	Effect of SFN on the expression of GSTM1, GSTP1, NQO1, HO-1 in the upper airway of human subjects	Safe and effective induction of mucosal Phase 2 enzyme expression in the upper airway of human subjects, in a dose-dependent manner.	125 g dose (64 µmol SFN) → > 50% in NQO1 activity 200 g dose → > 200% NQO1 activity
Yanaka et al. (2009)[428]	48 H. pylori-infected patients	70 g/day broccoli sprouts for 8 weeks (glucoraphanin 420 µmol/day) vs alfalfa sprouts	Effect of SFN on gastric H. pylori infection	Markers of H. pylori diminished in the broccoli sprout group only, remaining low for 8 weeks after cessation.	HO-1 increased 2 to 3-fold 24 hours after single 50 g dose of sprouts 3 markers of H. pylori infection declined to below cut-off after 8 weeks Levels returned to baseline 8 weeks after end of study

Lead Author & Year of Publication	Subject Number	Intervention	Endpoint	Finding	Dosage Relevance
Hanlon et al. (2009)[385]	6	Single and repeated intake of raw broccoli vegetable. (Noted lower than expected SFN Yield in the commercial broccoli)	Pharmacokinetic study of SFN	Sulforaphane was rapidly absorbed with peak plasma levels within 1.5h, characterised by a long terminal elimination phase. No impact of repeated intake, nor accumulation.	Rapid decline in plasma levels to 50% after ~ 3 hours, whether as single or repeated doses Fresh broccoli from retail sources generates very low SFN, even when raw
Kumar et al. (2010)[429]	85	3 diet groups to include different dietary glucosinolates over 4 weeks	Develop a method to measure isothiocyanate albumin urinary adducts	Hb and albumin adducts are a useful marker of isothiocyanate intake.	More stable adducts for easier quantification
Egner et al. (2011)[430]	50	2 groups over 7 days. 2 broccoli sprout-derived beverages,	Comparison of bioavailability and tolerability of SFN from beverages with and without	Bioavailability of MYR-active SFN-rich beverage = 70% vs 5% for glucoraphanin-rich MYR-inactive beverage.	Inter-individual variability in excretion was considerably lower with SFR than with GRR beverage Bioavailability SFR=70% vs GRR 5%

Lead Author & Year of Publication	Subject Number	Intervention	Endpoint	Finding	Dosage Relevance
		one glucoraphanin-rich (GRR = 325 µmol glucosinolate/g powder) and the other SFN-rich (SFR (Sulforaphane)= 202 µmol SFN/g)	MYR		Taste preference significant for GRR 34% null for GSTM1 genotype 51% null for GSTT1 SFR better bioavailability and more rapid uptake GRR more stable but unpredictable and overall poor bioavailability
Clarke et al. (2011)[397]	12	40 g broccoli sprouts for 4 weeks vs 6 x broccoli sprout MYR-inactive pills (equiv. 3g dried sprouts)	To compare glucosinolate metabolites SFN and erucin in MYR-active broccoli sprouts and MYR-inactive pills	Broccoli supplements devoid of MYR activity do not produce equivalent plasma concentrations of bioactive isothiocyanate metabolites compared to broccoli sprouts.	Plasma level ~ 2.3 µM from sprouts 7-fold higher peak conc. from sprouts 74% recovery from fresh sprouts vs 19% from MYR-inactive pills Significant inter-individual variability SFN and another isothiocyanate erucin (ERN) are inconvertible but effect is variable between subjects

Lead Author & Year of Publication	Subject Number	Intervention	Endpoint	Finding	Dosage Relevance
Cramer et al. (2011)[398]	4	42 g fresh broccoli sprouts (74 µmol SFN); 2 g of MYR-inactive broccoli sprout powder (120 µmol SFN)	Can MYR-active broccoli sprouts enhance SFN Yield of a glucoraphanin-rich MYR-inactive broccoli sprout powder?	The 24 h urinary SFN-N-acetyl-cysteine recovery was 65, 60 and 24 % of the dose ingested from combination, broccoli sprout and GRN (Glucoraphanin) powder meals, respectively.	Enhanced conversion to SFN when MYR-inactive powder is combined with MYR-active whole sprout powder Synergistic effect of combination Fresh sprouts + MYR-inactive powder enabled earlier SFN conversion
Bahadoran et al. (2011)[197]	81 x Type 2 diabetic patients	10 g and 5 g daily of a MYR-active broccoli sprout powder	Can a MYR-active broccoli sprout powder reduce biomarkers of oxidative stress in type 2 diabetes?	Significant decrease in MDA, ox. LDL cholesterol, Oxidative Stress Index, and significant increase in TAC. No effects were found on Total Oxidant Status. Greater effects for higher dose.	5g dose → 112 µmol SFN 10g dose → 225 µmol SFN Higher dose → 9% lower MDA, ~5% lower Ox-LDL, ~16% higher TAC Lower dose → 4.6% lower MDA, no change in ox-LDL and 10.3% higher TAC
Clarke et al. (2011)[431]	24	68 g broccoli sprouts for 4 weeks vs 6 x broccoli sprout MYR-inactive pills	Metabolic comparison between fresh broccoli sprouts and MYR-inactive	Broccoli sprout supplement results in significantly lower excreted isothiocyanate metabolites, compared to fresh sprout. (5-fold lower excretion of SFN metabolites).	Fresh sprouts demonstrate HDAC activity Supplement did not show HDAC activity

Lead Author & Year of Publication	Subject Number	Intervention	Endpoint	Finding	Dosage Relevance
		(equiv. 3g sprout powder in glucoraphanin content: ~220µmol)	supplements. Comparison of HDAC effects	Conversion of SFN metabolites to ERN metabolites. HDAC findings inconclusive.	
Bahadoran et al. (2012)[432]	81x type 2 diabetic patients	5 and 10g MYR-active broccoli sprout powder daily for 4 weeks	Cardiovascular Risk parameters	At the 10g dose, significant decrease in serum triglycerides, oxidised LDL: LDL and Atherogenic Index of Plasma (AIP).	Required 10g daily dose to achieve statistically-significant change

5.13 Characterisation of the Intervention Material

The intervention material used in some of the published SFN studies is not well-characterised, making it difficult in some cases to attribute relevance. For a food to be considered within the realm of medicine as either a functional food or a nutraceutical, the properties and effects of its bioactive constituents on human cellular function must be well understood. As described earlier in relation to GST polymorphisms, such an understanding should allow for the variable effects in individuals in whom gene variants impacting on the relevant pathways demonstrate a different response.[381,383] Development of a plant-derived medicine or supplement requires the knowledge that a certain concentration of the bioactive will result in a measurable change at the cellular level. In the case of studies using broccoli sprouts as the intervention material, it is essential that the factors responsible for generating bioactive SFN are assayed and quantified. Such characterisation should include at least the level of the precursor, glucoraphanin and the quantity of SFN released. Additional assays to quantify the MYR enzyme and ESP, as a modifier of SFN release may also be performed but are not essential if yield of SFN and perhaps the other broccoli sprout bioactive ITCs, erucin and iberin is known.[436] In the presence of ESP, variable amounts of glucoraphanin are enzymatically converted to the relatively inactive SFN nitrile,[253] so removal of ESP is a desirable goal in optimising a broccoli sprout ingredient for SFN. If such an ingredient is to be used as a nutraceutical, it must be standardised to one or more of the bioactives in order to provide within tolerance, clinical predictability. In a clinical trial, the outcome is less meaningful if the data do not exist to draw a relationship between the intervention material and the observable effects.

In a 2004 study, Murashima[37] used 100 g fresh sprouts in 12 subjects over seven days and reported positive outcomes in a number of parameters related to lipid metabolism. Sprouts vary widely in their composition[387,437] due to a range of factors; because the sprouts in this study had not been characterised, the study is of limited value as a basis from which to extrapolate its findings in the development of an efficacious supplement. Subsequent studies using fresh sprouts have more fully-characterised the intervention material. A study[397] which compared fresh sprouts against a MYR-inactive sprout powder in tablet form measured the plasma

concentration of SFN after a dose of 40 g fresh sprouts containing 150 µmols glucoraphanin; SFN Yield of the sprouts was not specified. An earlier unrelated study[438] using 68 g of fresh sprouts had shown that this dose released 105 mg SFN, so that 40 g of fresh sprouts may proportionately yield around 62 mg SFN.

The effect of a MYR-active broccoli sprout powder was investigated in type 2 diabetics.[432] Eighty-one patients were randomised to receive placebo, 5 g or 10 g powder daily over four weeks. The manufacturer of the powder specified it as yielding 22.5 µg/ gram but this was not confirmed by assay as part of the trial. Using these values, subjects received respectively 112 and 224 µmols SFN daily. The 10-gram dose resulted in a 9% reduction in markers of oxidative stress as measured by malondialdehyde (MDA) and ~ 16% increase in total antioxidant capacity (TAC). The lower dose resulted in 4.6% lower MDA values and 10.3% higher TAC. Effects on lipid peroxidation parameters were seen only with the higher dose. Fasting blood glucose dropped by 11.3% at the higher dose and 4.9% at the lower dose.

In a dose-escalation study[41] investigating the effects of SFN in nasal cells in the upper airways, subjects were given from 25 – 200 g of fresh sprout homogenate daily. Increased Phase 2 enzyme expression in nasal lavage cells occurred in a dose-dependent manner, with maximal enzyme induction observed at the highest dose. The sprouts used in the study claimed a glucosinolate content of 6 µmol/ gram. The SFN levels of the 175g and 200g doses were assayed at 89 and 102 µmols, respectively. At doses yielding > 51 µmol SFN (by calculation), a significant dose-response in Phase 2 enzyme expression was observed. At the highest dose (102 µmols SFN), there was a 200% increase in NQO1 activity with a 50% increase at the dose yielding 64 µmols SFN. These findings may imply a protective effect of SFN in diseases of the airways such as asthma and chronic obstructive pulmonary disease and also in countering the effects of airborne pollutants.

5.14 What Micromolar Concentrations are Possible?

Based on the available, albeit limited clinical data, we have estimated the micromolar concentration which may be possible using a MYR-active whole broccoli sprout supplement. These data are based on the effects of single doses and so do not

adequately reflect the clinical situation and the significant effect of cellular accumulation of SFN and its metabolites.[439]

Ye et al[411] and Cornblatt[183] showed that 200 µmols SFN was capable of producing a 2 µM concentration in breast cells. A dose of 200 µmols corresponds to ~ 35 mg SFN. Our lab's own unpublished tapped density data on a whole sprout powder show that a Size '00' capsule is capable of holding 700mg of powder, with allowance for a small quantity of encapsulation processing aids.[440] Bahadoran's 2011 study on type 2 diabetics showed that 5 g of their powder contained a dose of 112.5 µmols SFN, or 19.7 mg SFN (3.94 mg per gram or 0.4% SFN Yield).[197] Had they used capsules instead of powder in their study, each subject would have needed more than 12 x 700 mg capsules daily to supply 35 mg SFN, a number which is not clinically practical.

To provide 35mg SFN, 8.9 g of the grade of broccoli sprout powder used by Bahadoran would be required. Therefore, to achieve ~2 µM using Bahadoran's powder in human breast cells, around 13 capsules would be required, again impractical. It is likely that other grades of MYR-active broccoli sprout powder contain higher levels of glucoraphanin and yield more SFN; in such cases, fewer capsules would be required to achieve the same outcome. If a daily dose of 4 capsules is practical and a powder with a 1% SFN Yield were available, an intracellular concentration of around 2.0 µM may be achieved.

Clarke et al used 68 g of fresh sprouts, which they claim contained 105 mg SFN.[397] With a drying ratio typically of ~12:1, the fresh sprouts would be calculated to be equivalent to ~5.7 g of powder. For comparison and (unrealistically) assuming no processing losses, SFN Yield of such a powder could be 18.4 mg SFN per gram (~1.8 % SFN Yield). The powder used in Bahadoran's trial yielded 0.4%, so that Clarke's fresh sprouts could have provided around 4-fold more SFN. There are not sufficient available data to make similar calculations for other cell types represented in Table 5.1 but it is clear that there can be wide variability in the SFN Yield from different forms. Clarke et al compared their fresh sprout product against a MYR-inactive broccoli seed extract capsule to demonstrate the marked contrast in SFN Yield between the two.[397] They conclude that their findings have implications for

people who purchase supplements devoid of MYR, believing incorrectly they are receiving a dose equivalent to that of fresh broccoli sprouts.

5.15 Toxicity Issues

Many of the *in vitro* studies (Refer Table 5.1) show that micromolar concentrations of SFN in various cell types needed to activate cellular defences via Nrf2, range from 0.2 – 5 µM.[209,222,208,219,225,220,221,218] *In vitro* studies using endothelial cells show that SFN is cytotoxic to these cells at concentrations of 20-40 µM and that such cytotoxicity is mediated through death receptors and apoptotic signalling.[295] These levels are well in excess of anything that can be achieved through diet or indeed via the broccoli sprout supplements currently available.[441] Toxicology studies utilising 32 different haematology and chemistry tests in human subjects have supported the safety of MYR-active broccoli sprouts.[426]

5.16 Detoxification Interactions

As phytochemicals have grown in popularity as supplements, the issue of drug-phytochemical interactions must be considered.[442] In particular, phytochemicals which modulate detoxification pathways can have unpredictable adverse effects on patients by either increasing or decreasing the available concentration of prescribed pharmaceuticals.[443] As an added complication, the patient may fail to inform the attending medical practitioner that one or more supplements is being ingested concomitantly.[442]

The Cytochrome P450 (CYP P450) family are drug-metabolising enzymes involved in the activation and detoxification of a large number of pharmaceuticals, with the CYP3A sub-family involved in the metabolism of more than 50% of all therapeutic drugs in clinical use.[444] Perhaps surprisingly, naturally-occurring chemicals, at dietary levels of intake, can modulate the hepatic and extra-hepatic expression of cytochrome P450 levels, resulting in marked changes in the metabolism of pharmaceutical drugs.[443] Once thought to be relatively benign in their effects on drug metabolism, phytochemicals can potentially increase the risk of an adverse drug reaction.

A well-studied example of such drug-phytochemical interactions is *Hypericum perforatum* (commonly known as St John's Wort) which can adversely affect the immunosuppressant effect of the drug cyclosporine, thus potentially putting the well-being of the transplanted patient at risk.[442]

Similarly, grapefruit and its bioactive component, *naringenin* have been known for over 20 years to inhibit CYP3A4 and patients are often advised to avoid its consumption when taking pharmaceuticals.[445,446] To illustrate the uncertainty associated with trying to classify these phytochemical-drug responses, a series of reviewed clinical studies were unable to verify green tea's known potent *in vitro* inhibition of CYP3A4.[447]

If the metabolism of a pharmaceutical is significantly inhibited, it can accumulate to higher levels than intended, thereby providing a greater than expected dose. Phytochemicals have the potential to both elevate and suppress cytochrome P450 activity, so that both over- and under-dosing effects can be observed.[442] Coupled with CYP3A4 inhibition, there are other documented effects of phytochemical-drug reactions; the effects on the rate or amount of absorption and interaction with ATP-dependent transporters can modify the clinical response. Adding to the complexity is the fact that genetic polymorphisms and environmental factors have been shown to alter CYP activities, resulting in inter-individual differences in drug effects.[448]

If the degree of inhibition is significant, the clinical implications may be relevant for the many patients chronically ingesting commonly-prescribed pharmaceuticals such as statins, benzodiazepines, antihypertensives, antimicrobials and numerous others.[445]

Whilst SFN can be an inhibitor of the Phase 1 enzyme CYP3A4, it is also a potent inducer of the Phase 2 detoxification enzymes, including GST and NQO1.[449] SFN typically does not induce Phase 1 enzymes but does induce Phase 2; this is thought to retard the activation of a presenting toxin to its more toxic intermediate, enabling metabolic conversion to excretable non-toxic compounds via the Phase 2 enzymes.[391]

There are limited data on the extent to which SFN inhibits CYP3A4. However, a series of experiments using both broccoli extracts and SFN evaluated CYP3A4 protein levels in human hepatocytes; there was a reduction of CYP3A4 protein to 33 ± 5% and 37 ± 8% for the 10 and 25 μM SFN concentrations respectively.[393] The authors consider whether it is possible for SFN to reach a concentration in the liver sufficient to inhibit CYP3A4 expression following dietary exposure, given that a single dose of 200 μmols (35 mg SFN) has been shown to be necessary to achieve a plasma level of 2 μM.[411] They comment that substantially higher peak concentrations are likely to be seen in the liver because of extensive first pass clearance of SFN by the liver.[393] SFN in this study was shown to reduce DNA adduct formation following exposure to aflatoxin B1. Where repression of genes involved in CYP3A4 gene activation can lead to undesirable interactions with pharmaceuticals, such repression can also have beneficial effects; in this case, reduction of aflatoxin B1 bioactivation with subsequent reduction in DNA damage. In a tissue culture study using Caco-2 cells, Lubelska et al. recently described the variable effects of different concentrations of SFN in combination with several pharmaceutical drugs; SFN was shown to alter the metabolism and transport of the drug. How this might translate to the *in vivo* situation is not known.[450]

Clearly, this complex area is in need of much more investigation and clinicians should be aware that when any phytochemical supplement is ingested in conjunction with a pharmaceutical, the patient's response should be monitored with a view to modifying dosages if necessary.

5.17 Anticoagulants Reliant on Vitamin K1 Inhibition

The pharmaceutical drug, coumadin (Warfarin) is an anti-coagulant drug with a narrow therapeutic index. As such, too much or too little of the drug can lead to either haemorrhage or formation of vascular blood clots. Its principal mechanism is reliant on inhibition of endogenous synthesis of vitamin K-dependent clotting factors. Because vitamin K is found abundantly in plant foods and especially green leafy vegetables including crucifers, patients taking this drug are typically advised to avoid consuming these vegetables and to ensure that such intake is consistent.[451] In considering whether or not to recommend a broccoli sprout supplement to a patient

prescribed coumadin, the effect of such a supplement on the drug's vitamin K-related metabolism is of clinical relevance.

Where a standard 80 gram (1/2 cup) serve of broccoli vegetable contains around 110 µg of vitamin K,[452] a 28 gram (½ cup serve) of fresh sprouts contains ~ 38 µg.[453] Since no direct data are available regarding the vitamin K content of dried whole broccoli sprouts, hypothetically assuming no losses, an estimate could be made as follows: A half cup of fresh sprouts is equivalent to about 2.3 g of powdered broccoli sprout, assuming a typical 12:1 drying ratio. In most instances, a daily dose of the powder would be less than 2.3 g, providing less than one-third of the vitamin K found in a single serve of broccoli vegetable.

A dose-response study examined the effect of broccoli vegetable on the stability of oral anti-coagulant treatment in healthy adults.[454] The relative bioavailability of vitamin K from broccoli was 29% compared with that of vitamin K supplements, illustrating the unlikelihood that vegetables consumed in dietary quantities would adversely affect coagulation.[454] Furthermore, it was shown that the threshold dose causing a statistically-significant lowering of the International Normalised Ratio (INR) occurred at a dose of 150 µg daily.

It is not known how the bioavailability of dried whole broccoli sprouts compares with fresh sprouts or fresh mature broccoli vegetable. Even though it might appear that an amount of less than 38 µg obtained from less than 2.3 g of broccoli sprout powder might be unlikely to destabilise the activity of the drug, the evidence does not exist and further investigation is needed.

As a separate issue, any biochemical which induces the expression of CYP2C9 can increase warfarin clearance, thereby reducing the antithrombotic response to a given warfarin dose. Whereas watercress-derived phenyl-ethyl isothiocyanate (PEITC) can inhibit CYP2C9, it is not clear if SFN does the same.[455] Given the growing popularity of broccoli sprouts as functional foods and supplements, knowledge of the relationship between regular consumption of the dried sprouts and possible interactions with pharmaceuticals including coumadin is of clinical importance.

5.18 Thyroid Dysfunction

A toxic effect of certain glucosinolates on livestock has been known for decades[261,456] and animals consuming rapeseed meal as a major food source have been especially susceptible to the adverse physiological effects of various degradative products of glucosinolates. In humans, such products can block iodine uptake by the thyroid gland and retard synthesis of T3 and T4 hormones in the gland; hypothyroid goitre may be the outcome.[457] Just how significant this is in humans regularly consuming broccoli or other crucifers is not clear. A study on thyroid cancer-prone Melanesian women concluded that relatively high crucifer intake of ~ 70 g/day increased the risk of thyroid cancer only in those women who were moderately iodine deficient (daily iodine intake below 96 µg/day).[458] The same effect was not demonstrated in European women.

Clinicians tend to be mindful of the need for caution when counselling 'thyroid patients' about the consumption of cruciferous vegetables[457]; however, little is known about the threshold levels requiring such caution. It may be that *at risk* individuals unnecessarily avoid cruciferous vegetables which could provide more benefits than risk.[459] In the absence of more definitive guidelines, it would seem prudent to consider the possibility of iodine deficiency.

In a mechanistic study, it was recently shown that the administration of thyroid hormone, T3 to rats triggers a redox-mediated translocation of Nrf2 from the cytosol to the nucleus in rat liver.[460] This may represent a cytoprotective mechanism of T3 to counter electrophile toxicity. The induction of Nrf2 in this rat model was blocked by pre-treatment with NAC. This has clinical implications since NAC is being consumed with increasing frequency as a dietary supplement promoted as providing a precursor compound for GSH synthesis. Such negative implications of NAC are not just relevant to thyroid function but also to the numerous cytoprotective compounds generated in response to Nrf2 activation.

5.19 Classical 'Antioxidant' Supplements

It may not be intuitive to consider that vitamins A, C and E could counter the effects of Nrf2-activating phytochemicals. However, several lines of evidence converge on

the notion that modulation of the intracellular redox environment by use of *supraphysiological doses* of 'antioxidant' vitamins significantly interferes with Nrf2 signalling mechanisms.[36,461-463] That the results of numerous clinical trials using 'antioxidant' vitamins have been disappointing is well-known.[464] The mechanism to explain this failure is not yet certain but it would seem reasonable to hypothesise that interference with Nrf2 signalling may offer one possible explanation.[461] It could be hypothesised that SFN-initiated gene expression of cytoprotective genes via activation of Nrf2 can be inhibited by altered redox state due to the presence of direct-acting antioxidants. How this translates to the clinical environment is not known but may signify the need for caution when combining supplements. In the context of a SFN-yielding broccoli sprout supplement, concomitant ingestion with supraphysiological doses of redox-modulating supplements such as vitamins A, C, E and NAC might negate the potential benefits of the broccoli sprout supplement.

5.20 Criteria for a Sulforaphane-Releasing Supplement - both preventive and therapeutic

For a broccoli sprout supplement to be suitable as a preventive and/or therapeutic agent, it must meet a number of criteria. These criteria include demonstrating retention or presence of both glucoraphanin and MYR in quantifiable amounts, elimination of factors which favour SFN nitrile formation over SFN, standardisation for a specified glucoraphanin content and/or SFN Yield, a potency capable of significantly activating Nrf2, low residue of particulate seed matter and oil, compliance with specified microbiological species/concentration for supplements and it must maintain stability for a period of at least 24 months.

5.21 Current Status of Broccoli Sprout Supplements

A number of broccoli-derived supplements are already commercially-available and fall into several categories, each with its own properties.

5.21.1 Broccoli Sprout/Seed Extracts Delivered as Powders

An extract is a common delivery form for phytochemical supplements. To produce a powder, typically only the fibrous plant material and water are removed, with or without added solvent(s). Although extraction is a time-honoured method of

concentrating bioactive compounds, in the case of cruciferous plants, extraction is not appropriate. Because the reaction between the precursor glucoraphanin and the enzyme MYR is initiated in the presence of moisture, the conversion to SFN may be complete before the extraction process is finished. Although glucoraphanin is stable over a long period, SFN is not.[365] Consequently, the final extract is not likely to contain the bioactive components.

To avoid total loss of glucoraphanin content in producing an extract, MYR in the broccoli sprout material must be completely deactivated prior to extraction.[465] The product of this process is a water-soluble glucoraphanin-rich powder devoid of MYR and as such has no intrinsic ability to generate SFN on ingestion. Such products abound in the market where the label typically specifies a quantity of '*sulforaphane glucosinolate'*. However, 'sulforaphane glucosinolate' is not a scientific term and consumers and clinicians alike understandably but erroneously assume that the quantity of the material shown on the label refers to SFN, when in fact, it refers to glucoraphanin.[466] Any reference to 'sulforaphane' is inappropriate for extracts, especially given the fact that no SFN is present in *any* broccoli sprout powder; it is generated only with the addition of water and then only in a MYR-active powder. MYR-inactive products such as extracts claim to rely on the uncertain composition of the colonic microflora to produce limited quantities of SFN.[467,387] It is claimed extracts have the advantage of being water-soluble and have a milder flavour than a whole sprout powder.[466]

5.21.2 Broccoli Seed or Minimally-sprouted Powders

Some products labelled as 'broccoli sprout powder' are derived from minimally-sprouted seed (less than 3 days' sprouting). These powders appear yellow-brown, coarse and contain a high proportion of particulate matter including visible seed husks. Such products tend to contain a high proportion of oil, making the finely-milled sprout powder difficult to handle if it is to be used for manufacturing ingestible capsules. Because this oil may also be a source of potentially toxic erucic acid, such seed powders are unsuited to production of encapsulated supplements. Seed powders are sometimes marketed as food supplements where bioactives are not

named or quantified. These powders typically retain both glucoraphanin and MYR, although these values are typically not assayed.

5.21.3 Broccoli Whole Sprout Powders

Although whole broccoli sprout products have been developed with the aim of optimising SFN Yield, there are other bioactive substances in the broccoli plant with similar and supporting activities.[468] The value of a wholefood supplement has been highlighted by studies that compare the SFN Yield of a whole sprout with that of an extract. The recent work by Clarke et al.[397] comparing the in vivo effects of SFN ITCs in whole sprouts versus those in a MYR-inactive supplement in humans not only reaffirms that erucin and SFN are capable of interconversion but also provides further evidence that the bioavailability of SFN and erucin is dramatically lower when subjects consume MYR-inactive broccoli supplements versus fresh broccoli sprouts.

Although there do exist some whole broccoli sprout powders retaining both glucoraphanin and MYR, they are not as widely-available as are the extracts. Their appearance is typically more olive-green than yellow-brown and they may or may not be finely-milled. Most are available as unspecified food powders, although some manufacturers produce higher-grade finely-milled material, optimised for SFN bioactivity. These ingredients are much better suited to medicinal applications. The dilemma for the clinician is in identifying those commercially-available supplements capable of delivering the required clinical outcomes.

5.21.4 What Can a Clinician Expect of a MYR-active Supplement?

Clinicians are aware that there are many factors which can prevent the patient from adopting changes in eating behaviour; failure is common. If a patient simply does not consume the recommended diet, is there a case for appropriate supplementation, not just of nutrients but also of phytochemicals such as SFN, known to enhance cellular defences?

At this incomplete stage of SFN research, clinical trial data regarding therapeutic interventions are limited. Nevertheless, a large body of epidemiological evidence shows that even several serves per week of (presumably cooked) broccoli vegetable

may confer measureable cytoprotection.[419] Therefore, even small amounts of a MYR-active broccoli sprout supplement may offer at least the same degree of protection. As a preventive, especially in those whose poor vegetable intake is unresponsive to clinical advice, appropriate SFN supplementation may be indicated.

However, for therapeutic purposes, a relevant therapeutic dose is much more difficult to determine. Examining the *in vitro* data shown in Table 5.1 and the *in vivo* data shown in Table 5.2, it is clear that there is wide variability in the response to SFN in different tissue types. For example, the neural cortex is protected against dopamine toxicity at intracellular SFN concentrations as low as 0.1 µM.[218] Pancreatic β-cells respond to slightly higher doses, with SFN at 2.5 µM increasing cell viability to ~80% under conditions of oxidative stress.[223] Aortic levels of GSH and NQO1 levels increased 25% and 66% respectively at just 0.25 µM.[219] However, cardiomyocytes required a SFN level of 5 µM to retain 92% cell viability over a 48-hour period.[225] Table 5.3[208,209,218-226] summarises the effects of increasing sensitivity to SFN in different tissues or cell types.

Doses required for HDAC inhibition and direct cancer cell kill appear to be much higher. Tan et al[221] showed that malignant lung adenocarcinoma cells were unresponsive to doses up to 2 µM, even though normal lung cells showed up to a 7.5-fold increase in cytoprotective NQO1 at this dose. Inhibition of HDAC activity required 15 µM in prostate epithelial cells.[232]

5.21.5 Clinically-relevant Doses

The earlier estimate indicated that ~ 0.6 µM SFN could be achieved in breast cells with 4 capsules daily of the powder used in Bahadoran's trial. This may be sufficient for cytoprotection in pancreatic β-cells, neurons, aortic cells and other cell types.

Table 5.1 shows that induction of cytoprotective genes coding for NQO1 and GST can occur at low intracellular concentrations in a range of tissues. This should be readily achievable with modest intake of a whole sprout powder supplement. However, SFN has been shown to accumulate in cells and to conjugate with GSH so that both SFN and its metabolites are bioactive. Accumulation can lead to millimolar concentrations.[193] Ye and Zhang[417] show that Area Under Curve (AUC) calculations

based on multiple doses over an extended period may better correlate with inducer capacity than that derived from a single dose. In this way, multiple dosing in a fixed time period may enhance induction by some ITCs with initially low activity. This is clinically-relevant in that multiple dosing has a different effect from the single doses used in many *in vitro* studies.

5.22 Future Research

Development of a pharmaceutical is a multi-faceted, complex, protracted and expensive process. Many of the same steps are essential in the development of a functional food or nutraceutical supplement but since the FDA does not formally approve a supplement prior its market release,[469] a consumer or a clinician cannot be assured of either its quality or the potential efficacy.

There is a popular perception[470] that dietary supplements are inherently safer than pharmaceuticals and in 1994, the U.S. Congress enacted the Dietary Supplement and Health Education Act (DSHEA)[471] to authorise the regulation of such supplements. The implementation of DSHEA however has led to considerable confusion for manufacturers and marketers. Aware of the growing consumer interest in supplements together with the burgeoning costs of delivering health care to the community, the FDA recently released proposed guidelines which, if implemented, will significantly affect the supplement industry.[472] The guidelines specify that new ingredients in supplements must be proven to be safe, a situation likely to incur considerable extra manufacturing costs.[469] However, if inexpensive dietary supplements are found to be safe and effective, the requirement for additional research could yield significant community cost savings as well as health benefits.[473]

The number of published clinical trials for SFN is comparatively small, although 25 planned or in-process studies are currently listed with the U.S. National Institutes of Health.[474] Consideration of the existing SFN clinical trial data against a background of epidemiological and *in vitro* data for cruciferous vegetables and SFN itself would seem to indicate that an appropriately-produced MYR-active broccoli sprout supplement is safe and could have preventive and clinical value. In order to give clinicians the confidence they need to recommend such supplements, standardisation of both nomenclature and assay protocols is essential.

Not only is labelling of the currently available broccoli sprout supplements inconsistent but there is no reliable way for a clinician or consumer to know if the product will yield SFN on ingestion. The ability to influence cell function is entirely due to the yield of SFN and other ITCs, so that reporting other parameters is largely irrelevant.

5.23 Conclusion

Nutrition professionals and public health authorities encourage individuals to consume a diet with the appropriate proportion of plant foods; however, clinician experience and large-scale survey data continue to show that adherence to such recommendations is generally poor. Even though no supplement can replace the value of a balanced and varied diet, a SFN-yielding supplement capable of nutrigenomically inducing the expression of cytoprotective genes may provide a health benefit not achievable with the more popular ‘antioxidant’ vitamin supplements. A MYR-active SFN-yielding standardised whole broccoli sprout supplement may make the health-promoting benefits of the unpopular cruciferous vegetable more widely and conveniently available.

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Declaration of interest: One of the authors, Christine Houghton is the Director of Cell-Logic Pty Ltd, a company which manufactures a broccoli sprout ingredient.

CHAPTER 6

Sulforaphane and other Nutrigenomic Nrf2 Activators: can the Clinician's Expectation be Matched by the Reality?

This chapter was published in *Oxidative Medicine and Cellular Longevity* as a Review Article. The abbreviations, formatting and referencing of this document have been altered slightly to more closely reflect the formatting of other chapters and published work in this thesis.

A pdf version of the manuscript is attached as Appendix C.

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6.0 ABSTRACT

The recognition that food-derived non-nutrient molecules can modulate gene expression to influence intracellular molecular mechanisms has seen the emergence of the fields of nutrigenomics and nutrigenetics. The aim of this review is to describe the properties of nutrigenomic activators of transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2), comparing the potential for SFN and other phytochemicals to demonstrate clinical efficacy as complementary medicines. Broccoli-derived SFN is emerging as a phytochemical with this capability, with oral doses capable of favourably modifying genes associated with chemoprevention. Compared with widely-used phytochemical-based supplements like curcumin, silymarin and resveratrol, SFN more potently activates Nrf2 to induce the expression of a battery of cytoprotective genes. By virtue of its lipophilic nature and low molecular weight, SFN displays significantly higher bioavailability than the polyphenol-based dietary supplements that also activate Nrf2. Nrf2 activation induces cytoprotective genes such as those playing key roles in cellular defence mechanisms including redox status and detoxification. Both its high bioavailability and significant Nrf2-inducer capacity contribute to the therapeutic potential of SFN-yielding supplements.

KEYWORDS: glucoraphanin, myrosinase, nutrigenomics, antioxidant, oxidative stress.

6.1 INTRODUCTION

Whilst early 20th century nutrition science resolved issues related to micronutrient deficiency states and the latter part focused more on macronutrient excesses,[475] the first decade of the 21st century has already seen old paradigms challenged and new theories proposed. The recognition that food-derived non-nutrient molecules can modulate intracellular molecular mechanisms has seen the emergence of the fields of nutrigenomics and nutrigenetics, disciplines derived from the interweaving of the sciences of nutrition, biochemistry, molecular biology and genomics. It has been estimated that there are more than 5000 different phytochemicals present in food[476] and our current knowledge is limited to a reasonable understanding of the function of just a few.

Against this background sits the quest to identify biomolecules with significant nutrigenomic potential. A growing body of research highlights one such biomolecule, SFN, an isothiocyanate (ITC) derived from the cruciferous vegetable family and in

particular from Brassica oleracea.[477] Although the plant kingdom is the source of thousands of phytochemicals, little is known about the way in which food-derived phytochemicals support the maintenance of human health and especially those associated with cellular defence mechanisms. As the science of nutrigenomics evolves and our understanding of the many interactions between phytochemicals and endogenous cytoprotective mechanisms grows, the significance of plant foods in human health becomes clearer.

A critical review of the formulations of some available supplements reveals numerous flaws, shedding doubt on their potential efficacy.[478] There are few published clinical trials using phytochemicals as the intervention material and only a small number of these withstand scientific scrutiny. However, even when benefit for a compound has been demonstrated, it is common for a commercial product to include the ingredient at a dose many-fold lower than that shown to be efficacious in either clinical trials or as it was traditionally employed by cultures of the past. As a further trap for the unwary consumer or uninformed clinician, supporting commentary may include citations for in vitro and animal studies, giving the reader a false impression of the product's likely efficacy as a supplement for humans.

Because it appears that many consumers have accepted a role for complementary medicines in their personal health management, it is important to review the evidence on whether plant-derived supplements can assist in modifying various biochemical and physiological risk factors for disease. The aim of this review is to describe the properties of nutrigenomic activators of Nrf2, focusing on the potential for SFN and other activators of gene expression to demonstrate clinical efficacy as complementary medicines.

6.2 BEYOND NUTRITIONAL DEFICIENCIES AND EXCESSES

6.2.1 Nutrigenetics and Nutrigenomics

The interlinked sciences of nutrigenetics and nutrigenomics provide the clinician with a more targeted opportunity to personalise a patient's treatment programme,[369] revealing those genetic polymorphisms which may compromise individual biochemical function. Even without access to sophisticated genome profiling, a clinician's knowledge that

potent food-derived biomolecules can interact with intracellular signalling pathways provides another dimension to clinical management and disease prevention processes. The realisation that food-derived molecules are in constant conversation with complex intracellular control systems via signalling pathways, has unveiled the role of food as so much more than a source of micro- and macro-nutrients.[479] What becomes immediately apparent in this model is that no multi-nutrient supplement can substitute for the enormous diversity in phytochemicals present in a balanced human diet. Also evident is that the health benefits of the popular polyphenolic phytochemicals such as those found in green tea, grape seed, red wine, curcumin, pomegranate and olives are unlikely to be due to direct-acting antioxidant effects demonstrated by these molecules in numerous in vitro studies.[480,481] Polyphenols are typically large bulky molecules which are poorly absorbed and poorly-bioavailable,[184] so that it is unlikely that the intracellular micromolar concentrations necessary to scavenge free radicals can be achieved. Polyphenols can also behave as either antioxidants or pro-oxidants depending on the experimental conditions.[482] In addition, newer evidence suggests polyphenols and other phytochemicals may function hormetically, whereby dose-response is characterised by low dose stimulatory response and high dose inhibition.[483]

In a bioactive-specific approach, a recent comprehensive review of phytochemicals indicated for cardiovascular disease, focused on both preclinical and clinical beneficial effects of four commonly-supplemented compounds.[484] The review concluded that there are few definitive trials in this area and in some studies the exact dose used is not clear. However, the authors confirm the findings of others in that the use of a very high dose is associated with the most protective effects for a few phytochemicals, whereas the lowest dose turns out to be the most effective for other compounds.

As with vitamin ‘antioxidants’, the notion that ingested polyphenol supplements act as ‘antioxidants’ in human cells is called into question.[480] Emerging evidence suggests that polyphenols or their metabolites exert their systemic intracellular effects not as direct ‘antioxidants’ per se but as modulators of signalling pathways.

6.2.2 Cruciferous Vegetables Harbour Nutrigenomic Potential

The classification, cruciferous vegetables (crucifers) includes species predominantly from the Brassicaceae family and the more common members are cultivars of the Brassica oleracea genus including broccoli, cabbage, cauliflower, Brussels’ sprout and

kale but also of the Raphanus genus which includes various types of radish. Although these vegetables are good sources of micronutrients, their value to human health would seem to be at least partly due to the nature of the phytochemicals they contain and in particular the glucosinolates,[485] the enzymatic hydrolysis products of which are capable of modifying gene expression.[486] Although vegetables such as broccoli are not popular dietary choices,[258] the unique health-promoting value of crucifers continues to be reaffirmed.[487] A recent review[488] investigating the effect of crucifers on total and cardiovascular mortality, found that several prospective studies showed no association for total vegetable consumption but did show a significant inverse association for cruciferous vegetable consumption. The potential benefits of green leafy vegetables in general and cruciferous vegetables in particular are not limited to their effects in cancer and cardiovascular disease. In a 27-year prospective cohort study on cognitive decline in ageing women (n=15,080), those in the highest quintile of cruciferous vegetable intake declined more slowly than those in the lowest quintile, with a linear dose-response evident.[489] Those in the highest quintile of green leafy vegetable intake also experienced slower cognitive decline. The association did not change when data for participants with cardiovascular disease and diabetes were excluded.

Most research on crucifers has focused on broccoli, Brassica oleracea (both vegetable and sprouts) as a source of bioactive compounds with nutrigenomic potential. The last two decades have seen accelerating interest in the role of broccoli in human health following evidence that induction of detoxification enzymes might be responsible for the majority of the observed health benefits of vegetables.[27,490] After isolating broccoli-derived SFN, Zhang's group showed that SFN was a major and very potent Phase II enzyme inducer. The group of induced enzymes includes NAD(P)H:NQO1 (quinone reductase) and the family of GSTs, both of which are required for the detoxification of steroids and the ubiquitous environmental toxin, benzo(a)pyrene.[491-493] Zhang et al. concluded that the induction of detoxification enzymes by SFN may significantly contribute to the anticarcinogenic action of broccoli. The way that SFN demonstrably increased target enzymes is indicative of a nutrigenomic effect, even though the precise mechanism to explain such gene expression was not known at the time. It would be another two years before the mechanism to explain the effect of SFN would be elucidated.[146]

6.3 INFLUENCING SIGNALLING PATHWAYS

6.3.1 Nrf2 as 'Master Regulator' of Cell Defence

Although SFN interacts in a number of mammalian biochemical pathways, its effect on the redox-sensitive transcription factor, Nrf2 (nuclear factor erythroid 2-related factor 2) appears to be responsible for its greatest clinical potential when administered at practical oral doses.[494] Reference to Nrf2 first appeared in the scientific literature in 1994 and has subsequently been the subject of over 5,500 MEDLINE published papers.[146] In the ensuing two decades, Nrf2 has emerged as a key modulator of the cell's primary defence mechanism, countering many harmful environmental toxicants and carcinogens.[495] Considerable research has focused on Nrf2's role in preventing the activation of carcinogens to toxic metabolites, especially by induction of the Phase II detoxification enzyme, NAD(P)H:Quinone reductase (NQO1).[496]
The elucidation of the mechanism by which Nrf2 acts as a cytoplasmic 'switch' to activate a battery of cytoprotective genes arguably heralds a new paradigm in nutrition science. Identification of Nrf2 gave the first real clue that bioactive diet-derived compounds like SFN had the potential to co-ordinately influence large banks of function-specific genes.[497]

Nrf2 has been variously described as an activator of cellular defence mechanisms,[150] the master redox switch[151] and a guardian of health span and gatekeeper of species longevity.[152] As a mediator for amplification of the mammalian defence system against various stressors, Nrf2 sits at the interface between our prior understanding of oxidative stress and the endogenous mechanisms cells use to deal with it. What has become clear is that although attempts to counter oxidative stress by 'antioxidant' vitamin supplementation have been disappointing,[34] many phytochemicals have the capacity to activate Nrf2 and thereby induce genes[192] which collectively regulate much of the cell's endogenous defence system, enhancing its survival.[498] This finding may be clinically significant in that diseases known to be underpinned by oxidative stress may prove to be more responsive to such amplification of cellular defences via Nrf2 activation than by the administration of direct-acting antioxidant supplements.[154]

6.3.2 Sulforaphane - an Inducer of Nrf2 Target Genes

Notably and perhaps surprisingly, given its significant cytoprotective potential, SFN does not exhibit a direct antioxidant effect; instead it is weakly pro-oxidant.[499] As further evidence to support the critical role of redox signalling in cellular defence mechanisms, the ability of SFN to induce NQO1 and cell cycle arrest in prostate cancer cell lines was shown to have been completely abrogated by pre-treatment with the glutathione (GSH) precursor, N-acetyl-cysteine.[209,226] This finding has implications for the regular ingestion of readily-available supplements of NAC.

Sulforaphane [1-isothiocyanato-(4R)-(methylsulfinyl) butane: $CH_3S(O)(CH_2)_4$ -N =C =S] is a small (M.W. = 177.29) aliphatic lipophilic organosulfur molecule which is not present in cruciferous or other plants (Supplementary Data; Figure 1). Instead, plants of the Brassica genus contain a biologically inactive precursor compound, glucoraphanin (GRN) which is contained within a plant cell vacuole together with an enzyme, MYR which is separately compartmentalised.[500] It is when the plant cell ruptures and the GRN and MYR come into contact that SFN is enzymatically produced.[501] (Supplementary Data; Figure 2). Compared with its stable GRN precursor, the resulting SFN aglycone is relatively unstable[366]; this has implications for culinary applications of broccoli and other cruciferous vegetables. Broccoli is not the only crucifer which yields SFN but it yields the highest amounts, with its GRN content around 75%[431] of total glucosinolates. Notably, glucosinolate-containing plants contain variable quantities of both precursor and enzyme.[502] As a result, the yield of SFN and other isothiocyanates can vary widely.

Cutting, chewing or otherwise disrupting the broccoli plant cell structure initiates the synthesis of SFN which, compared to its stable GRN precursor, begins degrading soon after synthesis.[365] For consumers to take advantage of the cytoprotective benefits of broccoli and other crucifers, steps must be taken to conserve the integrity of the SFN released.

SFN belongs to one of nine identified classes of chemical Nrf2 activator.[503] Structurally varied, the only property shared by all inducers is their ability to react with sulfhydryl (-SH) groups. Nrf2 therefore is intimately tied to sulfur chemistry and provided dietary protein is adequate, a balanced diet should furnish sufficient sulfur. However, there are concerns that sulfur intake in many may be marginal,[504] with some researchers

suggesting that deficiency of sulfur amino acids can compromise GSH synthesis to a greater extent than for protein synthesis in both the presence and absence of inflammatory stimuli.[505] Whilst vegan diets may provide significant levels of phytochemicals,[506] there may be a need for vigilance regarding sulfur adequacy, given that the sulfur-containing amino acids are least abundant in plant proteins and that vegans typically consume about half of the sulfur consumed by those consuming a mixed balanced diet.[504]

6.3.3 Broccoli Sprout vs. Broccoli Vegetable

Much of the clinically-relevant Brassica research relates to broccoli sprouts[169] rather than to the mature vegetable, with most of the early work in this field done by a group at the Johns Hopkins University beginning in the early 1990s. The group found that 3-day-old sprouts of cultivars of certain crucifers contained 10–100 times higher concentration of GRN than the corresponding mature plants.[169] With a focus on identifying plants with cancer chemopreventive properties, they found that the sprouts were highly effective in reducing the incidence, multiplicity and rate of development of mammary tumours in dimethylbenz(a)anthracene-treated rats. Broccoli sprouts also had the added advantage of containing mostly the methylsulfinylalkyl glucosinolate (75% of the total) and very little of the indole glucosinolate found in the mature plant and which is a potential tumour promoter.[278] Their realisation that small quantities of broccoli sprouts may protect against cancer as effectively as much larger quantities of the vegetable stimulated subsequent research.

6.3.4 How Nrf2 Activators Influence Gene Expression

Although the complexity of Nrf2- pathways has not yet been fully elucidated, the principal elements are depicted in Figure 6.1.[252] Essentially, Nrf2 is sequestered in the cytoplasm by the actin-bound cytosolic repressor Keap-1 (Kelch-like ECH-associated protein 1), a cysteine-rich protein which also acts as a sensor of variations in cytoplasmic redox status. When the appropriate signal is detected by cysteine thiols within Keap-1, its ability to bind and retain the transcription factor Nrf2 in the cytoplasm is lost. Keap-1 typically responds to an electrophilic or oxidative stress signal.[252]

Thus released, Nrf2 translocates to the nucleus where it aligns with a short nucleotide base sequence in the promoter region of its target genes; this sequence is commonly

known as the Antioxidant Response Element (ARE) or the Electrophilic Response Element (EpRE), the latter considered a more correct descriptor, although the terms are used interchangeably.[507] To bind, Nrf2 dimerises with other basic leucine zipper (bZIP) proteins such as small Maf proteins (MafG) to form a transactivation complex that binds to AREs.[508]

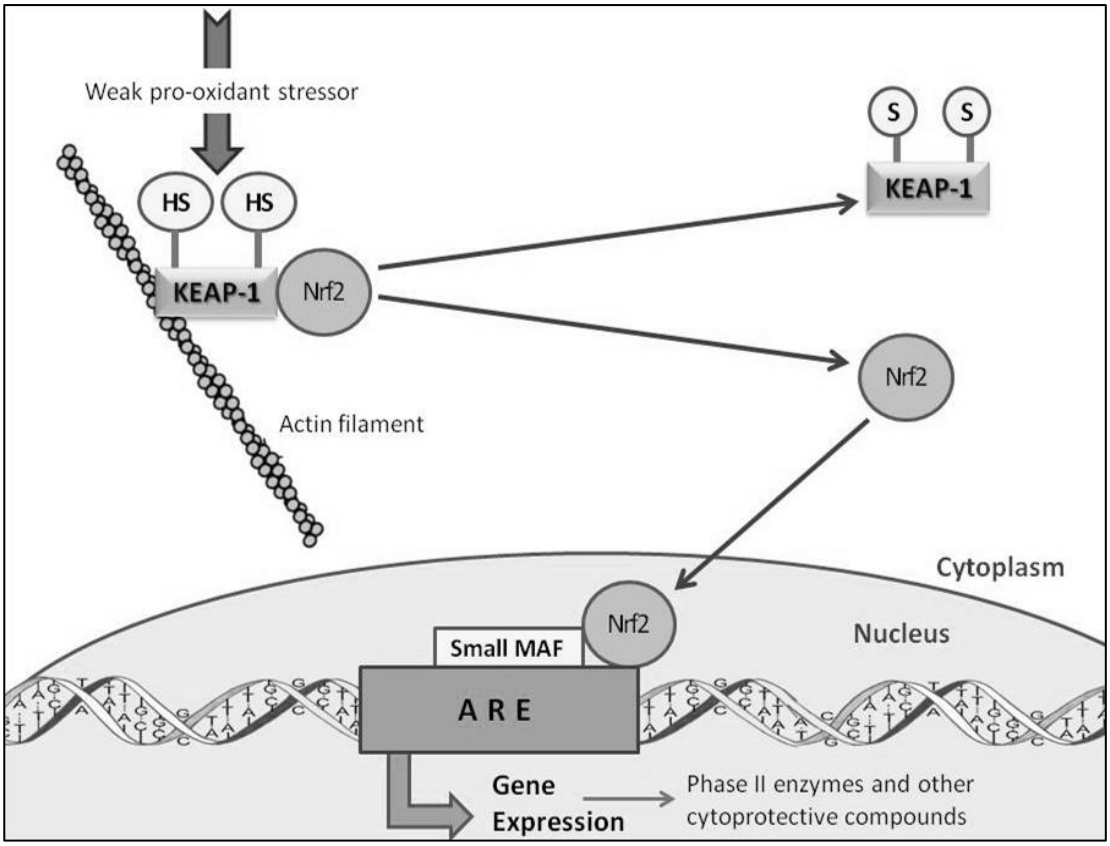

Figure 6.1 The mechanism by which Nrf2 activation increases the expression of genes with an ARE in their promoter regions.

Human Keap-1 contains 27 cysteine residues providing sulfhydryl groups (-SH) which act as sensors of ARE inducers including oxidative stress.[509] Small Maf proteins are essential for Nrf2 function.[510] Figure adapted from Kensler, 2003 with permission.[50]

When an electrophilic or oxidative stressor challenges the cell, Keap-1 senses the disturbance to its cytoplasmic redox equilibrium. After release from Keap-1, Nrf2 levels rapidly rise in the nucleus, upregulating a battery of cytoprotective genes, each containing at least one ARE. Of significance is the effect of Nrf2 on induction of the

rate-limiting enzyme for (GSH) synthesis, γ-glutamyl-cysteine synthetase, thereby elevating tissue GSH levels.[511]

For the Nrf2-Keap1 pathway to such a play a key role in cytoprotection, its activity must be capable of being regulated in tandem with the ever-changing cellular environment. Under basal non-stressed conditions, Nrf2 is continuously degraded via the ubiquitin-proteasome pathway.[512] With a half-life of around 20 minutes,[513] Nrf2 is maintained at a low cellular level.[514] Exposure to stressors inactivates Keap1 by direct modification of cysteine thiol residues, thereafter releasing Nrf2 in a derepression-type stress response.[515]

The clinical significance of this mechanism is apparent when considering the hepatotoxic effects of acetaminophen, a drug responsible for considerable drug-induced liver injury.[516] Excessive doses of this common analgesic/ antipyretic drug rapidly deplete intracellular GSH reserves. However, the cell activates an adaptive response whereby Keap-1 senses the acetaminophen metabolite, N-acetyl-p-benzoquinoneimine (NAPQI), subsequently activating Nrf2.[517] GSH is synthesised rapidly along with a battery of other Nrf2 target genes. This mechanism may not be adequate to increase GSH levels in an acute care setting, given that translation times for protein synthesis of various Nrf2 target genes can take hours. A study investigating the effect on gene expression of cytoprotective hemoxygenase-1 (HO-1) in neurons after subarachnoid haemorrhage showed that Nrf2 levels increased ~ 4-fold at 12 hours, peaking at > 4.5-fold at 24 hours, with HO-1 levels increased to >3-fold at 12 hours and peaking at >4.5-fold at 24 hours.[518]

6.3.5 Phase II Enzymes and the Detoxification Mechanisms

The mechanisms that cells use to detoxify potentially-harmful compounds, often carcinogens,[157,271] can involve a Phase I component associated with monoamine oxidases of the Cytochrome P450 family and a Phase II component where the intermediate compound produced by Phase I is metabolised in a way that permits ready excretion. A compound which activates Phase I and Phase II enzymes is known as a bifunctional inducer; however, if it activates only Phase II enzymes, it is a monofunctional inducer.[519] Phase II enzymes are induced by Nrf2 and as such are integral to this discussion. For safe and efficient detoxification, a toxin will ideally

undergo a relatively slow Phase I reaction followed by a more rapid Phase II; this tends to prevent accumulation of the Phase I metabolite which can be more toxic than its precursor.[13]

Therefore, for an optimal cellular detoxification environment, Phase II reactions should be at a rate which prevents intermediate products of Phase I from accumulating. Aliphatic SFN acts as a monofunctional inducer, whereas the indole ITCs from mature broccoli are bifunctional inducers derived from the glucosinolate glucobrassicin.[169] Of clinical significance is the finding that Phase II enzymes have a relatively long half-life, so that upregulated expression of these proteins can remain for several days. In a study using human adult retinal pigment epithelial cells (ARPE-19), NAD(P)H:Quinone reductase remained active for more than 5 days.[520]

6.4 INDUCERS OF Nrf2 TARGET GENES

Nrf2 can be activated by a variety of inducers, not all of which are obtained orally. For example, the pro-oxidant signals generated by the ROS released during exercise[521] or from inhaled environmental chemicals[522] are capable of upregulating the cellular endogenous defences, provided exposure is sufficiently modest that it does not overwhelm the cell's defences.

6.4.1 Diet-derived Nrf2 Inducers

Although a number of phytochemicals have been investigated in relation to their Nrf2-inducer ability, the mechanistic studies to explain the nature of the induction are limited. A 2008 review paper which focused on molecular mechanisms of phytochemicals in chemoprevention listed a number of natural and synthetic Nrf2 inducers; many others are known. The authors showed that a number of these have been mechanistically investigated but the mechanisms are not known for all. Of the three naturally-occurring molecules they discuss, only SFN, carnosol and quercetin have been mechanistically investigated on the basis of their Nrf2 nuclear accumulation. Furthermore, they state that only SFN has been studied for its roles in multiple mechanisms.[509]

Given the more extensive literature on SFN, we hereafter consider its potential as a supplement of clinical significance and where the data exist, comparing its potential with that of popular and widely-available phytochemical supplements.

6.4.2 Sulforaphane – in Vitro Effects

SFN is a potent Nrf2 inducer with consequent induction of cellular defences.[168] The effect is rapid in cell culture with activation by SFN occurring within 30 minutes in human bronchial epithelial BEAS-2B cells.[523] Using microarray analysis to investigate the effect of SFN in the wild-type murine liver, Hu et al. showed that expression levels of 1725 genes were increased after 3 hours' exposure and 3396 genes were changed after 12 hours.[192] Comparing expression patterns at different time points, they also showed that maximal change occurred 12 hours after a single administration of SFN, based on fold changes greater than 2-fold. The identified Nrf2 target genes can be classified broadly as those coding for a range of cytoprotective proteins, including antioxidants (enzyme and non-enzyme), drug-metabolising enzymes, drug-efflux pumps, heat shock proteins, NADPH regenerative enzymes, growth factors and growth factor receptors, heavy metal binding proteins and various nuclear receptors including PPAR-γ as well as for Nrf2 itself.[192]

Vitamin D's protective effects on human cells are well-recognized[524]; it may be nutritionally significant that the vitamin D receptor (VDR) is a Nrf2 target gene inducible by SFN[525]; in turn, vitamin D can increase Nrf2 expression.[526] To further illustrate this diversity, Nrf2 target genes include those coding for β-defensin-2 (HBD-2), an antimicrobial peptide associated with innate immunity, protecting the intestinal mucosa against bacterial invasion. HBD-2 can be induced by SFN[525] and was shown in a cell culture study using human Caco-2 cells to be significantly induced 1.6-fold at 24 hours and 2-fold at 48 hours by SFN concentrations of > 5 μM. These results may have relevance in disorders of the intestinal epithelium but systemically, an intracellular concentration of 5 μM is probably higher than can be readily achieved by diet or even via practical doses of available oral SFN-yielding supplements.

The downstream enzyme products of Nrf2 target genes are efficient and versatile. They include those which constitute the glutathione and thioredoxin systems, the major cellular reducing systems in the body.[191] Several reasons explain their efficiency and versatility:[28] *1)* they are not consumed stoichiometrically, as are direct-acting antioxidants such as ascorbate and tocopherols; *2)* their duration of action is long with half-lives measured in days, so their induction need not be continuous; *3)* they restore the endogenously-produced direct-acting antioxidants like coenzyme Q10 and the

tocopherols by returning them to the reduced state (in particular via NQO1 because both coenzyme Q10 and tocopherols are quinones). Major products of Nrf2 target genes and their roles in cytoprotection are listed in Table 6.1.

Table 6.1 Major Products of Nrf2 Target Genes

PRODUCTS OF Nrf2-TARGET GENES	ROLE IN CYTOPROTECTION
Glutathione (non-enzyme) (GSH)	Abundant intracellular sulfur-containing direct antioxidant – predominant intracellular thiol.[264] Essential in function of Glutathione peroxidase and GST for redox balance and detoxification.[264]
Haemoxygenase-1 (HO-1)	Redox-regulating, broad protection against oxidative stress.[527] Metabolises haem, also producing bilirubin which scavenges peroxyl radicals. Anti-inflammatory and immune-modulating properties. [528]
Thioredoxin (Trx) (non-enzyme)	Ubiquitous intracellular sulfur-rich protein. Singlet oxygen quencher and hydroxyl radical scavenger.[529]
Thioredoxin reductase (TrxR)	An oxido-reductase which regenerates Trx and GSH.[530]
Glutathione-S-transferase (GST)	A Phase II detoxifying enzyme with broad spectrum of activity, depending on subclass.[28]
Quinone reductase NAD(P)H:Quinone oxido-reductase (NQO1)	A multifunctional redox-regulating and detoxifying enzyme, including protection against oestrogen quinone metabolites.[531] Directly scavenges superoxide but less efficiently than SOD.[315] Stabilises the p53 tumour suppressor protein,[30] especially under exposure from γ-irradiation or other oxidative stress. Protective

	against dopamine cytotoxicity where SOD and Catalase were not.[532]
Ferritin	Binding of free iron to prevent its reaction with superoxide to produce hydroxyl radical.[533]
Metallothionein	Removal of heavy metals such as mercury and cadmium.[534]
Peroxisome proliferator-activated receptor (PPAR-γ)	Regulator of adipogenesis and central integrator of glucose metabolism, energy homeostasis and skeletal metabolism.[535]
Nuclear factor erythroid 2-related factor 2 (Nrf2)	Nrf2 induces its own synthesis.[409]
NADPH regenerative enzymes	Restores reducing equivalents and reduces oxidised GSH to its reduced form.[30]

6.7 QUINONE REDUCTASE (NQO1) – A TOOL TO EVALUATE INDUCER CAPACITY

Initially considered as an Nrf2-activated Phase II enzyme associated with detoxification pathways, the function of NQO1 is now considered to be much broader[30]. NQO1 has been described as a "quintessential cytoprotective enzyme" and is coded by what is considered 'one of the most consistently and robustly inducible genes within its class'.[30] Furthermore, its activity declines with age whilst upregulation of its activity by Nrf2 induction is described as an avenue for maintaining cellular defences with advancing age.[152] Furthermore, animal studies show significant decline in Nrf2 activity between youth and old age.[536-538] Humans genetically deficient in NQO1 are more susceptible to the carcinogenicity of benzene exposure.[314] NQO1 is highly active in pulmonary tissues[539] as well as in epithelial and endothelial cells in general,[494] suggesting that it could act defensively against compounds absorbed via the airways, gut and bloodstream. NQO1 activity is used as a rapid screening procedure and a biomarker of the anticarcinogenic activity of phytochemicals.[253,503] The assay[490] uses cells defective in Phase I function to provide the means for selectively distinguishing monofunctional inducers that elevate Phase II enzymes.[540]

6.7.1 The CD Value as a Comparative Marker

The term 'CD value' describes the concentration required to double NQO1 activity in murine hepatoma cells.[541] A CD value is also useful for comparing the potential in vivo nutrigenomic effect of an ingestible bioactive compound. The CD value has also been used [27,253] to classify Brassica spp. according to their relative 'anti-cancer potential'. When several crucifers were compared for their Nrf2-inducer effect,[542] ITCs of cabbage, kale and turnips exhibited less NQO1 inducer capacity than broccoli-derived SFN. SFN returned ~33,000 units NQO1 inducer activity/g fresh weight for broccoli, cabbage returned ~11,000 units, kale ~10,000 units with turnip ~2,000 units. This property may partly explain why broccoli is researched more extensively than are other Brassica spp.

6.7.2 Clinical Significance of CD Value

In data from studies comparing CD values of well-known phytochemicals, SFN showed the highest potency, with a concentration as low as 0.2 µM required to double the activity of NQO1.[27,541] The comparative CD values of other phytochemicals have been

documented by others, [32,543-545] with lower micromolar concentrations representing those with the higher inducer activity. (Figure 2)

CD values are available for phytochemicals used in common oral supplements [32,253,543,544,546]: SFN (0.2 µM), andrographolides (1.43 µM), quercetin (2.50 µM), β-carotene (7.2 µM), lutein (17 µM), resveratrol (21 µM), indole-3-carbinol from mature broccoli vegetable (50 µM), chlorophyll (250 µM), α-cryptoxanthin (1.8 mM) and zeaxanthin (2.2 mM), An earlier study conducted in a different laboratory[546] had shown curcumin (2.7 µM), silymarin (3.6 µM), tamoxifen (5.9 µM), genestein (16.2 µM), epigallocatechin-3-gallate (EGCG) (>50 µM) and ascorbic acid (>50 µM). The comparative NQO1 inducer activity of these phytochemicals is: SFN> andrographolides> quercetin> curcumin> silymarin> tamoxifen> beta-carotene> genestein> lutein> resveratrol> I-3-C> chlorophyll> α-cryptoxanthin > zeaxanthin.

Notably, the CD value of SFN is 13.5-fold greater than that of curcumin, 18-fold greater than silymarin and 105–fold greater than resveratrol, all phytochemicals which are extensively promoted for their claimed health-promoting properties. It may be useful for relevant oral supplements to be evaluated in relation to the CD value of their primary ingredient(s), given that an internet search will readily reveal many self-select and clinician-recommended supplements claiming to ‘enhance detoxification’ and ‘promote longevity’, even though supporting evidence is not apparent. Many such supplements claiming to target ‘detox’, are based on ingredients such as chlorophyll and vitamin C, both of which have comparatively low NQO1 inducer capacity.

It is also important to note that the CD values discussed above are generated from *in vitro* cell culture data and that both the ranking and the magnitude of these values can change substantially when the same compounds are investigated in an *in vivo* environment. When considering the clinical relevance of such findings, it is useful to do so in the context of bioavailability as discussed later in 6.10.

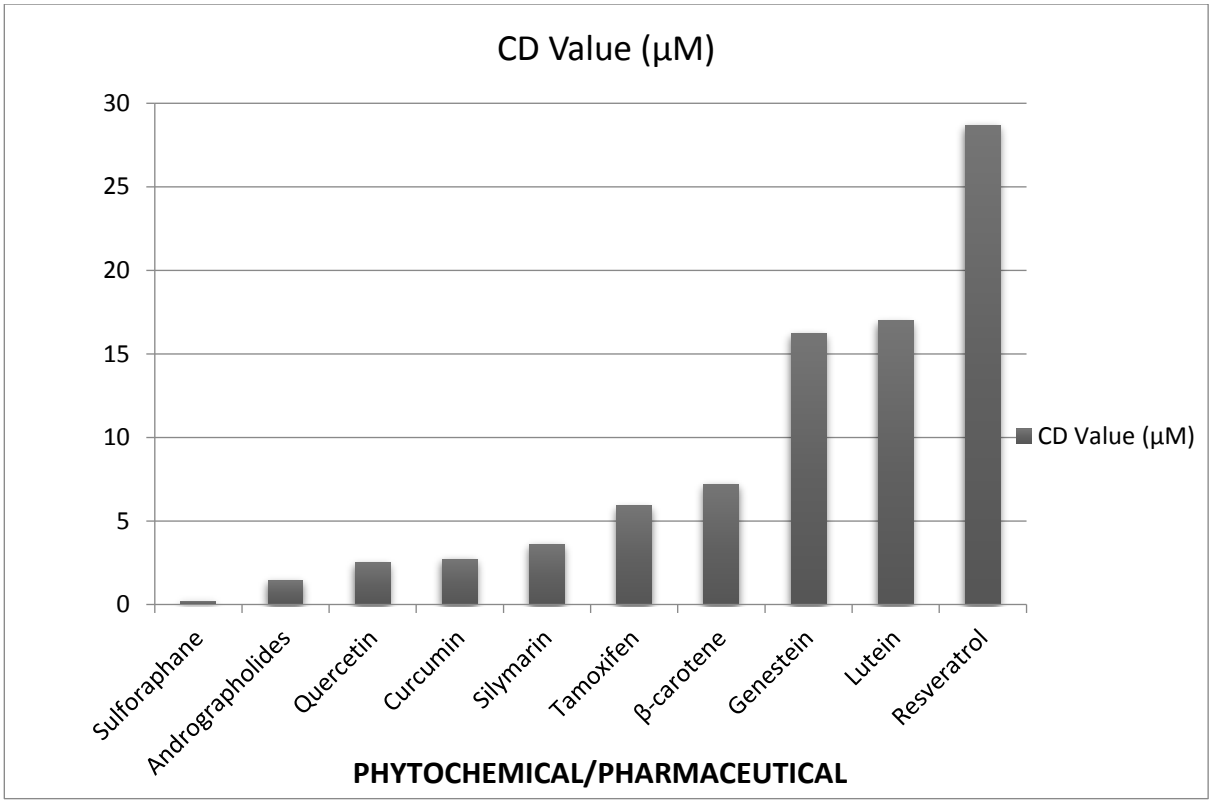

Figure 6.2 CD Values of popular phytochemicals used as supplements and a commonly-prescribed pharmaceutical.

CD Values refer to the concentration of a compound required to double the activity of the Phase II detoxification enzyme, Quinone reductase.[32,253,543,544,546]

6.7.3 Comparing Effects of Indole Glucosinolates

Indole-3-carbinol (I-3-C), the ITC found in mature broccoli vegetable (but not significantly in the sprout) required > 50 µM to double NQO-1 activity[253]. In vivo, I-3-C must be dimerised in the acidic environment of the stomach to 3,3'-diindolymethane (DIM) to be active.[547] This has certain clinical implications as synthetic molecules of both I-3-C and DIM are available as supplements. With significantly lower inducer capacity than SFN,[546] it bears mention that DIM is also a bifunctional inducer of the detoxification pathway, thus limiting its cytoprotective potential. Early research on broccoli sprouts suggested potential limitations to the use of indole glucosinolates such as I-3-C as chemoprotectors in humans.[169] Not only are they weak inducers of Phase II enzymes but as bifunctional inducers, they simultaneously activate Phase I enzymes. They may also have estrogen receptor binding activity, adding to their potential as tumour promoters.[169]

Interestingly, DIM is sometimes recommended clinically for patients with compromised estrogen metabolism, the theory being that DIM inhibits CYP1B. Inhibition of CYP1B1 shifts estrogen metabolism towards 4-hydroxyestrone, a metabolite which can contribute to carcinogenesis.[531] Not all data agree; a 2007 cell culture study analysed gene expression using microarray profiling and quantitative real-time–polymerase chain reaction in MCF7 breast cancer cells treated simultaneously with estradiol and DIM.[548] CYP1B1 was upregulated with a fold-change of 3.93 ± 0.25. Such findings would tend to suggest that DIM may not protect against the metabolism of estrogen to the 4-hydroxy metabolites. Such conflicting data indicates that clinical trials are required to establish the in vivo effects of such an intervention when using a clinically-relevant dose of a readily-available supplement.

To illustrate the differences in potency between SFN and I-3-C in a study using a prostate cell line, it was found that both compounds inhibited the proliferation of the prostate cancer cells in a dose-dependent manner but the inhibitory concentration of SFN required was just 10% that of I-3-C.[549] They may also be safety issues which require caution in the recommendation of I-3-C supplements, available at many times the quantity of I-3-C achievable from broccoli vegetable consumption. Although I-3-C administered one-week after the last dose of the carcinogen has been shown in rats to result in a latency delay of mammary tumour formation, it did not alter tumour incidence or multiplicity among survivors.[550] Any research showing a preventive benefit of this compound must be considered against the risk that it may promote liver and colon cancer.[550]

6.8 OTHER MODES OF ACTIVATING Nrf2

Although our focus is to compare the inducer capacity of phytochemicals, Nrf2 in human cells is activated by a range of stressors, not all of which are chemical in nature. The diverse nature of Nrf2 activators is highlighted in the three examples which follow. We use several examples of pharmaceuticals with pleiotropic Nrf2-inducing effects. Furthermore, we illustrate that when pharmaceutical Nrf2 activation occurs at supraphysiological levels, the outcome may be unexpected, indicating that the significantly lower inducer capacity of diet-derived Nrf2 activators may represent a hormetic effect.[551]

6.8.1 Mechanical Effects

The mechanical effect of blood flow in regions where arteries are exposed to high shear stress are protected from inflammation and atherosclerosis. By contrast, low-shear regions are susceptible and this effect has been shown to be due to the effect of Nrf2 in reducing activation of the endothelium at atherosusceptible sites.[173]

6.8.2 Pharmaceutical Drugs

The pharmaceutical tamoxifen, commonly-prescribed to women following treatment for breast cancer is an NQO1 inducer but its CD value is 30–fold lower than that for SFN.[552] Nrf2 inducer activity may play some role in this drug's therapeutic profile in addition to its primary role as a selective estrogen receptor modulator (SERM).[552] These comparative data may be clinically significant when considering the potential value of a drug or supplement with cytoprotective potential. A number of other pharmaceuticals activate Nrf2. The redox-modulating activity of the frequently-prescribed statins and Angiotensin Converting Enzyme (ACE) inhibitors has been attributed to their Nrf2-inducer ability.[553] Similarly, gold salts, once the mainstay of treatment for rheumatoid arthritis, are Nrf2 inducers.[554] Indomethacin, now seldom used in reducing the symptoms of inflammatory joint diseases has Nrf2-inducing properties, illustrating that non-steroidal anti-inflammatory drugs (NSAIDs) exhibit properties other than their anti-inflammatory effects.[555]

A relatively new pharmaceutical, Bardoxolone Methyl (BARD) was shown to enhance estimated glomerular filtration rate (eGFR) in patients with chronic kidney disease, a disease characterised by significant oxidative stress.[556,557,558] BARD is a synthetic analogue of oleanolic acid, a triterpenoid found extensively in edible plants[559] and with broader cytoprotective properties attributed to Nrf2 induction.[179] The Phase 3 BEACON Trial[560] was halted in October 2012 following adverse events including 57 deaths out of 2185 participants in the BARD arm.[561] In comparing the inducer activity of BARD with that of SFN, a 2005 study comparing a range of triterpenoids showed that BARD was 230-fold more potent than SFN as a NQO1 inducer.[562] The adverse effects demonstrated by the synthetic triterpenoid analogue in the BEACON trial may be representative of a hormetic response at the upper end of a bifunctional dose-response. By contrast, phytochemicals at the doses provided by foods are typically non-toxic.[551]

6.8.3 Exercise

Exercise is associated with an increased flux of glucose and oxygen through the mitochondria, a process which increases levels of ROS such as superoxide. An essential role for exercise-induced ROS formation in activating transcription factors and co-activators has been proposed.[119] Ristow et al demonstrated that typical exercise-related changes in gene expression were almost completely abrogated by daily ingestion of supplements of vitamins C and E at doses of 1000 mg and 400 IU respectively.

A review highlighted 23 studies showing that antioxidant supplementation interferes with exercise training-induced adaptations.[463] An emerging theme[375] supports the view that because Nrf2 is activated by a mild pro-oxidant signal, high doses of antioxidant supplements may blunt signals required to activate endogenous defences.[563,564] Ristow's assertion that antioxidant supplementation blocks many of the beneficial effects of exercise is supported by such evidence.

6.9 OTHER ACTIONS OF NQO1 WHICH CAN BE INFLUENCED BY SULFORAPHANE

NQO1 exhibits broad substrate specificity extending well outside its better-known role as a Phase II inducer; its other roles as described in the following section may contribute to its cytoprotective capacity. Its actions include: *1.)* Protection against benzene-derived quinones such as benzo(a)pyrene, a carcinogen found commonly in petrochemical exhaust gases and in barbecued meats [565]; *2.)* NQO1 can reduce catechol estrogen quinones to catechol estrogens, a process associated with lowering breast cancer risk due to elevated estrogen metabolites.[566] *3.)* NQO1 can scavenge superoxide, albeit at a lower order of magnitude than does SOD.[567] *4.)* NQO1 stabilises p53, the tumour suppressor gene[30]; *5.)* NQO1 restores oxidised coenzyme Q10 (ubiquinone) and the tocopherols to their reduced forms.[30]

Several NQO-1 polymorphisms exist and these have been associated with risk of carcinogenesis. The C609T gene variant is one of very few common SNPs known to almost completely eliminate enzymatic activity; consequently, NQO1 is attracting considerable research attention given its multiple effects in cellular defences.[568]

6.9.1 Other Mechanisms – Animal Studies

Although a large volume of the published SFN research is associated with its Nrf2-inducer potential, some studies point to other mechanisms. A recent study used broccoli sprout juice as the intervention material in stroke-prone spontaneously-hypertensive rats to investigate possible effects on renal damage.[569] After 4 weeks, the animals were shown to have been largely protected against renal damage. Mechanistically, the effect was shown to be independent of systemic blood pressure but to parallel stimulation of the AMPK/SIRT1/PGC1a/PPARa/UCP2 axis. Whether this can be replicated in humans at practical doses has not yet been investigated.

6.10 THE ISSUE OF BIOAVAILABILITY

6.10.1 Comparative Effects of Popular Phytochemical Supplements

Aside from wide variation in Nrf2 inducer capacity, a second barrier to clinical efficacy is bioavailability. When bioavailability is low, cell culture studies may significantly over-estimate the intracellular concentration that ingestion of such a compound can achieve, being unlikely to demonstrate the expected clinical benefit indicated by the in vitro work.[570,571] In considering the potential clinical efficacy of a phytochemical, the active compound and/or any of its active metabolites must reach the cells of the target organ(s) in appropriate concentration. Oral bioavailability of polyphenols is typically < 10%, ranging between 2 and 20%,[572] with many closer to 1%; cooking and processing significantly reduce polyphenol content.[352] By comparison, a pharmacokinetic animal study showed that SFN was rapidly absorbed with its absolute bioavailability 82%.[573] Many phytochemical-containing supplements contain polyphenolic molecules such as curcumin (turmeric), catechins (green tea), resveratrol (grapes), and ellagic acid (berries and pomegranate), hydroxytyrosol and oleuropein (olives). Much of the evidence used to promote these supplements is either from in vitro or animal studies, with limited clinical evidence to support the assertions. Supplements of these phytochemicals frequently bear an ‘antioxidant’ claim, even though the amount of polyphenol reaching the circulation or target cells is seldom adequate to alter redox status,[480,574] gene expression studies have helped in quantifying likely systemic responses. Preclinical cell culture or animal studies may involve very high doses of an isolated polyphenol. Such doses are seldom clinically practical, considering average dietary intake of mixed

polyphenols in food is approximately 1gram per day of poorly-bioavailable compounds.[181]

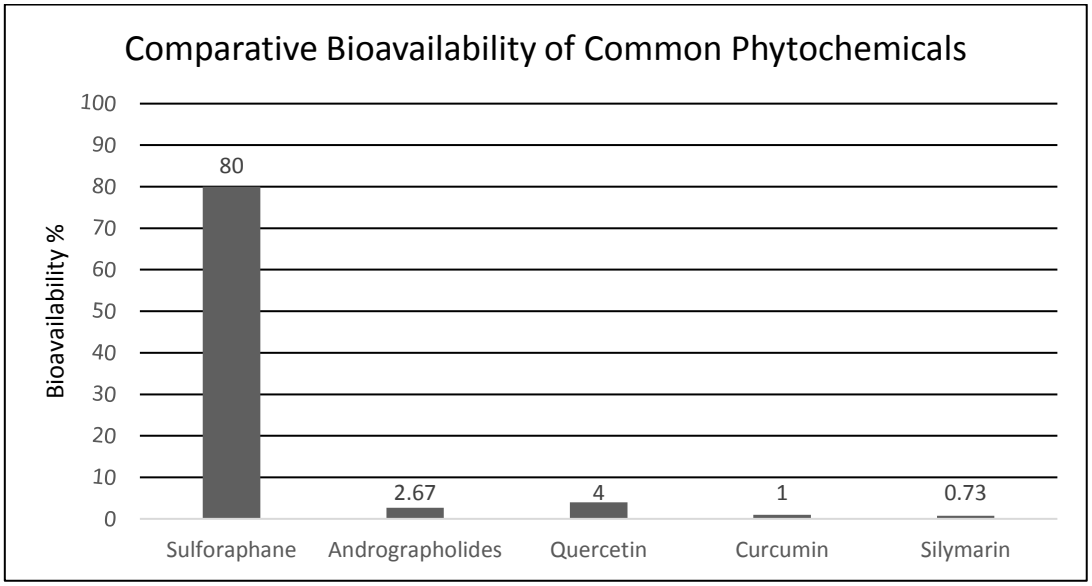

Figure 6.3. Comparative Bioavailability of Phytochemicals commonly used in supplements.[182,545,575-578]

Curcumin, resveratrol and silybin are examples of popular polyphenol supplements for which preclinical findings cannot be readily extrapolated to the clinical environment. Figure 6.3 compares the bioavailability of several polyphenols with that of SFN [native curcumin at ~ 1%,[575] resveratrol < 1%,[577] and silybin ~ 0.73% [576]]. In each case, the systemic bioavailability compares the plasma concentration of an oral dose to an intravenous dose and is expressed as a percentage, where F = Bioavailability.[545]

$$F^{oral} = (AUC^{oral}/Dose^{oral}) / (AUC^{i.v.}/Dose^{i.v.}) \times 100\%$$

The high intracellular concentrations of polyphenols required to replicate in vitro findings are difficult to achieve in humans with practical oral doses.

There is some evidence to suggest that the activity of some polyphenols may instead reside in their metabolites,[579] so that small quantities absorbed intra-cellularly act as signalling molecules and may act synergistically with other biomolecules.[580] It is likely that any direct antioxidant effects occur only within the lumen of the gut and not systemically. [482,581]

Quercetin naturally found in onions, watercress, tea and other plants, is a popular oral supplement typically promoted as an 'antioxidant' or 'anti-inflammatory' agent. Some studies suggest that quercetin may have anti-cancer potential[582] but other studies describe potential for risk,[583] given that quercetin may exhibit pro-oxidant effects, especially in a GSH-depleted cellular environment.[584]

Specifically, quercetin can exert an inhibitory effect on the metabolism of catechols via the catechol-O-methyltransferase enzyme (COMT).[585] This may have implications in estrogen-related disorders where inappropriately-metabolised estrogens can form DNA adducts.[531] Whether oral doses of quercetin have these effects in humans is not known but the issue has been flagged as 'concerning' since readily-available quercetin supplements represent up to 100 times the quantity typically available in a Western diet.[586]

6.10.2 Curcumin

Curcumin is regarded as having in vitro anti-inflammatory activity by virtue of its ability to inhibit the transcription factor, NF-κB.[587] In a study investigating inflammation in human tenocytes, high concentrations of 5–20 μM were required to inhibit IL-1β-induced inflammation.[588] However, very high oral doses in humans (up to 8 g) yielded curcumin peak intracellular levels of only 0.5 – 2.0 μM, clearly not attaining a concentration of the same order; commonly-recommended supplemental doses of up to 180 mg were undetectable in plasma.[589,590] Laboratory findings demonstrating an impressive and diverse array of cytoprotective effects for curcumin may not generally apply to practical oral doses in humans.[591]

By contrast, there is evidence for an effect in gastrointestinal tissue, where transport occurs across a single enterocyte membrane.[592,593] Patients with colorectal cancer were administered doses up to 3.6 g curcumin daily.[594] M_1G, a marker of DNA damage, decreased 38% in the colorectal tissue, showing that a dose of 3.6 g daily achieves pharmacologically efficacious levels in colonocytes but with negligible distribution outside the gut, confirming its poor systemic bioavailability.

When considering both CD value and bioavailability, native curcumin with bioavailability of ~ 1% would appear to be less clinically-relevant than SFN which shows both high inducer activity and high bioavailability. Even enhanced forms of curcumin with ~ 7-fold

higher bioavailability still exhibit comparatively low bioavailability.[595] Investigating physiologically-achievable doses of curcumin, Lao et al, administered from 500 - 12,000 mg of a curcumin powder; no curcumin was detected in any of 74 participants taking up to 8,000 mg; low serum levels in the ng/ml range were detected only for doses > 8,000mg, with doses below 4,000 mg barely detected.[596] Similarly, curcumin was not detected in normal liver or colorectal liver metastases in patients receiving 3.6 g/d for 1 week.[594] Howells et al conclude that in vitro studies with curcumin in the high 10 µmol/L range or below might have human physiological relevance but that its role as a chemopreventive agent may lie primarily within the gastrointestinal tract.[590]

6.10.3 Resveratrol

Resveratrol achieved international acclaim after studies in mice and lower organisms indicated that it was responsible for a longevity effect.[597] Only mice administered resveratrol from birth lived longer; those started at middle age had no longevity benefit.[598] The benefit appeared due to enhanced expression of survival genes, a number of which are also expressed during caloric restriction.[599]

The longevity effect has never been tested in humans, so an appropriate dose is not known nor even if a longevity benefit is likely.[600,601] Although well-absorbed, resveratrol displays low bioavailability; at least 70% of an oral 25 mg dose in human subjects was shown to appear as resveratrol metabolites in plasma, with most of the oral dose subsequently recovered in the urine.[577] Like curcumin, resveratrol is readily absorbed by enterocytes/ colonocytes,[578] showing potential benefit to intestinal tissues. A daily resveratrol dose of 3000 mg administered to overweight or obese men with non-alcoholic fatty liver disease (NAFLD) over 8 weeks, did not significantly improve any of the features of NAFLD over placebo.[602]

A review of 3650 publications on resveratrol concluded that the evidence is not sufficiently strong to justify a recommendation for resveratrol to humans beyond the dose which can be obtained from dietary sources, estimated to be ~ 4mg daily for adults.[603]

6.10.4 Silymarin

Silymarin, the major flavonoid complex in Silybum marianum has a long history of traditional use in liver disorders.[604] Silymarin supplements claiming to target human liver detoxification mechanisms are readily-available. Silibinin, the most bioactive of the complex is insoluble in water, not lipophilic and with low bioavailability of 0.73% in rats.[576] Its CD value ranks next below curcumin and third after SFN. Where optimising Phase II detoxification is the desired outcome, there may be value in considering both CD values and bioavailability. Such evidence sheds considerable doubt on the likely efficacy of many such phytochemicals at doses typically found in commercially-available supplements. Nevertheless, published trials show that silymarin exhibits hepato-protective properties in humans, indicating that other mechanisms may be responsible.[604,605]

6.10.5 Sulforaphane

SFN's lipophilic nature and low molecular weight readily enable passive diffusion into cells.[573] It is rapidly absorbed, peaking in plasma as early as 1 hour after ingestion.[318] Predictably, dose-dependent pharmacokinetics in rats reveals that bioavailability decreases with increasing dose.[573] The doses corresponded to ~ 0.5mg, 1.0 mg and 5.0 mg/kg of pure SFN, a relatively high for humans who typically consume a Brassica vegetable and not pure SFN. It is unlikely that humans, through diet would ingest such high quantities of SFN. By calculation, a MYR-active whole broccoli sprout supplement yielding 1% SFN could deliver 10 mg SFN per gram of powder, corresponding to ~ 12 g of fresh broccoli sprouts (dried powder retains ~ 8% moisture). Administering 5.0 mg/kg of SFN to a 70 kg human at the upper end of the animal dose range represents an intake of 350 mg or 35-fold the quantity that a human might reasonably ingest from dietary the fresh sprouts. Clearly, these quantities are not a practical means of providing a broccoli sprout supplement for human use.

6.10.6 Dose Considerations in Humans

An indication of what might be practically achievable with supplementation is illustrated by several human studies. Ye et al. showed that after a single 200 µmol oral dose of SFN, both SFN and its metabolites were detected in plasma and erythrocytes in just 15 minutes, peaking in all four subjects at ~ 2.00 µM after 1 hour and declining with first-

order kinetics, with a mean half-life of 1.77 ± 0.13 hours.[411] To investigate effects in systemic tissue, Cornblatt et al. showed that one hour after a single 200 µmol oral dose of SFN administered to 8 women, metabolites were detected in resected left and right breast tissue at concentrations of 1.45 ± 1.12 and 2.00 ± 1.95 pmol/mg tissue respectively. (Section 4.14.5 queried the validity of the units shown here; more likely, the correct units were pmol/g.) This proof-of-principle study observed a significant induction of NQO1 enzymatic activity in the same tissue.[318] In another example, a dose-escalation placebo-controlled study investigated Phase II gene expression in human airways mucosa, showing that a 200 gram broccoli sprout homogenate delivering 102µmol of SFN increased NQO1 mRNA expression by almost 200%.[41]

Given that oral doses appear capable of increasing NQO1, we consider whether it may be possible that a SFN-yielding broccoli sprout powder might deliver a plasma concentration of ~ 2.00 µM. By calculation, a 1% powder yields 56.4 µmol SFN per gram. Ye et al. showed that a single 200 µmol dose resulted in a peak plasma concentration of ~ 2.0 µM after 1 hour. As Ye[411] et al. had shown that a 200 µmol oral dose had resulted in a plasma concentration of ~ 2.0 µM and Riedl[41] had shown that 102 µmol had increased NQO1 mRNA expression by ~ 200%, these orders of magnitude could be achievable with a SFN-yielding broccoli sprout powder. Theoretically and by calculation, an individual could consume around 2 g of a 1% SFN-yielding broccoli sprout powder to achieve what Riedl et al. achieved with 200 g broccoli homogenate and 4 g to achieve what Ye et al, achieved with a single 200 µmol dose.

6.11 FACTORS GOVERNING SULFORAPHANE YIELD

6.11.1 The Role of MYR

Glucosinolates as Brassica-derived precursor compounds are converted to their bioactive forms only under the action of MYR because GRN has no inherent bioactivity. Investigating the metabolic fate of ingested broccoli phytochemicals, Shapiro et al. showed that MYR-inactivated broccoli resulted in 10-20% lower conversion to ITCs. When the colonic microflora were reduced, recovery of ITCs in a MYR-free environment was negligible. It may be inferred that MYR is essential for SFN synthesis and that the colonic microflora may exhibit MYR-like activity.

The colonic microflora appear capable of limited MYR activity, with conversion to the bioactive ITC varying from 1% to 40% of the dose.[606] Several genera of human microflora such as Bifidobacterium, Lactobacillus and Bacteroides have been reported to possess MYR-like activity [607] but with wide variability in their population, the ability to hydrolyse glucosinolates cannot be reliably estimated. So unpredictable is this factor that a large clinical trial using a MYR-inactive BSE could not achieve statistical significance.[38] Many available broccoli sprout supplements are MYR-inactive extracts which claim their clinical benefit is due to the alleged conversion to SFN by the colonic microflora. Neither consumers nor clinicians have any way of knowing if an individual harbours MYR-active microflora.

6.11.2 The Nitrile Factor

Among crucifers, broccoli contains significant amounts of Epithiospecifier protein (ESP), a non-catalytic inhibitor of MYR activity.[251] ESP produces inactive SFN nitrile. Under certain conditions, the nitrile pathway is favoured, with the hydrolysis product constituting as much as 75% nitrile (Figure 6.4). The colonic microflora also support nitrile formation, thereby further limiting the potential of a MYR-inactive supplement[608] ESP deactivation can significantly enhance SFN Yield, illustrating that broccoli and broccoli sprout products cannot be meaningfully evaluated on the basis of their GRN content alone.

It is likely that clinical trials using either fresh or powdered broccoli sprouts may give conflicting results when the presence or absence of nitrile has not been considered. The presence of ESP means that assayed measurement of the SFN Yield is critical in order to estimate the real efficacy of a broccoli sprout powder intended for a supplement; measurement only of GRN and MYR does not allow for the effect of ESP on enzyme activity.

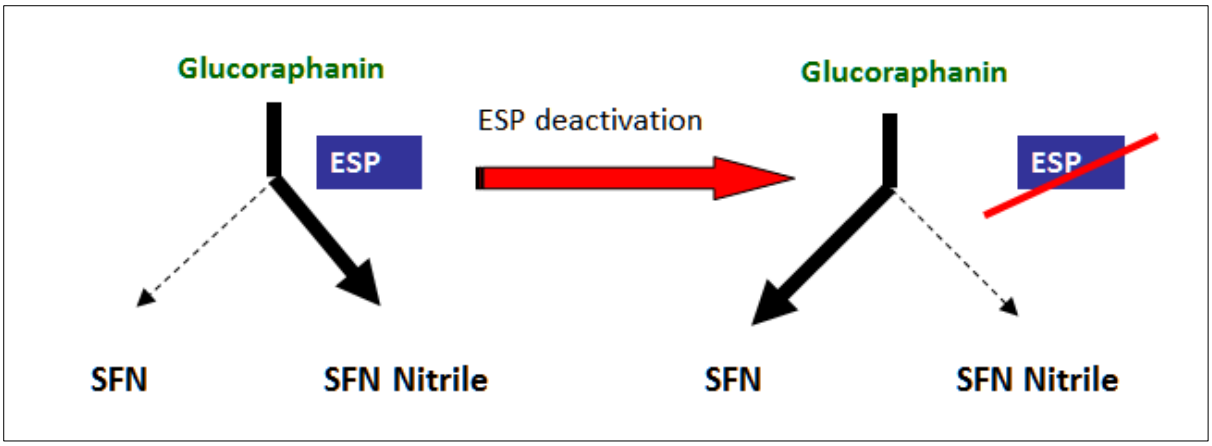

Figure 6.4 Epithiospecifier protein (ESP), an inhibitor of myrosinase enzyme

The presence of Epithiospecifier Protein (ESP) prevents complete conversion of glucoraphanin to sulforaphane. Instead, part of the glucoraphanin is converted to inactive sulforaphane nitrile. As much as 75% of the product of MYR activity on glucoraphanin can be sulforaphane nitrile.

6.12 CLINICAL IMPLICATIONS

6.12.1 Cruciferous Vegetable Consumption

The presence of unquantified amounts of ESP in raw broccoli has clinical implications; as a salad vegetable, raw broccoli may not be an efficient means of obtaining the benefits conferred by SFN. Similarly, cooking has been shown to destroy the enzyme in as little as 3 minutes of steaming.[609]. Five minutes of microwave cooking resulted in 74% loss of glucosinolates from broccoli florets with high-pressure cooking and boiling leading respectively to 33% and 55% losses.[253]

Even consumers and clinicians conscious of the importance of cruciferous vegetables in the diet may be unaware that open-air storage of broccoli as occurs during transport and in retail environments may lose 55% of its glucosinolates after 3 days and storage in plastic bags at 22 degrees C may result in similar losses over 7 days.[253]
Also of significance is the fact that broccoli cultivars for vegetable production are not selected on the basis of their SFN Yield. It is possible that the cultivars available to consumers are not good sources of cruciferous bioactives. Until Food Law allows appropriate health claims to be associated with cruciferous vegetables, there is no incentive for growers to select higher yielding cultivars. In short, neither a clinician nor a consumer has the information needed to make an appropriate choice.

6.12.2 Supplements Derived from Cruciferous Vegetables

Similarly, it is not generally known if a producer of broccoli sprout powder as a supplement ingredient has deactivated the ESP. If two supplements contain high levels of GRN but one has had the ESP deactivated, the comparative SFN Yield from these broccoli sprout powders may be markedly different. Ideally, a SFN-yielding supplement would be characterised on the basis of the various determining factors; the presence of quantifiable GRN, active MYR together with the inhibitory ESP.

These concerns are reflected in a recent study which compared a commercially-available supplement labelled as containing 30 mg 'sulforaphane glucosinolate' per dose with a quantity of fresh sprouts containing the same amount of GRN.[397] The study showed that consumption of a MYR-devoid broccoli supplement when compared with broccoli sprouts produced 7-fold lower plasma concentrations of the bioactive ITC metabolites in the subjects. Clarke et al concluded that these findings have implications for people who consume the recommended dose of such a MYR-inactive broccoli supplement believing they are obtaining equivalent doses of ITCs. This is significant in that the available broccoli sprout supplements are dominated by the MYR-inactive 'extracts', even though MYR-active whole broccoli sprout supplements do exist.

There is a further strong case for a whole food broccoli sprout supplement on the grounds that although GRN is the primary glucosinolate found in broccoli and broccoli sprouts, it is not the only one; erucin (ERN) and iberin comprise most of the remaining 25% of the glucosinolate content of broccoli. Although it has been known for two decades that ERN and SFN are interconvertible[610], Clarke's more recent study suggested that the clinical effects are likely to be due to the combined effects of all the glucosinolate hydrolysis products.[397]

6.13 STANDARDISATION

To compound the difficulties associated with determining the clinical potential of a SFN-yielding supplement, variations in nomenclature add to the problem. The term, *'sulforaphane glucosinolate'* which has recently appeared in the scientific literature is now associated with and specified for commercially-available MYR-inactive extracts derived from broccoli seed or sprout extracts.[144,397] Since 'sulforaphane glucosinolate' describes only the quantity of 'glucoraphanin', this nomenclature could erroneously lead

both clinicians and consumers to believe that the material will deliver SFN when consumed.

6.13.1 Commercial Assay Protocols

Various methods to describe a SFN supplement are commonly used in industry. To evaluate and compare different broccoli sprout powders intended as supplements or for use in clinical trials, assay methodologies must be standardised. There are several common practices for reporting the SFN derived from a broccoli sprout sample but because the assay protocol is almost never specified for a commercial product there is no way to reliably compare these values from one product to another.

6.13.2 Sulforaphane Potential

SFN Potential is a calculated value by measuring GRN and then assuming 100% conversion to SFN, whether or not MYR has been retained after processing. Based on relative molecular weights, the measured amount of GRN is multiplied by 0.406 to arrive at a SFN Potential. No provision is made for the presence or absence of either MYR or ESP. Where ESP has not been fully deactivated, calculating SFN Potential will over-estimate the amount of SFN that could be produced on ingestion. Broccoli sprout powdered ingredients or supplements which claim SFN Potential and for which the ESP has not been deactivated may yield limited SFN.

6.13.3 Sulforaphane Yield with Addition of Exogenous MYR

By adding enough exogenous MYR to ensure full conversion of GRN to SFN, this method overcomes the possibility that the starting material may contain only GRN and may be completely or partially MYR-inactive. The assay results may not specify that exogenous MYR was added, so that the reader may incorrectly conclude that the material will yield SFN on ingestion.

6.13.4 Sulforaphane Yield Due to Endogenous MYR

This method more closely resembles the in vivo situation after ingestion of the supplement, in that conversion to SFN is entirely dependent on the quantities of ESP and MYR retained after processing. It may provide a lower SFN value when compared

with the other methods, even though it may be the method which most reliably approximates SFN's metabolic fate in human physiology.

The same supplement assayed by each of these procedures is likely to produce quite different results and more importantly, only supplements which have retained MYR activity are likely to demonstrate in vivo effects. Methods which assess SFN's inducer capacity in cell culture may more reliably evaluate the clinical potential of a supplement or enable comparison of supplements. RT-qPCR-array and pathway analysis studies provide gene expression data which is another step closer to establishing the clinical effects of a supplement.[611]

6.14 CONCLUSION

The evolving science of nutrigenomics is in many ways legitimising the important role of plant foods in human health, not just as sources of nutrients but as a huge library of phytochemicals capable of interacting with intracellular biomolecules to influence gene expression. Of the many thousands of phytochemicals in the food supply, SFN exhibits properties which may make it an ideal cytoprotective biomolecule, deliverable in practical doses as a whole food supplement. Attempts to produce SFN-releasing supplements have resulted mostly in forms with little or no bioactivity, such as extracts of the seed or sprout, rather than as MYR-active whole food products. When compared with other phytochemicals widely-used in dietary supplements, SFN is significantly more bioavailable than polyphenols such as curcumin, resveratrol and silymarin. It is also significantly more able to induce NQO1, a Phase II enzyme essential in the metabolism of a number of exogenous toxins, oxidised nutrients and endogenous metabolites. Such comparative findings call into question the clinical efficacy of many of the supplements popular among consumers. Alleged benefits of such supplements appear to require much higher intracellular concentrations than can be achieved with reasonable oral intake.

Initial attempts to produce high-potency pharmaceutical Nrf2 inducers have so far been unsuccessful. Given the prevalence of diet-related disease and the evidence that many consumers have accepted a role for complementary medicines in their personal health management, appropriately validated SFN-releasing supplements may provide another avenue for supporting human health. Such supplements will need to demonstrate

sufficient nutrigenomic potential that they can modify key biochemical and physiological risk factors for disease.

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CHAPTER 7

Methodological Considerations in Investigating the Effects of Sulforaphane on Gene Expression - referencing the EASYGENEX Study

7.0 BACKGROUND

The EASYGENEX Study (**E**ffect of **a S**ulforaphane-**Y**ielding supplement on **Gen**e **Ex**pression) was initially planned by the candidate to investigate the effect of a SFN supplement on a population of otherwise apparently healthy type 2 diabetic men not using insulin replacement. As discussed in Chapter 2, type 2 diabetes has been mechanistically associated with perturbed cellular redox status in human cells including pancreatic beta-cells.[6,281] It seemed reasonable that because antioxidant vitamin supplements had not been shown overall to favourably change the course of disease progression,[5,612] it may be possible to help restore redox status and related disease biomarkers by supplementing with a phytochemical Nrf2 activator such as SFN. Induction of Nrf2 target genes is known to generate a range of cytoprotective compounds including antioxidant enzymes and non-enzymic biomolecules such as glutathione and thioredoxin.

Interest in type 2 diabetes was partly influenced by Bahadoran's 2011 trial[197] and subsequent publications[432,613] in which her group had used a manufacturer-specified 0.4% SFN-yielding whole broccoli sprout powder to demonstrate positive changes in metabolic syndrome/ cardiovascular biomarkers. Their study used large dose volumes of an unpleasant-tasting powder, making this clearly impractical as a recommendation for regular use by individuals when compared to a standard Size '00' capsule of the same material which can contain no more than 700 mg. By calculation, the 5- and 10-gram powder doses would have yielded daily SFN doses of approximately 20 and 40 mg respectively. Bahadoran showed some benefits from a 5-gram daily dose and greater benefits from a 10-gram dose. In a low-density powder with a tapped density of around 0.4[e], 10 g occupies a volume of 25 millilitres, clearly impractical for long-term regular therapeutic use.

[e] Typical tapped density measured on the samples used in our university laboratory from sprouts grown using Caudill seed stock. http://www.caudillsprouting.com/Seeds/index.html

As the powder used in the EASYGENEX trial had been separately assayed by an independent commercial laboratory[f] to yield around 1.8% SFN, it was postulated that the higher yield would enable supplementation with just a few daily capsules as a more practical option. A small pilot study using real-time quantitative polymerase chain reaction (RT-qPCR Array) had been planned to investigate gene expression in a range of around 40 redox and inflammation-sensitive genes expected to be relevant in individuals with type 2 diabetes as well as to investigate plasma SFN metabolites and relevant disease biomarkers. Thereafter, the plan had been to review the PCR gene expression findings in conjunction with the polymorphisms obtained separately for each participant for a number of the same genes on the PCR test panel. In a further step, the plan had been to investigate protein levels for several of the genes showing greatest expression.

Originally named the EASYDIAB2 Study, it was planned to recruit around six participants for this pilot study, expecting that the outcomes would provide pharmacokinetic and dose-response relationship data that would inform a larger study.

It later became apparent that in order to select as homogeneous a population as possible, the selected exclusion criteria were so narrow that it was difficult to recruit suitable individuals to the study. Consequently, the study design was modified to investigate the effect of similarly high SFN-yielding broccoli sprout capsules on a population of twenty healthy young men. To broaden the potential findings of the study by accessing a larger gene set, it was decided to utilise microarray instead of the more restrictive RT-qPCR platform, even though it is well-established that microarrays tend to generate a significant noise-to-signal ratio.[614] The detailed methodology for the EASYGENEX Study is described in Chapter 8.

7.1 DESIGNING A STUDY TARGETING Nrf2 MODULATION

A recent Special Issue editorial[615] highlights the role of Nrf2 as an emerging therapeutic target. A number of small-scale clinical trials utilising SFN as the intervention compound in various doses and forms have shown positive outcomes,

[f] Eurofins Supplement Analysis Center. http://www.eurofinsus.com/food

with authors typically describing the observed responses being associated with activation of the transcription factor Nrf2. (Table 7.1)

In conducting these trials, other known Nrf2 activators and modulators must be considered and ideally excluded. Typically excluded are dietary glucosinolates from cruciferous and related plant foods, phytochemical supplements such as green tea, pomegranate or turmeric and other polyphenols,[200] antioxidant vitamin supplements which may attenuate SFN's weak Nrf2-activating pro-oxidant signal,[616] medications known to activate Nrf2 such as statins[617] and metformin[618] and environmental stressors such as cigarette smoke.[619] The one significant and likely Nrf2 activator that had not been excluded is exercise and this factor is addressed in some detail later as it appears that the failure to exclude regularly-exercising individuals may have impacted the expected findings of the EASYGENEX Study.

7.2 DOSE CONSIDERATIONS

What has emerged in recent years is the fact that Nrf2 activation appears to act hormetically in that whilst low Nrf2 activity can compromise normal cellular function, persistently high Nrf2 activity can also be detrimental to cells.[620,621] The latter aspect has been harnessed in chemotherapy in which specific pharmaceuticals have been developed to inhibit Nrf2 activity in cancer cells, since it is now known that cancer cells too can take advantage of the cytoprotective properties of Nrf2 activation.[622,166] Pharmaceutical Nrf2 activation in non-cancer cases must be equally considered with caution; the failed Bardoxolone methyl trial[561] in patients with chronic kidney disease acts as stark warning that attempting to exceed normal physiological limits can have serious and sometimes fatal consequences. As discussed in Chapter 6, the trial had to be stopped prematurely when increased number of heart failure events including some deaths became apparent. Chapter 6 referred to the fact that Bardoxolone methyl has more than 200-fold greater ability to activate Nrf2 than does SFN.[560]

Therefore, what becomes of critical importance in clinically utilising the beneficial effects of Nrf2 activators is that an appropriate dosage range can be established for both preventive and therapeutic applications. The review material in Chapters 4, 5 and 6 addresses many of the issues associated with the significant variability in Nrf2 inducer potential by phytochemicals as well as the large observed differences in bioavailability when attempting to translate *in vitro* effects into *in vivo* responses.

SFN has been shown to be a potent inducer of Nrf2 as well as being highly-bioavailable, especially when compared to some popular phytochemical supplements.[153]

In Chapter 6, it was suggested that it may not be possible that oral dosing of existing SFN-yielding supplements could achieve the high intracellular concentrations described in the various *in vitro* studies or in those using large daily quantities of fresh broccoli sprouts or sprout homogenates.[623] More recently, some supplements have been shown to yield higher levels of SFN, such that it may be important to more cautiously consider supplement dosage. Holmstrom et al. highlight similar cautions in the conclusion to their paper investigating the role of Nrf2 in mitochondrial function by suggesting that both "insufficient and persistently high Nrf2 activity can have detrimental consequences".[621] SFN has been shown to induce Nrf2 in cancer cells as well as in normal cells, highlighting the need for further *in vivo* investigation, since Nrf2 activation is just one of multiple synergistically-acting mechanisms by which SFN may confer chemoprotection.[157]

7.2.1 EASYGENEX Study – Selecting the Dose

Because it is well-established that SFN is a potent activator of Nrf2, in most clinical trials in which SFN is the intervention material, pathways and outcomes associated with Nrf2 activation are the primary focus. Even so, SFN's pleiotropic nature has been noted in a range of other less well-understood cellular events.[157] In this context, SFN is also known to exhibit epigenetic effects which include post-translational histone modification, DNA methylation and post-transcriptional gene expression regulation by non-coding microRNAs.[163]

A number of *in vitro* studies have used microarray analysis as their platform for investigating gene expression via Nrf2. A relatively recent study used human retinal pigment epithelial cells to examine the effect of SFN on the expression of oxidative stress-related genes. The study found that 10μM SFN was the optimal dose needed to enable the cells to counter the oxidative assault. Mechanistically, SFN was shown to activate Nrf2 and induce a number of its target genes.[624]

In determining the dose for the EASYGENEX Study, consideration of the range of doses utilised in *in vitro* studies played little part. Instead, the focus was on the more

relevant doses used in the published clinical trials in which SFN has been used as the primary intervention. Comparison was difficult due to the variety of forms in which SFN had been administered; some studies used the synthetic form, others generated SFN *in situ* by combining GRN with a plant source of MYR, whilst others used fresh broccoli sprouts, an aqueous homogenate of fresh sprouts or a powder of dried whole sprouts. One study claimed to be using a broccoli sprout BSE but the Supplementary Materials describing the material showed that it was, in fact a dried whole broccoli sprout powder.[198] Daily doses of SFN in these studies ranged from 4.4 mg daily as the lower dose in the chronic obstructive pulmonary disease (COPD) study by Wise et al. to 105 mg in the prostate xenograft study by Myzak et al. in 2007, with others studies selecting doses in between (Table 7.1).

Where fresh sprouts or their minimally-processed derivatives are used, SFN is not the only bioactive compound being investigated, even though it is the most abundant. Other isothiocyanates (ITCs) and a range of other phytochemicals and micronutrients may all impact gene expression. The second most abundant ITC generated from MYR hydrolysis of broccoli sprouts is erucin which has been shown to readily interconvert with SFN. When a whole broccoli sprout or whole plant derivative is used as the intervention material, converted erucin may contribute to the total SFN yield and subsequent effect on gene expression.[397]

Table 7.1 Selection of clinical trials utilising various forms of broccoli sprouts or sulforaphane as the primary intervention to evaluate their effect on disease biomarkers.

Disease and/or Biomarker	Daily Dose	Form	Reference
Metabolic Syndrome	SFN not specified	Fresh sprouts (100 g)	Murashima et al.; 2004[37]
Helicobacter pylori infection	14 – 56 g fresh sprouts (SFN not specified)	Fresh broccoli sprouts (14-56 g)	Galan et al.; 2006[425]
Prostate xenograft – HDAC activity in PBMCs	68 g fresh broccoli sprouts, yielding 105 mg SFN (as a single dose)	Fresh broccoli sprouts (68 g as a single dose)	Myzak et al; 2007[438]
SFN metabolite effects in female breast tissue	35 mg SFN	Fresh sprout extract + daikon MYR	Cornblatt et al;2007[183]

Chronic Obstructive Lung Disease	19 mg SFN	Broccoli sprout homogenate	Riedl et al.; 2009[41]
Type 2 diabetes	40 mg SFN (calculated)	Broccoli sprout powder	Bahadoran et al.; 2011and 2012[197,432,613]
Autism	9 - 14 mg SFN based on body weight	Stabilised SFN supplement	Singh; 2014[199]
Detoxification (Atmospheric pollutants)	262 mg GRN + 7 mg SFN	GRN + daikon MYR in juice	Egner et al.; 2014[202]
Nasal Allergic Response	18 mg SFN	GRN extract + daikon MYR in mango juice	Heber et al.; 2014[203]
Off target effects of SFN and HDAC inhibition	Claimed to be 18 mg SFN per gram fresh sprouts [g]	Fresh broccoli sprouts (34, 68 and 102 g)	Baier et al; 2014[204]
Asthma	18 mg SFN	GRN extract + daikon MYR in mango juice	Brown et al.; 2015[203]
Prostate – PSA doubling time	60 mg SFN	Stabilised SFN from seed extract	Cipolla et al.; 2015[200]
Breast tissue (biopsied)	SFN not specified. SFN metabolites measured	GRN supplement (180 mg daily)	Atwell et al.; 2015
Sickle Cell Disease	SFN not specified	Broccoli sprout homogenate (50, 100, 150 g)	Doss et al.;2016[205]
COPD – effects on two types of respiratory cells via Nrf2	Daily SFN at 4.4 mg and 26.6 mg	SFN extracted from fresh broccoli sprouts	Wise et al; 2016[206,207]
Type 2 diabetes – hepatic glucose production	Daily SFN of 27 mg in a 5-gram powder dose	Whole broccoli sprout powder, described as a broccoli sprout extract (BSE)	Axellson et al.;2017[198] (Supplementary material)

[g] The SFN yield claimed by Baier et al is in stark disagreement with those stated by Myzak et al (2006) using the same fresh sprouts (Broccosprouts®). Myzak et al. claim that 68 g fresh sprouts yield 105 mg SFN, whereas Baier et al. claim that their sprouts yield 18 mg/ gram fresh weight. It is unlikely that Baier et al are correct when comparing their values with those used in other studies. Baier et al state later in their paper that their dose is similar to that available in a BroccoMax® supplement that lists one serving of the product to contain '30 mg sulforaphane glucosinolate'. Baier et al. don't comment that such a supplement is devoid of myrosinase and is therefore not equivalent to a supplement that yields 30 mg sulforaphane.

The highest daily dose used in the studies listed in Table 7.1 is 102 g of fresh broccoli sprouts. If it is assumed that Baier's stated SFN Yield is incorrect (explained in Footnote g above) and instead use Myzak's stated SFN Yield for fresh commercially-available sprouts carrying the same brand name, then Baier's highest dose of 102 g fresh sprouts would then have been equivalent to 158 mg SFN. Myzak's stated SFN Yield from fresh broccoli sprouts is in agreement with the order of magnitude stated by Riedl et al[41] in their study which used a homogenate of fresh broccoli sprouts.

Myzak and Baier both use HDAC inhibition as their outcome measure. Two earlier cell culture studies by Myzak et al[230,232] showed that 15 µM SFN significantly inhibited HDAC in human embryonic kidney cells, colorectal cancer cells and three different prostate epithelial cell lines. The group's working hypothesis was that it was not so much SFN itself that was responsible for the observed HDAC inhibition but more the SFN metabolites produced through the mercapturic acid pathway; the EASYGENEX Study therefore includes measurements of the SFN metabolites.

Chapter 5 queried whether it is possible to achieve the higher intracellular levels shown to be associated with HDAC inhibition in tissue culture. In 2002, Ye et al[411] reported that in four human volunteers, a single total ITC dose of 200 µmols (~ 35 mg, most of which is likely to be SFN) from a MYR-hydrolysed extract of 3-day broccoli sprouts resulted in 0.943 ± 2.27 µmol/l in plasma, serum and erythrocytes at one hour after feeding. They further comment that peak plasma levels reached 2 µM. The authors of the study comment that the ITC and ITC metabolite levels in erythrocytes contained only low *micro*molar concentrations in contrast to the much higher *milli*molar concentrations that can accumulate in tissue culture studies.[625] They suggest that this is because most of the ITCs in the blood were in the form of conjugates rather than as free ITCs, given that most conjugates are absorbed very slowly in tissue culture studies compared with the rapid absorption of ITCs.

In the EASYGENEX Study, participant blood samples were collected at 0, 7 and 14 days of continuous twice daily dosing, whereas the pharmacokinetic study by Ye et al discussed above used a single dose with sampling at several time points within hours of dosing. The participants were expected to accumulate SFN and its

metabolites over the extended time frame of the study, increasing the likelihood that the intracellular concentrations in the EASYGENEX Study would be higher. Accumulation of metabolites has been shown to be particularly cytoprotective in epithelial cells lining the gut[626] (especially the stomach) and the urinary bladder.[627] In both types of epithelia, a single membrane is the only barrier to SFN absorption into these cells. In the urinary bladder, concentration of SFN metabolites in the urine for extended time periods prior to voiding may explain the observed cytoprotective effects of SFN in this organ.[628]

Myzak et al.[629] report that HDAC inhibition was apparent in murine colonic mucosa and peripheral mononuclear cells, suggesting that dietary SFN is capable of inhibiting HDAC *in vivo*. It is worth noting that in general, HDAC inhibitors exhibit greater effects on cancer cells than on normal cells,[630] raising the possibility that such differential effects may provide an opportunity to utilise SFN in cancer therapy as well as in prevention.

Where *in vitro* HDAC studies typically employ 15μM SFN, the human retinal pigment epithelial cell culture study referred to in 3.1 used the lower 10μM SFN concentration.

A concentration of 10μM SFN was found to be the optimal dose needed to enable the cells to counter an oxidative assault. Mechanistically, SFN was shown to activate Nrf2 and induce of a number of its target genes.[624] Whether a lower 5μM more typical Nrf2-activating dose would have also been effective in modulating gene expression in these cells is not known.

In practice, there are fewer *in vivo* opportunities to study the concentrations of SFN and its metabolites in cells compared with those of the *in vitro* situation, so that the available evidence for intracellular SFN concentrations is limited. Muscle biopsy is one mode of investigating such concentrations following SFN dosing but the procedure carries enough risk as well as participant discomfort that it is not often a test method of choice. Cornblatt et al,[183] in a proof-of-principle pilot study of eight women, were able to show the presence of SFN and its metabolites in epithelial/stromal-enriched mammary tissue excised during reduction mammoplasty 60-90 minutes after a 200 μmol (35 mg) SFN dose of a freeze-dried powder. Where

the pre-dose plasma metabolite concentration was 0.01µM ± 0.02, the post-dose dose level was much higher at 0.92µM ± 0.72. The post-dose level shown here is in agreement with the peak plasma SFN concentration shown by Ye et al as 0.943µM ± 2.27.

To demonstrate that the metabolites had significantly accumulated in urine at the same time as plasma was sampled post-surgery, urinary metabolite concentrations were assayed. The pre-dose metabolite concentration was shown to average 4.07µM ± 5.22, with the post-dose level much higher at 158.85µM ± 93.89. The accumulation of SFN metabolites in the urinary bladder is considered to provide a cytoprotective environment for the prevention of bladder cancer. The potential for SFN as a therapy in the context of bladder cancer prevention is discussed in some detail in a recent review paper.[628]

Overall, review of the published literature on SFN shows that the doses required for Nrf2 induction are lower than those for HDAC inhibition and a number of other putative cytoprotective processes.[157] When considering the higher dose levels, one must consider the potential for toxicity. Although Shapiro et al.[426] showed that SFN is safe and generally well-tolerated in humans, a more recent study[631] raised the question of safety in comparing desirable and potentially toxic cellular effects of SFN in Hepa1c1c7 cells. When comparing increasing intracellular concentrations of SFN with curcumin, quercetin and resveratrol on this hepatic cell line, it was found that only SFN and curcumin significantly increased NQO1 protein expression and activity without triggering G_2/M cell arrest or mitotic catastrophe. Quercetin was found to disrupt mitosis at a concentration 100-fold higher than that required to induce NQO1. Since quercetin has been a popular dietary supplement over many decades, one might question whether this *in vitro* study is applicable to the *in vivo* situation. Nevertheless, it highlights the need for caution when using doses much higher than it is practically possible to obtain from food, especially of isolated compounds extracted from their whole plant sources.

7.2.2 The Dose Employed in the EASYGENEX Study

Having considered the range of doses used in studies focused separately on Nrf2 induction and HDAC inhibition, a dose in the higher range but in keeping with other studies was selected for the EASYGENEX Study. Myzak showed in a human study

that 68 g of fresh broccoli sprouts yielding 105 mg SFN strongly inhibited HDAC activity in PBMCs 3 and 6 hrs following consumption.[438] Two dose levels were selected, with the highest dose of capsules approximating the SFN Yield of Myzak's 68 g of fresh broccoli sprouts.

The EASYGENEX Study participants were therefore administered 4 capsules (~ 50 mg daily SFN Yield) in the first week and 8 capsules (~ 101 mg daily SFN Yield) in the second week. The capsules had been produced from whole fresh dried broccoli sprouts using the same seed stock used by Myzak et al. as described in Footnote g.

7.2.3 Ingestion of dried broccoli sprouts from powder vs capsules

The reaction between GRN and MYR is so rapid that SFN is released in the mouth when a SFN-yielding broccoli sprout powder is ingested. As a result, supplementing with such a powder may generate different *in vivo* responses from supplementing with a capsule containing exactly the same quantity of the same powder. An innovative study[632] used an *in vitro* digestion model to compare, among other things, the conversion and bioaccessibility of SFN in the mouth, stomach, and small intestine. In minimally-steamed (one minute) broccoli vegetable, approximately 30% as much SFN was generated in the mouth as in the stomach and with only around 23% as much SFN nitrile produced in the mouth.

The capsules typically used in phytochemical supplements are manufactured from vegetable cellulose. The Size '00' Vcaps selected for the EASYGENEX Study and described in 8.2.2 are specified by the manufacturer as disintegrating in no more than 30 minutes (NMT < 30 mins). As a consequence, studies where powder has been used are likely to reveal different levels of SFN conversion and absorption from those where a capsule is the intervention dose form.

7.3 THE ROLE OF EXERCISE IN Nrf2 ACTIVATION

When designing the EASYGENEX Study, the role of exercise as a significant contributor to Nrf2 activation had not been considered; in retrospect, it should have been listed for exclusion along with dietary factors, particular supplements and pharmaceuticals, especially since the participants recruited were young, healthy men predominantly from the university's School of Human Movement and Nutrition

Sciences. As described in Chapter 8, the participants were in the habit of undertaking significant regular aerobic and resistance exercise sessions. When the differential gene expression microarray data from baseline to day 7 and day 14 was reviewed, the expected induction of the Nrf2 target genes was not detected. However, plasma analysis showed that there were significant levels of SFN and its metabolites in the samples, confirming that the intervention material did yield SFN *in situ*. What appeared to be the study's major response was related to inhibition of inflammation-related genes associated with the Nuclear Factor-kappa B (NF-κB) network.

In reviewing what might have led to failure to demonstrate Nrf2 activity, consideration should have been given to whether the participants at baseline and throughout the study had already optimised their Nrf2 activity in some other way; the effect of exercise seemed the most likely possibility

7.3.1 Known Association Between Exercise and Nrf2 Activation

When reviewing the literature on the relationship between Nrf2 and exercise, it was apparent that although there were limited human data to quantify such a relationship, there were, nevertheless many peer-reviewed publications that focused on relevant mechanistic aspects. Aerobic exercise in particular increases respiration which results in greater generation of the stressor, superoxide anion. Since such stressors are the signal detected by Keap-1 to release Nrf2, it should not have been surprising that these participants at baseline might already have been exhibiting significant expression of the Nrf2 target genes. If Nrf2 had already been maximally activated in these young men, the addition of another weak stressor in the form of SFN would not have changed the status quo. Two of the EASYGENEX participants had lower exercise output than the average of the study group (but still averaged 30 minutes of moderate intensity walking daily). When considering if they might have displayed a different response to SFN, no difference could be demonstrated.

Although the relationship of exercise to Nrf2 activation has not been extensively reported in humans or in animals, a recent review paper[633] summarises what is known about the effects of acute and regular exercise on Nrf2 activity and downstream targets of Nrf2 signalling. The authors raise the possibility that because

some phytochemicals have also been shown to activate Nrf2, there may be synergistic effects between exercise training and ingested phytochemicals.

The authors highlight human studies which showed that the response to exercise differed significantly between younger and older individuals, between the trained and untrained and between acute bouts of exercise and regular exercise. In addition, even where Nrf2 had been activated, some Nrf2 target genes and their protein expression products did not always respond as expected. Of significance when considering the results of the EASYGENEX study, the authors state that there are no studies to date that investigate the role of exercise *intensity* and Nrf2 activation.

Some of the salient points which emerge from the review by Done et al. are these: a) regular exercise in rodent models consistently results in Nrf2 signalling and protein abundance across multiple tissues. b) more active older individuals had significantly greater Nrf2 protein and a higher Nrf2/Keap-1 ratio in muscle tissue, suggesting that there is the potential to restore Nrf2 signalling in older age. c) short, intense bouts of exercise as in interval training deliver repeated shifts in redox balance, differentially affecting the frequency of Nrf2 activation. d) both animal and human studies in this area are almost exclusively on males. e) nuclear accumulation of Nrf2 was demonstrated only in younger men, showing that ageing is associated with impairment to nuclear import of Nrf2. f) Nrf2 mRNA was increased 2 hours after acute exercise in middle-aged women who were regular exercisers but not in sedentary women who showed no change in Nrf2 or its target genes. g) there may be an upper limit to the stimulatory effects of exercise. h) there may be a synergistic effect when combining exercise with Nrf2-activating dietary supplements. i) direct-acting antioxidant vitamin supplements may attenuate the beneficial effects of exercise.

Overall, Nrf2 activation varied with acute vs regular exercise, with age, with fitness status and possibly with sex. What becomes immediately apparent is that there is a dearth of research on the role of exercise in Nrf2 signalling. The ability to quantify this effect would contribute greatly to our understanding of how to more effectively apply exercise prescriptions.

In the EASYGENEX Study, wherein the participants were young males, most of whom exercised regularly, it may not have been possible to detect differential gene

expression across the two one-week trial periods because these young men had already achieved maximal Nrf2 activation. Nevertheless, the study did show that a range of other genes not associated with Nrf2 activation were differentially-expressed. It is likely that gene expression in these networks may have been substantially altered by the effect of exercise on other transcription factors and their downstream pathways, perhaps limiting the ability to detect the real effect of the SFN intervention.

It is likely that a potential weakness in some other studies may have been uncovered and where exercise is not typically considered in the list of exclusion criteria when considering the effects of a dietary intervention on Nrf2. Just one peer-reviewed published SFN-based study could be located (also conducted by a Nutrition and Exercise Science department) wherein those engaged in vigorous activity for more than 6 hours per week were excluded.[397]

7.3.2 Immune-related Effects of Exercise.

Brief bouts of exercise are widely-known to lead to robust increase in circulating PBMCs in humans[634] but it is not known whether this might impact on the simultaneous effect of SFN. An acute bout of exercise can cause tissue injury and the release of both pro- and anti-inflammatory cytokines, whereas regular physical activity seems to promote an anti-inflammatory environment in the body, attenuating the inflammatory effect.[635] T-lymphocytes are sometimes grouped as T helper-1 (Th1) cells or T helper-2 (Th2) cells; Th1 cells are primarily pro-inflammatory in their actions whilst Th2 cells are anti-inflammatory. This is significant in the context of the EASYGENEX Study because the Th1-Th2 balance is largely determined by NF-κB activity which was shown to be significantly downregulated by SFN. Clinically, this may also be relevant in that Th1-dominated responses are involved in the pathogenesis of organ-specific autoimmune disorders but in contrast, allergen-specific Th2 responses are responsible for atopic disorders in genetically susceptible individuals.[636]

The effect of exercise is also affected by sex and pubertal phase. A study[637] investigating the effect of brief bouts of exercise on young, healthy individuals showed that the genes commonly regulated by exercise in all tested groups are related to growth, apoptosis, inflammation and tissue repair. Using the same

Affymetrix U133 + 2.0 arrays for gene expression that was selected for the EASYGENEX Study, the group identified 894 genes significantly altered by exercise in PBMCs. They also noted distinct gene alterations likely to affect monocytes in an anti-inflammatory, anti-atherogenic pathway, including downregulation of TNF-α and TLR4. SFN is also known to downregulate TLR4, an effect in common with that of exercise and discussed further in Chapter 8.[638]

7.4 NF-κB INFLAMMATION-RELATED PATHWAYS

Of the exercise-related studies, there is extensive reference to the mechanistic relationship of exercise to inflammation and as such to the transcription factor, NF-κB. In relation to its observed anti-inflammatory activity, SFN has been shown to modulate the activity of the transcription factor NF-κB, well-known for its role in the immune system. Whereas SFN directly activates cytosolic Nrf2, its action on NF-κB is to inhibit NF-κB binding to the DNA.[639] Such anti-inflammatory mechanisms are suggested to contribute to SFN-mediated cancer chemoprevention. Since the EASYGENEX Study detailed in Chapter 8 highlights the effect of SFN on downregulating in the NF-κB network, the next section describes relationships and pathways that may be relevant to the study's findings.

7.4.1 Regulation of NF-κB

Members of the NF-κB family of transcription factors function as dominant regulators of inducible gene expression in virtually all cell types in response to a broad range of stimuli, with particularly important roles in coordinating both innate and adaptive immunity.[640] NF-κB is controlled by various mechanisms of post-translational modification and subcellular compartmentalisation as well as by interactions with other cofactors or corepressors.[641] The NF-κB family of transcription factors includes RelA (p65), RELB and others and as a complex, NF-κB mediates immune responses to cellular challenges that include bacterial and viral infection and inflammation.[188] These transcription factors play critical roles in both adaptive and innate immunity, inflammatory responses, cell differentiation, proliferation and apoptosis. The EASYGENEX Study identified *RELB* as one of the significantly downregulated genes in the NF-κB network. The activity of NF-kB is tightly regulated at multiple levels, a factor that may be associated with its influence on the expression of numerous

genes.[642] Nuclear translocation of NF-κB is primarily controlled by signalling associated with IkB kinase (IKK) in two related pathways associated respectively with the NF-κB classical (canonical) and alternative pathways.

Among the most potent NF-κB activators are tumour necrosis factor (TNF-α), interleukin (IL)-1β and bacterial lipopolysaccharide (LPS), with TNF-α activation being one of the best-characterised of the NF-κB signalling pathways.[643] Furthermore, several cross-talk relationships associated with NF-κB have been identified and three clinically-relevant relationships are described below.

7.4.2 Cross-talk Between Nrf2 and NF-κB.

Given that SFN is associated with cellular defences via the essentially opposing mechanisms of Nrf2 and NF-κB, it is not unexpected that molecular cross-talk between these transcription factors has been reported.[188] Imbalance between Nrf2 and NF-κB is associated with a significant number of diseases across various body systems and these relationships are the subject of extensive research in cancer biology.[644]

Although the complex interplay between Nrf2 and NF-κB has been highlighted, there remains much to be explored in order to understand how such relationships may impact on disease pathophysiology at the molecular level. As part of the cross-talk between these two transcription factors, NF-κB has been shown to regulate Nrf2-mediated ARE expression. Several mechanisms exist by which p65 (the canonical NF-κB subunit) can exert negative effects on ARE-linked gene expression.[645] It would seem that the cross-talk between Nrf2 and NF-κB enables cells to more finely regulate their responses to cellular stressors.

7.4.3 Cross-talk Between NF-κB and CEBPB

CCAAT-enhancer-binding proteins (C/EBPs) are activated by a high-fat diet or a diet especially when high in the fatty acid palmitic acid. C/EBPβ governs a network of genes crucial for dietary-driven inflammatory activity and insulin resistance.[646] *C/EBPβ* overexpression induces NF-κB DNA binding, JNK activation and pro-inflammatory cytokine expression but when C/EBPβ expression is downregulated, (as in murine C/EBPβ $^{-/-}$ knockdown), palmitic acid-induced inflammation was

inhibited. C/EBPβ is known to be central to the pathogenesis of several inflammatory and metabolic disorders.[646] The two families of transcription factor, NF-κB and C/EBPβ have been considered as a complex that may act synergistically.[647] More recently, C/EBPβ overexpression has been shown to promote cardiomyocyte hypertrophy and that C/EBPβ knockdown protects these cells from hypertrophy via inhibition of NF-κB transcriptional activity.[648]

7.4.4 Cross-talk Between NF-κB and Phase 1 Detoxification.

Other forms of molecular cross-talk have been investigated with the NF-κB RELB subunit closely interacting with the Acyl hydrocarbon receptor (AhR). Numerous exogenous compounds have been shown to bind to activate the AhR.[649] The ArH/Rel B dimer can bind to DNA response elements as well as NF-κB. *RELB* has been implicated in breast cancer and is also a critical factor in the function and differentiation of dendritic cells. Furthermore, AhR can regulate the generation of regulator T cells (T_{reg}) or pro-inflammatory T cells, positioning it as a key factor in the immune system.[649]

The AhR plays a key role in the cellular defence mechanisms in that it regulates a number of the *CYP450* genes, particular *CYP1A1, CYP1B1* and *CYP1A2* which respectively encode the Cytochrome P450 enzymes 1A1, 1B1 and 1A2. Many substrates for CYP1 enzymes are AhR ligands.[650] These reactions occur not only in Phase 1 detoxification of environmental toxicants but also in the metabolism of endogenous compounds such as steroid hormones.[651] The product of the Phase 1 reaction may be a compound with greater carcinogenicity than the initial CYP450 substrate. Many of the enzymes active in the subsequent Phase 2 detoxification reactions are products of Nrf2 target genes, such as NQO1, GST and UDP-glucuronosyl transferase (UGT), linking Nrf2, NF-κB and the AhR in the initial stages of detoxification.[531]

7.4.5 NF-κB and clinical implications of dysregulation

As the relationship between NF-κB and AhR has been only recently known, there is as yet, little data to support the relationship. Several observations by Vogel et al (2009) suggest that *RELB* contributes to cancer cell survival and tends to be overexpressed in the inflammatory breast cancer phenotype, the form with highest

metastatic potential. Perhaps surprisingly, the AhR binds to the NF-κB consensus site, suggesting that the AhR may affect the canonical NF-κB pathway. This suggests that the AhR may function not only on AhR signalling but also NF-κB signalling that is activated by classical NF-κB inducers such as LPS.[649] In addition, *RELB* has been shown, by inducing manganese superoxide dismutase (*MnSOD*) to act as a major cancer cell survival factor by conferring radiation resistance and by inhibiting cancer cell apoptosis.[652]

7.5 MICROARRAY vs RT-qPCR PLATFORMS IN STUDYING GENE EXPRESSION

Any study investigating gene expression must select the most appropriate platform on which to do so. When it had been initially planned to use SFN to investigate differential gene expression in type 2 diabetes, the RT-qPCR platform was chosen as it is considered most accurate when expression of a relatively small number of specific process-related genes is under investigation. This technique is generally considered the 'gold standard' in precision when quantifying gene expression.[653] The plan had been to use probes associated with around 40 Nrf2-target genes, including those coding for antioxidant/detoxification enzymes, non-enzymic glutathione and inflammation biomarkers because their roles in the redox imbalance associated with glucose dysregulation has been well-documented. Given what may now be presumed to be the effect of exercise in the EASYGENEX Study, it is fortuitous that this platform was not adopted, as none of the Nrf2-target genes was differentially-expressed by the SFN intervention.

Instead, an oligonucleotide microarray platform, the Affymetrix HG U133 plus 2.0 Genechip was selected to enable investigation of a large number of human genes. The manufacturer[h] describes this chip as *"a single array representing14,500 well-characterised human genes that can be used to explore human biology and disease processes."* Although microarrays permit the simultaneous investigation of a very large number of genes, they lack the precision of RT-qPCR. Microarray is a multi-stage process in which the accuracy of each step may influence the gene expression estimates.[654] The weakness of microarrays is generally considered to be

[h] www.affymetrix.com/support/technical/datasheets/human_datasheet.pdf

their low accuracy, precision and specificity[655] and have been criticised as a method that fails to identify clinically-relevant information.[654]

Their usefulness in the EASYGENEX Study lay in the ability to identify genes and pathways which may not have been expected. In Chapter 8, the steps needed to enhance the signal-to-noise ratio are described as a way of providing more meaningful data. Having identified that the genes expressed in the EASYGENEX Study were primarily related to the NF-κB network, a further study might utilise RT-qPCR as the platform to focus on the role of SFN in attenuating inflammation in human cells.

CHAPTER 8

Effect of a Sulforaphane-Yielding supplement on Gene Expression in Healthy Young Exercising Men; a Microarray Analysis

(THE EASYGENEX STUDY)

8.0 Abstract

The broccoli-derived phytochemical, sulforaphane (SFN) has been extensively studied for its beneficial effects on human health, exerting many of its known effects by activation of the transcription factor, Nrf2. Its additional Nrf2-independent effects are of increasing interest. A primary aim of this study was to investigate SFN gene expression using two SFN dose levels with the expectation that Nrf2-dependent genes would express at the lower dose and that Nrf2-*in*dependent genes would also express at the higher dose. SFN is metabolised extensively by the gut microflora with *GSTP1* genotypic differences largely determining the nature and extent of the metabolites produced. A further aim of this study was to investigate the effects of the two doses of a broccoli sprout-containing supplement on plasma SFN and its mercapturic acid metabolites and to determine if the responses in plasma SFN and metabolites vary between participants with different *GSTP1* genotypes. An open-label dose-escalation study was conducted with 21 young, healthy, physically-active men. Plasma was collected before and after consuming encapsulated whole broccoli sprout supplements over two 7-day continuous periods (53 g SFN/day during week 1 and then 106 g SFN/day in week 2). Genotypes were identified from buccal swab samples. Liquid chromatography with tandem mass spectrometry was used to measure plasma SFN and its metabolites. Gene expression analysis was conducted using Ingenuity Pathway Analysis on PBMCs using a microarray platform.

The presence of significant levels of plasma SFN and its metabolites confirmed that SFN had been generated *in situ* by ingestion of the broccoli sprout capsules, with SFN reaching 0.08 μM in those carrying the wild-type genotype and 0.02 μM in those with polymorphisms for *GSTP1.* The SFN-NAC metabolites reached 0.03 μM in wild-type *GSTP1* genotypes and 0.06 μM in those with polymorphisms. Over the 14-day supplementation period there was a significant downregulation of the NF-kB network. There were 10 upregulated and 26 downregulated genes, 8 of which have not

previously been associated with SFN (*DDC, ACSM2A, HERC6, PDIA4, ZBTB2, IGF2B2, DDX3X and GK5).* All 36 differentially-expressed genes can be broadly grouped into 6 overlapping categories related to their cellular functions; immune modulation (anti-inflammatory), metabolism (adipogenesis, glucose metabolism, insulin sensitivity), neurotransmitter synthesis, cytoprotection, cardioprotection and redox modulation.

This study provides evidence that whole MYR-active broccoli sprout capsules yield significant SFN and that in the study population, the intervention resulted in significant differential expression of Nrf2-independent genes only.

8.1 Introduction

Epidemiologically, cruciferous vegetable consumption is associated with reduced cancer risk,[180] whilst vegetables in general are associated with chronic disease prevention.[656] In the last 25 years, interest in the chemopreventive properties of broccoli-derived SFN has grown significantly, such that it has been employed as the intervention compound in a number of human clinical trials. The range of disease processes targeted in these clinical trials is diverse, providing evidence that the application of bioavailable SFN is potentially much broader than chemoprevention; the nature of these studies and the range of forms and doses employed were discussed in Chapter 7.

When reviewing the available clinical trials utilising different forms of SFN intervention, it is clear that some of these may not be appropriate for practical daily dosing, even though studies using fresh sprouts and their homogenates or powdered forms have demonstrated significant clinical responses. One of the goals in designing the current study was to gain greater insight into SFN's dose-response in healthy individuals ingesting an encapsulated SFN-yielding dietary supplement. In working with nutraceuticals and functional foods as interventions, one must be mindful that the techniques being utilised are largely designed for pharmaceuticals rather than for foods and that there are important differences that are not easy to separate and appropriately quantify.[657]

What is emerging is that diseases known to be underpinned by redox imbalance and uncontrolled inflammation are proving to be more responsive to amplification of

cellular defences via Nrf2 activation than to administration of direct-acting antioxidant supplements.[154,35] Cellular Nrf2 levels are under strict control by multiple mechanisms, the best-characterised of which is mediated as described in Chapter 4 by interaction with Kelch-like ECH-associated protein 1 (Keap-1).[188]

The greater focus of SFN research has been on its nutrigenomic role as a potent activator of the transcription factor Nrf2 (coded by the gene *NF-E2-related factor 2*), even though it is known to participate in other cellular processes.[157] Where many studies have focused on plasma disease biomarkers and clinical disease outcomes, others employ gene expression platforms as a means of discovering more about mechanistic aspects of Nrf2 activation and its downstream targets as well as to search for novel processes and molecular pathways.

Nrf2 and its target genes can be activated at the lower end of the SFN dose continuum[631] but other effects such as HDAC inhibition are thought to require higher intracellular concentrations; the SFN concentration reported in *in vitro* HDAC studies[39,412,435] is typically 15μM but there is limited information on how this might compare with oral doses required in human populations.[658] Similarly, the doses required to modulate inflammation via NF-kB are poorly-defined. A PubMed search on the grouped terms ‘sulforaphane’ and ‘NF-kappa B’, at time of writing returned more than 80 publications but when the limit, ‘clinical trial’ is applied, there are none.

Further confounding the ability to quantify a dose-response relationship, it has been long known that polymorphisms in genes of the *GST* families of Phase 2 detoxification enzymes modulate SFN metabolism *in vivo* but the effect appears to be inconsistent.[659,660] The primary route of *in vivo* metabolism of SFN and other ITCs is by the mercapturic acid pathway, a major pathway for elimination of many xenobiotics, ultimately via the urine.[661] Of the three major human *GST* families, cytosolic *GSTs* form the largest of the seven classes.[662] The most efficient GST isozyme catalysts of ITC metabolism are GSTM1-1 and GSTP1-1.[663]

Polymorphisms of *GSTP1* are common but are not distributed equally by ethnicity. In a study[664] reviewing *GSTP1* polymorphisms across population groups, the wild-type *GSTP1* (Ile/Ile) was represented in 47% of Europeans, 61% of Asians and 50% of Indians. Additionally, the *GSTP1* heterozygote (Ile/Val) was carried by 43%, 34%

and 44% of these ethnic groups respectively, with the homozygote (Val/Val) carried by just 10%, 5% and 6% of the same groups.

Human GSTP1 enzyme catalyses thioether conjugation of reduced glutathione (GSH) with potentially toxic electrophile reactive intermediates, acting as a detoxifying enzyme.[665] A key enzyme in the Phase 2 detoxification pathways, the GSTP1 enzyme selectively detoxifies the toxic epoxide of benzo(a)pyrene, a highly carcinogenic metabolite of polycyclic aromatic hydrocarbon (PAH). The role of the *GST* in its various forms plays a key role in protecting cells from a wide range of endogenous and exogenous potentially toxic chemical entities.[664]

This fact highlights an important aspect of the *GSTP1* gene and its enzymatic product in human health.[666] Furthermore, it is overexpressed in tumours following chemotherapy and has been associated with poor prognosis in breast cancer. A recent study identified a novel regulatory function of *GSTP1* in which it modulates Estrogen Receptor-alpha (ERα) signalling events in a non-enzymatic manner, thereby closely associating it with estrogen-dependent cancers such as breast, uterine and prostate cancer.[667]

As discussed in some detail in Chapter 5, SFN has been identified as a molecule with potential as an effective chemopreventive for bladder cancer by multiple mechanisms including its ability to induce genes of the *GST* family, even though not all studies agree on whether *GST* polymorphisms are generally cytoprotective.

In a 2011 study considering bladder cancer susceptibility in relation to *GST* polymorphisms, it was shown that patients carrying the *GSTP1* homozygous genotype were at increased risk for developing high-grade and muscle invasive bladder cancer.[668] Another meta-analysis two years later confirmed this by concluding that the presence of the homozygous *GSTP1* polymorphism is a strong predisposing risk factor for bladder cancer.[669] Contrary to these findings, a more recent 2016 meta-analysis showed no association between *GSTP1* polymorphisms and bladder cancer susceptibility.[670]

SFN's effects are pleiotropic such that it is not known to what extent *GSTP1* contributes to its observed chemopreventive effects. Interestingly and in support of SFN's chemopreventive potential, a very recent 2017 review article suggested that,

based on available evidence from epidemiological, *in vitro*, preclinical, and early phase trials, phytochemicals such as ITCs and specifically SFN, represent a promising potential chemopreventive agent in bladder cancer.[628]

Clearly, many factors are involved and to further confound opportunities to draw any definitive conclusions, it has been suggested that genotypes associated with more favourable handling of carcinogens may be associated with less favourable handling of phytochemicals.[659]

Knowing that SFN is a substrate for the gut microflora which produce SFN mercapturic acid metabolites, a further aim was to determine if the responses in plasma SFN and metabolites vary between participants with different *GSTP1* genotypes. Usually considered as a key Phase 2 detoxification enzyme, *GSTP1* codes for the glutathione-S-transferase enzyme, known also to be pivotal to SFN conjugation.

It was anticipated that the findings might be clinically-relevant in that individuals known to carry *GSTP1* polymorphisms could expect a higher levels of plasma SFN and its metabolites in response to SFN supplement from those expressing the wild-type *GSTP1* gene.

Whether or not regular consumption of broccoli vegetable as the primary dietary source of SFN is sufficient to achieve the higher doses indicated by *in vitro* studies and preliminary clinical trials continues to be a key research consideration.[384,671,672] Broccoli sprouts as a more concentrated source of broccoli bioactives are commonly the intervention material in this context. The EASYGENEX Study selected a SFN-yielding broccoli sprout capsule as its intervention; a detailed rationale for the selected form and dosage is discussed in Chapter 7.

Chapter 7 also describes the way in which SFN has been shown to exhibit anti-inflammatory effects which are at least in part due to its ability to modulate the activity of the transcription factor NF-kB, well-known for its role in immune function. Unlike its direct activation of Nrf2, SFN has no direct effect on release of NF-kB from its complex with the IκB kinase enzyme (IKK) but instead acts to inhibit the binding of NF-kB to the DNA.[639]

Microarray techniques provide an avenue to investigate gene expression and in some cases, to discover hitherto unknown target genes and their related pathways. This study therefore utilised a microarray platform as its means of investigating the effects of SFN on gene expression in young, healthy men.

8.1.1 The Aims of the EASYGENEX Study

The primary aim of this dose-escalation study was to investigate two doses of SFN on gene expression, with the second dose double that of the first. A further aim was to determine if the responses in plasma SFN and metabolites vary between participants with different *GSTP1* genotypes. As detailed in Chapter 7, the higher dose has been used in other studies utilising broccoli sprout forms other than orally-ingestible capsules. Since other studies have shown that SFN induces expression of Nrf2-dependent target genes, it was expected that this would similarly occur in the EASYGENEX Study.

Furthermore, there was the expectation that Nrf2-*in*dependent genes would also be expressed at the higher dose and that the higher dose would reveal different and more quantitatively significant effects. The study's hypothesis extended to consideration that genes associated with HDAC inhibition and inflammation might be negatively expressed. SFN is also known to be associated with other processes like cell cycle regulation, apoptosis, angiogenesis, metastasis and microtubule disruption[673] and so it was considered that the expression of these genes might be modulated by the intervention.

8.2 MATERIALS AND METHODS

8.2.1 Study Design

The study was an open-label dose-escalation clinical trial conducted in accordance with the ethical guidelines laid down by the Declaration of Helsinki, 1975 and approved by the Ethics Committee of the University of Queensland (Clearance Number: 2013000222).

Healthy, non-medicated, young adult men with BMI in the normal range and of average age 25 ± 4 years were recruited by advertisement from the university campus and neighbouring areas. Prospective participants were excluded if they

were female, aged under 18 or over 35, a smoker or regularly took dietary supplements considered to potentially interfere with the treatment. This intervention study was conducted over the period from 14th September, 2016 to 7th October, 2016 in two 7-day blocks with the intervention for the second week at double the dosage administered in the first week. Following a 3-day washout period during which cruciferous vegetables, other known Nrf2 activators and redox-modulating foods and supplements were excluded from the diet, participants attended the university laboratory in the fasted state for collection of baseline anthropometric data, blood and buccal swab sampling; questionnaires were used to collect details of each participant's usual dietary intake and physical activity. Items for the Active Australia Survey were used to quantify physical activity in METmins This self-report approach has been shown to be valid.[674] Details appear with Table 8.1 which also lists participant anthropometric data, participant ethnicity and *GSTP1* genotype.

Participants were given a 14-day diary in which to record daily details of diet and sleep as well as any adverse effects. Each participant was given sufficient capsules for the following 7 days with instructions that these be taken as two capsules in the morning and two capsules in the evening (a total of 28 capsules). The dietary and other exclusions undertaken during the 3-day washout period were to be continued throughout the 14-day intervention period. Participants returned in the fasted state on the 7th day for blood sample collection and to return any unused capsules. Sufficient capsules to cover Day 7 to Day 14 with instructions to take 4 capsules night and morning were provided (a total of 56 capsules). On Day 14, the final fasting blood sample was taken. Any remaining capsules were collected and counted for the Day 7 and Day 14 visits respectively. On Day 14, participants were asked to provide feedback on the treatment, including any adverse or other effects experienced. Adherence to the protocol was assessed through interview and by counting the number of capsules returned at the end of the study.

8.2.2 The Intervention Material

The broccoli sprout powder used to make the capsules was supplied by Cell-Logic Pty Ltd (Queensland, Australia). The sprouts had been grown, dried and milled to a fine powder, suitable for encapsulation. The material (Batch EUCAPE 00080573) was assayed by Eurofins Supplement Analysis Center, 1365 Redwood Way,

Petaluma CA. 94954, USA to contain 3.77 ± 0.52% GRN and to yield 1.89 ± 0.30 % SFN per gram. The SFN assay was specified to be performed without the addition of an exogenous source of MYR, so that the SFN Yield is determined only on the basis of the endogenous presence of both glucoraphanin (GRN) and MYR. The powder was stored in a sealed foil bag in a dark, low-temperature, low-humidity environment. Size '00' vegetable capsules purchased from CapsuGel® in Sydney Australia were used to produce the study capsules, using the Cap-M-Quik hand-filling device. (Cap-M-Quik.com. 40950 Bouvier Court, Murrieta, CA 92562). Each capsule contained on average 700 ± 11 mg of broccoli sprout powder. By calculation, each capsule contained 26.4 mg GRN and would yield 13.2 mg SFN. Assuming full conversion of GRN to SFN, participants in Week 1 of the study ingested 52.8 mg SFN daily and in Week 2, ingested 105.6 mg SFN daily.

8.2.3 Sample Collection

Blood samples from 21 apparently healthy human donors were obtained after informed consent was given. Blood used for RNA extraction was collected in PAXgene (Qiagen) tubes and frozen at −80°C after a 2 h incubation at room temperature. Serum and plasma samples were obtained after centrifugation of blood collected in vacutainer tubes (with and without EDTA).

8.2.4 Plasma Extraction for Metabolite Analysis

Plasma was first defrosted and 200µL transferred to a 1.5mL micro centrifuge tube. To this, 50µl of the internal standard solution (sulforaphane-d8 in methanol) was added followed by vortex mixing. To this solution, trifluoracetic acid (50µL) (TFA) was added to precipitate plasma proteins in the sample followed by brief vortex mixing and centrifugation at 11,600 x g for 10 min. 100µL of the resulting supernatant was removed and 10µL injected for analysis. Throughout the extraction process, samples were kept at 4°C.

8.2.5 SFN Metabolite Assay Using LC–MS–MS

An assay of plasma SFN and its metabolites was undertaken to establish the absorption and subsequent metabolism of the intervention material to its major metabolites and the difference in these concentrations at two dose levels.

Chromatographic separation was carried out on an Agilent 1260 Infinity HPLC system (Agilent Technologies, CA, USA) using a Kinetex 5µm C18, 250 x 4.6mm with an AQ C18 4 x 3mm Security Guard cartridge, all purchased from Phenomenex, Unit 35, 2-6 Chaplin Drive, Lane Cove West, NSW, 2066 Australia. The mobile phase consisted of solution A: distilled water with 0.1% formic acid, and solution B: 75% acetonitrile, 25% methanol and 0.1% formic acid run with a gradient with a flow rate of 1mL/min. Starting with 10% solution B and increasing to 50% over the first 6 minutes. This flow was held for 2 minutes before increasing to 90% solution B at 10 minutes and holding for 2 minutes. The flow was returned to 10% solution B by the end of the run at 13 minutes. The column temperature was maintained at 30°C and the analytes were quantified with an Agilent 6460 triple quad mass spectrometer with transitions as follows: sulforaphane 179.2→115.2; sulforaphane-d8 187.2→123.2; sulforaphane NAC 342.0→179.0; sulforaphane glutathione 487.0→136.0; sulforaphane-cysteine 301.0→136.0. This method is based on and modified from a previously-published SFN method[675] and an Agilent protocol for curcumin extraction and analysis.[i]

8.2.6 RNA Extraction

RNA was isolated from whole blood collected in PAXgene tubes using the PreAnalytiX PAXgene blood RNA kit (Qiagen) according to the manufacturer's instructions. RNA was eluted in RNase-free water. RNA quality was determined by spectrophotometry and by using the RNA 6000 NanoChip kit on an Agilent 2100 Bioanalyzer (Agilent Technologies). RNA passing quality control criteria were used for further analysis. Samples were shipped on dry ice to the Ramaciotti Centre for Genomics (The University of New South Wales) for microarray analysis.

8.2.7 Reverse Transcription

Reverse transcription reactions were performed using the AffinityScript QPCR cDNA Synthesis kit from Agilent Technologies and following the manufacturer's instructions.

[i] Obtained from Agilent and accessed 18th May, 2017. https://www.agilent.com/cs/library/applications/5991-3340EN.pdf

8.2.8 Microarray Procedures

Microarray analysis was performed in the Ramaciotti Centre for Genomics (The University of New South Wales) using the Affymetrix Human Genome U219 Array Plate. Microarray data were analysed for each of the 3 times points of the study (Day 0, Day 7 and Day 14) using R (v3.4.0) (R Development Core Team 2005) and Bioconductor.[676] The '.CEL' data file was imported using the 'affy' package (v1.54.0).[677] Custom microarray probe set definition (v21), updated according to the latest genome sequence and gene annotations, were used to map each probe to its corresponding gene.[678] The data were normalised using the Robust Multi-array Average (RMA) function[679] from the affy package. The quality of the normalised data were assessed using the 'arrayQualityMetrics' package (v3.32.0)[680] and any outliers identified were excluded from subsequent analyses. Three samples were identified as outliers based on the gene expression distances between microarrays; these samples include participant 6 day 7, participant 5 day 14, and participant 20 day 0. Principal component analysis was performed using the 'arrayQualityMetrics' package and the 'mixOmics' package.[681] The heat map was drawn using the 'iheatmapr' package (https://github.com/AliciaSchep/iheatmapr). Information regarding the repeated measure of the subjects across the three time points was included as factors in all differential gene expression and unwanted variation analyses.

Unwanted variations from the microarray data were identified using the *remove unwanted variation* (ruv) package (v0.9.6).[682] Unwanted variations were identified from a set of negative control genes for which expression is not known to change with respect to the biological factor of interest.[683] In this study, negative control genes were identified empirically from a preliminary analysis of significantly differentially-expressed genes, using the 'limma' package (v3.32.2).[684] The 1,402 empirical negative control genes consisted of those commonly found among the 30% least significant genes from all pairwise time points comparisons (day 0 – 7, day 7 – 14, and day 0 – 14). The 1,402 empirical negative control genes consisted of those commonly found among the 30% least significant genes from all pairwise time points comparisons (day 0 – 7, day 7 – 14, and day 0 – 14).

The optimum number of unwanted factors was identified using the 'getk' function; there were 13 for unwanted factors for day 0 – 7 and 14 unwanted factors for both

day 7 – 14 and day 0 – 14. The ‘ruv4’ function was used to identify the top 14 unwanted factors. The unwanted factors were removed using the linear model during a subsequent ‘limma’ analysis and significantly differentially-expressed genes were identified for each pair of time. The log fold-change in gene expression was calculated as the difference between the latest date and the earliest date. The *p*-values were adjusted using the Benjamini-Hochberg procedure and a gene was deemed significantly differentially-expressed if the adjusted p-value was < 0.05.

Two samples from participants who reported being unwell between Day 0 and Day 7, participants #21 and #23, were suspected of having a viral infection since they showed an increased expression of genes involved in response to viral infection. Since removing these two samples would have decreased the number of significantly differentially-expressed genes across all time points, it was decided to focus the analyses on the comparison of day 0 versus day 14.

8.2.9 Ingenuity Pathway Analysis (IPA)

Significantly differentially-expressed genes were analysed using the IPA software (Ingenuity Systems Inc., Redwood City, CA). IPA was used to identify enriched canonical pathways and to identify significantly differentially-expressed genes in the context of the IPA Global Molecular Network. All 17,684 genes defined by the custom CDF file were used as the background gene set for the analysis of enriched canonical pathways.

8.2.10 Buccal Swab Collection and Analysis

Sample collection kits for analysis of polymorphisms of genes associated with cellular redox and cytokines were purchased from Fitgenes Pty Ltd who supplied the Isohelix T-Swabs manufactured by Cell Projects Limited.

Samples collected using these kits were analysed by DNAiQ Genetic Testing Laboratories, using the following procedure. For DNA extraction & quality control, Genomic DNA from buccal swabs was extracted and purified using a paramagnetic particle automated method according to the manufacturer’s instructions (Maxwell 16 instrument and AS1020 DNA purification kit, Promega). The DNA concentration and 260/280 absorbance quality ratio for all samples extracted was determined using

spectrophotometry according to the manufacturer's instructions (Nanodrop One instrument, Thermofisher). Samples with DNA concentration >=35 ng/μL and purity >=1.7 were used in the study. DNA was either diluted with RT-PCR grade water or evaporated to obtain a final concentration in the range of 50ng/μL. DNA was stored at -20C until use.

For real-time PCR, genotyping from purified DNA was determined using a real-time PCR and fluorescence detection method according to the manufacturer's instructions (QuantStudio 12k instrument Life Technologies). Each SNP was targeted using TaqMan SNP genotyping assay probes (ThermoFisher Scientific) in an OpenArray Genotyping plate format (Life Technologies). Standard cycling conditions were configured for SNP genotyping assays on an OpenArray Genotyping format according to the manufacturer's instructions (QuantStudio 12k instrument Life Technologies).

8.2.11 Data Analysis

Analysis of the microarray data has been included in each relevant section above. The plasma SFN and metabolite concentration data were first checked for normality using the Shapiro-Wilk test. If data were not normally distributed, they were log-transformed and rechecked. SFN data were normally distributed. The metabolite data were normally distributed after log transformation. A 2-way (group*time) repeated measures multivariate analysis was used to assess main effects (group and time) and any interaction. Statistical analysis was done with SPSS (version 24)

8.3 RESULTS

8.3.1 Study Flow, Adherence and Supplement Tolerance

A total of 23 men gave written consent and were enrolled in the study. Figure 8.1 shows the flow of participants through the study, in which three were subsequently withdrawn, two were unqualified (Participants #3 and #4) by not meeting the age criterion of 18 years and the other (Participant # 1) dropping out after 7 days, citing abdominal discomfort due to the supplement as the reason.

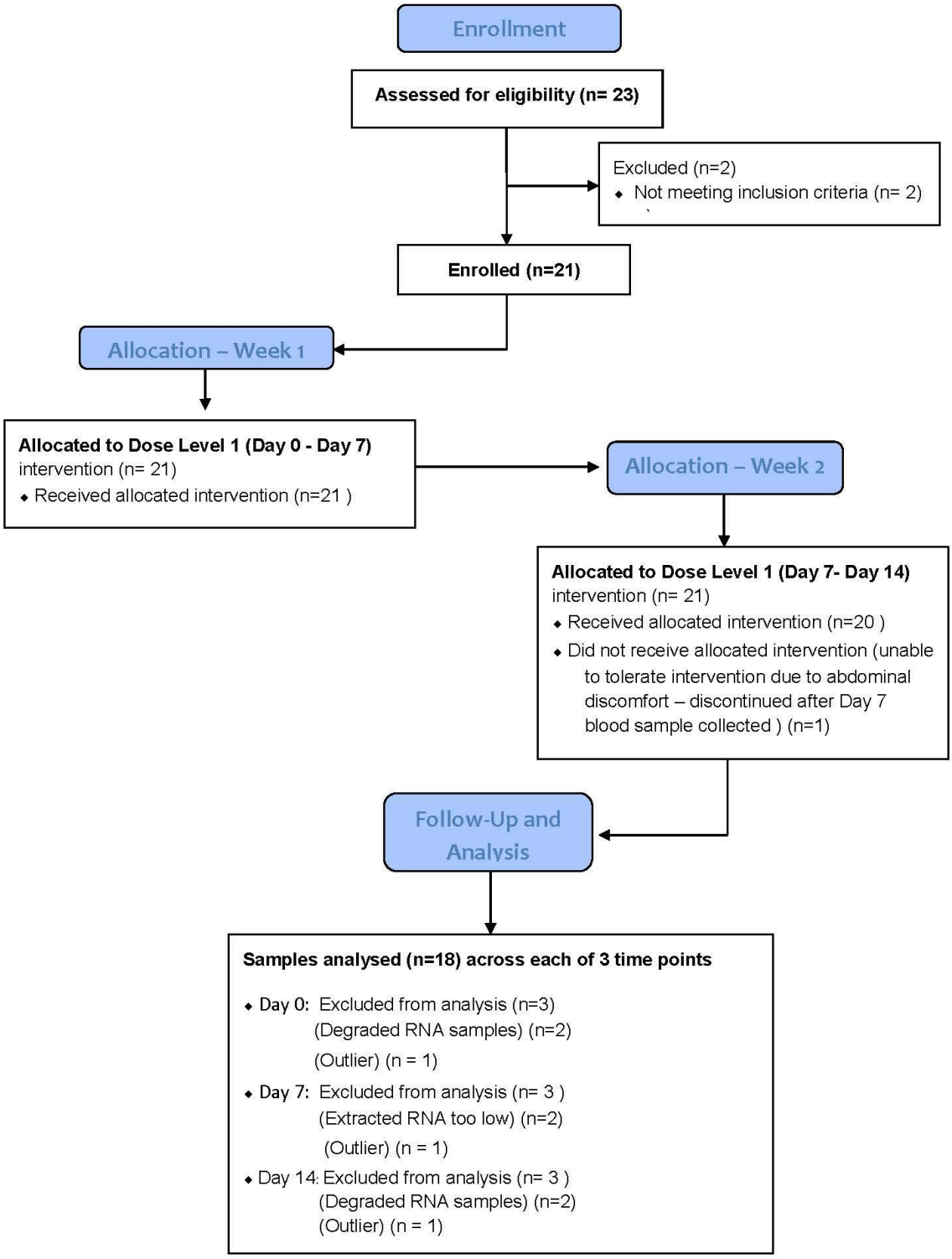


Figure 8.1 EASYGENEX Study Flow Chart

Most participants took capsules as instructed; however, there were exceptions; participants # 6, 9 and 25 each missed one dose, with #9 missing the last evening dose before the end of the study. Nevertheless, over the two 7-day periods, the overall amount ingested approximated the specified dosages, with the exception that the timing of the missed dose by #9 may have affected his final plasma metabolite level.

Furthermore, participant # 18 exercised at the gym before attending for his 7-day blood sample collection and failed to fast for the second blood test. Four participants reported that the capsules caused bloating, one reported that this eased in the second week and one reported nausea if the capsules were taken on an empty stomach. Reported adherence with dietary exclusions appeared to be very good apart from one participant who reported a single occasion during which he consumed mustard, a food from the 'excluded foods' list. However, plasma SFN data as described later in 8.6 indicated that actual adherence appeared to be different from reported adherence.

From the Outlier Detection for Distances between Arrays (Table 8.2), 3 outlier samples were identified, these were participant 20 in day 0, participant 6 in day 7, and participant 5 in day 14. Outlier samples were not included in the analyses of significantly differentially expressed genes. Table 8.3 classifies all participants as to their inclusion or exclusion from the final datasets analysed.

8.3.2 Participant Characteristics

Baseline data was collected for each participant with the demographic characteristics of the 20 men shown in Table 8.1. The results of the genotyping analysis for GST polymorphisms is also recorded in Table 8.1.

Table 8.1. Individual Participant Characteristics

Anthropometric data, average physical activity output and GSTP1 genotype.

Participant #	**Age** (years)	**Height (m)**	**Weight (kg)**	**BMI**	**Ethnicity**	**Average Daily Exercise Physical Activity (METMins)** [a]	***GSTP1* Genotype**
							Wild-type (Normal) +313AA **Homozygous Variant** +313 GG **Heterozygous Variant** +313 A>G (Ile105V)
~~**1**~~	25	1.72	73	24.7	Caucasian	N/A	N/A
2	24	1.81	74.5	22.7	Asian	407	
~~**3**~~	~~17~~						N/A
~~**4**~~	~~17~~						N/A
5	30	1.78	78	24.6	Caucasian	1067	
6	20	1.81	88	26.9	Caucasian	467	
7	28	1.7	73	25.3	Asian	270	
8	20	1.8	95	29.3	Asian	643	
9	20	1.77	65	20.7	Caucasian	259	
10	31	1.7	74.8	25.9	Caucasian	150	
11	25	1.74	77	25.4	Caucasian	285	
12	28	1.67	72	25.8	Asian	764	
13	20	1.75	51	16.7	Asian	433	
14	23	1.7	70	24.2	Asian	120	
15	23	1.69	69	24.2	Asian	N/A	
16	25	1.78	73	23.0	South Asian	1131	
17	23	1.8	90	27.8	Caucasian	694	
18	22	1.94	94	25.0	Caucasian	154	
19	29	1.86	83	24.0	South Asian	370	
20	31	1.78	78	24.6	South Asian	345	
21	30	1.85	86	25.1	Caucasian	360	
22	28	1.82	79	23.8	Caucasian	424	
23	29	1.65	65	23.9	Asian	287	

[a] *1 MET = the resting metabolic rate, equivalent to oxygen uptake of 3.5 mL/kg/hr.*[674] *Walking and moderate activities were defined as having an energy expenditure rate equivalent to 3.5 METs and vigorous activities as 9.0 METs. According to the Australian government recommendations, adults are recommended to undertake at least 500 MetMINs per week or total time (150 minutes of moderate activity). 2012 Development of Evidence-based Physical Activity Recommendations for Australian Adults (18-64 years) www.health.gov.au/internet/main/publishing.../DEB-PAR-Adults-18-64years.pdf*

8.3.3 RNA Sample Integrity

The measured RNA Integrity Number (RIN) for all samples was above 7.5, demonstrating that the RNA integrity was not ideal but satisfactory for analysis. Table 8.2 shows that in 3 samples, RNA had been degraded; these samples were Day 0 for participants # 10 and 12 and on Day 14 for participant # 20. Insufficient RNA had been extracted for two samples (Participant #5 on Day 7 and Participant # 7 on Day 7). As a consequence, not all participants have data for all 3 time points.

Table 8.2 RNA Quality by Sample at each Time Point.

Collection data participant at each time point showing missing data points, low RNA concentration and degraded samples.

RNA Collection and Quality Record - Samples analysed by MicroArray																							
	Participant #																						
Time	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
0	Y	Y	Y	Y	Y	Y	Y	Y	Y	d	Y	d	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
7	Y	Y	n	n	L	Y	L	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
14	n	Y	n	n	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	d	Y	Y	Y

Y	Microarray results available. Samples stored at -80 degrees C.
L	Not analysed - RNA concentration too low
d	RNA degraded
n	No blood collected for RNA extraction

8.3.4 Microarray Data Quality Analysis

The arrayQualityMetrics tool generated a heatmap image that demonstrated Distance between Arrays Figure 8.2, with subsequent detection of outliers for the distances between arrays, Figure 8.3.

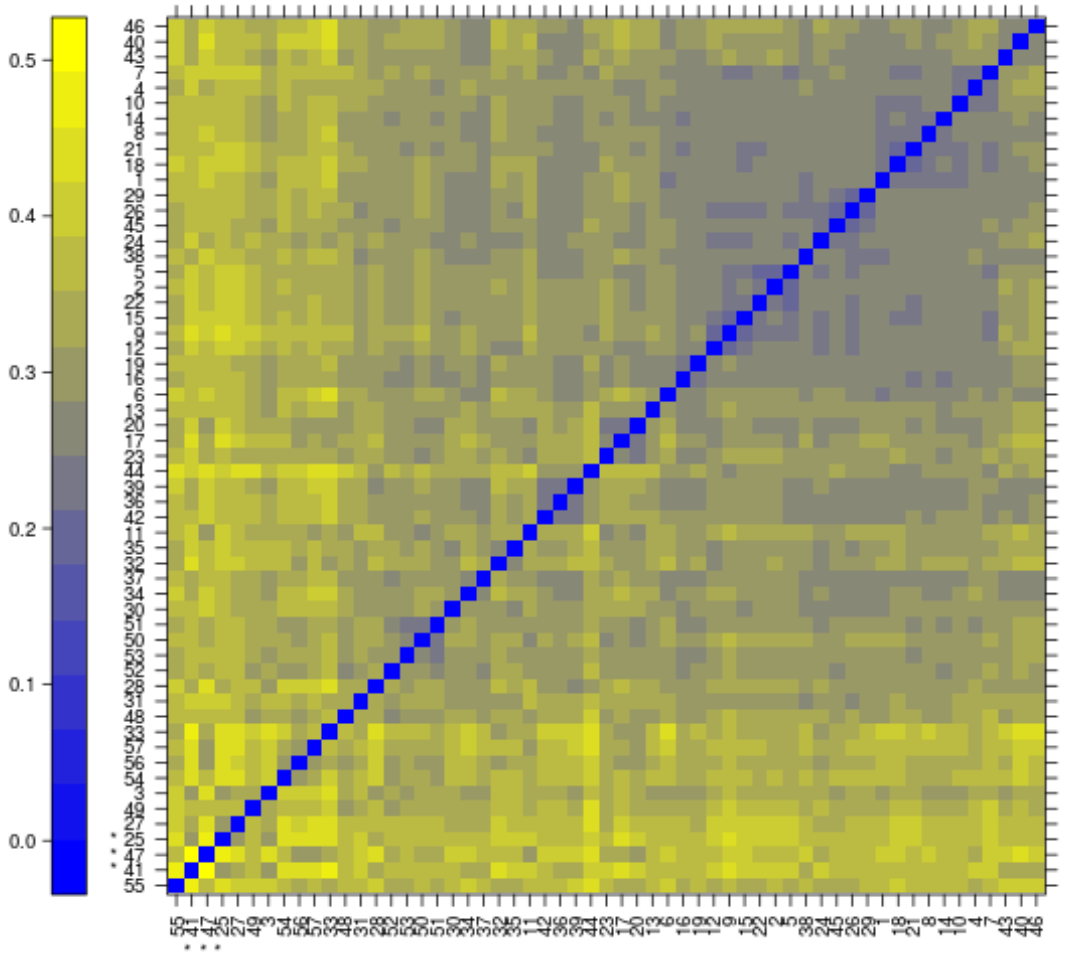


Figure 8.2 Distances between arrays

This figure shows a false colour heatmap of the distances between arrays. The colour scale is chosen to cover the range of distances encountered in the dataset. Patterns in this plot can indicate clustering of the arrays either because of intended biological or unintended experimental factors (e.g. batch effects). The distance between two arrays a and b (dab) is computed as the mean absolute difference between the data of the arrays (using the data from all probes without filtering). Outlier detection was performed by looking for arrays for which the sum of the distances to all other arrays, ***Sa = Σb dab*** *was exceptionally large. Three such arrays were detected, and they are marked by an asterisk* as 25, 41 and 47. (Shown lower left – both axes)*

8.3.5 Outlier Detection for Distances Between Arrays

The heatmap shown in Figure 8.2 highlighted the three arrays, 25, 41 and 47 as being outside the determined threshold of 21.1 for outlier analysis. Figure 8.3 identifies the participant and time period which classifies them as outliers.

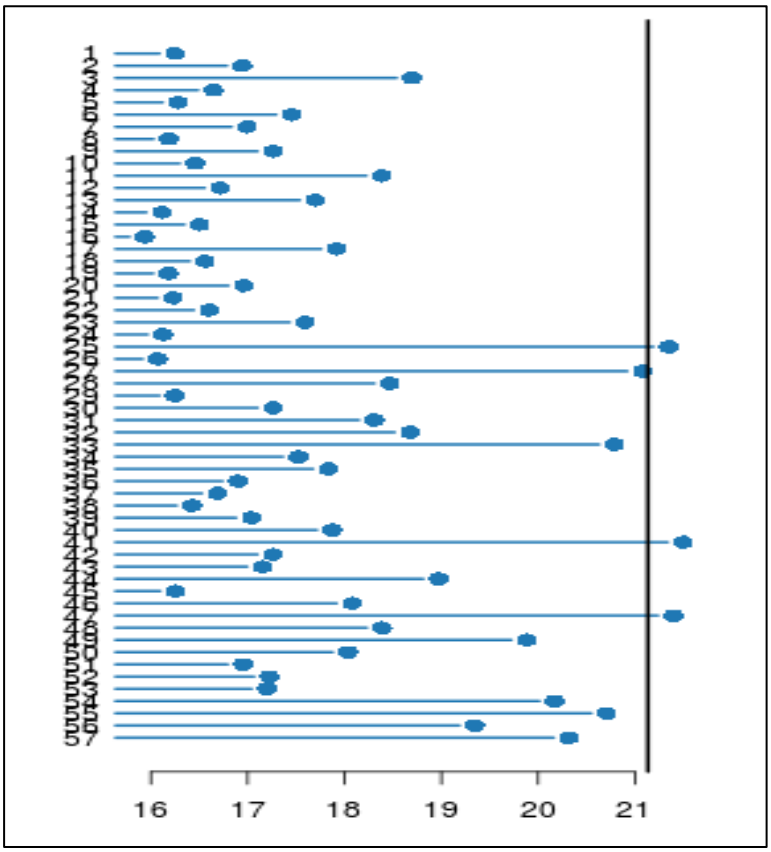


Figure 8.3 Outlier Detection for Distances between arrays.

This figure shows a bar chart of the sum of distances to other arrays ***Sa****, the outlier detection criterion from Figure 8.2. The bars are shown in the original order of the arrays. Based on the distribution of the values across all arrays, a threshold of 21.1 was determined, which is indicated by the vertical line. Three arrays exceeded the threshold and were considered outliers.*

The following three samples were identified as the outliers: Participant 6 day 7; Participant 5 day 14; Participant 20 day 0

8.3.6 Principal Component Analysis (PCA)

PCA is an method for grouping the samples based on their similarity in the expressions of all the transcripts. To explore genes for which expression changed due to SFN in two different doses from baseline, genome-wide expression profiles for the two dose levels were investigated. The PCA data, illustrated graphically in Figure 8.4 do not show a visually-apparent PCA relationship. The absence of clusters indicates that there were few significantly differentially-expressed genes across the time periods Days 0 – 7 and Days 7-14.

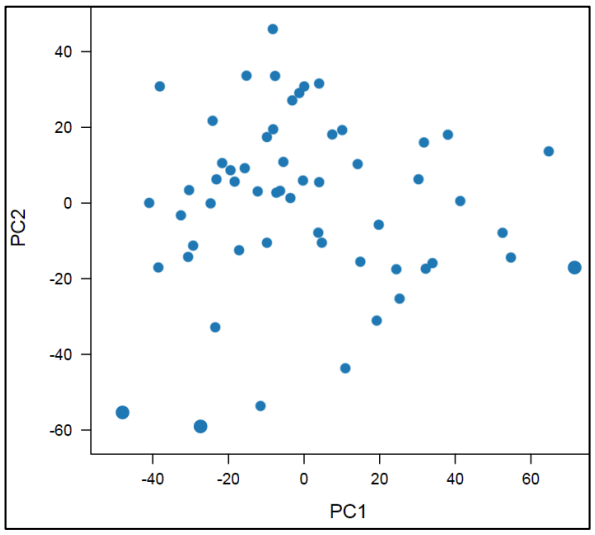


Figure 8.4 Principal Component Analysis.

This figure shows a scatterplot of the arrays along the first two principal components, reflecting the overall data (dis)similarity between the arrays, with the larger points indicative of the outliers.

8.3.7 Number of Differentially-expressed Genes

Overall, when comparing the numbers of genes significantly differentially-expressed from Day 0 to Day 14, 36 genes with adjusted p-values <0.05 were identified; 10 of these genes were upregulated and 26 were downregulated. For the positively-expressed genes, the fold-change ranged from 1.1 to 1.3 and for the negatively-expressed genes from 0.8 to 0.9.

The results of the study appear to have been confounded by the presence of viral infection in two of the participants (#21 and #23) during Week 1. Had the outliers and the subjects carrying the Day 7 viral load data been removed, there would have been an overall decrease in the number of significantly differentially-expressed identified genes. (Refer Table 8.4)

Participants for whom one or two data points were missing were retained in this set of analyses. This is illustrated in Table 8.3 and Figure 8.1, showing that the final sample number for each time period was 18.

Review of the data overall and consideration of the sparseness of the differentially-expressed genes shown by the Volcano Plots for each of the time periods confirmed the need to focus on the period Day 0 – Day 14.

Table 8.3 Samples that are included in this analysis.

Of the 23 enrolled participants, 18 at each time point were included in the data analysis

Time Point (Days)	Participants	Number of Samples
0	1, 2, 5, 6, 7, 8, 9, 11, 13, 14, 15, 16, 17, 18, 19, 21, 22, 23	18
7	1, 2, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23	18
14	2, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 21, 22, 23	18

Table 8.4 shows the list of significantly differentially-expressed genes comparing two time points with baseline. These three periods are: Day 14 versus Day 0, Day 14 vs Day 7 and Day 7 vs day 0. The study's primary focus as explained earlier is on the Day 0-Day 14 period.

Table 8.4 The number of significantly differentially-expressed genes based on viral load.

The numbers of differentially-expressed genes were reduced when the subjects with viral infections were included or excluded. (adjusted p-value <0.05). The fold-change for positively-expressed genes ranged from 1.1 to 1.3 and the fold-change for negatively expressed genes ranged from 0.7 to 0.9. Refer Table S 8.1 for data.

Comparisons	Number of Genes with Positive fold-change	Number of genes with Negative fold-change
Excluding the outliers		
Day 7 vs Day 0	17	83
Day 14 vs Day 0	10	26
Day 14 vs Day 7	36	90
Excluding the outliers and subjects (virus) 21 and 23 from Day 7		
Day 7 vs Day 0	10	64
Day 14 vs Day 0	8	13
Day 14 vs Day 7	8	15

8.3.8 Revised PCA With Outliers Excluded

After removal of outliers, the revised Ingenuity PCA data are graphed below wherein Figure 8.5a represents PCA of samples with the subject ID indicated. The first PCA plot showed that samples from the same subject tend to have similar gene expression and were clustered close together in the graph. Figure 8.5b represents the PCA of samples with the sample collection day indicated.

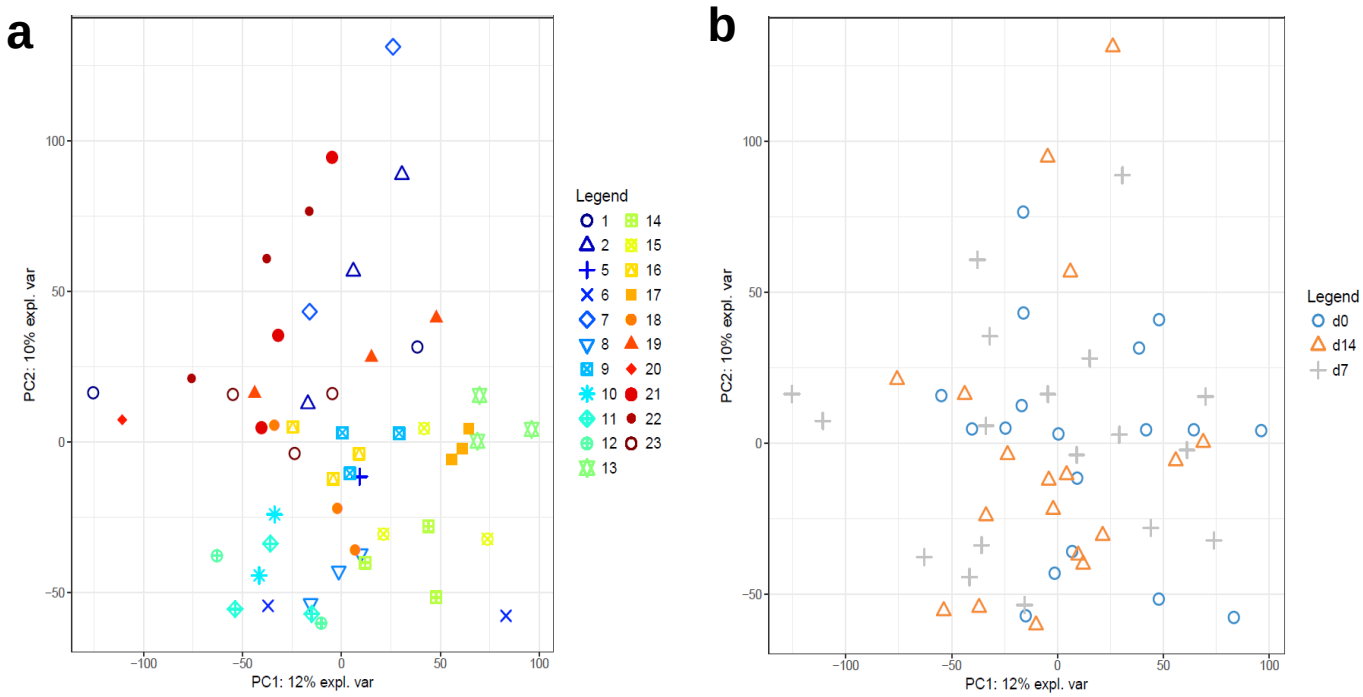


Figure 8.5 Principal component analysis (PCA) of the microarray data without outliers.

There were no clearly defined clusters for Day 0- 7and Day 7-14, so that comparison of gene expression between the individual time periods is not expected to generate reliable data. This suggested that there were few significantly differentially expressed genes when comparing the gene expression from different time points.

Review of the two principal components on the x and y axes respectively, showed that there was an absence of distinct clusters when considering the samples from Days 0, 7, and 14. This suggests that there were few significantly differentially-expressed genes across Days 0 – 7 and Days 7-14.

Each point in the scatter plot represents one microarray sample. Samples that have similar gene expression profiles will be close to each other. Figure 8.5a shows that for participant #17, the samples for all three time points had similar gene expression profile and where therefore close together on the PCA plot. Some other participants

also have their samples from different time points clustered closer together (e.g. #18, #9, and #13 as examples). This suggests that for the most part, the gene expression profile from different time points for the same person are similar.

8.3.9 Summary of Microarray Quality Analysis Data

The various quality control measures described above reaffirm that there are relatively few significantly differentially-expressed genes across the two 7-day time points.

8.4 ANALYSIS OF SIGNIFICANTLY DIFFERENTIALLY-EXPRESSED GENES

8.4.1 Day 0 – Day 14 Heatmap

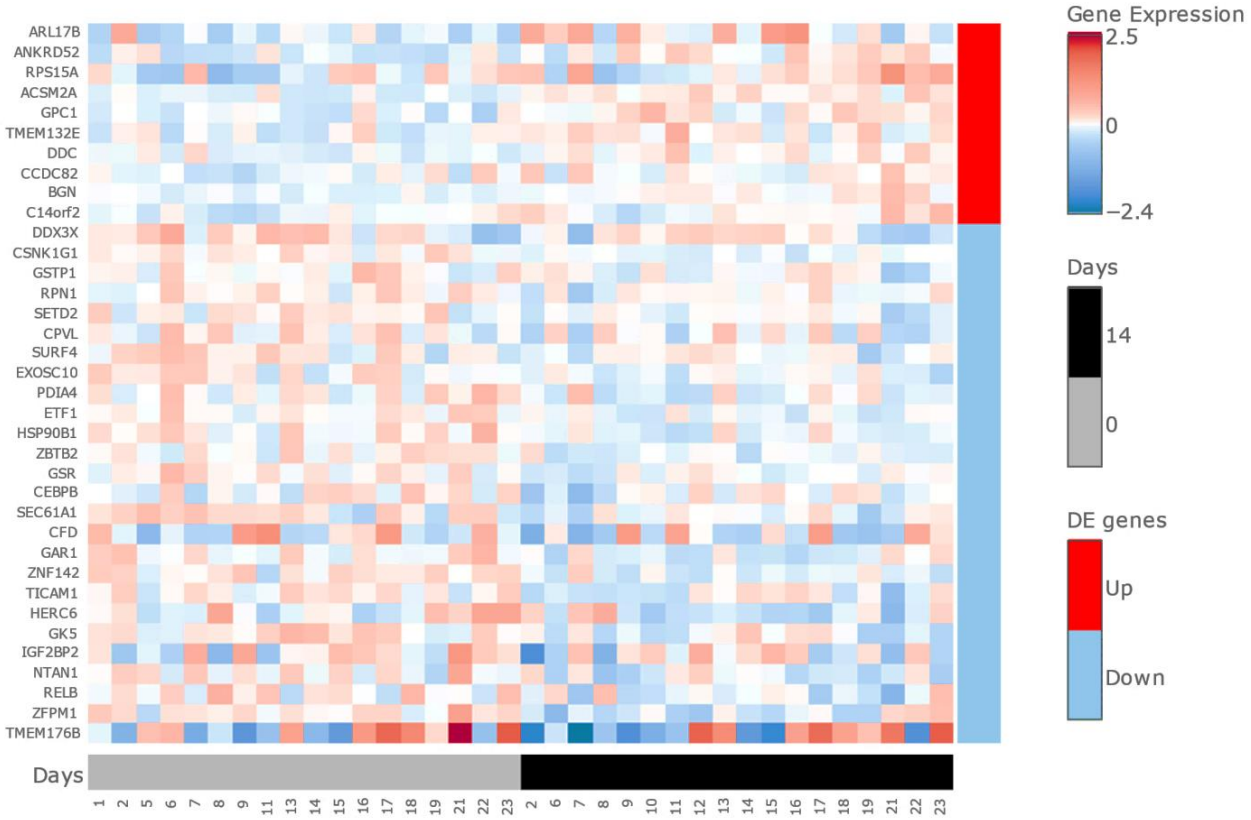


Figure 8.6 Heat map showing the expression level of the significantly differentially-expressed genes for the 23 sets of participant data for Day 0 - Day 14.

The rows represent the significantly differentially-expressed genes and the columns represent the identification numbers of the participants. Each cell of the heat map represents the gene expression values normalised using the RMA algorithm, in which red represents high gene expression, blue represents low gene expression and white represents medium expression. The bar on the right side of the heat map represents the significantly differentially-expressed genes, red = significantly increased expression, light blue = significantly decreased expression. The bar below the heat map indicates the number of days of SFN treatment, grey = 0 days and black = 14 days. The bottom left

region of the heat map highlighted a group of genes which had significantly increased expression in day 0 as compared to day 14. It also highlighted another group in the top right region of the heat map, showing genes with statistically significantly increased gene expression from Day 0 to Day 14.

8.4.2 Further Confirmation of Differential Gene Expression via Volcano Plot.

To further illustrate significant differential expression from Day 0 to Day 14, the R statistical programming language was used to generate a Volcano Plot (Figure 8.7).

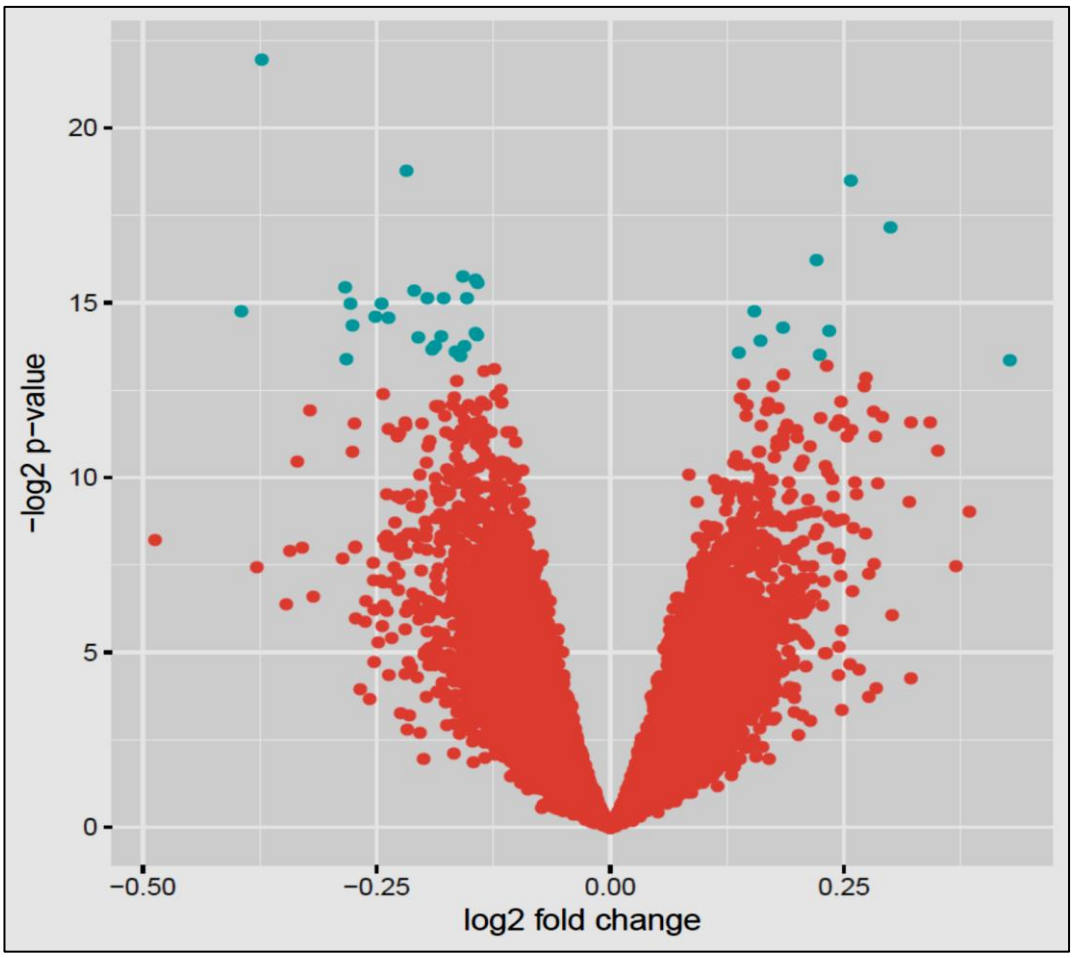


Figure 8.7 Volcano plot showing the significantly differentially-expressed genes when comparing changes from Day 0 to Day 14.

The y-axis represents the negative log adjusted p-values and the x-axis represents the log fold-change, visually separating upregulated and downregulated genes. Blue dots = significantly differentially-expressed genes (adj. p-value < 0.05) and Red dots = other genes.

8.4.3 Differentially-expressed Genes Day 0 – Day 14

Table 8.5 Significantly differentially-expressed genes when comparing Day 0 to Day 14. (adjusted p < 0.05)

Those marked as BOLD, are indicated as significant within the Ingenuity Network #1.

Gene Symbol	Log Fold-change	Fold-change	Adjusted p-value	Description
Increased Gene Expression at Day 14 compared with Day 0				
ARL17B	0.43	1.3	0.047	ADP ribosylation factor like GTPase 17B
ANKRD52	0.30	1.2	0.030	ankyrin repeat domain 52
RPS15A	0.26	1.2	0.016	ribosomal protein S15a
ACSM2A	0.23	1.2	0.041	acyl-CoA synthetase medium-chain family member 2A
GPC1	0.22	1.2	0.045	glypican 1
TMEM132E	0.22	1.2	0.037	transmembrane protein 132E
DDC	0.18	1.1	0.041	dopa decarboxylase
CCDC82	0.16	1.1	0.042	coiled-coil domain containing 82
BGN	0.15	1.1	0.037	biglycan
C14orf2	0.14	1.1	0.045	chromosome 14 open reading frame 2
Decreased Gene Expression at Day 14 compared with Day 0				
TMEM176B	-0.39	0.8	0.037	transmembrane protein 176B
ZFPM1	-0.37	0.8	0.004	zinc finger protein, FOG family member 1
RELB	-0.28	0.8	0.037	RELB proto-oncogene, NF-kB subunit
NTAN1	-0.28	0.8	0.047	N-terminal asparagine amidase
IGF2BP2	-0.28	0.8	0.037	insulin like growth factor 2 mRNA binding protein 2
GK5	-0.28	0.8	0.041	glycerol kinase 5 (putative)
HERC6	-0.25	0.8	0.038	HECT and RLD domain containing E3 ubiquitin protein ligase family member 6
TICAM1	-0.24	0.8	0.037	toll-like receptor adaptor molecule 1
ZNF142	-0.24	0.8	0.038	zinc finger protein 142
GAR1	-0.22	0.9	0.016	GAR1 ribonucleoprotein
CFD	-0.21	0.9	0.037	complement factor D
SEC61A1	-0.21	0.9	0.041	Sec61 translocon alpha 1 subunit
CEBPB	-0.20	0.9	0.037	CCAAT/enhancer-binding protein beta
GSR	-0.19	0.9	0.045	glutathione-disulfide reductase
ZBTB2	-0.19	0.9	0.044	zinc finger and BTB domain containing 2
HSP90B1	-0.18	0.9	0.041	heat shock protein 90 beta family member 1
ETF1	-0.18	0.9	0.037	eukaryotic translation termination factor 1
PDIA4	-0.17	0.9	0.045	protein disulfide isomerase family A member 4
EXOSC10	-0.16	0.9	0.045	exosome component 10

Gene Symbol	Log Fold-change	Fold-change	Adjusted p-value	Description
SURF4	-0.16	0.9	0.037	surfeit 4
CPVL	-0.16	0.9	0.044	carboxypeptidase, vitellogenic like
SETD2	-0.15	0.9	0.037	SET domain containing 2
RPN1	-0.14	0.9	0.037	ribophorin I
GSTP1	-0.14	0.9	0.041	glutathione S-transferase pi 1
CSNK1G1	-0.14	0.9	0.041	casein kinase 1 gamma 1
DDX3X	-0.14	0.9	0.037	DEAD-box helicase 3, X-linked

8.4.4 Differentially-expressed Genes for Periods Day 0 – Day 7 and Day 7 – Day 14

The Supplementary Materials contain the tables of differentially-expressed genes for the periods Day 0 to Day 7 and Day 7 to Day 14. These data have been removed from the Results section as they failed to meet the criteria for significance; even so, for completeness, they are included as supplementary material because elements of these data form part of the Discussion section in 8.7.

8.5 NETWORK ANALYSIS

8.5.1 Network #1 - NF-kB Interactive Network

Based on the input dataset, the IPA library of canonical pathways identified the NF-kB network as the most significant network, illustrated in the interactive network diagram in Figure 8.8. Chapter 7 discussed in some depth the NF-kB network and its cross-talk relationships with both Nrf2 and the Aryl hydrocarbon receptor, the latter being integral to the Phase 1 detoxification pathway.

Of the genes identified as interactive in the NF-kB pathway, IPA highlights *RELB*,[685] a component of the NF-kB sub-family and *CEBPB*,[686] both of which are downregulated in the EASYGENEX Study. The two aforegoing literature references suggest that the NF-kB complex also regulates *RELB* and *CEBPB*. What this study has identified is possibly evidence of a negative feedback mechanism, wherein NF-κB requires *CEBPB* and *RELB* subunit to function whilst NF-κB is a regulator of *RELB* and *CEBPB* expression.

This is further evidenced in that a cross-talk relationship between NF-κB and *CEBPB* has been identified showing that these transcription factors can act synergistically.[647]

These transcription factors acting together have been shown to mediate the expression of the major acute phase reactant, serum amyloid A2 via IL-1 and IL-6. Downregulated *CEBPB* has been shown to protect cardiomyocytes from hypertrophy via inhibition of p65-NF-κB, further highlighting the close inter-relationships between these transcription factors.[648]

These relationships are considered to be clinically significant in that a diet high in saturated fat, especially palmitic acid directly activates CEBPB protein expression in liver, adipocytes and macrophages, providing a putative link between dietary fat and the known inflammatory state associated with adiposity and type 2 diabetes.[646]

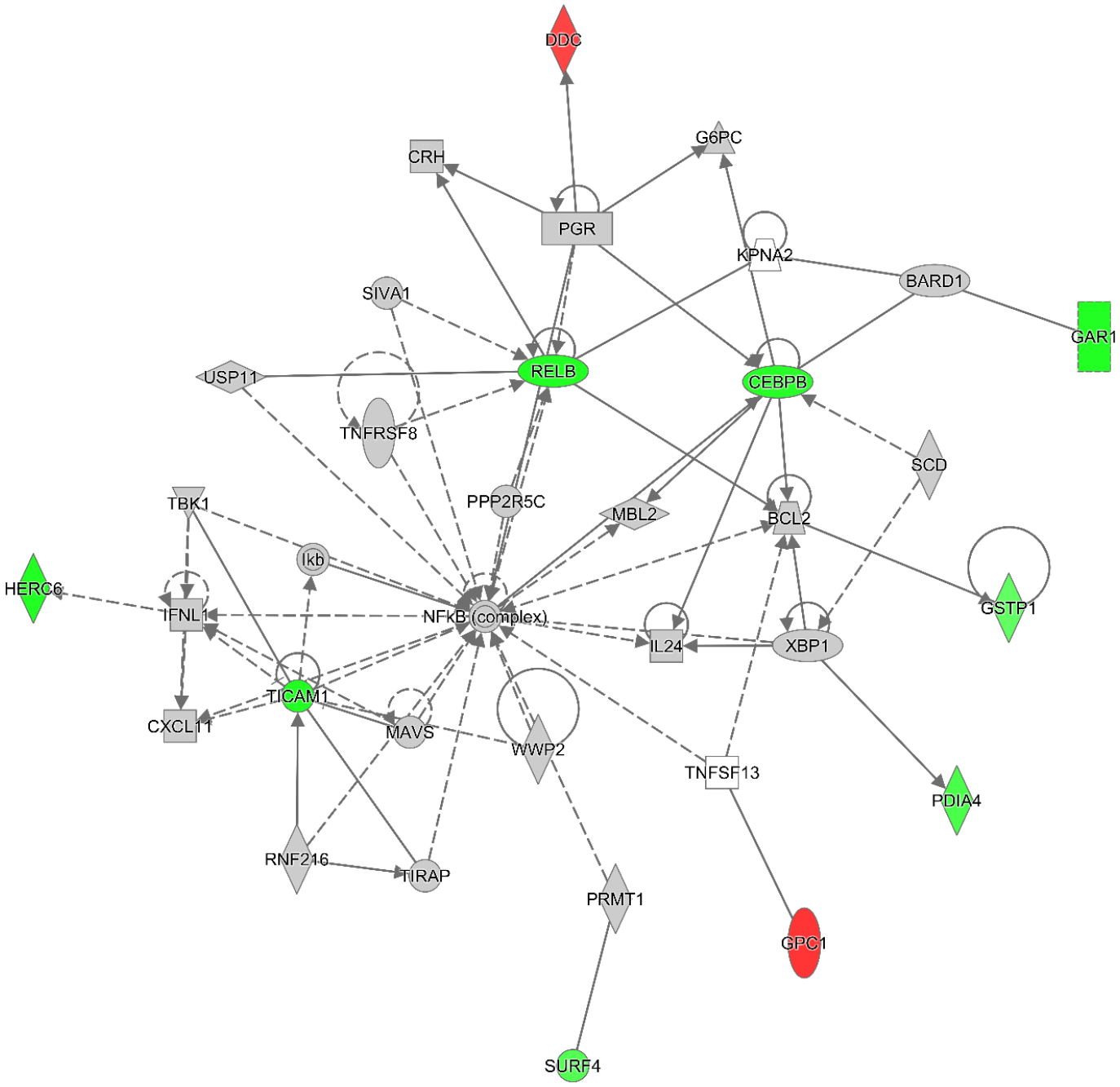


Figure 8.8 NETWORK 1. The highest scoring interaction network comparing Day 0 to Day 14 highlighted a possible negative feedback mechanism for the activity of the NF-kappa B (NF-kB) complex.

Genes that have significantly increased expression are highlighted in red and genes with significantly decreased expression in Day 14 are highlighted in green. The shape of each node in the network references the following: trapezium = transporter; diamond = enzyme; concentric circles = protein complex; square = cytokine; triangle = phosphatase; tall rectangle = ion channel; long rectangle = ligand-dependent nuclear receptor; tall ellipse = transmembrane receptor; long ellipse = transcriptional regulator. Genes in this network are known to be involved in cell death and survival, protein synthesis, and inflammatory response. The network highlights the decreased expression of genes whose protein product is known to interact in the NF-kB complex.

8.5.2 Canonical Pathways

IPA generated the *Top 15 Enriched Canonical Pathways* across the two 7- day periods and the overall 14-day period. The Canonical Pathway for Day 0 - 14 complements Network #1 by identifying the pathways highlighted by the statistically-significant degree of enrichment above a *-log (adjusted p-value)* threshold. For each of the Figure 8.9 enriched pathways expressed as *-log(p-value)*, seven are significant and above the threshold.

IPA identifies the relevant genes in the canonical pathway which had significantly increased gene expression compared to the average level of all genes but because these are expressed as grey bars, there is insufficient data to determine whether expression is up- or downregulated. Those pathways for which the ratio was low (i.e. less than 0.1) are indicative of too few statistically-significant differentially-expressed genes compared with the ratio to the total number of genes in the canonical pathway. Table 8.6 identifies the genes contributing to the pathways.

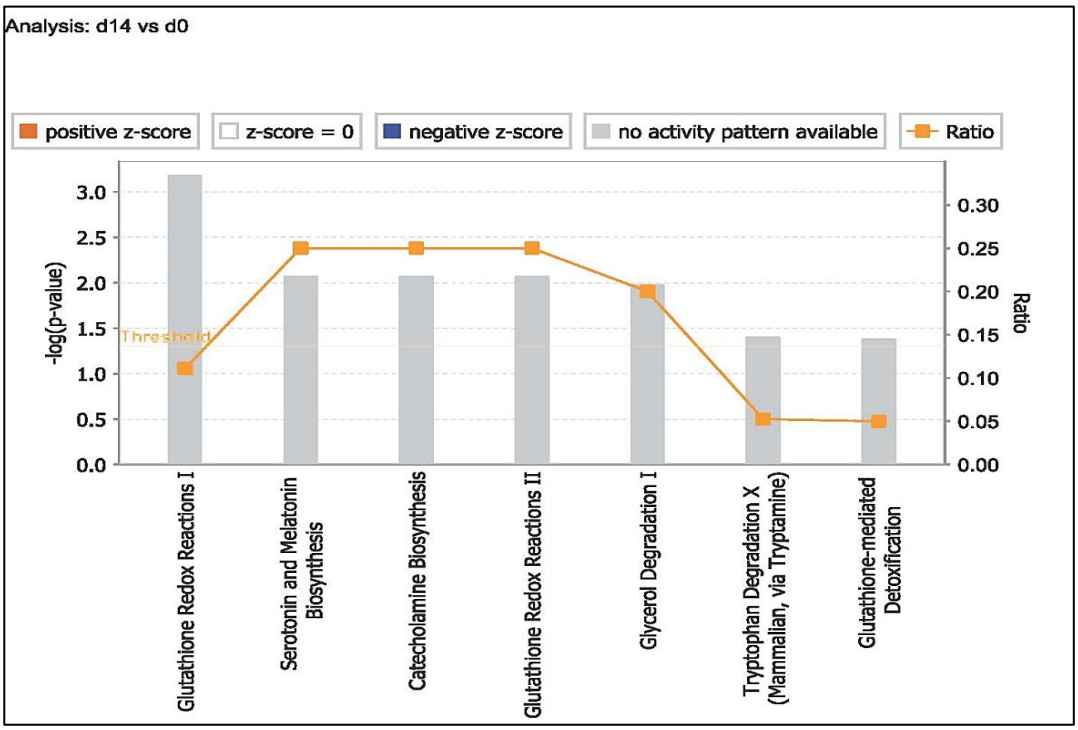


Figure 8.9 Enriched canonical pathways for day 0 vs day 14 comparison.

The left y-axis represents the significance of the canonical pathways as shown as the -log adjusted p-value; the right y-axis represents the enrichment ratio. The enriched canonical pathways are shown as labels on the x-axis. The bars represent the significance of each of the pathways and the threshold represents the adjusted p-value level of < 0.05. The enrichment ratio (orange line) represents the proportion of genes in the canonical pathways that are also the query genes.

Table 8.6 The five top Canonical Pathways identified by IPA for Day 0 - Day 14.

Those pathways shown in bold and above the threshold are significant, with the higher bars indicating greater enrichment.

The expression column represents the up- or downregulated genes, indicated in Table 8.5.

Ingenuity Canonical Pathways	-log (p-value)	Ratio	z-score	Genes	Expression
Glutathione Redox Reactions I	3.17	0.11	NaN	GSR, GSTP1	↓
Serotonin and Melatonin Biosynthesis	2.07	0.25	NaN	DDC	↑
Catecholamine Biosynthesis	2.07	0.25	NaN	DDC	↑
Glutathione Redox Reactions II	2.07	0.25	NaN	GSR	↓
Glycerol Degradation I	1.97	0.2	NaN	GK5	↓

8.5.3 IPA Interpretive Summaries

IPA provides summaries of the microarray data, grouped to include *Top Canonical Pathways* and *Top Networks*. The highlighted networks for Day 0 – 14 are listed as relating to processes associated with the inflammatory response, cell death and survival, protein synthesis, cell-to-cell signalling, haematological system function, cell cycle, replication, recombination and repair, connective tissue, cancer, endocrine and gastrointestinal disorders. The top up- and downregulated molecules are identified in this context; however, review of the literature reveals that limited data exist for a number of those genes and their molecular products.

Of those Network #1 genes for which data is more extensive, a number bear mention, especially in relation to their differential expression following the intervention in this study.

8.5.4 Differentially-expressed Genes

Of the significantly differentially-expressed genes across the Day 0 – Day 14 period, two upregulated and eleven downregulated genes have been listed in Table 8.7. As each is described in the scientific literature in relation to its known function in humans, it has been selected for more detailed discussion.

Table 8.7 Differentially-expressed genes from Network #1 and their key properties and functions

Gene	***Properties and Functions***
Key Upregulated genes (Defined in Table 8.5)	
DDC	*DDC* is required for synthesis of dopamine as well as epinephrine and norepinephrine. *DDC* can also decarboxylate 5-hydroxytryptophan to produce serotonin.[687]
ACSM2A	*ACSM2A* belongs to a family of mitochondrial Acyl-CoA synthetase enzymes essential for *de novo* lipid synthesis, fatty acid catabolism and remodelling of membranes.[688]
Key Downregulated genes	
RELB	The NF-kB or Rel protein family plays critical roles in adaptive and innate immunity, inflammation, cell differentiation, proliferation and apoptosis. *RELB* is overexpressed in various cancers.[649]
CEBPB	Plays an essential role in cell proliferation (pro- or antiproliferative), differentiation and growth arrest in specific cell types, including adipocyte differentiation.[689]
HERC6	HERC proteins are key components of a wide range of cellular functions – neurodevelopment, DNA damage repair, cell growth and immune response.[690]
TICAM1	TICAM1 is an adaptor protein in TLR3 and TLR4 signalling pathways that mediate pro-inflammatory cytokine and interferon (IFN) responses.[691]
PDIA4	*PDIA4* inactivation restores a classical mitochondrial apoptosis

	pathway.[692]
ZBTB2	A potential proto-oncogenic master control gene of the p53 pathway.[693]
IGF2BP2	Associated with decreased insulin secretion and type 2 diabetes.[694]
DDX3X	The helicases are nucleic acid-dependent ATPases, which unwind and remodel DNA or RNA[695]
GSR	GSH homeostasis is regulated by *de novo* synthesis (via *GPX1*) as well as the status of the GSH:GSSG redox couple (GSR). Both *GSR* and *GPX* are Nrf2-dependent genes.[696] Downregulation of *GSR* was observed but *GPX* was not.
GSTP1	Codes for a key Phase 2 detoxification enzyme which uses SFN as a substrate for the synthesis of mercapturic acid metabolites. Polymorphisms are associated with cancer susceptibility.[697]
GK5	Active in triacylglycerol biosynthesis and glycerol degradation pathways, especially high in hepatocytes but limited in adipocytes under normal conditions.[698,699]

8.5.5 Enriched Canonical Pathway Comparisons.

Figures 8.9, 8.10 and 8.11 illustrate the marked differences in the canonical pathways generated across the 3 study periods. Although the Day 0 – Day 7 and Day 7 – Day 14 periods indicate there was significant differential expression, the enrichment ratio representing the proportion of query genes within the total number of genes in the canonical pathways is limited (orange line). Furthermore, Day 7 - Day 14 shows the likely possibility that the significantly enriched canonical pathways such as Integrin Signalling and Vascular endothelial growth factor (VEGF) signalling were the result of viral infection in Participants #21 and #23 rather than the effect of the intervention. Figure 8.12 shows the genes with significantly decreased gene

expression involved in response to viral infection as well as their upstream regulatory partners (IPA generated image).

8.5.6 Day 0 – Day 7. Top 15 Canonical Pathways

IPA classifies the identified pathways as being related primarily to the signalling processes of fundamental cellular functions; movement, function and maintenance, development, growth and proliferation are compromised. Since the enrichment ratio is low (i.e. ratio less than 0.1) for the canonical pathway query genes, no significance can be attached for this period.

Figure 8.10 illustrates a number of downregulated pathways within the initial 7 days of the study. Since all participants were included in the analysis, it is not possible to differentiate the observed effects of SFN or viral burden. However, several of the identified pathways are known to be SFN targets. Of the four signalling pathways listed below (*Integrin, ERK/MAPK, Extravasation and VEGF)* and shown in Figure 8.10, for three of these, SFN is already known to interact; these pathways are included for this reason.

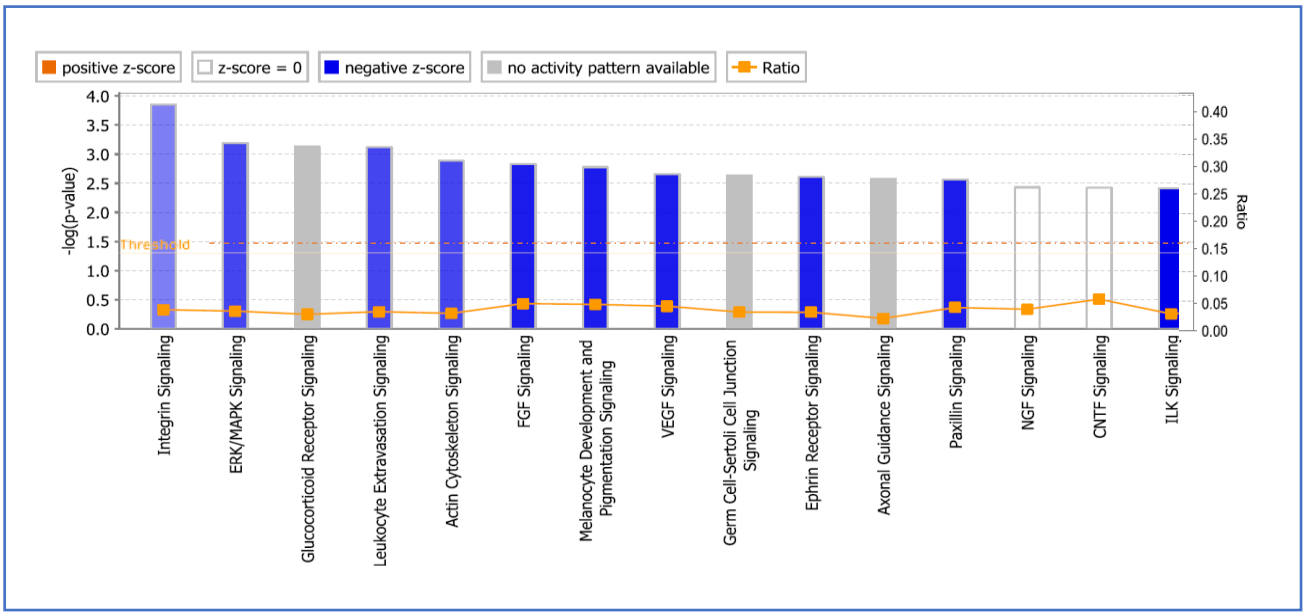


Figure 8.10 Day 0 – Day 7. Top 15 enriched canonical pathways

For each, the left y-axis represents the significance of the canonical pathways shown as -log adjusted p-value; the right y-axis represents the enrichment ratio and the enriched canonical pathways are shown as labels on the x-axis. The bars represent the significance of each of the pathways and the threshold represents the adjusted p-value level of < 0.05. The enrichment ratio (orange line) represents the proportion of genes in the canonical pathways that were also the query genes.

8.5.7 Day 7 – Day 14. Top 15 Canonical Pathways

Day 7 vs day 14 analysis: As with the Day 0 – Day 7 period, the data for the IPA Enriched Canonical Pathway are not sufficiently robust to be considered significant. Reference to this period has been made as it reflects the effect of the virus in two participants on the entire dataset for this period. The strong negative z-score identifies significant enrichment in *Interferon Signalling* but the ratio of query genes to the total for this pathway prevents us from drawing any conclusion. It is likely that the significantly decreased downregulation of interferon was due to resolution of the virus and not related to SFN. The analysis of the regulator effects supports the decreased expression of genes involved in response to viral infections (Figure).

Heat map analysis (not shown) indicated that two samples, subjects 21 and 23 from day 7, had increased expression of known viral infection-related genes as described earlier. Although Participant #2 was suspected to have had a viral infection during the study, the expression of genes involved in response to viral infection were comparatively low for subject 2.

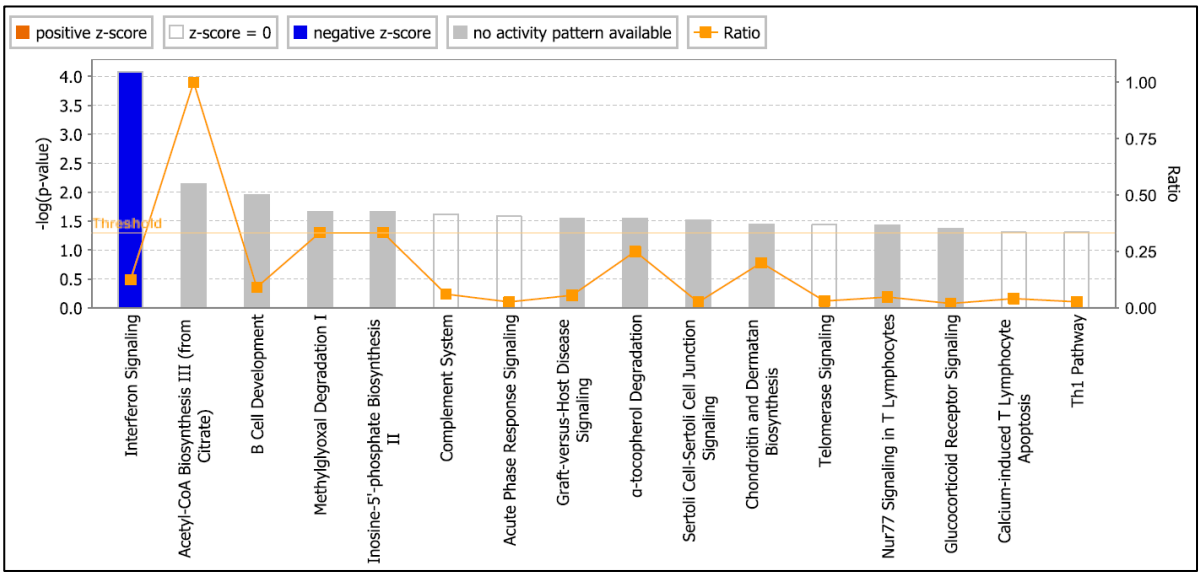


Figure 8.11 Day 7 – Day 14. Top 15 enriched canonical pathways.

Interferon signalling was highlighted as being significantly downregulated during the second 7-day period of the study. The enrichment ratio (orange line) represents the low proportion of genes in the canonical pathways that were also the query genes.

8.5.8 Viral Infection – a Possible Confounder

Our rationale for considering that two of the study participants exhibited symptoms of infection as well as changes in gene expression was described earlier. IPA identified the genes *MX1, IFITM3, OAS1, PLSCR1* and *OAS3* as being downregulated due to the presence of a virus. Downregulation was due to their upstream regulatory partners, *USP18, IFNL1, IFNA2 and EIF2AK2.*

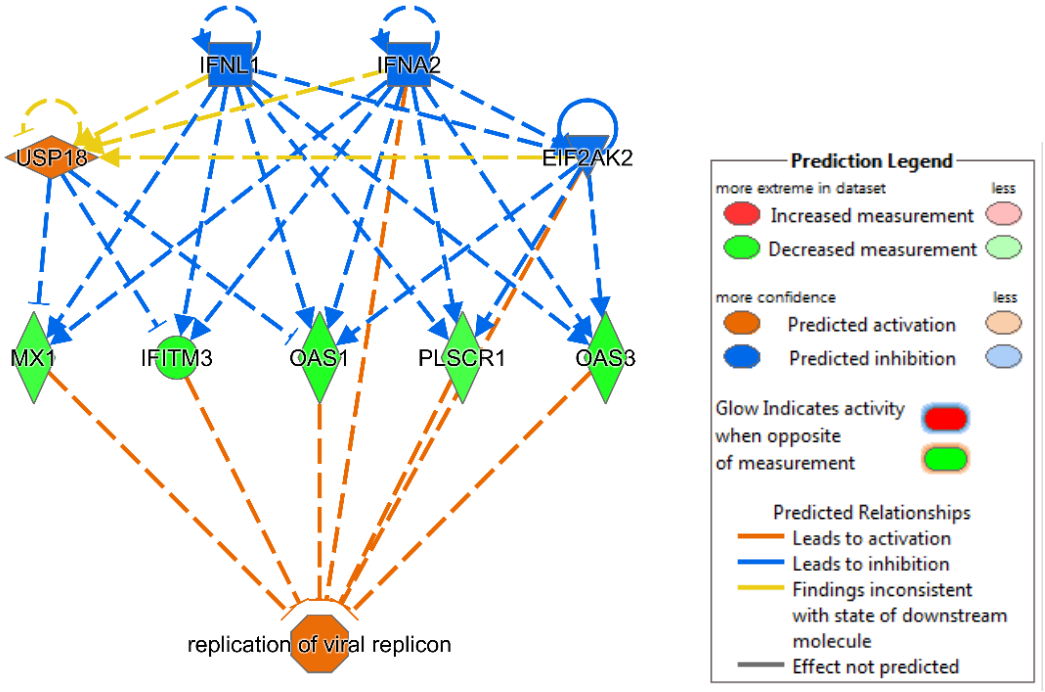


Figure 8.12 Genes with significantly decreased gene expression involved in response to viral infections and their upstream regulatory partners.

The shape of each node in the network references the following: tall diamond = enzyme; long diamond = peptidase; square = cytokine; octagon = biological process.

8.6 PLASMA SFN AND ITS MERCAPTURIC ACID METABOLITES.

Participants #2, #8 and #9 had baseline SFN levels well above the average of 3.19 ± 5.3 ng/ml indicating that they had not adhered to the study instructions. Data from these 3 participants was removed; all three carry the normal *GSTP1* gene. Table 8.8 lists plasma concentrations of SFN and its mercapturic acid metabolites of the remaining participants across each time period expressed in ng/ml as measured. The standard deviations of each of these indicated very large inter-individual variability.

Table 8.8. Plasma concentrations of SFN and its mercapturic acid metabolites of all participants mean ± SD.

SFN = sulforaphane; SFN-NAC = sulforaphane N-acetyl cysteine metabolite; SFN-GSH = sulforaphane glutathione metabolite; SFN-CYS = sulforaphane-cysteine metabolite. High levels for SFN at baseline are a likely indicator that dietary exclusions were not followed by Participants #2, #8 and #9.

Day	**SFN** (ng/ml)	**SFN-NAC** (ng/ml)	**SFN-GSH** (ng/ml)	**SFN-CYS** (ng/ml)
0	**10.36** ± 19.7	**13.62** ± 12.7	**3.57** ± 7.3	**0.60** ± 0.4
7	**37.25** ± 68.7	**64.05** ± 83.9	**3.77** ± 3.8	**4.98** ± 9.8
14	**64.50** ± 65.1	**97.15** ± 147.4	**8.32** ± 21.2	**7.52** ± 16.1

8.6.1 Presentation and Analysis of the Data according to Genotype

The data for all participants were separated according to the nature of *GSTP1* genotype (derived from Table 8.1) and expressed initially as nanograms per millilitre (ng/mL) (Table 8.9) and later converted to micromolar concentrations (µM). (Utilised in Figures 8.13, 8.14 and 8.15) for ease of comparison with other studies.

Table 8.9. Plasma SFN Concentrations via genotype

Plasma concentrations of SFN and its metabolites considering GSTP1 genotype in accordance with the 3 time periods for which samples were collected. SFN data is reported as Mean ± Standard Deviation with data for the three metabolites reported as Median Values with their Interquartile Range.

SFN and SFN metabolite levels during the trial for all participants and according to *GSTP1* polymorphism				
Metabolite	**Gene or Variant**	**0 days**	**7 Days**	**14 days**
SFN (ng/ml)	All	14.8± 22.3	53.2 ±77.3	82.3±65.9
	Normal	22.7 ± 26.9	36.0 ±38.7	78.2±56.8
	Homo/Hetero	4.2 ± 6.1	76.2±111.1	87.6±82.0
SFN-NAC (ng/ml)	All	0.93 [0.50]	1.48 [1.27]	1.58 [1.14]
	Normal	0.88 [0.29	0.82 [1.30]	1.52 [1.01]
	Homo/Hetero	1.22 [0.72]	1.81 [0.64]	1.73 [1.74]
SFN-GSH (ng/ml)	All	1.79 [0.85]	1.60 [3.52]	2.04 [2.34]
	Normal	1.91 [0.81]	1.54 [3.46]	1.81 [3.97]
	Homo/Hetero	1.69 [1.64]	1.78 [6.53]	2.58 [1.03]
SFN-CYS (ng/ml)	All	0.52 [0.41]	0.86 [4.55]	0.52 [1.73]
	Normal	0.41 [0.45]	0.53 [2.77]	0.38 [6.48]
	Homo/Hetero	0.67 [0.36]	4.3 [21.95]	0.66 [1.96]

8.6.2 Summary of Findings

Overall, there was a significant ($p<0.05$) effect of time for SFN, SFN-NAC and SFN-CYS. There were no significant ($p>0.05$) effects of group (wild type vs homo/hetero) and no significant group*time interactions. There was a trend for a group effect in SFN-NAC ($p=0.09$) indicating a greater number of participants would be needed to find a significant group effect.

Figures 8.13, 8.14 and 8.15 show the differences in the micromolar concentrations and proportions of SFN and the measured metabolites, SFN-NAC, SFN-GSH and SFN-CYS. Of the metabolites, SFN-NAC was significantly the highest of the three and in those carrying polymorphisms, SFN-NAC was higher than SFN itself.

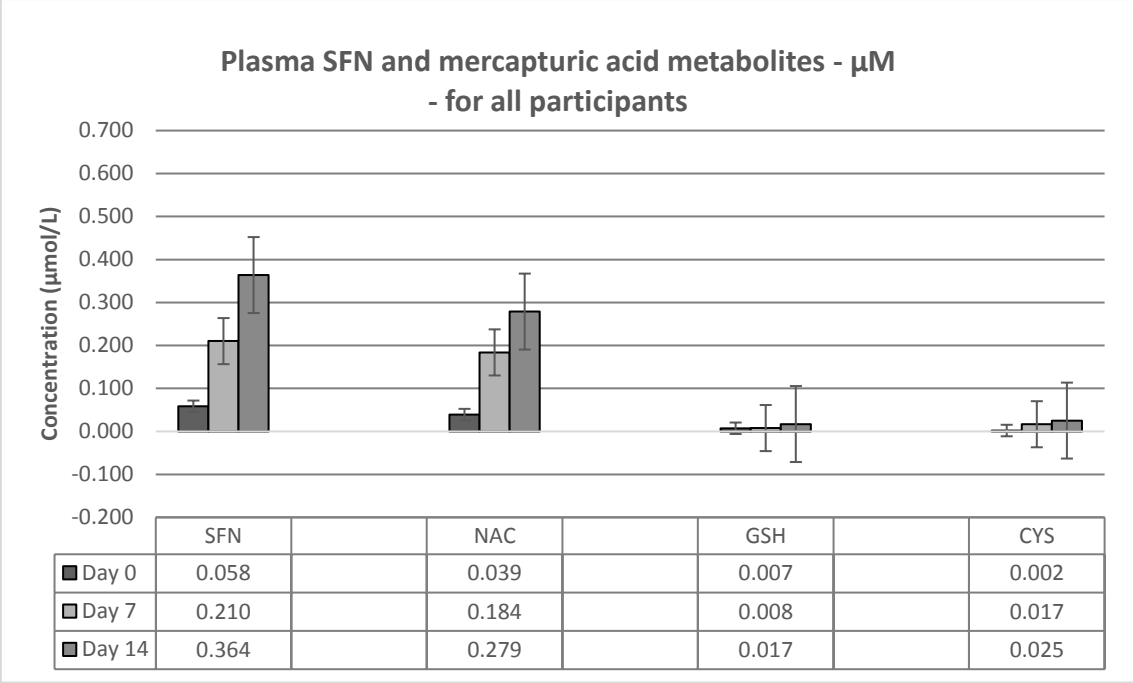


Figure 8.13 Plasma SFN and mercapturic acid metabolites (µM) for all participants, combining wild-type, homozygous and heterozygous polymorphisms, with error bars indicating standard deviation.

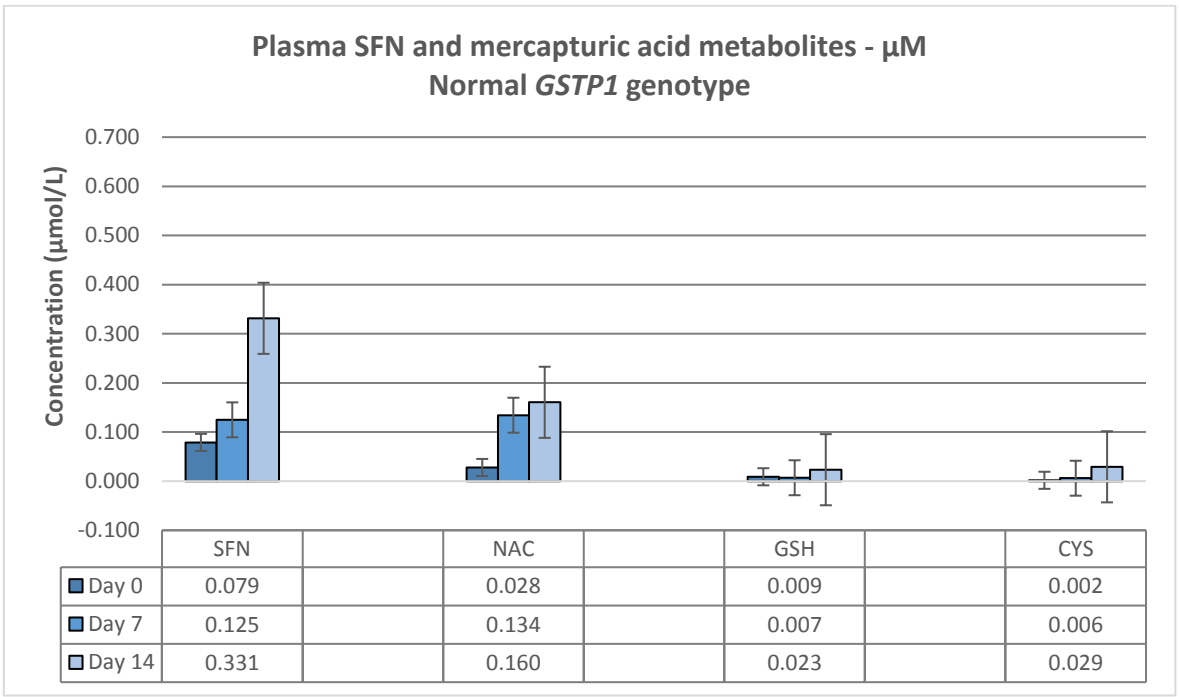
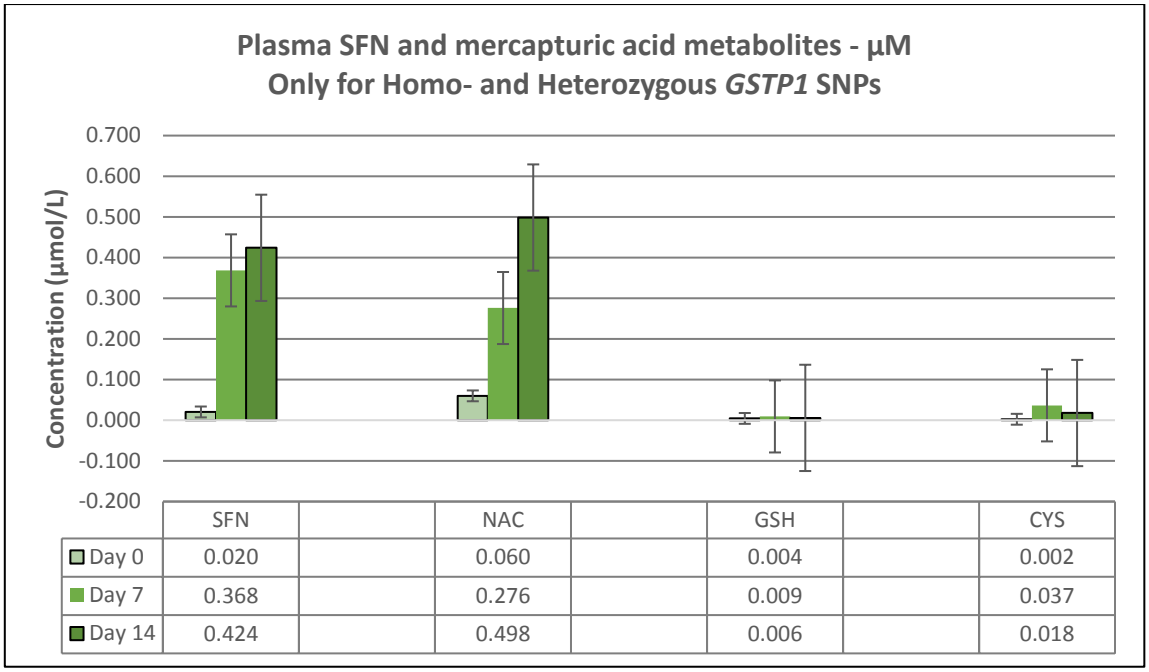


Figure 8.14 Plasma SFN and mercapturic acid metabolites (µM) for participants with wild-type GSTP1 genotype, with error bars indicating standard deviation.



	SFN		NAC		GSH		CYS
Day 0	0.020		0.060		0.004		0.002
Day 7	0.368		0.276		0.009		0.037
Day 14	0.424		0.498		0.006		0.018

Figure 8.15 Plasma SFN and mercapturic acid metabolites (µM) for participants with combined homozygous and heterozygous GSTP1 polymorphisms, with error bars indicating standard deviation.

8.7 DISCUSSION

SFN is the primary bioactive compound generated from broccoli sprouts following the enzymatic hydrolysis of GRN by MYR. As a food-derived nutraceutical, the human genome is adapted to this readily-bioavailable low M.W. lipophilic molecule. Both SFN-yielding and GRN supplements are available to consumers, even though the dose-response for each form is not fully-characterised. The broccoli sprout form utilised in this study provides the convenience of a capsule, designed to be taken daily over extended periods, thereby more closely approximating the regular dietary intake of a cruciferous vegetable. As a whole food ingredient and not an extract of a specific bioactive compound, this intervention is more likely to provide the full spectrum of micronutrients and other phytochemical bioactives naturally-occurring in broccoli sprouts.

In this study, a microarray platform was utilised to investigate the differential gene expression at two dose levels of a SFN-yielding encapsulated supplement. The advantage of using a microarray platform lies in the ability to simultaneously evaluate

the effect of a specific intervention on many hundreds of genes. The results of this study are intended to highlight important directions for future research in this field.

SFN derived from food and supplements is best-known for its role in Nrf2-dependent effects associated with the induction of a large battery of cytoprotective genes. Clinical trials using SFN and broccoli sprouts have generally shown Nrf2-dependent effects. However, SFN also exhibits other effects for which gene expression data and related mechanistic findings are emerging.[157,204] Surprisingly, there was no observed expression of Nrf2-dependent genes; instead, the study highlighted a series of Nrf2-independent effects, some of which may give preliminary indication of SFN's involvement in new clinically-relevant areas such as neurotransmitter synthesis.

8.7.1 Lack of Nrf2-dependent Effect on Differential Gene Expression

The lack of an Nrf2-dependent effect in this study is hypothesised as being related to the fact that the participants in the intervention group were all young, healthy and physically-active. Therefore, it might be expected that inflammation and other disease markers at baseline would already be in the normal range, potentially limiting any changes due to the intervention. In Chapter 7, some of the beneficial effects of physical activity on human health were discussed, highlighting the fact that little has been published on the role of exercise on Nrf2 and in particular that there is no quantitative data for comparative consideration.

Others have reported lack of effect of other phytochemical interventions under similar conditions to ours in that the trial participants were healthy.[700] A study using the isolated phytochemical quercetin ± antioxidant vitamins in a healthy population failed to observe the expected response on cardiovascular lipid and coagulation markers, even though blood levels of quercetin were as expected.[701] By contrast, they observed the predicted benefits of quercetin when health status was compromised by hypertension in a different study population.

Other SFN studies showed that participants with chronic obstructive airway disease (COPD)[206,207] and asthma[702] failed to respond to SFN. Here, it was postulated that upregulation of Nrf2 may have already been present at baseline as a compensatory mechanism for the disease process itself, making it difficult to further stimulate Nrf2

with SFN. In these cases, SFN had been shown to be well-absorbed using measurement of SFN and its metabolites in plasma, just as demonstrated in the EASYGENEX Study.

Similarly, SFN was not effective on glucose control biomarkers in metabolically well-regulated animals on a high-fat diet compared to its positive effects on obese metabolically-dysregulated animals on the same diet, indicating that responses may differ between normal and abnormal states.[198]

Chapter 7 described the process for determination of dosage in the EASYGENEX Study. In summary, histone deacetylation (HDAC) is known to be inhibited by an Nrf2-unrelated mechanism and typically responds to higher doses than for Nrf2 activation. As such, it was expected that HDAC inhibition would be observed at the higher of the dosage levels as administered during the Day 0 – Day 14 period.

However, little is known about the effect of exercise on HDAC inhibition. A 2009 study showed equivocal findings on the effect of 60 minutes of cycling on HDAC inhibition in human skeletal muscle tissue.[703] During the Day 7 – 14 period, HDAC downregulation with a fold-change of 0.9 at the higher dose was observed but this effect was not seen in comparing Day 0 with Day 14. As a consequence, it was not possible to determine whether failure to demonstrate significant HDAC inhibition was due to lack of effect of SFN or whether this group of young, healthy, regularly-exercising men might already have optimised HDAC activity.

Considering these data together with the known effects of exercise on Nrf2 activation, the effect of the intervention in this study might therefore be more difficult to detect than had an older, less healthy, less active study population been selected.

8.7.2 SFN's Effect on Differential Gene Expression

This study revealed relationships between SFN and a number of genes for which there are no reports in the scientific literature. Notably are the genes *DDC, ACSM2A, HERC6, PDIA4, ZBTB2, IGF2B2, DDX3X* and *GK5*.

From consideration of all the differentially-expressed genes across Day 0 – Day 14, a picture emerges of interactions in intricate signalling associated with many core cellular processes, particularly those associated with immune dysregulation. In

addition to downregulation of pro-inflammatory processes, others appear to be related to inhibition of cancer cell development by downregulating genes associated with angiogenesis and metastasis and by protecting tumour suppressor genes such as *p53* and *p21, (RELB, HERC6, ZBTB2).*

Two of the genes negatively expressed (*IGF2BP2* and *CEBPB*) may impact on glucose metabolism, potentially reducing changes that contribute to insulin resistance and adiposity. These effects of SFN are already known; however, this study has uncovered specific genes not previously associated. These genes can be broadly grouped into 6 overlapping categories with some genes known to exhibit multiple effects: immune modulation (anti-inflammatory), metabolism (adipogenesis, glucose metabolism, insulin sensitivity), neurotransmitter synthesis, cytoprotection, cardioprotection and redox modulation.

8.7.3 Immune Modulation (Anti-inflammatory Effects)

IPA highlighted the NF-kB Network as the most significant during Day 0 – Day 14, with the NF-kB subunit, *RELB* significantly downregulated.[649] NF-kB subunits are known to strongly act in synergy with members of the C/EBP family of transcription factors[704] and this study showed that both were downregulated.

Toll-like receptor (TLR4) pathways mediate pro-inflammatory cytokine and interferon responses, with the adaptor protein TICAM1 essential in stopping TLR-mediated IFN production.[691] SFN has been shown in a thiol-dependent manner to suppress TLR4 oligomerization, an event that is critical to TLR4 activation in inflammation. SFN's suppression of TLR4 oligomerization was shown to downregulate NF-kB activation,[705] potentially further regulating inflammation. Saturated fatty acids are known to act as ligands for toll-like receptor 4 (TLR4) in macrophages and adipocytes, with these signals in turn regulating various pro-inflammatory transcription factors.[646] IPA's Network #1 shows *TICAM1* to be downregulated by the SFN intervention, thereby highlighting the possibility that SFN was responsible for dysregulation in the expression of genes in the TLR4 pathway and consequently limiting a possible inflammatory response.

8.7.4 Dopamine Expression and its Little-Known Effect on Immune Function

A lesser-known effect of dopamine is that it may inhibit cytokine production[706] and is an endogenous regulator of inflammasome activation, bridging the nervous and immune systems.[706] It was shown that SFN upregulated *DDC*, so that it may contribute an anti-inflammatory effect by this additional mechanism. Substantial synthesis of neurotransmitters including dopamine occurs in the gastrointestinal tract[707] but it is not known whether SFN might exhibit anti-inflammatory or other effects here by this mechanism.

8.7.5 Metabolism – Adipogenesis, Glucose Metabolism, Insulin Sensitivity

Our study showed upregulation of *CEBPB*, a gene associated with adipogenesis. Activation of an associated transcription factor PPARγ, in turn activates expression of adipogenic genes.[689] SFN (20 μM) has been shown to decrease the expression of *CEBPB* and *PPARγ* during early stage adipogenesis in a cell culture model.[708] An animal study concluded that SFN may induce anti-obesity activity by inhibiting adipogenesis through downregulation of *PPARγ* and *C/EBPα* and by suppressing lipogenesis through activation of the AMP-activated Protein Kinase (AMPK) pathway.[709] The study's data did not identify *PPARγ.*

Upregulation of *ACSM2A* was observed*;* this is a gene that codes for the mitochondrial Acyl-CoA synthetase enzymes abundant in human liver and specific for medium-chain triglycerides (MCT). MCTs are known to fuel the lipid β-oxidation and ATP-generating pathway to induce thermogenesis and reduce *de novo* lipid synthesis.[710] SFN was shown here for the first time to upregulate *ACSM2A*. How this might impact regulation of adiposity is not known but since several association studies have linked *ACSM2A* polymorphisms to traits of insulin resistance.[711], this may be an avenue for further investigation of the properties of SFN, especially given that SFN has already been shown to have positive effects in patients with type 2 diabetes.[197,432] *ACSM2A* is also associated with enhanced glycine conjugation in detoxification of xenobiotics like benzoate, ibuprofen and aspirin.[712] The possible role of SFN in this context deserves further investigation.

Another metabolically-related gene*, IGF2BP2* is associated with decreased insulin secretion but effects on type 2 diabetes risk of its polymorphisms are unclear.[694] It

was hypothesised that SFN's observed downregulation of *IGF2BP2* may assist in restoring insulin secretion.

SFN was shown to downregulate *GK5.* Glycerol is a primary factor determining plasma glucose,[713] with catecholamines inducing lipolysis, thereby increasing the release of free fatty acids and glycerol from fat into the bloodstream.[714] This study showed upregulation of *DDC*, inferring enhanced catecholamine synthesis with a possible tendency towards lipolysis; it is not possible to say whether downregulation of *GK5* was in response to SFN. Aberrant function of the glycerol kinase enzyme has implications for adiposity and insulin resistance but as this study investigated PBMCs, its significance in relation to broader physiological function is not known.

A recent *in vitro* study investigating brown vs white adipocytes showed that SFN enhanced glucose uptake and oxidative utilisation, lipolysis and fatty acid oxidation together with adipocyte 'browning' in an Nrf2-related manner.[715] Another study showed that SFN stimulates lipolysis via a decrease in energy-sensitive AMPK signalling.[716] However, the genes identified in these studies were not differentially-expressed in the EASYGENEX Study. Clearly, SFN is affecting a number of adipose-related genes although the mechanisms are as yet poorly-characterised.

8.7.6 Neurotransmitter Synthesis and Neuroprotection.

The effect of an increase in Dopa Decarboxylase activity (*DDC*) may have implications for neuropsychiatric effects related to upregulation of synthesis of neurotransmitters dopamine, serotonin, epinephrine, norepinephrine and melatonin (a downstream product of serotonin). The relationship to neurotransmitter synthesis is reflected in the IPA Enriched Canonical Pathway (Figure 8.9) which shows Serotonin, Melatonin and Catecholamine pathways as enriched. This is the first time a relationship between SFN and *DDC* has been reported, although SFN's effect on dopamine is anti-glycative[717] and in other ways neuroprotective in an Nrf2-dependent manner[718]. SFN can prevent oxidation of dopamine in cells of the *substantia nigra* of the brain, with implications for patients with Parkinson's Disease.

In relation to neuroprotection, the Day 7 – 14 canonical pathway data highlighted the Methylglyoxal Degradation I pathway which is close to the threshold for significance but for which the activity pattern is not known. Methylglyoxal is the most potent

precursor of the AGEs, oxidant molecules that contribute to the etiopathogenesis of various neurodegenerative diseases. In a 2015 study, high levels of methylglyoxal have been found in the cerebrospinal fluid of Alzheimer patients.[719] In the same study, SFN was shown to enhance the methylglyoxal detoxifying system, increasing the expression and activity of *GLO1* (glyoxalase -1) in a glutathione-dependent manner. As SFN demonstrated a protective anti-glycative effect in that study, one might postulate that the direction of expression of the Methylglyoxal pathway in the EASYGENEX Study was downregulation.

In Day 7 – 14, *GLO1* was shown to be downregulated with a fold-change of 0.9. A related gene, *GLOD4* was also downregulated to a similar degree during Day 7 – 14. However, these genes were not present in the Day 0-14 data listed in Table 8.5.

8.7.7 Cytoprotection

This study highlighted a number of downregulated genes associated with cancer. The role of *CEBPB* in relation to adiposity overlaps its role in cancer. Increased *CEBPB* expression is implicated in development of some tumours with expression correlated with invasive activity.[720] Similarly*, IGF2BP2* described earlier in relation to insulin secretion has been shown also to contribute to colorectal carcinogenesis.[721]

Loss of function of the tumour suppressor protein p53 is known to be a frequent and early event in cancer.[722] *ZBTB2* is a potential proto-oncogenic master control gene of the p53 pathway, potently repressing the cell cycle arrest genes, *p21* and the tumour suppressor gene *p53*.[693] The EASYGENEX Study showed for the first time, downregulation of *ZBTB2*, further illustrating the known cytoprotective effect of SFN by reducing or removing the suppression of *p53*.[157] Response to chemotherapy has been shown to be associated with polymorphisms in the *GST* gene family, including *GSTP1*.[723] Knowledge of an individual patient's *GST* polymorphisms may inform dose determination when administering these highly potent pharmaceuticals.

Downregulation of *HERC6* was observed and there is currently no published association between SFN and *HERC6*. Small *HERCs* such as *HERC6* are related to male fertility, antiviral responses and cancer development. Where *HERC2* regulates BRCA1 stability during the cell cycle and regulates p53 signalling, much less is known of the physiological functions of the small *HERCs* (3-6).[690]

For the first time SFN's effect in downregulating *DDX3X* was observed; this gene that can promote metastasis with worse overall survival in patients with high *DDX3X* expression in metastatic tissue.[724] *DDX3X* regulates the NF-kB pathway but its interaction with NF-kB in cancer is controversial.[695,725]

There is no published relationship between SFN and *PDIA4*, a gene downregulated in this study. The protein disulfide isomerases are a superfamily of oxidoreductases localised in the endoplasmic reticulum (ER), nucleus, cytosol, mitochondria and cell membrane. *PDIA4* is intimately tied to the switch between pro-survival and pro-death pathways during ER stress, with *PDIA4* shown to play dual roles. This effect has been demonstrated in secretory cells such as pancreatic beta-cells, B-lymphocytes, hepatic cells and osteoblasts. Failure of this pathway to restore ER homeostasis results in the activation of apoptotic pathways.[726] Such *PDIA4* inactivation restores a classical mitochondrial apoptosis pathway, suggesting a possible association with chemoresistance.[692] How this relates to the observed *PDIA4* downregulation is not known but is likely to be associated with redox-related disulphide chemistry.

Dopamine can suppress systemic inflammation.[706,727] As it was shown that SFN upregulated *DDC* to increase dopamine synthesis, this may also contribute to cytoprotection because low *DDC* mRNA has been correlated to poor tumour prognosis in head and neck cancers.[728]

8.7.8 Cardioprotection

CEBPB may participate in cardiac hypertrophy with *CEBPB* knockdown protecting cardiomyocytes from hypertrophy via inhibition of p65-NF-kB.[648] As discussed in some detail in the previous chapter, NF-κB and C/EBPβ have long been known to function synergistically as a complex.[647] The significance of the observed downregulation of SFN in this context is not known.

8.7.9 Redox-related Effects

IPA identified Glutathione Redox Reactions as being significant overall for Day 0 – Day 14, highlighting two genes *GSR* and *GSTP1.* *GSR* is one of two Nrf2-dependent genes associated with GSH homeostasis;[696] the other is *GPX1*. Together,

the enzymes for which these two genes code are responsible for maintaining the GSH:GSSG couple, often used as a marker for oxidative stress. Here, GSH is oxidised by GPx and GSSG is returned to its reduced state by GSR.[729] The study showed downregulation of *GSR* but did not identify *GPX*. The reason that *GSR* was downregulated in this study is not clear, given that in a resting cell, the molar GSH:GSSG ratio exceeds 100:1[730] and it might be expected that upregulated *GSR* would tend to maintain this ratio.

Since Nrf2 is typically activated by SFN, *GSTP1* too might be expected to be upregulated. Here it is downregulated at Day 14. When the participants with a viral burden were excluded, *GSTP1* remained downregulated. Decrease in *GSTP1* gene expression in PBMC may be specific for these cells, since other studies have reported a reduction in *GSTP1* on mRNA and protein level in dietary intervention studies. PBMCs may be also adversely affected by cryopreservation.[731]

Juge et al[673] in their comprehensive review of SFN in cancer refer to a similar glutathione-related counter-intuitive finding in that glutathione reductase (*GSR*) needed for the regeneration of glutathione was consistently inhibited by SFN in several cell culture lines.[732] Nevertheless, this study showed that the intervention resulted in increased plasma GSH conjugates, the presence of which is dependent on the action of enzymes of the glutathione-S-transferase family of which *GSTP1* is one member. One might question whether this anomalous finding might be resolved by closer examination of the simultaneous effect of exercise in the young, healthy study population and the use of a different cell type for gene expression analysis.

8.7.10 Review of Day 0 – Day 7 and Day 7 – Day 14 Canonical Pathways

Although IPA identified a number of pathways by degree of enrichment in the study's sub-groups, there were proportionately few genes representative of this pathway. Even so, SFN-related processes possibly deserving further investigation are highlighted later.

8.7.11 Integrin Signalling Pathway: Day 0 – Day 7

The Integrins are a family of transmembrane proteins expressed in almost every cell type that mediate attachment to the extracellular matrix. They are important

regulators of cell survival, proliferation, adhesion and migration with established relationships in cancer.[733] In an *in vitro* study on renal carcinoma cells, SFN was shown to downregulate integrins, demonstrating reduced tumour growth and proliferation in a drug resistance model.[734] Integrins form numerous cellular signalling relationships, some of which are associated with metabolism. A recent review described integrins being interwoven in a network of interdependent regulatory pathways with cell metabolism, highlighting the emerging control of cell physiology by metabolic cues.[733] Integrins are also intimately associated with viral penetration of cells, being physiologically important receptors that have been usurped by viruses for attachment and/or cell entry.[735]

8.7.12 ERK/MAPK Signalling Pathway: Day 0 – Day 7

The molecular events linking cell surface receptors to activation of ERKs are complex and are closely linked to the process of metastasis in cancer, implicating SFN in the inhibition of cell motility. Various cellular stresses, as well as pro-inflammatory cytokines such as IL-1β and TNFα, can activate multiple MAP kinase signalling pathways which then play major effector roles in numerous cellular responses.[736] Small GTP-binding proteins activate MAP kinase kinase kinases (MAP3K) and the phosphorylation reactions which activate the MAP kinases (MAPK) to elicit cellular responses. Inhibitors of these pathways are effective in preventing induction of pro-inflammatory genes.[737]

In a cell culture model, SFN inhibited glioblastoma cell migration and invasion.[738] SFN (20 µM) reduced the formation of cell pseudopodia by phosphorylating ERK1/2 in a sustained way, contributing to the downregulated *MMP-2* (matrix metalloproteinase) expression and activity associated with metastasis.

As with the Integrin signalling pathway, viruses interact with and hijack some of the cell's core defence mechanisms. The influenza viral infection modifies the signalling pathways, ERK-MAPK, NF-kB, MAPK and others important for viral entry, viral replication, viral propagation and apoptosis.[739] Activation of the NF-kB pathway is a primary requirement for influenza virus infection and its efficient replication.[739] SFN and viruses impact some of the same pathways but the expression data revealed in this study are insufficient for any conclusion to be drawn.

8.7.13 VEGF: Day 0 – Day 7

VEGF is a potent angiogenic factor which is known to be suppressed by SFN, thereby limiting the spread of cancer.[740] Still below the threshold for significant enrichment, this study also highlighted inhibition of Leukocyte Extravasation Signalling which does not appear to be hitherto related to SFN. Leukocyte extravasation is a critical step in the inflammatory response, involving the migration of leukocytes from the bloodstream towards target tissues. The process promotes the rapid influx of leukocytes to inflammatory foci without compromising the integrity of the endothelial barrier[741]; no conclusions can be drawn.

In a 2007 review paper, SF showed time- and concentration-dependent inhibitory effects on expression of VEGF and two angiogenesis-associated transcription factors on human endothelial cells (HUVECs) in a concentration range of 0.8–25 mM; this is higher than the concentrations typically employed in cell culture studies.[157]

8.7.14 Acetyl-CoA III Biosynthesis (from Citrate): Day 7-14

Although no activity pattern for the pathway shown as Acetyl-CoA III Biosynthesis (from citrate) is available, it is above the threshold for significance and the ratio of the query genes to the total in this pathway is significantly higher than for other identified pathways. The ATP Citrate Lyase gene (*ACLY)*, essential for synthesis of Acetyl-CoA from citrate produced in the Kreb's Cycle was shown to have been downregulated. The lack of significant data for this pathway prevents any conclusion but given the central role of Acetyl-CoA in many aspects of metabolism, it would be of interest to explore this further in relation to the SFN intervention.

8.8.15 SFN and Plasma Mercapturic Acid Metabolite Data Quality

An initial review of the Table 8.8 data on SFN and its metabolites reveals an anomaly. It would be expected that the Day 0 SFN levels shown in Table 8.8 would be close to zero, given that the 3-day washout period was intended to remove SFN and its metabolites from the system before the introduction of the capsules.

The plasma data showed that three participants had relatively high plasma SFN levels at baseline, indicating either likely failure to adhere to the dietary exclusions

during the preliminary washout period or that our list of Dietary Exclusions had overlooked a significant contributor; the participant dietary intake diaries did not clarify the issue. It is not possible to say whether this had any significant effect on the overall outcome, especially if the participants had continued to consume the food or foods and thereby still enabling detection of a differential effect of the intervention from their baseline. These participants were removed from the analysis, further limiting the opportunity to observe the effect of the *GSTP1* polymorphism on SFN metabolism.

8.8.16 Comparison of SFN Metabolite Data with Other Studies

There are several limitations associated with quantification of SFN metabolites and described by Al Janobi et al.[675] These limitations are related to rapid plasma clearance, the potential for irreversible reactions with plasma proteins and possible instability of the metabolites during sample processing.

In addition, it is difficult to compare our metabolite data with that from other studies, most of which examine the effect of a single dose on the pharmacokinetics over several hours, to record peak values. Al Janobi et al[675] showed in their validation study that SFN and its metabolites all peaked between 1 and 2 hours with SFN's peak value of 0.8 μmol/L. All samples declined rapidly to 6 hours from where they declined more slowly thereafter.

In our study which involved twice-daily dosing over 14 days, we did not record measurements on time from last dose to blood collection, so that in our study no data on peak values are available for comparison. The inclusion of a pharmacokinetic step in our study would have been useful in enabling comparison with data from other studies, especially in relation to the plasma peak SFN value and the time point at which SFN peaked.

8.8.17 The Effects of GSTP1 polymorphisms in the EASYGENEX Study

Of the 20 participants (of diverse ethnicity), one was found to be homozygote, six were heterozygote and the remaining 13 carried the normal *GSTP1* gene showing that roughly one-third of participants carried a *GSTP1* variant. As described in the Introduction to this chapter, GSTP1 variants vary by ethnicity but the homozygote

form is the least prevalent across all ethnicities examined.[664] Table 8.1 lists the EASYGENEX study population by ethnicity, grouping participants by Caucasian, Asians and Southern Asian.

There was a significant effect of time on plasma levels of SFN and its NAC and CYS conjugate. In those with the *GSTP1* variants, with the > 3-fold increase in the NAC metabolite greatest for the *GSTP1* variant group at 14 days. Such an effect is considered in some studies to confer a chemopreventive advantage for those carrying either the homo- or heterozygote *GSTP1* polymorphism.

8.9 LIMITATIONS and FUTURE PERSPECTIVES.

The EASYGENEX Study showed several limitations likely to have affected the outcome. Participant numbers were initially small but reduced even further by the presence of a viral infection in some and degradation of RNA samples for others. The presence of the virus made it difficult to separate the effect of SFN from that of the virus. Selection of a young, healthy, physically-active male population may have limited the potential for expression of genes also modulated by exercise. By selecting this population, the study had inadvertently failed to exclude all significant Nrf2 activators; nor was any allowance made for sex differences which may or may not have had an effect. Gene expression data were taken from PBMC cells which are not necessarily representative of all cells, even though this cell type is considered a satisfactory surrogate tissue in dietary investigations. PBMCs are known to give less sensitive responses and reveal high inter-individual differences. They may also be adversely affected by cryopreservation.[731] The nature of microarray studies is that they query large gene datasets but lack the accuracy of platforms such as RT-qPCR. In a next step, it would be advantageous to select and investigate a number of the genes of interest from the EASYGENEX Study, using this more sensitive tool.

This investigation of plasma SFN and its mercapturic acid metabolites confirmed that SFN had been generated in *vivo* after ingestion of the broccoli sprout capsules. It was expected that participants would achieve steady state by taking these capsules over a number of days. As no pharmacokinetic studies in the minutes and hours after ingestion of the capsules were undertaken, it was not possible to identify the peak plasma levels. Given that two different doses were tested, it would have been

useful to have identified the peak plasma level for each dose. This is a limitation in that it was not possible to draw any conclusions regarding a dose-response. This further limited any ability to compare these findings with those of other studies where peak values and other pharmacokinetic markers had been recorded.

A number of other identified genes did not reach statistical significance. As some of these are known to be related to the functions identified in IPA Canonical Pathways, it would be advantageous to explore these further in relation to SFN but in a different population. The genes *TNFSF8, IL6R, IFI6, IRS2, LDLR, HDL, ACLY* and *HLA-DRA* are all directly clinically-relevant with relationship to inflammation and autoimmunity, lipid metabolism, glucose metabolism and energy synthesis.

Future studies could further investigate these effects on a more sensitive platform such as RT-qPCR and might also select an older, less healthy population to study these and other effects on disease markers.

8.10 CONCLUSION

As a phytochemical, low molecular weight, lipophilic SFN is structurally quite different from the more extensively-researched higher molecular weight, bulky polyphenol molecules abundant in plant foods. Where the polyphenols as a group are limited by their low bioavailability, SFN does not have this limitation. As a result, there is greater likelihood that its *in vivo* effects will more closely approximate what is observed in *in vitro* studies. The EASYGENEX Study showed that an ingested whole broccoli sprout capsule yielded sufficient SFN *in situ* to cross several membranes to be detected in white blood cells, the PBMCs. This study also supported the known effect of *GSTP1* polymorphisms on SFN metabolism.

Furthermore, it was demonstrated that SFN differentially-modified the expression of genes associated with core cellular processes related to immune modulation (anti-inflammatory), metabolism (adipogenesis, glucose metabolism, insulin sensitivity), neurotransmitter synthesis, cytoprotection, cardioprotection and redox-regulation; a number of genes is known to be associated with more than one process. Of the differentially-expressed genes in this study, eight were identified for which no prior reference in association with SFN could be found. This finding might reaffirm the known pleiotropic character of the SFN molecule, suggesting the requirement for

more targeted investigation of its nutrigenomic effects and their potential clinical significance.

8.10 SUPPLEMENTARY MATERIALS

Table S8.1 Significantly differentially-expressed genes on Day 0 compared to Day 7. (p<0.05)

Gene Symbol	Log Fold-change	Adjusted p-value	Description
Increased Gene Expression on Day 7, compared to Day 0			
SIGLEC1	0.40	0.0303	sialic acid binding Ig like lectin 1
GDPD5	0.28	0.0419	glycerophosphodiester phosphodiesterase domain containing 5
C20orf173	0.27	0.0419	chromosome 20 open reading frame 173
DYRK1B	0.24	0.0331	dual specificity tyrosine phosphorylation regulated kinase 1B
MEPE	0.21	0.0419	matrix extracellular phosphoglycoprotein
RIC8B	0.21	0.0303	RIC8 guanine nucleotide exchange factor B
CWC15	0.20	0.0392	CWC15 spliceosome associated protein homolog
SYNDIG1L	0.19	0.0248	synapse differentiation inducing 1 like
CENPX	0.18	0.0240	centromere protein X
MLF1	0.17	0.0248	myeloid leukemia factor 1
MT2A	0.17	0.0191	metallothionein 2A
ATP6V1F	0.17	0.0232	ATPase H+ transporting V1 subunit F
TGM2	0.16	0.0240	transglutaminase 2
ATG9A	0.16	0.0400	autophagy related 9A
CCDC82	0.16	0.0347	coiled-coil domain containing 82
TUBA1B	0.13	0.0459	tubulin alpha 1b
HDLBP	0.13	0.0347	high density lipoprotein binding protein
Decreased Gene Expression on Day 7, compared to Day 0			
TNFSF8	-0.45	0.0050	tumour necrosis factor superfamily member 8
F2RL1	-0.37	0.0255	F2R like trypsin receptor 1
ZNF92	-0.32	0.0115	zinc finger protein 92
CYP4F3	-0.30	0.0065	cytochrome P450 family 4 subfamily F member 3
DUSP1	-0.29	0.0322	dual specificity phosphatase 1
IL6R	-0.28	0.0003	interleukin 6 receptor
MAT2A	-0.26	0.0065	methionine adenosyltransferase 2A
HIST1H2AC	-0.26	0.0111	histone cluster 1 H2A family member c
CD46	-0.26	0.0017	CD46 molecule

PIK3IP1	-0.26	0.0068	phosphoinositide-3-kinase interacting protein 1
IRS2	-0.25	0.0393	insulin receptor substrate 2
EIF3L	-0.25	0.0179	eukaryotic translation initiation factor 3 subunit L
PHC2	-0.25	0.0069	polyhomeotic homolog 2
ATP6V1A	-0.25	0.0485	ATPase H+ transporting V1 subunit A
PTTG1IP	-0.24	0.0021	pituitary tumour-transforming 1 interacting protein
CEACAM4	-0.24	0.0065	carcinoembryonic antigen related cell adhesion molecule 4
SRSF1	-0.24	0.0073	serine and arginine rich splicing factor 1
TM6SF1	-0.21	0.0065	transmembrane 6 superfamily member 1
ROCK1	-0.21	0.0395	Rho associated coiled-coil containing protein kinase 1
VASP	-0.21	0.0111	vasodilator-stimulated phosphoprotein
LGMNP1	-0.21	0.0347	legumain pseudogene 1
PRNP	-0.20	0.0179	prion protein
RAB6A	-0.20	0.0471	RAB6A, member RAS oncogene family
CSF2RA	-0.19	0.0065	colony stimulating factor 2 receptor-alpha subunit
IL7R	-0.19	0.0232	interleukin 7 receptor
PELI1	-0.19	0.0208	pellino E3 ubiquitin protein ligase 1
EP300	-0.19	0.0322	E1A binding protein p300
SACM1L	-0.19	0.0234	SAC1 suppressor of actin mutations 1-like (yeast)
DDX3X	-0.19	0.0021	DEAD-box helicase 3, X-linked
SGK1	-0.19	0.0155	serum/glucocorticoid regulated kinase 1
CRK	-0.19	0.0069	CRK proto-oncogene, adaptor protein
USO1	-0.19	0.0287	USO1 vesicle transport factor
TANGO2	-0.19	0.0155	transport and golgi organization 2 homolog
RTN3	-0.18	0.0065	reticulon 3
SURF4	-0.18	0.0065	surfeit 4
SHOC2	-0.18	0.0391	SHOC2, leucine rich repeat scaffold protein
ABTB1	-0.18	0.0232	ankyrin repeat and BTB domain containing 1
CFL1	-0.17	0.0419	cofilin 1
TPRG1L	-0.17	0.0127	tumour protein p63 regulated 1 like
HMGB1	-0.17	0.0322	high mobility group box 1
RTN3P1	-0.17	0.0208	reticulon 3 pseudogene 1
LBR	-0.17	0.0234	lamin B receptor
PRKAR1A	-0.17	0.0239	protein kinase cAMP-dependent type I regulatory subunit alpha
CD82	-0.17	0.0322	CD82 molecule
PTAFR	-0.17	0.0355	platelet activating factor receptor
SLBP	-0.17	0.0232	stem-loop binding protein
RPS6KA5	-0.17	0.0069	ribosomal protein S6 kinase A5
CDC42SE2	-0.17	0.0419	CDC42 small effector 2
POLR2C	-0.17	0.0127	RNA polymerase II subunit C

NAP1L4	-0.17	0.0239	nucleosome assembly protein 1 like 4
GRN	-0.17	0.0347	granulin precursor
SDCBP	-0.17	0.0118	syndecan binding protein
RAD21	-0.16	0.0347	RAD21 cohesin complex component
ORAI2	-0.16	0.0419	ORAI calcium release-activated calcium modulator 2
FBXL3	-0.16	0.0239	F-box and leucine rich repeat protein 3
ACTB	-0.16	0.0118	actin beta
IFNGR1	-0.16	0.0207	interferon gamma receptor 1
CAPZB	-0.16	0.0303	capping actin protein of muscle Z-line beta subunit
STAT3	-0.16	0.0322	signal transducer and activator of transcription 3
TMEM154	-0.16	0.0069	transmembrane protein 154
STX3	-0.16	0.0342	syntaxin 3
RAB11FIP1	-0.16	0.0065	RAB11 family interacting protein 1
VNN2	-0.16	0.0240	vanin 2
RPN1	-0.16	0.0069	ribophorin I
PTPRE	-0.16	0.0234	protein tyrosine phosphatase, receptor type E
SNAP23	-0.15	0.0240	synaptosome associated protein 23
TOR1AIP1	-0.15	0.0322	torsin 1A interacting protein 1
CLRN3	-0.15	0.0455	clarin 3
CD164	-0.15	0.0347	CD164 molecule
CRLF3	-0.15	0.0275	cytokine receptor like factor 3
FPR2	-0.15	0.0248	formyl peptide receptor 2
TAGLN2	-0.14	0.0370	transgelin 2
NARS	-0.14	0.0155	asparaginyl-tRNA synthetase
TAB2	-0.14	0.0268	TGF-beta activated kinase 1/MAP3K7 binding protein 2
AKIRIN1	-0.14	0.0148	akirin 1
CAB39	-0.14	0.0419	calcium binding protein 39
NT5C2	-0.13	0.0431	5'-nucleotidase, cytosolic II
VMP1	-0.13	0.0337	vacuole membrane protein 1
ARF4	-0.13	0.0347	ADP ribosylation factor 4
ACTN1	-0.13	0.0303	actinin alpha 1
TBL1X	-0.12	0.0322	transducin beta like 1X-linked
LAMP2	-0.12	0.0419	lysosomal associated membrane protein 2
PPT1	-0.12	0.0419	palmitoyl-protein thioesterase 1

Table S8.2 Significantly differentially-expressed genes on Day 14 compared to Day 7. (p<0.05)

Gene Symbol	Log Fold-change	Fold-change	Adjusted p-value	Description
Increased Gene Expression on Day 14 compared to Day 7				
CYP4F3	0.29	1.2	0.0060	cytochrome P450 family 4 subfamily F member 3
RPS15A	0.27	1.2	0.0029	ribosomal protein S15a
PIK3IP1	0.24	1.2	0.0039	phosphoinositide-3-kinase interacting protein 1
EIF3L	0.23	1.2	0.0193	eukaryotic translation initiation factor 3 subunit L
IL7R	0.22	1.2	0.0058	interleukin 7 receptor
IL6R	0.22	1.2	0.0019	interleukin 6 receptor
USP10	0.22	1.2	0.0495	ubiquitin specific peptidase 10
KLRG2	0.22	1.2	0.0228	killer cell lectin like receptor G2
GOLGA8A	0.22	1.2	0.0435	golgin A8 family member A
VCPKMT	0.21	1.2	0.0315	valosin containing protein lysine methyltransferase
SRSF5	0.20	1.1	0.0428	serine and arginine rich splicing factor 5
CD46	0.20	1.1	0.0083	CD46 molecule
CSGALNACT1	0.20	1.1	0.0264	chondroitin sulfate N-acetylgalactosaminyltransferase 1
HAL	0.19	1.1	0.0427	histidine ammonia-lyase
TM6SF1	0.19	1.1	0.0120	transmembrane 6 superfamily member 1
DDX17	0.19	1.1	0.0040	DEAD-box helicase 17
TNFSF14	0.19	1.1	0.0380	tumour necrosis factor superfamily member 14
CD200	0.19	1.1	0.0482	CD200 molecule
LAT2	0.18	1.1	0.0315	linker for activation of T-cells family member 2
MME	0.18	1.1	0.0466	membrane metalloendopeptidase
OXNAD1	0.18	1.1	0.0123	oxido-reductase NAD binding domain containing 1
SRSF1	0.18	1.1	0.0432	serine and arginine rich splicing factor 1
CEACAM4	0.18	1.1	0.0131	carcinoembryonic antigen related cell adhesion molecule 4
SGK1	0.17	1.1	0.0228	serum/glucocorticoid regulated kinase 1
LUC7L3	0.17	1.1	0.0266	LUC7 like 3 pre-mRNA splicing factor
ARGLU1	0.15	1.1	0.0269	arginine and glutamate rich 1
RB1CC1	0.15	1.1	0.0495	RB1 inducible coiled-coil 1
MYO1F	0.15	1.1	0.0432	myosin IF
ATG16L2	0.15	1.1	0.0315	autophagy related 16 like 2

TAB2	0.15	1.1	0.0206	TGF-beta activated kinase 1/MAP3K7 binding protein 2
PTTG1IP	0.14	1.1	0.0459	pituitary tumour-transforming 1 interacting protein
NAP1L4	0.14	1.1	0.0334	nucleosome assembly protein 1 like 4
ZNF791	0.14	1.1	0.0466	zinc finger protein 791
CRLF3	0.13	1.1	0.0450	cytokine receptor like factor 3
SPSB3	0.13	1.1	0.0495	splA/ryanodine receptor domain and SOCS box containing 3
TMEM154	0.11	1.1	0.0479	transmembrane protein 154

Decreased Gene Expression on Day 14 compared to Day 7

Gene Symbol	Log Fold-change	Fold-change	Adjusted p-value	Description
LAP3	-0.50	0.7	0.0019	leucine aminopeptidase 3
PPIHP1	-0.49	0.7	0.0069	peptidylprolyl isomerase H pseudogene 1
GDPD5	-0.49	0.7	0.0019	glycerophosphodiester phosphodiesterase domain containing 5
SERPING1	-0.46	0.7	0.0241	serpin family G member 1
C12orf76	-0.45	0.7	0.0040	chromosome 12 open reading frame 76
SIGLEC1	-0.42	0.7	0.0233	sialic acid binding Ig like lectin 1
OAS3	-0.37	0.8	0.0083	2'-5'-oligoadenylate synthetase 3
KLF10	-0.36	0.8	0.0466	Kruppel like factor 10
IFITM3	-0.35	0.8	0.0019	interferon induced transmembrane protein 3
CDKN1C	-0.33	0.8	0.0281	cyclin dependent kinase inhibitor 1C
ETFB	-0.32	0.8	0.0459	electron transfer flavoprotein beta subunit
RBBP8	-0.32	0.8	0.0466	RB binding protein 8, endonuclease
ATP8B4	-0.32	0.8	0.0264	ATPase phospholipid transporting 8B4 (putative)
ACSF2	-0.32	0.8	0.0083	acyl-CoA synthetase family member 2
HERC6	-0.31	0.8	0.0040	HECT and RLD domain containing E3 ubiquitin protein ligase family member 6
VRK1	-0.31	0.8	0.0410	vaccinia related kinase 1
GPR84	-0.31	0.8	0.0176	G protein-coupled receptor 84
PFKM	-0.31	0.8	0.0228	phosphofructokinase, muscle
CENPU	-0.30	0.8	0.0450	centromere protein U
CDC42EP2	-0.30	0.8	0.0174	CDC42 effector protein 2
RCBTB1	-0.29	0.8	0.0466	RCC1 and BTB domain containing protein 1
IFI6	-0.28	0.8	0.0438	interferon alpha inducible protein 6
NTAN1	-0.27	0.8	0.0380	N-terminal asparagine amidase
LAG3	-0.27	0.8	0.0264	lymphocyte activating 3
CHCHD4	-0.26	0.8	0.0495	coiled-coil-helix-coiled-coil-helix domain containing 4

DDX60	-0.26	0.8	0.0241	DExD/H-box helicase 60
OAS2	-0.25	0.8	0.0181	2'-5'-oligoadenylate synthetase 2
ZFPM1	-0.25	0.8	0.0233	zinc finger protein, FOG family member 1
LDLR	-0.25	0.8	0.0113	low-density lipoprotein receptor
MTMR2	-0.25	0.8	0.0019	myotubularin related protein 2
OAS1	-0.24	0.8	0.0334	2'-5'-oligoadenylate synthetase 1
C12orf75	-0.24	0.8	0.0241	chromosome 12 open reading frame 75
IDH2	-0.23	0.9	0.0083	isocitrate dehydrogenase (NADP(+) 2, mitochondrial
CCDC162P	-0.23	0.9	0.0435	coiled-coil domain containing 162, pseudogene
FUNDC1	-0.23	0.9	0.0280	FUN14 domain containing 1
GBP4	-0.22	0.9	0.0046	guanylate binding protein 4
PPIH	-0.22	0.9	0.0148	peptidylprolyl isomerase H
HEG1	-0.22	0.9	0.0147	heart development protein with EGF like domains 1
ATIC	-0.22	0.9	0.0459	5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase
NUP93	-0.21	0.9	0.0086	nucleoporin 93
MX1	-0.21	0.9	0.0438	MX dynamin like GTPase 1
CDKN1A	-0.21	0.9	0.0255	cyclin dependent kinase inhibitor 1A
ACLY	-0.21	0.9	0.0123	ATP citrate lyase
TTC9C	-0.21	0.9	0.0227	tetratricopeptide repeat domain 9C
NAGA	-0.20	0.9	0.0065	alpha-N-acetylgalactosaminidase
THYN1	-0.20	0.9	0.0228	thymocyte nuclear protein 1
PLSCR1	-0.20	0.9	0.0327	phospholipid scramblase 1
TUBA1B	-0.20	0.9	0.0019	tubulin alpha 1b
KIF1C	-0.20	0.9	0.0315	kinesin family member 1C
CHST12	-0.19	0.9	0.0450	carbohydrate sulfotransferase 12
GLOD4	-0.19	0.9	0.0193	glyoxalase domain containing 4
SCARB2	-0.19	0.9	0.0193	scavenger receptor class B member 2
MT2A	-0.19	0.9	0.0086	metallothionein 2A
GPN3	-0.19	0.9	0.0269	GPN-loop GTPase 3
TJP2	-0.19	0.9	0.0131	tight junction protein 2
CIZ1	-0.19	0.9	0.0233	CDKN1A interacting zinc finger protein 1
CEBPB	-0.19	0.9	0.0206	CCAAT/enhancer-binding protein beta
NDUFA8	-0.18	0.9	0.0450	NADH:ubiquinone oxidoreductase subunit A8
CD300C	-0.18	0.9	0.0281	CD300c molecule
RIC8B	-0.18	0.9	0.0457	RIC8 guanine nucleotide exchange factor B
ARPP19	-0.18	0.9	0.0435	cAMP regulated phosphoprotein 19
TMEM41A	-0.18	0.9	0.0427	transmembrane protein 41A

GLO1	-0.18	0.9	0.0457	glyoxalase I
IL1RN	-0.18	0.9	0.0450	interleukin 1 receptor antagonist
FUCA2	-0.18	0.9	0.0233	fucosidase, alpha-L- 2, plasma
RUVBL1	-0.17	0.9	0.0280	RuvB like AAA ATPase 1
NEXN	-0.17	0.9	0.0233	nexilin F-actin binding protein
CTNNA1	-0.17	0.9	0.0311	catenin alpha 1
ZBTB2	-0.17	0.9	0.0426	zinc finger and BTB domain containing 2
HARS	-0.17	0.9	0.0391	histidyl-tRNA synthetase
HDAC1	-0.17	0.9	0.0438	histone deacetylase 1
SAMD9	-0.17	0.9	0.0469	sterile alpha motif domain containing 9
ZC3H4	-0.17	0.9	0.0450	zinc finger CCCH-type containing 4
GAR1	-0.16	0.9	0.0264	GAR1 ribonucleoprotein
GSE1	-0.16	0.9	0.0130	Gse1 coiled-coil protein
TAOK3	-0.16	0.9	0.0130	TAO kinase 3
DDX24	-0.16	0.9	0.0112	DEAD-box helicase 24
EIF4EBP1	-0.16	0.9	0.0315	eukaryotic translation initiation factor 4E binding protein 1
MRPL51	-0.16	0.9	0.0365	mitochondrial ribosomal protein L51
CDK5RAP2	-0.15	0.9	0.0438	CDK5 regulatory subunit associated protein 2
RUNX3	-0.15	0.9	0.0450	runt related transcription factor 3
SDF2L1	-0.14	0.9	0.0318	stromal cell derived factor 2 like 1
ARSB	-0.14	0.9	0.0162	arylsulfatase B
LRRFIP2	-0.14	0.9	0.0495	LRR binding FLII interacting protein 2
DTL	-0.14	0.9	0.0438	denticleless E3 ubiquitin protein ligase homolog
RAB8A	-0.13	0.9	0.0416	RAB8A, member RAS oncogene family
ANO6	-0.12	0.9	0.0432	anoctamin 6
HLA-DRA	-0.12	0.9	0.0438	major histocompatibility complex, class II, DR alpha
SMAP1	-0.12	0.9	0.0428	small ArfGAP 1
ELF4	-0.12	0.9	0.0499	E74 like ETS transcription factor 4

CHAPTER 9

Thesis Conclusion

9.0 Final Impressions

The major focus of this work has been on Brassica-derived SFN but in exploring this bioactive phytochemical, a much broader base has been traversed, a base which has examined principles that apply to many phytochemicals. This journey has highlighted controversies, investigated unresolved opposing views and has raised questions for which there are not yet answers; such are the challenges faced by a collaborative scientific community in any investigative endeavour.

9.1 The Role of Serendipity in Science

The clinical trials that were undertaken in delivering this thesis confirmed that there are no failed experiments in science; instead, these are valuable opportunities for enlightenment. The Fatigue Study described in Chapters 3 highlighted the importance of assaying the raw material to be used as an intervention rather than relying on the specification provided by the supplier. Had this been done, there would have been greater likelihood of determining whether the intervention had failed to provide a significant clinical response or instead that the enzyme-active intervention material simply did not meet its specification and as a result, failed to deliver the assumed bioactive content; an important lesson well-learned.

The EASYGENEX Study in Chapter 8 provided a different type of learning opportunity in that the study failed to exclude all the factors capable of activating Nrf2. Given that the intervention was expected to reflect similar findings to that of other studies investigating Nrf2 activation, this oversight had important ramifications. In reviewing the literature on exercise and Nrf2, it became apparent that, even though much is known about the health-promoting effects of exercise, little evidence exists to quantify its effect on Nrf2 activation.

This revelation may provide a valuable avenue for future investigation, especially since supraphysiological doses of antioxidant vitamin supplements are frequently ingested by sportspeople of all ages, even though it has been found that such

supplements can abrogate the beneficial effects of exercise.[119] It may be that quantifying the relationship between exercise and Nrf2 activation would more effectively deliver measurable benefit.

Chapter 8 postulated that exercise and SFN may have had similar effects in activating Nrf2 in the young, healthy males investigated. The genes differentially-expressed in this study were representative of Nrf2-*in*dependent targets and it would be valuable to know how many of these are also modulated by exercise and to what extent; existing data are limited.

9.2 Why Sulforaphane?

Of the many thousands of identified phytochemicals, SFN is just one. This single molecule is not the only Brassica-derived ITC with clinical potential but it is the one for which most evidence exists at this time, especially when few clinical trials have been conducted on other members. There may in fact be advantages in more intensively investigating other ITCs because although preservation of MYR activity is important for all ITCs, not all MYR-active GSNs contain the ESP inhibitor. Daikon radish for example does not contain ESP, whereas cabbage does. Regardless of the specific GSN precursor, the presence of ESP will impact the generation of the associated ITC.[406]

In the production of a SFN-yielding broccoli sprout raw material, the application of heat must be able to simultaneously retain MYR whilst degrading the ESP. It is perhaps for this reason that the MYR-inert broccoli sprout and broccoli seed extracts have been developed as a way to avoid addressing this challenging issue. A whole MYR-active broccoli sprout material is able to endogenously generate SFN, so that it is not reliant on the largely unknown and variable status of the gut microflora required to provide MYR-like activity when only GRN is ingested.

The EASYGENEX Study showed that daily ingestion of capsules of MYR-active dried broccoli sprouts resulted in the presence of SFN and its metabolites in the plasma, confirming that SFN in this form is bioavailable. It also confirmed that the presence of the metabolites was associated with the *GSTP1* genotype of the individual, showing that those carrying either homo- or heterozygous polymorphisms had higher plasma levels of both SFN and its metabolites than those with the normal

genotype. This may have implications for anticipating clinical responsiveness when employing SFN in both prevention and therapy.

9.3 Sulforaphane Amidst a Sea of Polyphenols

When comparing the clinical potential of SFN with other dietary phytochemicals such as the abundant polyphenols, SFN has clear advantages. Chapters 5 and 6 highlight the low bioavailability of the intact unmetabolised polyphenols as being a major barrier to their use systemically, even though they can function as direct-acting antioxidants in the gut, especially effective when consumed with carcinogen-containing foods. Figure 6.3 in Chapter 6 presents a comparison of SFN with the four polyphenols, andrographolides, quercetin, curcumin and silymarin, illustrating the far higher bioavailability of SFN over the four polyphenols, all of which are readily-available as dietary supplements.

The possibility that the metabolites of the polyphenols are both bioactive and bioavailable has been explored by some. However, the role of the gut microflora in producing an unpredictable range of possible metabolites is a largely unexplored but emerging research area. Greater clarity on the underlying processes may help to explain why there exist a few clinical trials showing positive outcomes for polyphenolic interventions with very low bioavailability, even though *in vitro* studies show that the intact polyphenols could not be detected in tissues at such doses. SFN by contrast can be readily detected in micromolar amounts in various cell types.

As a further significant difference, Figure 6.2 illustrates the superior capacity of SFN as an inducer of the cytoprotective Phase 2 detoxification gene *NQO1* by comparing the CD value of SFN with other phytochemicals, not all of which are polyphenols. On both counts, SFN is not only a more potent inducer of the Nrf2-dependent *NQO1* gene but is also more bioavailable than others that have been investigated, establishing SFN as a candidate for further independent or comparative research with clinical endpoints.

9.4 Sulforaphane and the Intricacies of Signalling

The NF-κB network was the predominant Canonical Pathway discussed in Chapter 8 and whilst some of the genes identified were already known to be associated with

SFN, others were not. The microarray data showed that the intervention resulted in the differential expression of 8 genes not previously associated with SFN. These genes are known to function in six overlapping pathways associated with a range of core cellular processes. This finding is worthy of more targeted gene expression techniques such as RT-qPCR in order to reject or validate the gene expression relationship to SFN. If one or more of these genes can be validated for its association with SFN, this may lead to new avenues for SFN research.

When exploring the functions of the genes found to be significantly differentially-expressed by SFN in this study, it becomes immediately apparent that there is substantial cross-talk and interactivity among their related biochemical pathways, such that each of these genes functions in multiple pathways. By way of example, two of the downregulated genes, *RELB* and *CEBPB* are known to act synergistically in downregulating inflammation. But *CEBPB* is also integral to the processes of adipogenesis, glucose metabolism and insulin sensitivity. Furthermore, the role of *CEBPB* in adiposity overlaps its role in cancer because increased *CEBPB* expression is implicated in the development of some tumours with expression correlated with more invasive activity.

Similarly, *DDX3X* as a member of the large DEAD-box protein family is known to regulate NF-kB, with dysregulation of this gene implicated in tumorigenesis. SFN was shown in the EASYGENEX Study to downregulate this gene along with four other cancer-related genes, *ZBTB2, HERC6, PDIA4 and IGF2BP2.* This suggests that there may be additional roles for SFN in chemoprevention.

Chapter 8 referred to a more recent published discussion of cancer cell survival mechanisms involving a redox-related process wherein cancer cells can switch between survival and death pathways. The EASYGENEX data showed for the first time that SFN may downregulate the *PDIA4* gene. Since *PDIA4* expression is upregulated in a variety of tumour cell lines, its yet-to-be-confirmed downregulation by SFN may be indicative of alternative signalling mechanisms used by cells to determine whether and when a cancer cell switches from death to pro-survival signals or *vice versa*.

An upregulated gene with multiple functions and identified in this study was *DDC*, the gene coding for dopa decarboxylase. Dopamine is usually considered for its role as a neurotransmitter and as the precursor for other catecholamine neurotransmitters. However, dopamine also has a lesser-known role in modulating the inflammatory process; the IPA data highlighted its presence in the NF-κB Network. If subsequent RT-qPCR and protein studies validate its induction by SFN, it would be intriguing to know whether this action is also applicable in neuropsychiatry and neurodegeneration. SFN is known to inhibit dopamine oxidation and has already been investigated in relation to Parkinson's Disease in which dopamine is both under-secreted and readily oxidised.

9.5 The Emerging Dual Roles of Nrf2 – a Certain Dilemma

Surely, one of the dilemmas of our time in this research field lies in attempting to unravel the dual role of Nrf2 activation in human cells. Where such activation has been demonstrated to be protective of healthy cells, the same may be equally protective of cancer cells, giving these cells a distinct survival advantage; it has already been shown that resistance to chemotherapeutic drugs (chemoresistance) could result from such induction. This leaves us to ponder how the observed positive clinical outcomes via Nrf2 activation can be reconciled against the potential adverse effects in individuals who may be in an undiagnosed preliminary stage of cancer cell progression.

Cancer cells are known to upregulate their own Nrf2 pathway so that strategies that attempt to enhance the cell's endogenous defences may not be advantageous in active disease. Although the precise signalling that governs Nrf2's pro-survival mode in cancer cells is not known, it is conceivable that induction of the Phase 2 detoxification genes via Nrf2 might reduce the effective dose of the chemotherapeutic agent. Pharmaceuticals designed to inhibit Nrf2 are already part of the cancer therapy armamentarium.

So, on the one hand, there is evidence to support the chemoprotective effects of Nrf2 activation[742] but on the other hand, Nrf2 inhibition is being already used to sensitise cancer cells to chemotherapeutic agents, thereby eliminating chemoresistance.[743]

9.6 So Many Questions – So Few Answers

To put these issues into perspective, it is important to consider them against the larger body of epidemiological evidence which connects chemoprevention with diet. A number of questions invariably arise from such consideration: Is there any documented evidence that dietary consumption of Nrf2 activators such as broccoli promotes cancer or retards recovery? Does a synthesised more potent Nrf2 activator behave in the same way as SFN and other phytochemical Nrf2 activators? Is there any evidence that dietary intake of plant foods promotes cancer in established disease? Could Nrf2 activation in healthy cells assist in restoring the endogenous mechanisms cells use to mount a successful challenge on a cancer cell? What does simultaneous activation of Nrf2 in healthy and cancer cells achieve? Is such plant food contra-indicated when a patient is undergoing chemotherapy? Does a diet high in plant food promote chemoresistance in such a patient? Do other benefits of a diet high in plant foods outweigh the possible adverse effects?

Given that cancer patients in particular are known to search for alternative therapies in addition to their conventional treatments, it is imperative that the answers to at least some of these questions are found. How do supplemental Nrf2 activators impact treatment with potent pharmaceuticals, many of which are designed to promote redox imbalance in the cancer cells? And if the answer to this question is not known, how should a medically- or even nutritionally-trained clinician advise a patient in relation to diet and/or particular supplements?

9.7 Multiple Mechanisms – Which are at Play?

One very important consideration is that Nrf2 is only one component of the cancer process and other mechanisms are known to modulate the process. Throughout this thesis, the pleiotropic nature of the SFN molecule has been highlighted and many of its Nrf2-independent effects are related to cancer. Is it possible that a phytochemical like SFN can simultaneously engage some or all of its multiple signalling pathways in healthy cells to over-ride the cancer cell's Nrf2 activation? Is there evidence that all of SFN's anti-cancer effects are similarly active in the cancer cell?

9.8 Concluding Remarks

This thesis set out to explore *the role of phytochemicals in modulating intrinsic human cellular defence processes.* Even though the focus is primarily on SFN, it is cast against a backdrop of other phytochemicals more abundant in the human diet. This has enabled the differences between this low molecular weight lipophilic molecule to be compared and contrasted with the typically larger, bulkier polyphenolic molecules in terms of their relative clinical potential.

An underlying theme running throughout this thesis is the awareness that the rapidly-evolving science of nutrigenomics holds promise as a more personalised model for health care. Phytochemicals are integral to this model with SFN arguably a promising future candidate for wider application.

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APPENDIX A

THE UNIVERSITY OF QUEENSLAND
AUSTRALIA

School of Human Movement Studies
HEAD OF SCHOOL
Professor Doune Macdonald

The University of Queensland
Brisbane Qld 4072 Australia
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CRICOS PROVIDER NUMBER 00025B

May 30, 2007.

Christine Houghton,
c/o School of Human Movement Studies,
The University of Queensland,
4072.

Dear Christine,

Re: ethical review of the following project:
Evaluation of the efficacy of an orally-bioactive form of superoxide dismutase (GliSODin) in reducing levels of fatigue in otherwise healthy women.

Thank-you for the opportunity to review your research proposal. I am pleased to let you know that provided you include the following paragraph in the Participation Information Document, your project will be cleared in accordance with the ethical review guidelines at The University of Queensland. Your approval number will be: HMS07/3005.

This study has been cleared by one of the human ethics committees of the University of Queensland in accordance with the National Health and Medical Research Council's guidelines. You are of course, free to discuss your participation in this study with project staff (contactable on 0417 728 458). However, if you would like to speak to an officer of the University not involved in the study, you may contact the Ethics Officer from the School of Human Movement Studies on 3365 6768.

Please note that:

(i) Adverse reaction to treatment by subjects, injury or any other incident affecting the welfare and/or health of subjects attributable to the research should be promptly reported to me.
(ii) Amendments to any part of the approved protocol (however minor) should be submitted to me for consideration.
(iii) Signed statements of informed consent should be kept secure in case we need to access them in the future.

I wish you well with your research.

Yours sincerely,

David Jenkins, PhD
Chair, HMS Ethics Committee
Deputy Chair, Medical Research Ethics Committee, UQ

APPENDIX B

THE UNIVERSITY OF QUEENSLAND

Institutional Human Research Ethics Approval

Project Title:	Effect Of A Sulforaphane - Yielding Encapsulated Product On Pharmacokinetics And Gene Expression In Healthy Men. The EASY GENEX Study - 29/04/2016 - AMENDMENT
Chief Investigator:	Ms Christine Houghton
Supervisor:	Prof Jeff Coombes, Prof Robert Fassett
Co-Investigator(s):	Prof Jeff Coombes, Prof Robert Fassett
School(s):	School of Human Movement Studies
Approval Number:	2013000222
Granting Agency/Degree:	Jointly RBWH Foundation, Cell-Logic Pty Ltd and HMS
Duration:	31st October 2016

Comments/Conditions:

- Extension of project duration to 31st October 2016
- Change of project title from "Effect Of A Sulforaphane - Yielding Encapsulated Product On Pharmacokinetics And Gene Expression In Healthy Men. The EASY GENEX Study" to "Effect of a Sulforaphane - Yielding Encapsulated Product on Pharmacokinetics and Gene Expression in Young Healthy Males. The Easy Genex Study"
- Changes to participants
- Patient Information Sheet and Consent Form v1.2, April 2016
- Changes to Protocol

Note: if this approval is for amendments to an already approved protocol for which a UQ Clinical Trials Protection/Insurance Form was originally submitted, then the researchers must directly notify the UQ Insurance Office of any changes to that Form and Participant Information Sheets & Consent Forms as a result of the amendments, before action.

Name of responsible Committee:
Medical Research Ethics Committee
This project complies with the provisions contained in the *National Statement on Ethical Conduct in Human Research* and complies with the regulations governing experimentation on humans.

Name of Ethics Committee representative:
Dr Jennifer Paratz
Chairperson
Medical Research Ethics Committee

Signature _____ Date 17/8/16

APPENDIX C

Educational Course Compilation (not Peer-reviewed)

Following recognition that the field of Nutrigenomics is professionally represented primarily by academics and researchers, there appeared to be a need for an evidence-based educational programme to address the growing requirements of practising clinicians in a number of health-focused disciplines. Self-paced online courses were developed at two levels and authored jointly by Dr Yael Joffe RD, PhD (genetics and nutrigenetics components) and Christine Houghton (nutritional biochemistry and applied clinical nutrition components). These courses have already been completed by clinicians from various locations across the globe.

Compilation Time Frame	Course Name	Online availability
Feb.,2015 – March, 2016	***ADVANCED TRANSLATIONAL NUTRIGENOMICS.*** Co-author of 10-module online self-paced professional education course for practising clinicians. Student input ~ 450 hours	**Centre for Translational Genomics (CTG)** www.ctgeducation.com originally Manuka Science www.manukascience.co.za
May, 2016 - Sep.,2016	***FOUNDATIONS IN NUTRIGENOMICS.*** Co-author of 4-module online self-paced professional education course for practising clinicians. Student input ~ 200 hours	**Centre for Translational Genomics (CTG)** www.ctgeducation.com

COMPLETION OF THESIS. The total of 307 pages comprising this thesis includes 35 preliminary pages designated as *Front Matter* and paginated separately by Roman numerals.