THE PROTECTIVE ROLE OF NRF2/KEAP1 IN NEUROLOGICAL DISEASE AND OXIDATIVE STRESS-INDUCED CELL DAMAGE

Petra Bergström

Department of Clinical Chemistry

Institute of Biomedicine

Sahlgrenska Academy at University of Gothenburg

UNIVERSITY OF GOTHENBURG

Gothenburg 2013

Till formor & forfar

THE PROTECTIVE ROLE OF NRF2/KEAP1 IN NEUROLOGICAL DISEASE AND OXIDATIVE STRESS–INDUCED CELL DAMAGE

Petra Bergström

Department of Clinical Chemistry, Institute of Biomedicine, Sahlgrenska Academy at University of Gothenburg, Göteborg, Sweden

ABSTRACT

Oxidative stress is a common feature in the pathogenesis of many diseases, including neurodegenerative diseases like Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS). Nrf2 and Keap1 regulate an inducible defence system against oxidative stress. In addition to oxidative stress, the Nrf2-dependent defence system is also triggered by reactive substances in our diet, such as the isothiocyanate sulforaphane from broccoli, and both broccoli and sulforaphane have been shown to protect from disease in a number of studies. The aim of this thesis has been to investigate the Nrf2 response after repeated, short stimulations with sulforaphane, simulating the brief Nrf2 stimulation expected after regular broccoli intake. Furthermore, genetic variation in the Nrf2- and Keap1-encoding genes *NFE2L2* and *KEAP1* were investigated for associations with PD and ALS. In **paper I**, we found that brief stimulation of Nrf2 with sulforaphane was enough to induce a prolonged Nrf2 response in astrocytes. We also found that repeated four-hour stimulations for several days resulted in sustained increase in the resistance to superoxide-induced cell death and an accumulation of one of the protective enzymes induced by Nrf2. The results of **paper II** indicate that brief sulforaphane treatment repeated for three consecutive days increased radioresistance in an Nrf2-dependent manner, suggesting that the Nrf2 system can be trained. In **paper III and IV**, we found that genetic variants of the *NFE2L2* gene may affect risk and phenotype of both PD and ALS. We also found that a genetic variant of the *KEAP1* gene may affect the phenotype of ALS. In conclusion, data presented in this thesis indicate that Nrf2 can be activated by brief, repeated stimulations to protect from oxidative stress-induced damage. In addition, *NFE2L2* may be a risk gene for both PD and ALS, while *KEAP1* may affect the phenotype of ALS.

Keywords: ALS, amyotrophic lateral sclerosis, astrocytes, haplotype, Keap1, *KEAP1,* neuroprotection, *NFE2L2*, Nrf2, oxidative stress, Parkinson's disease, risk factor, SNP, sulforaphane, genetic variation

SAMMANFATTNING PÅ SVENSKA

Vi omger oss dagligen med ämnen som hotar att reagera med och att skada våra celler. Fria radikaler är molekyler som på grund av sin kemiska struktur är särskilt reaktiva. Fria radikaler uppstår när vi röker, solar oss eller äter grillat kött, men även när vi andas syre. Vår omgivning hade alltså varit skadlig för oss om vi inte hade haft ett inbyggt skydd mot fria radikaler och andra reaktiva molekyler. När mängden fria radikaler överstiger cellens förmåga att försvara sig, uppstår ett tillstånd som kallas oxidativ stress. Om cellerna tvingas utstå oxidativ stress för länge uppstår skador som kan leda till olika sjukdomstillstånd, som till exempel cancer och neurodegenerativa sjukdomar.

Nrf2 och Keap1 är två viktiga proteiner involverade i cellens inbyggda försvar mot oxidativ stress. Nrf2/Keap1 utgör ett evolutionärt välbevarat försvarssystem som, lite förenklat, sätter igång cellens egen produktion av många olika typer av antioxidanter. Antioxidanter är molekyler som reagerar med fria radikaler och neutraliserar dem. Normalt sitter Nrf2 och Keap1 ihop i ett komplex som hindrar Nrf2 från att stimulera produktion av antioxidanter. När reaktiva molekyler träffar komplexet, lossnar Keap1 från Nrf2, som då blir aktiverat. Naturliga substanser i vår kost, såsom sulforafan från broccoli, kan också reagera med Nrf2/Keap1-komplexet och trigga igång Nrf2-aktivitet. Anledningen till det är troligen att de här ämnena är reaktiva till sin natur. Höga doser skulle alltså vara skadliga för våra celler, men lagom doser ger precis rätt stimulans av Nrf2/Keap1-systemet för att vi ska få ett ökat skydd – på så sätt hjälper de våra celler att stå emot även andra typer av skador.

Ett flertal studier har visat att broccoli skyddar mot DNA-skada och cancer och att detta skydd är beroende av ett fungerande Nrf2-protein. Vi ville undersöka om sulforafan från broccoli kan skydda mot cellskador även om man bara tillför ämnet sporadiskt, vilket skulle kunna motsvara hur det är i det vanliga livet då man kanske äter broccoli några gånger per vecka. Vi behandlade astrocyter – en celltyp som står för en betydande del av skyddet mot oxidativ stress i hjärnan – med sulforafan och mätte hur aktiveringen av Nrf2-genen påverkades. Vi upptäckte att korta, övergående stimuleringar (1-4 timmar) var tillräckligt för att Nrf2-systemet skulle vara kontinuerligt aktiverat i över två dagar. När cellerna behandlades med sulforafan fyra timmar per dag under fyra dagar, såg vi dessutom att vissa delar av Nrf2-systemet ökade successivt dag för dag. Detta ledde också till att cellerna var mindre känsliga för celldöd orsakad av oxidativ stress och kan vara en del av

förklaringen till att sporadiskt intag av broccoli skyddar mot cancer. Samtidigt såg vi att andra delar av responsen avtog under upprepad behandling, vilket visar på hur viktig doseringen av Nrf2-stimulerande ämnen är. Upp till en viss dos ökar den positiva effekten, men blir dosen för hög så avtar effekten igen. Riktigt höga doser kan till och med vara skadliga.

Den mest ödesdigra konsekvensen av oxidativ stress är att fria radikaler kan reagera med DNA och orsaka ett brott på båda DNA-strängarna på samma ställe – ett så kallat dubbelsträngsbrott. Ett dubbelsträngsbrott leder till att DNA-kedjan går helt av, vilket är särskilt svårt för cellen att reparera. Joniserande strålning används ofta i cancerterapi, just på grund av förmågan att effektivt döda celler genom att orsaka dubbelsträngsbrott med hjälp av fria radikaler. Ett problem med strålterapi är att bieffekterna varierar kraftigt från person till person vid samma stråldos. För att inte allvarligt skada de känsligaste patienterna, ger man en lägre stråldos till alla. Det leder till att vissa cancerpatienter istället får för låg dos med sämre möjlighet till bot som följd. Vi undersökte om kort, upprepad, stimulering med sulforafan från broccoli kunde skydda friska celler från celldöd efter joniserande strålning. Vi såg att celler som fått en 4-timmars behandling med sulforafan före strålning överlevde i större utsträckning än obehandlade celler, men bara om förbehandlingen hade upprepats tre dagar i följd. Fyndet antyder att Nrf2/Keap1-systemet kan tränas upp för att ge ett mer effektivt skydd mot fria radikaler.

Parkinsons sjukdom (PD) och amyotrofisk lateralskleros (ALS) är två relativt vanliga neurodegenerativa sjukdomar. Vid båda sjukdomarna föreligger ärftliga varianter med tydliga kopplingar till förändringar i specifika gener, men flertalet patienter med PD och ALS insjuknar utan att man kan identifiera den bakomliggande orsaken. Troligen samverkar flera faktorer som orsak till de icke-ärftliga (sporadiska) varianterna av sjukdomarna. Som exempel kan varianter av så kallade riskgener verka tillsammans med miljöfaktorer, vilket kan leda till sjukdom. Oxidativ stress tros vara en viktig faktor vid utveckling av neurodegenerativa sjukdomar och man har sett att nivåerna av Nrf2 och Keap1 är förändrade i hjärnan hos patienter med både PD och ALS. Vi ville undersöka om Nrf2 och Keap1 är riskgener för någon eller båda av dessa sjukdomar. Vi jämförde därför patienter och kontroller för att se om det fanns genetiska förändringar som kunde förklara varför man utvecklar PD eller ALS. Vi hittade varianter av Nrf2-genen som gav ett ökat skydd mot PD respektive ALS. Vi hittade även en variant av Keap1-genen som gav ett ökat skydd mot ALS. Tillsammans visar de här resultaten att Nrf2-genen kan vara en riskgen för både PD och ALS och vid det senare

tillståndet verkar även Keap1 vara en faktor av betydelse för tidpunkten när sjukdomen inträffar.

Sammanfattningsvis tyder våra resultat på att en kort retning av Nrf2 med sulforafan från broccoli kan vara tillräckligt för att ge ett ökat försvar mot oxidativ stress i minst 24 timmar. Dessutom verkar det som om Nrf2-systemet och skyddet mot oxidativ stress kan tränas upp genom upprepade, korta retningar. Vi hittade också varianter av Nrf2-genen som var kopplad till både risk och sjukdomsbild av PD och ALS. En variant av Keap1-genen påverkade också förloppet av ALS. Resultaten talar för att ett förändrat Nrf2/Keap1-system, i kombination med miljöfaktorer, över tid kan påverka risken att utveckla Parkinsons sjukdom eller ALS.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals:

I. **Petra Bergström**[1], Heléne C. Andersson[1], Yue Gao, Jan-Olof Karlsson, Christina Nodin, Michelle F. Anderson, Michael Nilsson, Ola Hammarsten. *Repeated transient sulforaphane stimulation in astrocytes leads to prolonged Nrf2-mediated gene expression and protection from superoxide-induced damage.* Neuropharmacology. 2011; 60: 343-353.

II. Sherin T Mathew[1], **Petra Bergström**[1], Ola Hammarsten. *Repeated transient Nrf2 stimulation protects primary human fibroblasts from radiation-induced damage.* Manuscript in preparation.

III. Malin von Otter[1], Sara Landgren[1], Staffan Nilsson, Dragana Celojevic, **Petra Bergström**, Anna Håkansson, Hans Nissbrandt, Marek Drozdzik, Monika Bialecka, Mateusz Kurzawski, Kaj Blennow, Michael Nilsson, Ola Hammarsten, Henrik Zetterberg. *Association of Nrf2-encoding NFE2L2 haplotypes with Parkinson's disease.* BMC Medical Genetics. 2010; 11:36:1471-2350.

IV. **Petra Bergström**[1], Malin von Otter[1], Staffan Nilsson, Ann-Charloth Nilsson, Michael Nilsson, Peter M. Andersen, Ola Hammarsten, Henrik Zetterberg. *Association of NFE2L2 and KEAP1 haplotypes with amyotrophic lateral sclerosis.* Submitted manuscript, April 2013.

[1]Equal contribution.

CONTENT

ABBREVIATIONS

A	adenine
AD	allelic discrimination
ALS	amyotrophic lateral sclerosis
ARE	antioxidant response element
ATP	adenosine triphosphate
C	cytosine
carboxy-DCFH-DA	(5-(and-6)-carboxy-2-7-dichlorodihydrofluorescein diacetate
CEU	Utah residents with Northern and Western European ancestry from the CEPH collection. (One of 11 populations in HapMap phase 3)
CO	carbon monoxide
ddNTP	dideoxynucleotide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DSB	DNA double-strand break
EBSS	Earle's Balanced Salt Solution
EdU	5-ethynyl-2´-deoxyuridine
G	guanine
GSH	glutathione, reduced
GSSG	glutathione, oxidized
GSTs	glutathione S-transferases
GWAS	genome-wide association study
H2AX	histone 2 variant H2A.X
γH2AX	H2AX, phosphorylated
Hmox1	heme oxygenase-1, protein (mouse, rat)
HO-1	heme oxygenase-1, protein (human)
IR	ionizing radiation
KEAP1	kelch-like ECH-associated protein 1, gene
Keap1	kelch-like ECH-associated protein 1, protein
LD	linkage disequilibrium

MCB	monochlorobimane
MEF	mouse embryonic fibroblast
mRNA	Messenger RNA
Nqo1	NAD(P)H dehydrogenase quinone 1, protein (mouse, rat)
NQO1	NAD(P)H dehydrogenase quinone 1, protein (human)
NFE2L2	nuclear factor (erythroid-derived 2)-like 2, gene
Nrf2	nuclear factor (erythroid-derived 2)-like 2, protein
OR	odds ratio
PBP	progressive bulbar palsy
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD	Parkinson's disease
PI	propidium iodide
PLS	primary lateral sclerosis
PMA	progressive muscular atrophy
p-value	probability value
p_c-value	probability value, corrected
qPCR	quantitative PCR
RNA	ribonucleic acid
RS	reactive species
SF	sulforaphane
SNP	single nucleotide polymorphism
SOD	superoxide dismutase
T	thymine
Txnrd1	thioredoxin reductase 1
X	xanthine
XO	xanthine oxidase

DEFINITIONS IN SHORT

Free radical   A molecule lacking an electron in its outer
                            shell, making it prone to react with other
                            molecules (electron acceptor).

Antioxidant   A molecule with an extra electron in its outer
                            shell. The extra electron can neutralize free
                            radicals (an electron donor).

Oxidative stress   A state of imbalance between the production
                            and cellular clearance of reactive molecules,
                            leading to cellular stress.

1 INTRODUCTION

1.1 OXIDATIVE STRESS

In our everyday lives we are constantly exposed to reactive molecules threatening to harm important biomolecules in our cells. These molecules can be referred to as **reactive species (RS)**. RS include **free radicals** and non-radicals easily converted to free radicals. The general feature of free radicals is that they lack an electron in the outer shell, making them unstable and prone to react with other molecules. This reaction, when one molecule or atom donates an electron to another, is called a **redox** reaction since one molecule (the electron acceptor) is reduced while the other (the electron donor) is simultaneously oxidized. The reactivity of reactive species varies [1].

Free radicals in a cell can originate from external sources, such as sunlight exposure or cigarette smoke [2, 3]. Cellular free radicals can also derive from internal sources, such as activated immune cells or microglia [4], the leaking of superoxide ($O_2\cdot^-$) during oxygen metabolism in the mitochondria, [5, 6], or by enzymatic reactions including reduction of oxygen. One well studied example is xanthine oxidase (XO), which catalyses reduction of hypoxanthine to uric acid via xanthine (X), producing both superoxide and hydrogen peroxide (H_2O_2) in the process [7]. Hydrogen peroxide is not a free radical, but it is regarded as an RS as it readily forms the very reactive hydroxyl radical (OH·) [8]. Interestingly, some radicals are also used as signalling molecules in some tissues, e.g. the nitric oxide radical (NO) controlling blood flow through its role in relaxation of blood vessel smooth muscles [9]. Excess nitric oxide and superoxide can react and form the extremely reactive peroxynitrate radical ($ONOO^-$), why the levels of cellular nitric oxide and superoxide need to be closely regulated. Our cells have developed refined systems to keep radical levels low. Examples of proteins involved in preventing radical formation are superoxide dismutases (SOD, reducing superoxide to hydrogen peroxide) [10] and catalases/peroxidases (reducing hydrogen peroxide to water). Cells also possess systems to remove damaged biomolecules and repair oxidative DNA damage, further limiting the toxic effects of RS. However, when the amount of reactive substances exceeds the capacity of these cellular defence systems, a potentially harmful state occurs. This state is referred to as **oxidative stress**.

1.1.1 Oxidative stress in cell damage and disease

Prolonged situations of oxidative stress, with radical levels exceeding the cellular clearing and repair capacity, will damage lipids, proteins and DNA. If the oxidative stress-induced damage is severe, it could eventually lead to disease. Mutations or other damages to proteins like SOD or catalases/peroxidises could decrease the cellular capacity to clear endogenous radicals and it has been suggested that increased mitochondrial leakage in combination with a declining radical defence over time is a contributor to cellular ageing [11, 12]. Since motor neurons have an unusually high energy demand, they may be especially sensitive to increase in mitochondria leakage [13]. It is still debated whether oxidative stress generates or is a consequence of disease. Either way, markers of oxidative damage are increased in patients with neurodegenerative diseases and oxidative stress is clearly a part of the pathogenesis of these diseases [14].

1.1.2 Oxidative stress protection

The first line of defence against radical damage is radical clearance, briefly mentioned above. One component of this defence is **antioxidants**, which are either synthesized within the cell or obtained from the diet. A cellular antioxidant is an electron donor with potential to neutralize harmful radicals and prevent oxidation of important biomolecules (Figure 1).
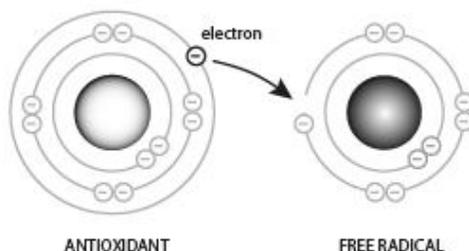
Figure 1. Simplified model of a redox reaction between a free radical and an antioxidant. A free radical lacks an electron in its outer shell and is chemically unstable. An antioxidant acts as an electron donor to the free radical to prevent oxidization of other more important molecules.

Some cellular antioxidants such as ascorbate, bilirubin and uric acid are consumed in the process, potentially producing new reactive end products. Other antioxidants are renewable and function as redox-sensing switches in response to RS. The antioxidant protein or molecule, oxidized by a radical, is immediately transferred back to its reduced state by a specific enzyme. Examples of proteins used in this fashion in the cell are glutathione (GSH)

and thioredoxin [15]. A common feature of redox-sensing proteins, such as GSH, is the presence of reactive cysteines, one of 21 amino acids constituting the building blocks in our proteins. The cysteine side chain consists of a **thiol** (-SH) group, which is often involved in redox reactions acting as an electron donor [16]. Diet-derived antioxidants have been attributed the beneficial effects associated with a diet rich in fruit and vegetables. However, global scientific evidence that intake of supplementary antioxidants increases health is lacking [17-19]. Instead, the tendency of many naturally occurring compounds to interfere with thiols in cellular redox proteins and thereby inducing the endogenous production of antioxidants, has gained much attention lately [20, 21].

1.2   GENES AND GENE EXPRESSION

All the information needed to create a living being is contained in the deoxyribonucleic acid (**DNA**) molecule [22]. During sexual reproduction, one DNA chromosome is inherited from the mother and one from the father but due to recombination, the genes in the new individual will be a mosaic of inherited genes [23]. How the nucleotide bases (A, T, G and C) are combined on the DNA strands to form **genes** defines the genetic properties of the DNA. The genes consist of protein-coding regions – exons – and non-coding regions – introns. The role of non-coding DNA is still largely unknown, but increasing data unravels a picture much more complicated than was originally assumed [24]. The DNA upstream of a gene contains the gene **promoter**. The promoter is the binding site for proteins necessary for transcription of a gene, such as **transcription factors** and RNA polymerase. In the mRNA resulting from gene transcription, non-coding regions are cleaved off in a process called splicing. The resulting mRNA constitutes a template for translation into a protein, which is the final gene product. Measurements of mRNA and protein levels can be used to assess the **gene expression** of a target gene in biochemical experiments.

1.3   THE NRF2/KEAP1 SYSTEM

Nuclear factor (erythroid-derived 2)-like 2 (**Nrf2**) [25] is an important protein in the cellular defence against oxidative stress. Nrf2 is a transcription factor and induces gene expression of response genes by binding to their promoters. Nrf2 specifically binds to a DNA sequence called the **antioxidant response element (ARE)** [26-28]. A number of genes harbour this sequence in their promoters, including the Nrf2 gene itself, and are thereby regulated by Nrf2

[26, 29-34]. The common property of these genes is that they all code for proteins involved in the protection against oxidative stress [35, 36].

Nrf2 is continuously synthesized in the cell, but the basal Nrf2-induced transcription is kept relatively low under normal conditions, since the majority of Nrf2 is repressed by the inhibitor kelch-like ECH-associated protein 1 (**Keap1**) [37, 38]. Keap1 is a redox-sensing protein containing cysteines with potentially reactive thiol groups. The human Keap1 contains 27 cysteines, of which several are biologically active. When free radicals and other RS react with Keap1 cysteines [39-42], the Keap1 conformation is altered. Nrf2 repression is then lost, resulting in accumulation of Nrf2 in the nucleus where the expression of Nrf2 response genes is induced [43-45]. Examples of Nrf2-regulated proteins are Heme oxygenase-1, NAD(P)H:quinone dehydrogenase 1 and GSH-regulating proteins (see below).

1.3.1 Glutathione (GSH)

Glutathione (**GSH**) is an abundant antioxidant protein, existing in mM concentrations in the cells. GSH is endogenously produced from the three amino acids glycine, glutamatic acid and L-glycine and functions as an electron donor in redox reactions. Reduction of the GSH thiol forms a reactive GS-S molecule. GS-S is immediately reduced by another GSH molecule to form GS-SG. Under unstressed conditions, GS-SG is reduced back to GSH by glutathione reductase. Several of the enzymes necessary for GSH synthesis, including the rate-limiting glutamate cysteine ligase subunits (modifying and catalytic), are regulated by Nrf2 [46, 47]. Consequently, Nrf2 activation increases the basal levels of GSH through increased transcription of GSH precursors. Nrf2 activation is also expected to increase the reduction capacity of GSH due to increased levels of glutathione reductase [48], the enzyme responsible for reducing oxidized GS-SG back to the reduced GSH form.

In paper I of this thesis, mRNA levels of genes involved in GSH synthesis were measured as an indicator of Nrf2 activation. Increase in GSH protein levels was also used as a measure of the Nrf2 response

1.3.2 NAD(P)H:quinone dehydrogenase 1

NAD(P)H:quinone dehydrogenase 1 (denoted **NQO1** in human and **Nqo1** in rat/mouse) confers cytoprotection through two-electron reduction of quinones to hydroquinone using NADH or NADPH, thereby avoiding formation of toxic semiquinones [49-52]. The *NQO1/Nqo1* promoter contains an ARE and

the gene is regulated by Nrf2 [26, 53]. Nqo1 is upregulated by broccoli seeds and sulforaphane treatment in an Nrf2-dependent manner [54].

In paper I and II, NQO1/Nqo1 mRNA and protein levels were studied after Nrf2 activation by sulforaphane.

1.3.3 Heme oxygenase–1

Heme oxygenase-1 (denoted **HO-1** or HMOX1 in human and **Hmox1** in rat/mouse) is another well characterized, cytoprotective protein regulated by Nrf2 [55-57]. HO-1 is an enzyme involved in heme degradation, catalyzing the conversion of heme to biliverdin. Biliverdin is subsequently conversed to bilirubin, which is a protein with antioxidant potential. During heme break down, carbon monoxide (CO) and iron (Fe^{2+}) are released. HO-1 is highly induced by Nrf2 stimulation with sulforaphane [58, 59].

In this thesis, HO-1 mRNA and protein levels were studied after sulforaphane stimulation of astrocytes (paper I) or fibroblasts (paper II).

1.4 NRF2 ACTIVATORS

Numerous substances from fruits and vegetables, so called **phytochemicals**, have been suggested to interfere with Keap1 cysteines and induce Nrf2-mediated transcription of genes harboring the ARE in their promoter (see [60] for a review). An example of a phytochemical associated with Nrf2 upregulation and disease protection in a number of studies is the isothiocyanate sulforaphane from cruciferous vegetables (see below) [54, 61-68]. Synthetic derivatives of natural phytochemicals are widely used in experimental setups and are also tested in clinical trials for treatment of various conditions. For example, the synthetic triterpenoid bardoxolone methyl is currently tested for chronic kidney disease in type 2 diabetes patients [69] and dimethyl fumarate was recently tested in a placebo-controlled phase III trial for relapsing multiple sclerosis [70].

Phytochemicals are commonly synthesized as a part of the plant defence [20]. The cellular response to many exogenous substances can be described with the term **hormesis** [71-73]. The hormetic response describes a dose-dependent increase in beneficial effects up to a certain concentration of the substance. Thereafter the benefits decline to a point after which the negative effects dominate [74] (figure 2). Even though many Nrf2 activators have antioxidant properties, research has not provided convincing evidence that supplementary intake of antioxidants is beneficial [17-19]. Instead, the

protective effects of substances from vegetables and fruits may be due to their reactive nature.
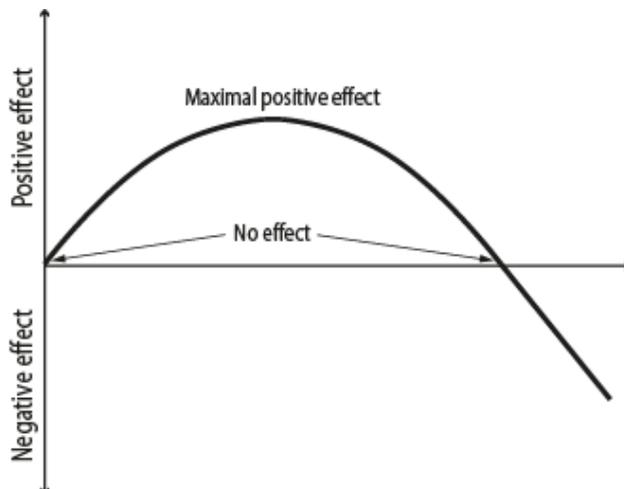
Figure 2. The concept of hormesis. The positive effects of a substance increases with dose up to a certain point, above which the positive effects decline with dose. Too high doses have a negative effect.

1.4.1 Sulforaphane

Sulforaphane is a substance naturally occurring in cruciferous vegetables and can be referred to as a phytochemical due to its well-known therapeutic effects. It is formed from the precursor glucoraphanin by the enzyme myrosinase. In an intact cell, glucoraphanin is kept protected from myrosinase, but if the plant cells are damaged, such as upon chewing, the two are mixed and the isothiocyanate sulforaphane can be produced [75]. Sulforaphane has been shown to interact with Keap1 thiols and protect from disease by Nrf2 mediated gene expression [39, 64, 76]. Importantly, sulforaphane was shown to cross the blood brain barrier and protect mice against brain inflammation in an Nrf2-dependent manner [77].
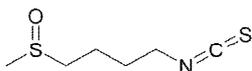
Figure 3. Chemical structure of the isothiocyanate sulforaphane.

1.5 MOLECULAR GENETICS

1.5.1 Genetic variation

Single nucleotide polymorphism

A single nucleotide polymorphism (**SNP**) is a variation in a single DNA base-pair within a population. This means that two individuals can have different bases on a specific position in the DNA. SNPs occur frequently and are more common in non-coding regions than in coding regions. If an SNP is situated in an exon it could alter the codon and subsequently the amino acid sequence of the resulting protein. Although an SNP could theoretically be either A, T, C or G, most often there are only two different variants. The alternative variants of a gene are called **alleles**. An individual can have two copies of either allele (homozygous carriers) or one copy of each (heterozygous carriers). The combination of alleles an individual carries is called a **genotype** [78] (figure 4).
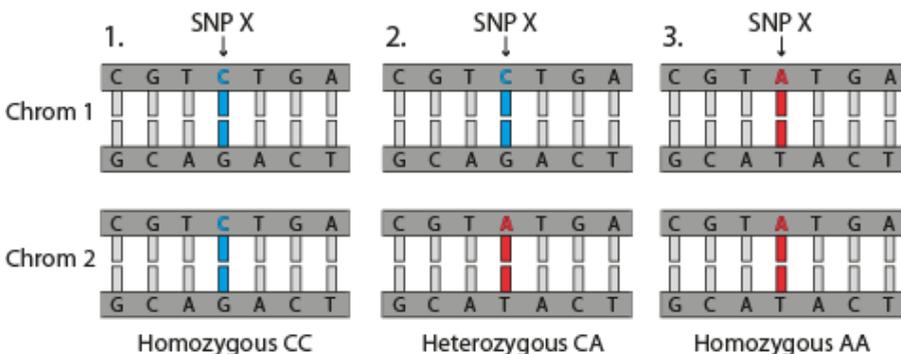
Figure 4. Single nucleotide polymorphisms. In this example there are two possible alleles - SNP X can be either C or A. Individual 1 is homozygous for the C allele (genotype CC), individual 3 is homozygous for the A allele (genotype AA), while individual 2 is heterozygous (genotype CA).

Tag SNPs

During meiosis, DNA regions in close proximity of each other are less likely to be broken up by recombination and therefore the probability that certain SNPs are inherited together can be predicted. This phenomenon is called linkage disequilibrium (LD) and is an advantage in genetic studies, since genotyping of only a few SNPs is sufficient to cover the total common genetic variation of a gene with high certainty [79]. SNPs used to tag for the total genetic variation of a gene in this fashion are called **tag SNPs**. The tag SNPs used in a study may not be biologically important for the studied

disease, but they could tag for other genetic variations that are [78, 80] (figure 5).

Figure 5. The concept of tag SNPs. Some regions of the DNA have been conserved during evolution and are likely to be inherited together. This is used in genetic studies, since the genotype in one SNP, a Tag SNP, can be used to predict the genotypes in other SNPs in LD with the tag SNP.

Haplotypes and haplotype windows

Genotyping of tag SNPs can be used to screen a gene to identify which area, if any, of the gene that is associated with a certain disease. The tag SNPs can tag for different types of genetic variation, such as other SNPs, insertions or copy number variations, which possibly affect gene transcription and consequently disease pathogenesis. The study of combinations of consecutive SNPs rather than single SNPs may be a better way to investigate the impact of a gene on a disease, since it covers a larger segment of the gene. The combination of neighbouring SNPs on the same chromosome is called a **haplotype**. Again, an individual can be homozygous or heterozygous for a certain haplotype. In the example in figure 6 below, one SNP position out of three is altered creating three possible genotypes: AGC/AGC, AGC/AGA or AGA/AGA. When all the possible combinations of different SNPs on all three positions are taken into account, a more complex image appears. Increasing the number of SNPs also increases the possible combinations of SNPs of that window (i.e. the number of possible haplotypes). However, because of LD patterns discussed earlier, a few haplotypes will be distributed over the majority of the population and the remaining haplotypes will each exist only in a few individuals [78, 80].
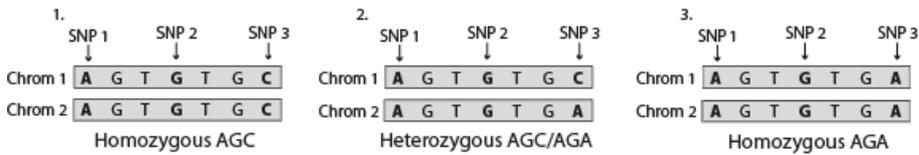
Figure 6. The concept of haplotypes. In this example, individual 1 is homozygous for haplotype AGC. Individual 2 carries an SNP on position 3 on one chromosome and is heterozygous for the haplotypes AGC/AGA. Individual 3 has the SNP on position 3 on both chromosomes and is homozygous for the AGA haplotype.

1.5.2 Genetic association studies

The International HapMap Project is an attempt to map common genetic variations, the linkage between them and their frequencies in African, Asian and European populations. Since this information is publically available, it provides a useful tool in genetic studies aiming at finding new genetic factors in common diseases [78]. Genome-wide association studies (GWAS) are commonly used today to screen the DNA for disease-gene associations without a pre-existing hypothesis, while a candidate gene approach is used to investigate the genetic association of a specific gene with a disease based on previous knowledge. In paper III and IV of this thesis, haplotypes in the candidate genes *NFE2L2* and *KEAP1* were analysed for associations with the two neurodegenerative diseases PD and ALS. To be able to investigate various combinations of SNPs and haplotypes of different sizes, a **sliding window** approach was used. In haplotype studies, sliding window means that all possible combinations of consecutive SNPs of interest are analyzed for associations with a disease. The SNPs are grouped into **haplotype windows** of different sizes and analyzed, sequentially moving forward along the gene one SNP at a time. The first analysis includes a window of two SNPs and the window size is successively increased in order to finally include all the SNPs investigated in the study (see figure 7 for an overview).

SNP 1   SNP 2   SNP 3   SNP 4
GENE

Haplotype window 1
(2 SNPs)
Step 1
Step 2
Step 3

SNP 1   SNP 2   SNP 3   SNP 4
GENE

Haplotype window 2
(3 SNPs)
Step 1
Step 2

SNP 1   SNP 2   SNP 3   SNP 4
GENE

Haplotype window 3
(All SNPs)
Step 1
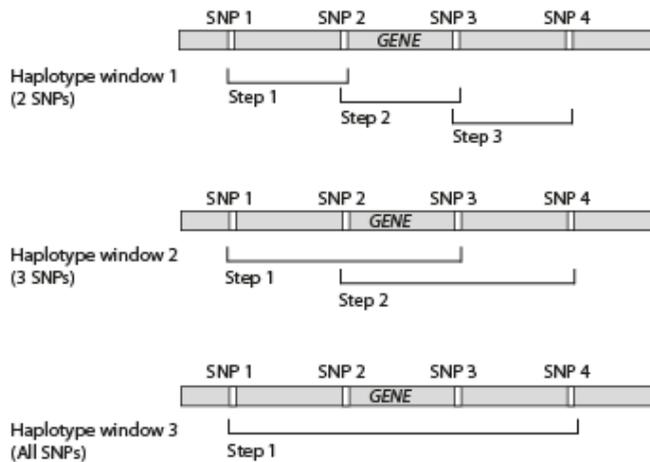
Figure 7. The sliding window approach in genetic association studies. The combination of consecutive SNPs is analyzed for associations with the disease, sequentially moving along the gene one SNP at a time. The haplotype window is increased with one SNP for each analysis.

Statistical significance

When testing an experiment statistically, the null hypothesis means there is no association between the investigated parameters. Rejection of the null hypothesis at a certain significance level is the basis of statistical significance testing. The **significance level** is usually set to 0.05 or 0.01, meaning that the risk of rejecting the null hypothesis if it is true (false positive or type I error) is less than 5 or 1 % respectively. Thus, the risk of making a type I error increases with the number of tests performed. In a genetic association study a type I error would mean concluding that there is a disease association with a genotype, even when there is not.

The probability value (*p*-value) of a significance test is the probability to achieve as extreme data as the observed data, if the null hypothesis is true. In a genetic association study, this would mean; concluding that there is no disease association with a genotype, even when there is (false negative or type II error). Thus, if the *p*-value is larger than the significance level, the null hypothesis is rejected. The **power** of a test is the probability to reject the null hypothesis when it is false. The power depends on the strength of the association as well as the sample size.

Correction for multiple testing

Correction for multiple testing is necessary to decrease the risk of type I errors in a study where many statistical analyses are performed. A general purpose method is **Bonferroni correction**, where the *p*-values are simply multiplied with the number of analyses performed. However, when the tests are as highly correlated as in haplotype sliding window analysis, this method is overly conservative. Instead, **permutation tests** can be used. In permutation tests the genotypes and phenotypes are randomly shuffled 10 000 times. The corrected p_c-value reflects how many simulated *p*-values that are smaller than the observed *p*-value. The ultimate way to test if an identified association is true is **replication** in additional populations [81].

Odds ratio

The odds ratio (**OR**) is used to describe the difference in risk of an outcome, e.g. a disease, between two groups where one group is exposed to a certain factor while the other group is not exposed. In genetic association studies investigating the association of a disease with a certain genotype, the OR describes the risk of getting the disease in individuals with the genotype compared to individuals without the genotype. An OR = 1 indicates there is no difference between individuals in the two groups, an OR > 1 indicates an increased risk, while an OR < 1 indicates decreased risk.

1.5.3 Genetic variation in *NFE2L2* and *KEAP1*

NFE2L2

NFE2L2, the gene coding for the transcription factor Nrf2 [25], is located on chromosome two. The gene is 34 kbp long and contains five exons. Nrf2 has three isoforms, meaning that the mRNA can be spliced in different ways to code for three variants of the protein, differing at the N-terminus. In this thesis, eight tag SNPs covering the common genetic variation of *NFE2L2* were analysed for associations with PD (paper III) and ALS (paper IV).

dbSNP does not report *NFE2L2* coding SNPs with a frequency > 5 % in the European (CEU) population. However, SNPs in non-coding regions like promoter or enhancer elements could also affect the properties or the efficiency of a gene. Three such SNPs were found in the upstream promoter region of the *NFE2L2* gene [82]. These SNPs (-653A>G, -651G>A and -617C>A) were shown to affect Nrf2 protein levels [83, 84]. The three functional SNPs in the Nrf2 promoter have since the discovery been associated with a number of diseases [83-89]. SNP -617 is situated in an ARE of the Nrf2 promoter and the minor SNP allele (-617A) was specifically found to decrease Nrf2 protein levels, resulting in enhanced risk of lung cancer [90].

In this thesis, the three functional promoter SNPs and eight tag SNPs were investigated for associations with PD (paper III) and ALS (paper IV) (figure 8).
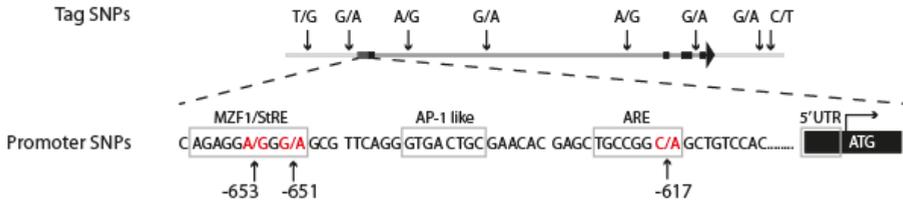
Figure 8. The tag SNPs and promoter SNPs in *NFE2L2* analyzed for associations with PD and ALS. Eight tag SNPs covered the common SNP variation in *NFE2L2*. Three functional SNPs are located in the promoter region. *Adapted from von Otter et al. 2010 [91] and Marczak et al.2012 [83].*

KEAP1

KEAP1 encodes the Nrf2 repressor protein Keap1 [38]. *KEAP1* is a 17 kbp long gene located on chromosome 19, and contains seven exons. *KEAP1* codes for two differently spliced mRNAs, which are both translated into identical proteins. The common genetic variation of Keap1 is covered by three tag SNPs, which were analyzed here for associations with PD (paper III) and ALS (paper IV). One of these SNPs is found in exon two and one is located in exon four, but neither SNP changes the amino acid sequence of Keap1. One *KEAP1* SNP, located in the intron between exon three and four, has previously been associated with increased lung capacity in a population-based cohort study [89] (figure 9).
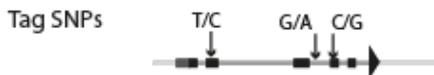
Figure 9. Three tag SNPs cover the SNP variation of the *KEAP1* gene. Two synonymous SNPs are located in exon two and four and one SNP is located in the intron between exon three and four. *Adapted from von Otter et al. 2010 [91].*

1.6 NRF2/KEAP1 IN DISEASE

Nrf2 primarily regulates the inducible defence against oxidative stress and Nrf2 and Keap1 are not essential for survival. Nrf2 deficient mice survive without an apparent phenotype, but are more susceptible to environmental insults and more prone to develop a range of diseases suggested to be influenced by oxidative stress [92-96]. Keap1 deficient mice die from starvation due to hyperkeratosis in the upper digestive tract, but mice lacking both Nrf2 and Keap1 survive [97] indicating that Keap1 deficiency is lethal due to constitutive Nrf2 activation. A slightly altered efficiency of the Nrf2 system would hence rather be apparent over time and *NFE2L2* and *KEAP1* should probably be regarded as potential **risk genes** for disease rather than disease-causing genes. This means that carrying certain variants of the genes could increase the risk of a certain disease, given that one or several other conditions are fulfilled. Those could be the combination of several risk genes or exposure to certain environmental factors.

1.6.1 Nrf2/Keap1 in Parkinson's disease

Parkinson's disease (PD) is a neurodegenerative disease affecting the central nervous system. PD is defined by motor symptoms, such as rigidity, slowness of movement, postural instability and a characteristic resting tremor. The motor symptoms result from a decrease in the neurotransmitter dopamine in the brain, due to extensive cell death of dopamine-producing cells in substantia nigra. Another important feature is the presence of aggregates of α-synuclein (Lewy bodies) in neurons. Mutations in several genes have been found in families with hereditary PD. However, the majority of PD cases are sporadic (idiopathic, without known cause) and age is the major known risk factor [98, 99] Oxidative stress may increase with an age-dependent decline in oxidative stress defence and oxidative stress likely plays an important role in PD pathogenesis [100, 101]. Several studies have suggested Nrf2 to be involved in the PD pathogenesis [102]. For example, the PD-associated protein DJ-1 has been shown to prevent Nrf2-Keap1 interaction, leading to Nrf2 activation [103]. Coffee and tobacco have been shown to protect from PD in epidemiological studies [104, 105] and recently, a study showed that substances from coffee and tobacco increased cell survival of neurons in two fly models of familial PD due to Nrf2 activation [106]. Intraperitoneal administration of sulforaphane has also been shown to protect against MPTP-induced cell death in nigral dopaminergic neurons through Nrf2-dependent upregulation of Hmox1 and Nqo1 [107]. A new model of sporadic PD was introduced recently, using cells derived from the olfactory mucosa of PD patients [108]. Using this model, it was shown that NQO1 and GSH levels

were decreased in PD patient-derived cells, but could be restored by sulforaphane-mediated Nrf2 activation [109]

1.6.2 Nrf2/Keap1 in amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease affecting the upper and lower motor systems. Mutations in twelve genes have been found to cause the inheritable form of ALS (familial ALS, FALS), of which mutations in the *SOD1*, *FUS* and *TRDBP* genes are the most common. The disease progression is rapid (mean survival time is 3-5 years) and approximately 95% of all ALS forms are considered isolated or sporadic (SALS). However, besides a lower age at onset of the inheritable form, SALS and FALS are clinically indistinguishable and all the genes found mutated in FALS have also been found in SALS. Familial ALS can be inherited in autosomal dominant, autosomal recessive and X-linked patterns and FALS rates are possibly underestimated due to low penetrance of the disease [110]. Riluzole is an inhibitor of glutamate uptake and the most efficient ALS treatment today. Even though riluzole has shown neuroprotective effects, it primarily delays the need of ventilator care and at best increases survival by 3-5 months [111, 112]. A mouse model overexpressing the human mutated *SOD1* G93A is widely used to mimic ALS. Several studies using this model have suggested Nrf2 as an important factor in ALS pathogenesis and the expression pattern of Nrf2 and Keap1 are altered in the brain and spinal cord of ALS patients [113, 114].

2 AIM

2.1 Overall aim:

The aim of the studies described in this thesis has been to investigate the Nrf2 response after repeated, short stimulations with sulforaphane, simulating the expected brief stimulation after regular broccoli intake. Furthermore, genetic variations in the Nrf2- and Keap1-encoding genes were investigated for associations with PD and ALS.

2.2 Aim of the individual papers:

1. To investigate how brief, repeated stimulations with sulforaphane affect the Nrf2 response in astrocytes, in order to test how intermittent intake of Nrf2 activators can protect from disease.

2. To investigate if Nrf2 activation in human fibroblasts can be amplified by repeated transient stimulation with sulforaphane, to render fibroblasts more resistant to ionizing radiation.

3. To investigate if common genetic variations in the genes encoding Nrf2 and Keap1 influence risk and/or progression of Parkinson's disease.

4. To investigate if common genetic variations in the genes encoding Nrf2 and Keap1 influence risk and/or progression of amyotrophic lateral sclerosis.

3 METHODOLOGICAL OVERVIEW

3.1 ETHICS

Patients and controls

The human studies of this thesis were approved by the Regional Ethics Committee at the University of Gothenburg, Sweden or the Ethics Committee of the Pomeranian Medical University, Szczecin, Poland (paper III) and the Regional Ethical Review Board for northern Sweden (Umeå) (paper IV). The studies were conducted in accordance with the Helsinki Declaration of 1975 and informed consent was given from all patients and control subjects.

Primary rat astrocytes

The experimental protocol was approved by the Ethical Committee of the University of Gothenburg (paper II).

3.2 STUDY SUBJECTS

3.2.1 Parkinson's disease

Initially, 165 Swedish patients diagnosed with idiopathic PD (according to the Parkinson's Disease Society Brain Bank [115] and 190 Swedish control subjects were included in the study. All individuals were of Caucasian origin. PD patients with an age at onset of <50 years were screened for PD-causing mutations in the DJ-1, Parkin, PINK1 and LRRK2 genes [116, 117].

The Polish replication study consisted of 192 PD patients and 192 sex-matched control subjects. To minimize the risk of control subjects developing PD later in life, they were chosen to be of as high age as possible. All individuals were of Caucasian origin and had no familial aggregation of PD.

3.2.2 Amyotrophic lateral sclerosis

The study included 522 patients diagnosed with sporadic ALS and 564 control subjects. For subgroup analysis, the patients were categorized according to sub-diagnosis; amyotrophic lateral sclerosis (*ALS*, n = 324), progressive bulbar palsy (*PBP*, n = 173), progressive muscular atrophy (*PMA*, n = 24) or primary lateral sclerosis (*PLS*, n = 1). The patients were diagnosed according to established criteria for ALS [118] and subjects with known familial ALS (defined as having a biological relative with ALS within

three generations) were excluded from the study. All subjects were of Caucasian origin and all patients, when applicable, were followed until death.

3.3 CELL CULTURES

Cell cultures are widely used in preclinical research and provide a convenient tool to study the response of a specific cell type to various stimuli or stresses. Yet, a cell culture is a simplified model of an organism, where interactions and signalling between different cell types and tissues are lost. The oxygen tension in cell cultures is also much higher than in tissue, which poses a problem when studying oxidative stress. Immortalized cell lines are often used because they are easy to keep in culture. Primary cells have an advantage over cell lines, since they are more likely to represent *in vivo* situations. However, since primary cells have a limited life span in cell culture, experiments are often repeated on cells from several individuals and the results may therefore reflect between-individual variations.

3.3.1 Primary rat astrocytes

Astrocytes are the most abundant cell type in the brain. They interact closely with neurons and supply them with energy metabolites and structural support [119, 120] and they are also active in the antioxidant defence of the brain. Compared to neurons, astrocytes are more sensitive to Nrf2 inducers and they provide neurons with cysteine and glycine necessary for GSH synthesis [121]. Astrocytes are proposed to play a role in neurodegenerative diseases, like ALS and PD. Nrf2 activation in astrocytes have been shown to protect neurons against degeneration in mouse models of these diseases [114, 122].

In paper I, the kinetics of the Nrf2 response after sulforaphane stimulation was investigated in primary rat astrocytes from newborn Sprague Dawley rats (an outbred multipurpose breed of albino rat used extensively in medical research).

3.3.2 Primary human fibroblasts

Fibroblasts are the most common cell type in connective tissue. Primary human cells can be complicated to achieve, but since fibroblasts are abundant in skin, they are easily accessible compared to other cell types.

The human primary fibroblasts, used in paper II to investigate the effect of Nrf2 activation in normal cells before exposure to ionizing radiation, originated from foreskin of a newborn and were purchased from ATCC.

3.3.3 Mouse embryonic fibroblasts

The possibility of developing knockout mice (lacking one or more target genes) through inbreeding has made the use of mouse-derived cell cultures widespread. Knockout cells can be used to investigate if a certain response is dependent on the studied gene. In that type of study, the experiment is performed on wild type cells (cells with an intact gene) in parallel with the knockout cells. Since all other features are supposedly consistent between the knockout and wild type cells, the results will indicate if the target gene is involved in the investigated process or not. However, the response of a gene may vary between species. The study of a gene response in mouse-derived cells is therefore not necessarily a good model for the response in human cells.

In paper II, we used a mouse embryonic fibroblast (MEF) cell type lacking a functional Nrf2 gene [26], to investigate if the increased radioresistance that we observed after sulforaphane treatment depended on a functional Nrf2 response.

3.4 OXIDATIVE STRESS GENERATION

3.4.1 Xanthine/Xanthine oxidase (X/XO)

As discussed in the introduction, the transformation of X to uric acid by XO is an endogenous source of RS. Treatment of cell cultures with X and XO introduces both superoxides, hydrogen peroxides and hydroxyl radicals and is a traditional way to induce oxidative stress in cell culture experiments [7].

X/XO was used in paper I to investigate if sulforaphane pretreatment could protect rat astrocytes from radical-induced cell death.

3.4.2 Ionizing radiation

Ionizing radiation (IR) is widely used in cancer therapy due to its ability to introduce cytotoxic DNA double-strand breaks. IR induces DNA damage through production of free radicals, either in direct action or through the ionization of water [123].

In paper II of this thesis, IR was used to induce DNA damage in primary human fibroblasts, in order to determine if Nrf2 activation could protect normal cells from IR exposure at doses commonly used in the clinic during radiotherapy.

3.5 NRF2 ACTIVATION

3.5.1 Sulforaphane

The isothiocyanate sulforaphane from cruciferous vegetables is a well-established Nrf2 activator. Broccoli as well as pure sulforaphane has been shown to induce the Nrf2 system both *in vitro* and *in vivo* [54, 64, 65], leading to increased radical protection.

Sulforaphane was used in paper I and II to induce the Nrf2 response in cell cultures.

3.6 ASSESSMENT OF NRF2 ACTIVATION

Nrf2 is translated continuously, but under normal conditions its activity is regulated through continuous degradation of the Nrf2 protein [37, 38, 124]. Because of this condition, there is only a limited increase of Nrf2 expression levels when Nrf2 is stimulated and Nrf2 gene expression is therefore unsuitable as a measure of Nrf2 activation. Instead, the expression of genes regulated by Nrf2 – Nrf2 response genes – is expected to increase when cells are exposed to Nrf2 activators and can be used to assess Nrf2 activation. Here, the two well-established Nrf2-regulated genes HO-1/Hmox1 [36] and NQO1/Nqo1 [26] were used as indicators of Nrf2 activation.

Upon Nrf2 activation, the intracellular levels of the radical scavenger GSH are expected to increase, since Nrf2 regulates the expression of several GSH precursor proteins [46, 47]. The GSH levels can therefore be used as an indirect measure of Nrf2 activation.

Nrf2 activation of the cellular defence presumably increases the cell's capacity to clear reactive species; the levels of peroxides inside the cells after a peroxide insult can thus be used as a marker of increased resistance to oxidative stress.

3.6.1 Gene expression

mRNA levels

As mentioned earlier, transcription of a gene results in a single-stranded copy of the gene - an mRNA. The amount of a certain mRNA reflects how frequently a gene is transcribed under the specific conditions. In this thesis, pure mRNA was extracted from the cells before and after treatment with Nrf2 activators, using poly d(T)-covered magnetic beads in high access. Every mRNA strand ends with multiple Adenine (A) bases. Since A base-pairs with Thymine (T), the mRNA tails will attach to the magnetic beads and can be pulled out using a magnet. After mRNA purification, the sample contains all mRNA from the cells, representing the gene transcription at the moment when the cells were harvested. To measure the amount of mRNA transcribed from a specific target gene, a method called quantitative polymerase chain reaction (**qPCR**) is applied [125]. In the initial step of qPCR, the unstable mRNA molecules are converted to more stable, double-stranded DNA (complementary DNA or cDNA) using an enzyme called reverse transcriptase (reversing mRNA back to DNA). After reverse transcription, the cDNA represents double-stranded DNA copies of the coding regions of all genes that are transcribed at the moment of cell harvest and constitute the foundation for PCR amplification. In qPCR, the doubling of DNA is visualized after each thermal cycle, using a fluorescent probe. The probe is specific for the region of interest, and the increase in fluorescence reflects the increase in DNA. The probe is labeled with a fluorescent reporter dye at one end and a quencher at the other end. As long as the probe is intact, the quencher is close enough to the reporter dye to quench the fluorescence. When DNA polymerase works its way along the DNA and encounters the probe, the probe is cleaved resulting in a fluorescence signal reflecting the gene expression from the target gene [126, 127]. In this thesis, the increase in gene expression was calculated using the $\Delta\Delta C_T$-method [128]. The principal behind this method is that the increase in fluorescence from the target gene is related to a reference gene, which is supposedly not affected by the test procedure. The reference gene is measured in each sample along with the target gene. Subtracting the fluorescence intensity for the reference gene from the target gene gives a relative increase value for each sample. This value is then compared between samples and correlated to a calibrator sample, often a non-treated control sample.

qPCR was used in paper I and II, to assess gene expression of target genes after sulforaphane treatment. RNA polymerase II was used as reference gene and cells treated with DMSO only were used as calibrators for sulforaphane treatment.

Protein levels

Immunoblot (or western blot) is a method for protein quantification, including several steps. The most common experimental procedure for immunoblot is summarized below:

1. **Cell lysis** using a buffer containing a detergent and proteinase inhibitors.

2. **Protein separation** depending on size, using gel electrophoresis.

3. **Transfer** of the proteins to a nitrocellulose membrane using an electric current, to make them accessible for antibody binding.

4. **Blocking** of unspecific antibody binding to the membrane by soaking it with bovine serum albumin or powdered milk solution.

5. **Antibody labelling**. A) A primary antibody is used for specific binding to the target protein. B) A secondary antibody labelled with a reporter enzyme is added for unspecific binding to the primary antibody.

6. **Detection**. The reporter enzyme creates luminescence in a chemical reaction. The luminescence can be detected using photographic film or a digital camera and analyzed as relative optical density.

The efficiency of immunoblot depends a lot on the size of the analyzed protein. The transfer is usually the limiting step and large proteins are more difficult to transfer than small proteins. To eliminate transfer errors, an internal control protein is usually included in the procedure. The luminescence intensity from the target protein is related to the internal control, and the relative amount of the target protein can be estimated.

Immunoblot was used in paper I to measure protein levels of Hmox1 and Nqo1 in astrocytes after sulforaphane stimulation.

Nrf2 knock down

Small interfering RNA (siRNA) can be used to knock down the mRNA expression of a specific gene. The method utilizes the cells own system to regulate double-stranded RNA, by introduction of short RNA strands that are complementary to the target mRNA. The siRNA strands will base-pair with the target mRNA producing short, double-stranded RNA molecules. The presence of short double-stranded RNAs will trigger the cellular RNAi machinery, leading to elimination of the mRNA and a resulting decrease in expression of the target gene [129].

In paper I, this method was used to knock down Nrf2 in order to investigate the role of Nrf2 in sulforaphane-induced upregulation of cytoprotective genes.

3.6.2 Glutathione levels

Nrf2 activation is expected to increase GSH intracellular levels due to increased gene expression of GSH precursor proteins. To assess the effect of Nrf2 stimulation with sulforaphane on astrocytic intracellular GSH levels (paper I), a monochlorobimane (MCB) probe was used. The non-fluorescent MCB forms a fluorescent conjugate with reduced GSH in a reaction catalyzed by glutathione S-transferase (GST). The fluorescence intensity from the MCB-GSH conjugates can then be measured using fluorescence microscopy and compared to non-treated control samples [130].

3.6.3 Peroxide levels

Changes in peroxide production in cells can be assessed with the non-fluorescent probe carboxy-H2DCFDA (5-(and-6)-carboxy-2-7-dichlorodihydrofluorescein diacetate) [131]. Carboxy-H2DCFDA easily diffuses through the cell membrane into the cytoplasm. There it is cleaved by esterases, resulting in polarized dichlorofluorescein carboxy-DCFH. Carboxy-DCFH is still non-fluorescent, but due to its changed chemical properties, it can no longer cross the cell membrane and is trapped in the cell. Inside the cell, carboxy-DCFH is oxidized by peroxides to form the fluorescent carboxy-DCF. Peroxide levels can then be measured as fluorescence using a spectrofluorimeter.

This method was used in paper I to assess the peroxide production in sulforaphane-treated astrocytes following hydrogen peroxide insult.

3.6.4 DNA damage response

One of the first cellular activities after a DNA double-strand break (DSB) is formed is the phosphorylation of histone H2AX at serine 139 (γH2AX). The phosphorylation spreads to neighbouring H2AX molecules flanking the lesion. Within 10 minutes, thousands of H2AX molecules are phosphorylated, forming a γH2AX focus [132, 133]. Each γH2AX focus most likely represents a single DSB and can be used as a biomarker of DSB formation. A fluorescent antibody is used to label phosphorylated H2AX, to visualize the γH2AX foci [134]. The foci are either counted manually or with foci-counting software using fluorescent microscopy. Since γH2AX foci vary in size and shape, manual counting is more accurate. However, to count hundreds of cells from each sample is time consuming.

γH2AX foci were measured in paper II of this thesis to assess DSB formation in sulforaphane-treated human fibroblasts after irradiation.

3.6.5 Cell survival

Intracellular ATP levels

Adenosine triphosphate (ATP) is present in all metabolic processes in the cells. During apoptosis and necrosis, ATP levels drop rapidly as the cells lose the ability to synthesize new ATP and the ATP present in the cells is degraded by ATPases. ATP levels measured as fluorescence intensity using a luciferase-based bioluminescence assay can therefore be used as an early marker of cell death.

In paper I, ATP levels were measured in astrocytes to investigate if sulforaphane treatment could protect from peroxide-induced cell death.

Propidium iodide exclusion

Propidium iodide (PI) is a fluorescent nucleotide-binding dye widely used to measure cell viability. PI cannot cross the intact cell membrane, but leaks into apoptotic or necrotic cells. The PI combines with nucleotides and emits fluorescence that can be detected using fluorescence microcopy.

PI was used in paper I to measure late stages of cell death in sulforaphane-pretreated astrocytes after hydrogen peroxide insult.

EdU assay

5-ethynyl-2´-deoxyuridine (EdU) is a nucleoside analogue to thymidine, which diffuses into the cell nucleus where it is incorporated into newly synthesized DNA during S-phase. A fluorescent azide binds to the EdU, allowing for detection of dividing cells in a cell population using flow cytometry. The advantage of the EdU assay over the traditional BrDU assay using antibodies for detection, is that incorporated EdU can be labeled without denaturing the DNA molecule [135].

The EdU assay was used in paper II to measure the relative cell survival after irradiation in cells pretreated with sulforaphane.

3.7 GENETIC ASSOCIATION STUDIES

3.7.1 DNA Sequencing

Several methods to determine the exact sequence of nucleotides in a stretch of DNA, such as a gene, are used today. In this thesis, sequencing was used as follows; in the initial step, the target DNA was amplified using traditional PCR. Next, specific terminator nucleotides (diddeoxynucleotide triphosphates, ddNTPs) were added to the DNA sample along with the ordinary dNTPs necessary for DNA replication. Each of the four nucleotides (A, T, C and G) was labeled with a unique dye, making it possible to distinguish them from each other. During DNA amplification the ddNTPs are randomly inserted, leading to termination of DNA replication at random sites. This generates DNA fragments of all possible lengths, each with a nucleotide-specific dye at the 3'-end. The length of each fragment is determined using capillary gel electrophoresis and the fluorescence from the specific dye of the ddNTP of each fragment is analyzed with a laser at the end of the capillary [136, 137].

Sequencing was used in paper III and IV to genotype three functional SNPs in the *NFE2L2* promoter in PD patients, ALS patients and control subjects.

3.7.2 Allelic discrimination

Allelic discrimination (AD) is used to discriminate between different SNPs in a DNA sample. The chemistry used in AD is basically the same as in qPCR described earlier; only in AD two labelled probes are used. The probes are complementary to one SNP allele each, making it possible to distinguish between the genotypes in a DNA sample. The fluorescent probes are added to the PCR reaction and attach to the SNP regions of the DNA. During amplification of the DNA in the PCR reaction, the probes are cleaved by the heat-resistant DNA polymerase, leading to a fluorescent signal. Depending in the wave length emitted, it can be determined if an individual is homozygous for either SNP, or harbours one allele of either SNP [138, 139].

AD was used in paper III and IV to genotype tag SNPs in *NFE2L2* and *KEAP1* in PD patients, ALS patients and control subjects.

4 RESULTS AND DISCUSSION

4.1 PAPER I

Distinct induction kinetics of Nrf2-dependent genes

Astrocytes are highly involved in the defence against oxidative stress in the brain. The response to Nrf2 inducers is more pronounced in astrocytes compared to neurons and the neurons rely on astrocytes for supply of GSH metabolites [119-121]. Astrocytes are also proposed to be involved in brain injury and diseases like stroke and neurodegenerative diseases [114, 140, 141] and are therefore interesting as potential targets for Nrf2-stimulating therapy. However, much information is still needed before Nrf2-stimulating drugs can be used in the brain, such as the specific response after Nrf2 activation with a certain drug. Many Nrf2-activating compounds, such as sulforaphane, induce hormetic responses and are only beneficial up to a certain concentration. At higher concentrations, the benefits decrease in a dose-dependent manner until the effects are even adverse. Because of this property, it is crucial to find the correct dosing before these compounds are used in therapy. We examined the kinetics of some aspects of the Nrf2 response after stimulating primary rat astrocytes with sulforaphane.

The two well established Nrf2-regulated genes Hmox1 [55, 59] and Nqo1 [53, 54] have both been suggested to play important roles in brain injury and disease [49, 56], motivating kinetic study of the two genes in astrocytes. As expected, continuous treatment with 1-10 µM sulforaphane induced dose-dependent increase in gene expression of both Hmox1 and Nqo1 in rat astrocytes. We found, however, that the optimal treatment time for maximal induction using the same sulforaphane concentration, differed between the two genes. While Hmox1 gene expression started to increase more or less immediately when 10 µM sulforaphane was added to the culture medium, Nqo1 induction was delayed. Hmox1 reached its maximal mRNA expression levels after 12 hours, while Nqo1 still accumulated at 24 hours. This was also confirmed on protein level, since Hmox1 protein reached maximal levels at 24 hours, while Nqo1 protein still accumulated at 48 hours. Interestingly, Hmox1 levels started to decrease soon after maximal induction, showing signs of feedback regulation, while Nqo1 protein continued to accumulate (figure 10). The HO-1 promoter contains multiple ARE motifs, likely contributing to the rapid induction upon Nrf2 activation. Toxic Fe^{2+} is released during HO-1 action, why it is tempting to speculate that HO-1 enzyme activity may be in need of negative regulation. Carbon monoxide (CO) is also released during heme degradation [142]. CO has cytoprotective

functions in some tissues [143] and a recent study showed that induction of HO-1 and CO release increased NQO1 levels through activating Nrf2-dependent transcription [144]. This could possibly explain the delay in Nqo1 expression compared to Hmox1 observed in astrocytes and further supports the notion that Hmox1 activity needs fine-tuned regulation.
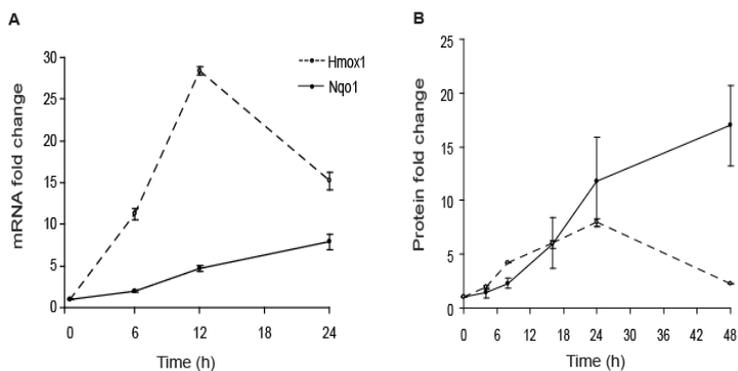
**Figure 10.** The two Nrf2-responsive genes Hmox1 and Nqo1 show different induction kinetics during 10 μM sulforaphane continuous treatment. Hmox1 mRNA levels (A) and protein levels (B) are maximally induced at 12 h and 24 h respectively and are then declining, while Nqo1 mRNA (A) and protein (B) is still increasing at 24 h and 48 h respectively. *Reprinted with permission from Bergström et al. 2011 [145].*

Brief sulforaphane stimulation leads to sustained protection against oxidative stress

Sulforaphane increases rapidly in both rat and human plasma after ingestion, reaching maximal levels already after approximately one hour. Decline of sulforaphane levels begins four hours after ingestion, with a half life of approximately two hours [146, 147]. We wanted to simulate this brief exposure expected after broccoli intake and see how it affected the Nrf2 response and the cellular capacity to withstand oxidative stress. Ten µM sulforaphane was added to the astrocyte culture media for 1-4 hours. Gene expression was measured at different time points after the sulforaphane was removed. We found that short stimulation with sulforaphane was enough to give sustained upregulation of Hmox1 and Nqo1 levels for several hours. When Hmox1 protein levels were measured fourteen hours after four-hour sulforaphane treatment, Hmox1 was still induced by 50 % compared to continuous 18 hour treatment (figure 11 A). Elevated GSH levels 20 hours after four-hour treatment corresponded with increased resistance to peroxide-induced cell death (figure 11 B, C). Closer examination of the Hmox1 and Nqo1 protein response after four-hour sulforaphane stimulation showed that the response correlated closely with continuous stimulation. Interestingly, the Hmox1 levels continued to accumulate for another 16 hours after the sulforaphane was removed from the media and Nqo1 protein still accumulated 48 hours after a four-hour treatment (figure 11 D, E).

These results suggest that brief sulforaphane stimulation, resembling the stimulation expected after a meal of broccoli, is enough to induce sustained protection against oxidative stress for at least 24 hours.
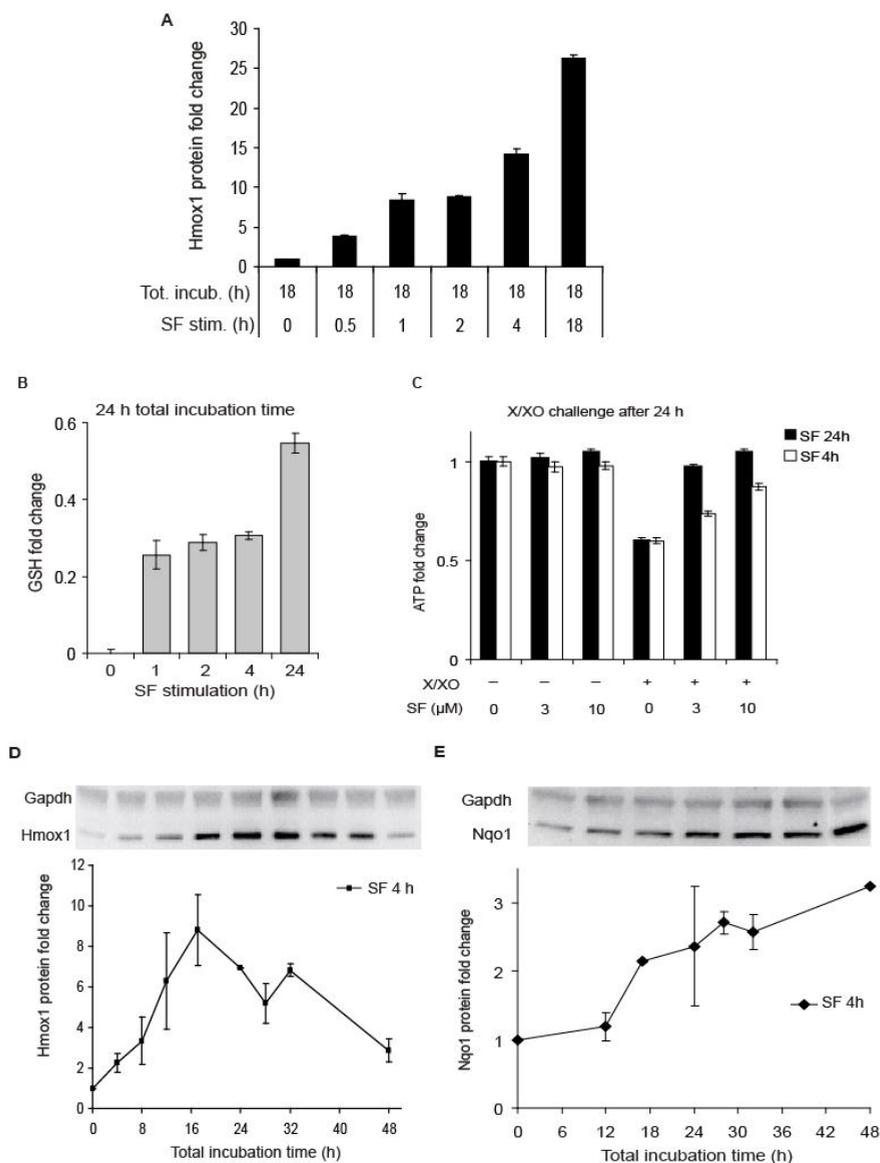
Figure 11. Short stimulation with sulforaphane is enough to induce sustained upregulation of the Nrf2 response. (A) Fourteen hours after four-hour stimulation with sulforaphane, Hmox1 protein is still induced to 50 % of the maximal induction capacity. This correlates with a sustained increase in GSH levels (B) and cell survival after peroxide challenge, as measured with ATP levels (C) 20 h after 4-h stimulation. Hmox1 protein levels increase up to 18 hours (D) and Nqo1 is still accumulating at 48 hours (E) after 4-h stimulation. *Reprinted with permission from Bergström et al. 2011 [145].*

Brief, daily sulforaphane stimulations mediate sustained protection against peroxide–induced cell death

Our data indicated that the Nrf2 response remains functionally elevated for more than 24 hours after brief sulforaphane treatment. We speculated that repeated brief stimulations could have a cumulative effect on the response. To test this, we treated rat astrocytes with 10 µM sulforaphane for four hours daily and measured the Nrf2 response. Under these conditions, Nqo1 mRNA and protein levels increased daily over a four-day period, resulting in an accumulation of Nqo1 over time (figure 12A-B). Because of the important role of Nqo1 in quinone detoxification, this could potentially increase the capacity of the astrocytes to resist oxidative stress. In contrast, Hmox1 mRNA and protein levels increased as expected after the first four-hour stimulation, but repeated treatment over the following days rather attenuated the Hmox1 response (figure 12C-D). GSH levels and protection from superoxides did not accumulate, but remained elevated after repeated sulforaphane stimulation (figure 12E-F).

In conclusion, repeated brief stimulations of the Nrf2 system with sulforaphane resulted in daily accumulation of Nqo1 and sustained upregulation of GSH and the resistance to peroxides. This suggests that brief, daily sulforaphane stimulations induce sustained protection against oxidative stress. However, the observed attenuation of the Hmox1 response following daily sulforaphane stimulations confirm the importance of finding the right dosing of Nrf2-stimulating drugs.

**Figure 12.** *Next page.* Brief, repeated stimulation with sulforaphane leads to daily accumulation of Nqo1 mRNA (A) and protein levels (B). Repeated stimulations attenuate the increase of Hmox1 mRNA levels (C) and diminish the induction of Hmox1 protein (D). GSH levels (E) and resistance to superoxides (F) remain elevated after repeated sulforaphane stimulations. *Reprinted with permission from Bergström et al. 2011 [145].*
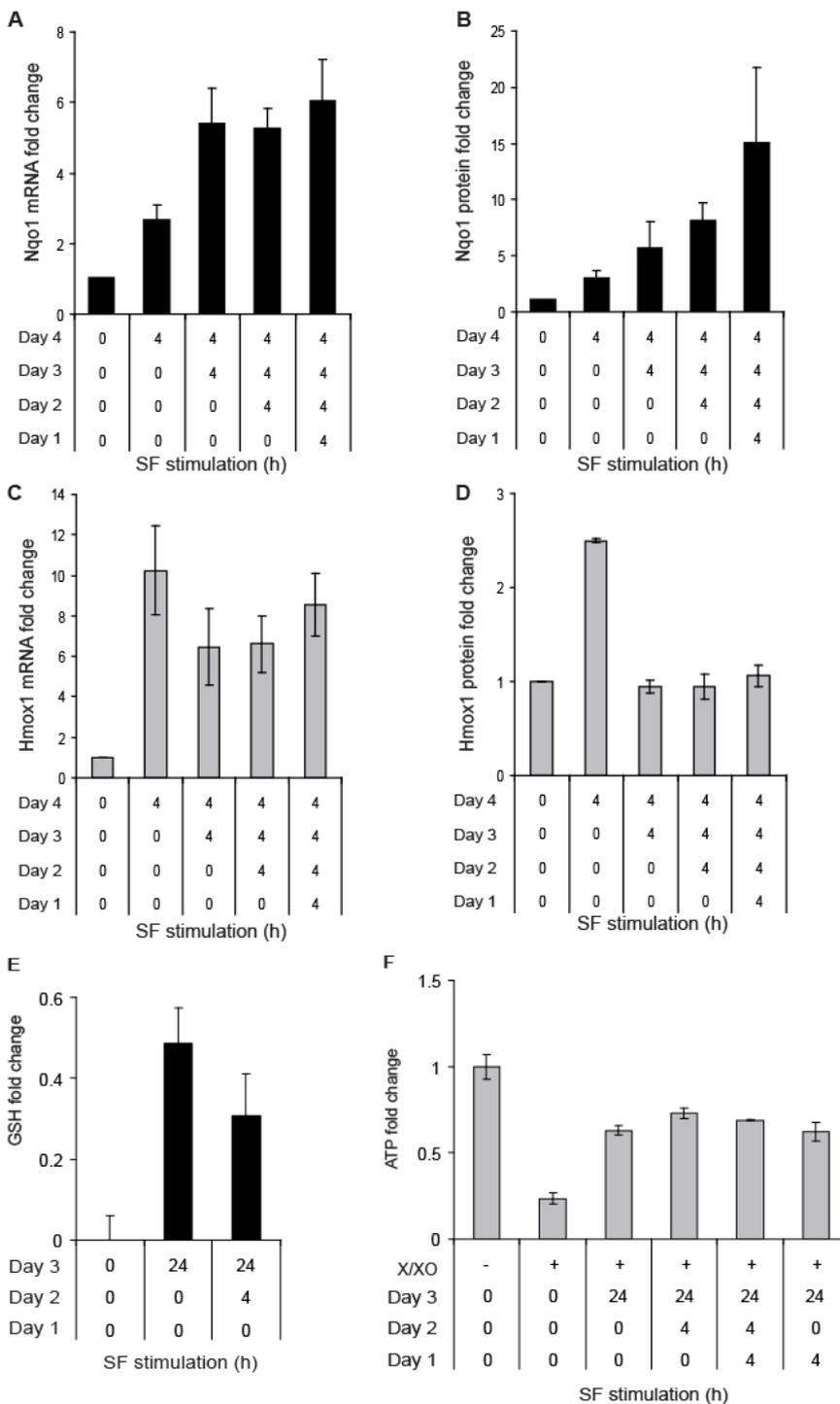
4.2 PAPER II

Repeated transient sulforaphane treatment induces hormetic radioresistance

Ionizing radiation (IR) induces toxic DNA double-strand breaks through introduction of RS and is commonly used in cancer therapy to kill off cancer cells [148]. To avoid severe side effects in radiosensitive patients, less sensitive patients may receive suboptimal radiation doses [149]. One way to protect normal tissue during radiotherapy would be to increase the oxidative protection in normal cells. With increasing radioresistance of normal cells, the radiation dose and the efficiency of radiotherapy could be increased. We wanted to investigate if repeated, daily sulforaphane treatment could improve the Nrf2 response in human primary fibroblasts and make them more resistant to ionizing radiation. We treated the cells with 0-30 µM of sulforaphane and measured cell survival after two or four Gy IR exposures. Single four-hour treatment did not increase cell survival significantly compared to non-treated control after irradiation. However, when the four-hour treatment was repeated for three consecutive days, sulforaphane concentrations up to 15 µM were increasingly protective. Higher concentrations seemed to decrease the beneficial effects of sulforaphane treatment, consistent with a hormetic response (figure 13).
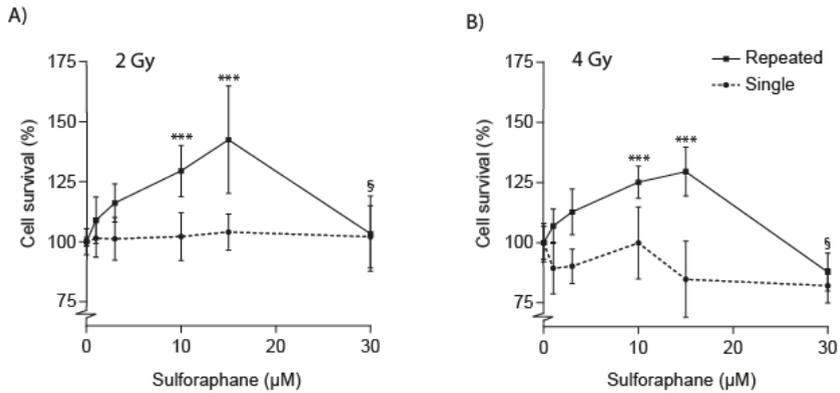
Figure 13. Repeated, brief stimulations with sulforaphane increase radioresistance in human primary fibroblasts. Treatment with 10 and 15 µM sulforaphane for four hours daily increases cell survival compared to non-treated control after both 2 Gy (A) and 4 Gy irradiation (B). At 30 µM sulforaphane, the effect is back to background levels, indicating a hormetic response to sulforaphane treatment. *** $p = \leq 0.001$, ** $p = \leq 0.01$, * $p = \leq 0.05$. § Significantly lower than maximum.

The increased radioresistance observed after repeated sulforaphane treatment is Nrf2-dependent

Several tumours with mutations in Keap1 or Nrf2 causing constant Nrf2 upregulation have been identified. Permanent activation of Nrf2 expression makes the tumours less susceptible to the increased oxidative stress typically surrounding rapidly dividing cells [150]. In addition, some cancer forms show inhibition of Nrf2 and the cytoprotective response [151]. Sulforaphane treatment has been reported to increase radiosensitivity in human HeLa cells in one study [152], while it can be expected to decrease the radiosensitivity in others [153]. However, since the Nrf2 system is already highly upregulated in cells with constitutive Nrf2 expression, sulforaphane stimulation should have a more substantial effect on normal tissue. Because of this, it is of interest to know whether the observed radioresistance after repeated sulforaphane treatment depended on a functional Nrf2 system.

To investigate if daily, four-hour sulforaphane treatments induced the Nrf2 response in human fibroblasts, gene expression of HO-1 and NQO1 was measured just prior to irradiation. Both single and repeated treatment with 3-15 μM sulforaphane induced a significant upregulation of HO-1 compared to non-treated control with an induction maximum at 10 μM. At higher sulforaphane concentrations, the HO-1 induction was dose-dependently attenuated suggesting feedback regulation in line with hormesis. As expected from our results in paper I, single four-hour stimulation failed to increase NQO1 mRNA, but repeated treatment induced a dose-dependent increase in NQO1 with no indications of downregulation even at 30 μM sulforaphane (figure 14).
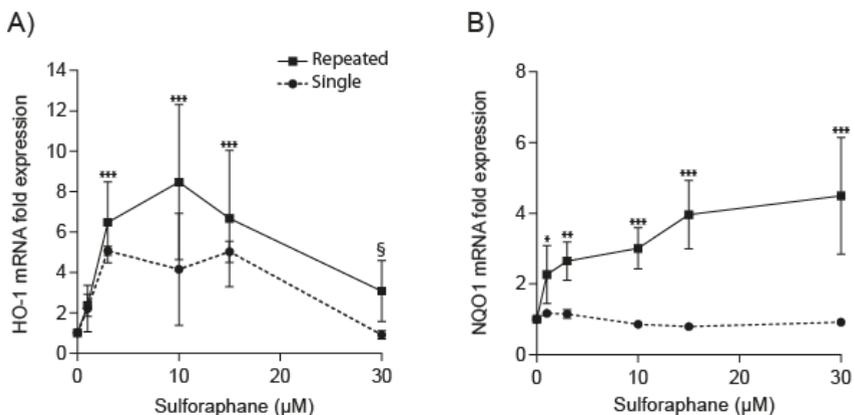
Figure 14. Repeated, brief stimulations with sulforaphane induce mRNA expression of HO-1 and NQO1 in human primary fibroblasts. A) Treatment with 3, 10 and 15 µM sulforaphane for four hours daily for three consecutive days increases HO-1 expression significantly compared to non-treated control. 10 µM sulforaphane results in maximal HO-1 induction, while the increase is attenuated at higher concentrations. (B) Repeated treatments with 1-30 µM sulforaphane increase NQO1 mRNA in a dose-dependent manner. *** $p = \leq 0.001$, ** $p = \leq 0.01$, * $p = \leq 0.05$. § Significantly lower than maximum.

Primary human fibroblasts treated with siRNA against Nrf2 did not survive repeated sulforaphane treatment followed by irradiation. Instead, we used Nrf2 wild type (WT) and Nrf2 knockout (KO) mouse embryonic fibroblasts to investigate if the increased radioresistance by repeated sulforaphane stimulations depended on a functional Nrf2 response. Nrf2 WT and KO cells were treated with sulforaphane for four hours once or for three consecutive days. Again, single four-hour treatment with sulforaphane did not increase radioresistance, while repeated treatments resulted in increased radioresistance in Nrf2 WT cells. The effect was observed after two or four Gy irradiation, but was slightly attenuated in cells exposed to four Gy IR. Interestingly, Nrf2 KO cells showed enhanced radiosensitivity after sulforaphane treatment and this effect was accentuated when the sulforaphane treatment was repeated or the IR dose increased (figure 15).
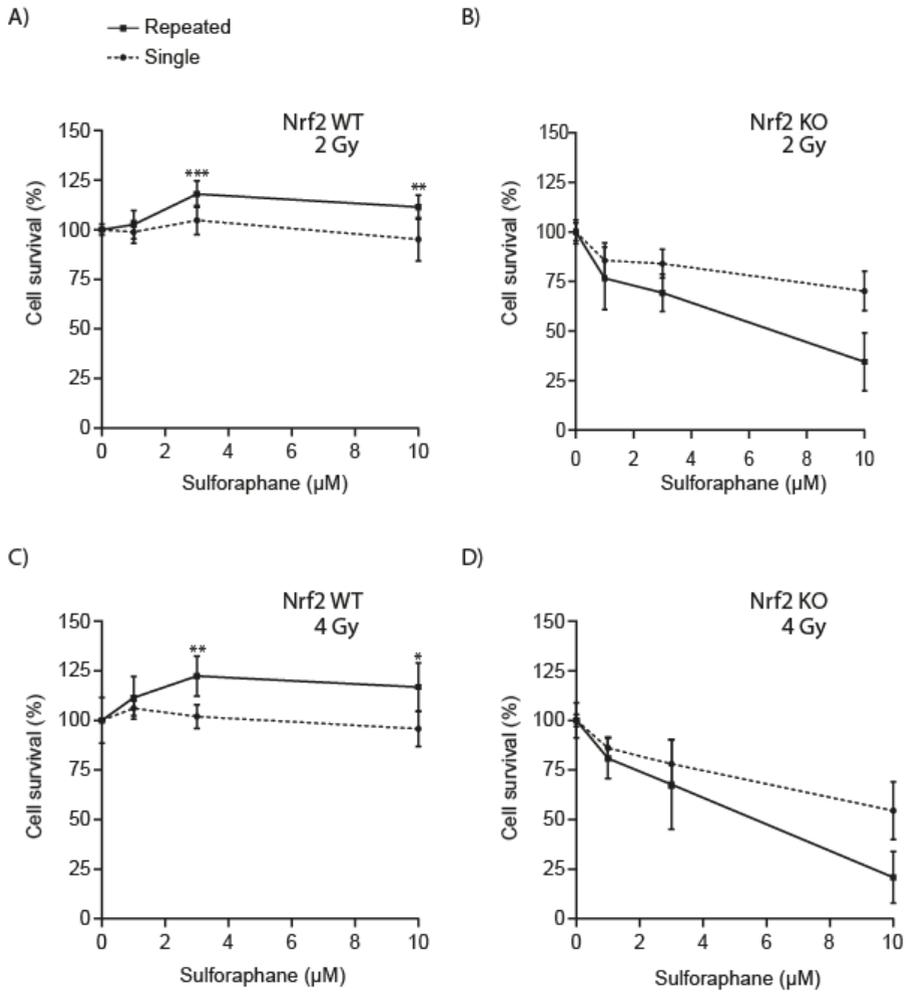
Figure 15. Increased radioresistance after sulforaphane treatment is Nrf2-dependent. A, C) Treatments with 3 or 10 µM sulforaphane for four hours daily during three consecutive days increase radioresistance significantly compared to non-treated control in Nrf2 WT MEF cells. B, D) Sulforaphane treatments enhance radiosensitivity in Nrf2 KO MEF cells, an effect that is accentuated in cells treated repeatedly for three consecutive days. *** $p = \leq 0.001$, ** $p = \leq 0.01$, * $p = \leq 0.05$

These results indicate that repeated treatments with sulforaphane prior to irradiation increases radioresistance of normal human fibroblasts in an Nrf2-dependent manner, possibly suggesting a new mechanism for protection of normal cells during radiotherapy. Further, repeated sulforaphane treatments enhance the toxicity of ionizing radiation in MEF cells lacking a functional Nrf2 response and this toxicity increases with sulforaphane concentration and/or irradiation dose. This supports the notion that phytochemicals can be beneficial due to their reactive nature rather than through antioxidant activity.

Summary of paper I and II

Many studies have shown protective effects of a diet rich in fruit and vegetables [154]. For decades, these beneficial effects were attributed the antioxidant properties of many natural dietary compounds, resulting in a widely accepted use of antioxidant supplements. Today, it is becoming increasingly clear that high supplementary intake of antioxidants rather increases the overall mortality [155, 156]. Instead, stimulation of the endogenous production of cellular antioxidants through reactive substances in the diet has gained much attention. According to the hormesis theory, a substance that is toxic at high concentrations may still provide beneficial effects within the right concentration span [74]. This applies to many everyday situations, such as physical exercise [157], intermittent fasting [158] and even to acute smoking [159]. Many naturally occurring substances react with thiols in the redox-sensitive protein Keap1, resulting in activation of the transcription factor Nrf2 and increased protection to a wide range of substances at the right dosing. The isothiocyanate sulforaphane from cruciferous vegetables is a typical and much studied example of a substance that triggers the Nrf2 system and induces protection against oxidative stress [160]. The results in paper II of this thesis showed that sulforaphane treatments increase the resistance to ionizing radiation in human and mouse-derived cell cultures at intermediate concentrations. On the contrary, high sulforaphane concentrations decrease the radioresistance in a typically hormetic fashion. Further supporting the hormetic action, sulforaphane was toxic to cells lacking the Nrf2 gene, especially in combination with radiation, and this effect was accentuated if the sulforaphane stimulations were repeated.

Ingested sulforaphane is absorbed within one hour, reaching maximal plasma levels after approximately four hours. It is then rapidly cleared from the body with a half-life of about two hours [146, 147]. Considering this, a meal of broccoli would likely result in a short boost of the Nrf2 system. The experimental setup in papers I and II of this thesis aimed at investigating how the Nrf2 system could be primed with sulforaphane to protect from radical-

induced cell damage. The results in paper I showed that brief stimulation with sulforaphane results in a functionally elevated Nrf2 response for at least 24 hours and that brief repeated sulforaphane stimulations lead to daily accumulation of Nqo1 mRNA and protein in astrocytes. Daily sulforaphane stimulations resulted in continuous increase in resistance to superoxides and hydrogen peroxides, indicating that the Nrf2 system can be exercised to improve performance. This notion was supported by the results in paper II, showing that four-hour daily sulforaphane stimulations for three consecutive days protected normal human fibroblasts from radiation-induced cell death, while single four-hour sulforaphane stimulation failed to increase radioresistance. Together, these observations may provide a molecular explanation to how repeated, short-term exposure to sulforaphane, such as after intermittent intake of broccoli, can provide prolonged protection against free radical-linked disease.

4.3 PAPER III

Association of *NFE2L2* haplotypes with Parkinson's disease

The majority of PD cases are sporadic, meaning that they occur without a clear family history. The genetic and environmental background to sporadic PD is unknown, but oxidative stress is a main feature of the PD pathogenesis [161]. Age is a major risk factor for PD and there are indications that the Nrf2 response is implicated in the PD process [101, 102]. Oxidation of dopamine to dopamine quinones is a contributing factor to increased oxidative stress in the PD brain. Induction of NQO1 with sulforaphane protected dopaminergic neurons *in vitro* [67], suggesting a potentially protective role for NQO1 upregulation in PD. A decreased function of the Nrf2/Keap1 system with age leading to accumulating oxidative stress could hypothetically have high impact on cellular functions over time. We wanted to investigate if common genetic variation in the genes encoding Nrf2 and Keap1 affects the risk of PD. To this end, common SNPs (present in > 5 % of the CEU population) in *NFE2L2* and *KEAP1* were genotyped in Swedish patients and controls, using sequencing and allelic discrimination. Both single SNP and haplotype analyses were performed using HelixTree 6.3 (www.goldenhelix.com).

In line with previously published data [162], no single SNPs were associated with risk or age at onset of PD. However, in the Swedish discovery study, we identified one haplotype window associated with Parkinson's disease (Figure 16). Within this window, the haplotype **G**AGC**AAAA** was protective and delayed PD onset by 4.6 years per allele ($p = 0.001$). The protective effect of this haplotype was confirmed in a Polish replication study, where it was associated with a decreased risk of PD, with an odds ratio of 0.4 per allele ($p = 2 \times 10^{-6}$). Three functional promoter SNPs are included in this haplotype (highlighted in bold). These SNPs were previously shown to affect Nrf2 protein levels and the promoter haplotype AGC was associated with high Nrf2 expression [83, 84]. As the SNP alleles A, G and C are the most common alleles on the respective positions, the promoter haplotype AGC could be regarded as the wild type variant [84]. One possible explanation to the observed protective effect of Nrf2 in this study would be that individuals with the full-expression (wild type) variant of the promoter have a better predisposition to cope with increased oxidative stress, which could in turn decrease the risk of PD. Supporting that the promoter region was responsible for at least a part of the association, the promoter haplotype AGC was associated with decreased risk of PD in the Polish study (OR = 0.6 per allele, $p = 0.003$) and tended to delay onset of PD in the Swedish patients with 2.1

years per allele, ($p = 0.061$). In the two independent PD populations studied in this thesis, we did not find any associations between genetic variation in *KEAP1* and PD.
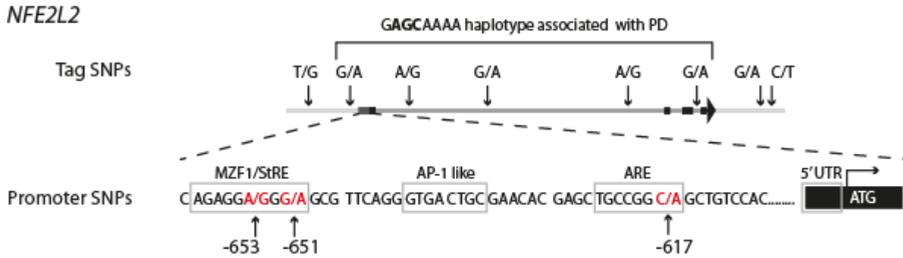
Figure 16. Tag SNPs and promoter SNPs in *NFE2L2*. The window associated with PD is indicated. This window consists of tag SNPs 2-6 according to gene direction and the three functional promoter SNPs -653A/C, -651G/A and -617 C/A. The haplotype **GAGC**AAAA (including the promoter haplotype AGC associated with high Nrf2 protein expression) of this window is protective in two independent European case-control studies. Adapted from von Otter et al. 2010 [91] and Marczak et al. 2012 [83].

In summary, these results suggest that *NFE2L2* may be a risk gene for PD, which along with other environmental or genetic factors affects the risk and progression of PD. Conversely, we have no data supporting *KEAP1* as a risk gene for PD.

4.4 PAPER IV

Association of *NFE2L2* and *KEAP1* haplotypes with ALS

Like PD, ALS is an age-related, neurodegenerative disease and 90-95 % of all patients have a form that is considered sporadic [110]. Markers of oxidative stress are increased in patients with ALS [163] and the levels of Nrf2 and Keap1 are altered in the brains of ALS patients [113]. Studies using the *SOD1* G93A mouse model have suggested Nrf2 and Keap1 to be important for ALS progression [114, 164, 165]. Fibroblast growth factor 1 (FGF-1) released from damaged neurons upregulates HO-1 expression in *SOD1* G93A rat astrocytes [166]. Together, this makes *NFE2L2* and *KEAP1* interesting as possible susceptibility genes for ALS. When common SNPs in *NFE2L2* and *KEAP1* were screened for associations with ALS, one window in *NFE2L2* consisting of tag SNPs 4-8 according to gene direction (see figure 17A) was associated with risk of ALS. The haplotype GGGAC within this window was protective and decreased the risk of ALS (OR = 0.6 per allele, *p* = 0.003).

ALS is a heterogeneous syndrome and early clinical diagnosis is not straight forward [167]. Initial symptom sites can be peripheral or lumbar and the rate of the disease progression has a tendency to differ depending on where the first symptom presents itself. Therefore, ALS patients are sub-diagnosed according to initial symptom site [168]. We wanted to investigate if ALS patients with different sub-diagnoses differed in the *NFE2L2* and *KEAP1* genes regarding age at onset and survival. When stratified by subgroup, the *NFE2L2* haplotype **G**A**GC**AGA, including three functional promoter SNPs associated with high Nrf2 protein expression [83, 84], was identified. This haplotype delayed ALS onset with 4.0 years per allele in subgroup *ALS (p = 0.008)*, but had no effect in subgroup *PBP*, or if the remaining subgroups (*PBP*, *PMA* and *PLS*) were pooled. In the *KEAP1* gene, we identified a protective haplotype in the window consisting of SNP 1-3 (figure 17B). The haplotype CGG within this window delayed ALS onset with 3.4 years per allele (*p* = 0.015). We found no evidence that genetic variations in *NFE2L2* or *KEAP1* affects the survival of patients with ALS.

Together, these findings suggest *NFE2L2* and *KEAP1* as possible risk genes for ALS and in addition indicate that patients clinically diagnosed with *ALS* may be genetically different from patients diagnosed with *PBP*.
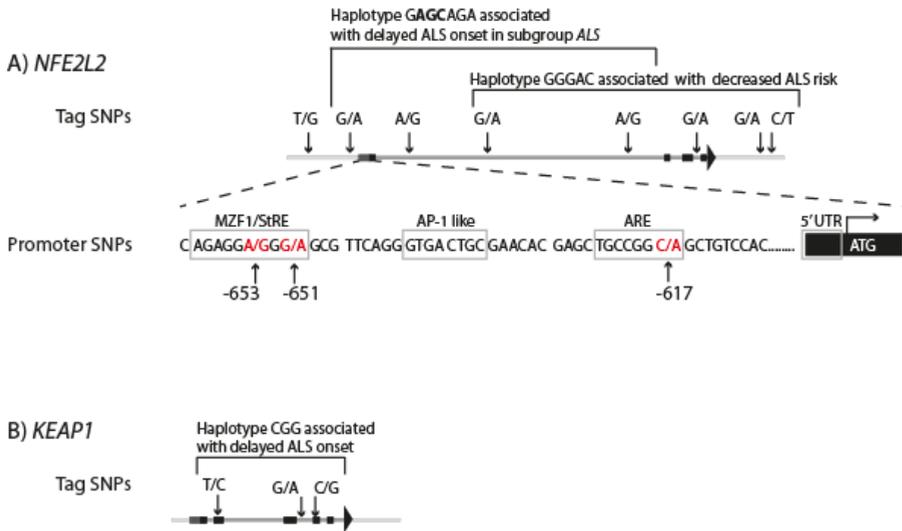
Figure 17. *NFE2L2* and *KEAP1* haplotype windows associated with ALS. A) The protective *NFE2L2* haplotype GGGAC in the window consisting of tag SNPs 4-8 according to gene direction decreases ALS risk. The protective haplotype G**AGC**AGA in the window consisting of tag SNPs 2-5 and the promoter SNPs -653, -651 and -617 is associated with delayed ALS onset in subgroup *ALS* only. B) The protective *KEAP1* haplotype CGG in the window consisting of tag SNPs 1-3 s associated with delayed ALS onset. Adapted from von Otter et al. 2010 [91] and Marczak et al.2012 [83].

Summary of paper III and IV

The relatively low concordance of PD and ALS in identical twins [169, 170] indicates that these diseases may be influenced by environmental factors [171]. Oxidative stress markers are increased in the tissue of patients with ALS and PD [100, 101, 163, 172, 173], but it is still debated whether oxidative stress is cause or consequence in the progression of neurodegeneration [14]. Either way, if the pathogenesis of neurodegenerative diseases is caused or accelerated by oxidative stress a mal-functional radical defence would most likely impact disease progression and severity. Several studies indicate that the efficiency of the Nrf2 system decreases with age. Nrf2 levels are reduced to about 50% in the liver of ageing rats [174] and the increase in vascular oxidative stress observed in ageing rats was linked to reduced Nrf2 activity [175]. The combination of a declining radical defence and an age-dependent increase in electron leakage from mitochondria would result in accumulation of radicals over time. In view of this, a genetic predisposition against a fully functional radical defence could have large impact on the progression of neurodegenerative diseases like ALS and Parkinson's disease, where age and possibly also oxidative stress are major risk factors. In line with this, we found protective haplotypes in the *NFE2L2* gene for both PD and ALS, suggesting a role for this central regulator of oxidative stress in the pathogenesis. Interestingly, one *NFE2L2* haplotype decreased the risk of PD in the Polish study, but delayed the onset of PD in the Swedish patients. In a similar fashion, a haplotype in *KEAP1* delayed ALS onset, supporting the notion that environmental factors, possibly such as dietary factors, could influence the time of onset of these diseases.

5 CONCLUDING REMARKS

The data in **paper I and II** of this thesis show that brief stimulation with sulforaphane is enough to induce continuous upregulation of the Nrf2 system in astrocytes for at least 24 hours. Furthermore, brief repeated stimulations with sulforaphane results in sustained increased ability of astrocytes to resist superoxide-induced cell death. This treatment also increases the radioresistance of human and mouse fibroblasts. Together, these results suggest that the Nrf2 system can be trained by brief, repeated treatment leading to increased protection against oxidative stress.

In **paper III and IV**, we identified genetic variants of the *NFE2L2* gene that protect against Parkinson's disease and amyotrophic lateral sclerosis. We also found a protective genetic variant of the *KEAP1* gene associated with delayed onset of ALS.

In conclusion, data presented in this thesis show that brief, repeated stimulations of Nrf2 induce prolonged protection from oxidative stress. In addition, *NFE2L2* may be a risk gene for the neurodegenerative diseases PD and ALS and the phenotype of ALS may also be influenced by *KEAP1*.

ACKNOWLEDGEMENT

My sincere thank you to all of you involved (in one way or another) in writing this thesis:

Ola Hammarsten, my supervisor; thank you for guiding me through this journey. I have truly learnt a lot since I first started in your group! Thank you also for teaching me how to always include *all* the necessary control samples in an experiment!

Henrik Zetterberg, my co-supervisor; thank you for being so very quick with the e-mail reply button, making me always feel prioritized.

Staffan Nilsson, my co-supervisor and "statistics guru"; thank you for making me feel like you have an eternity of time for our meetings, even though I know that you don't.

Malin von Otter, my informal co-supervisor; thank you for the most frictionless collaboration imaginable and thank you also for being my friend.

To all my friends and colleagues at Molekylärbiologi and Molekylär cellbiologi, many thanks for nice (and slightly crowded) lunch and coffee breaks.

Special thanks to my research group colleagues **Aida Muslimović**, **Susanne Nyström**, **Sherin Matthews**, **Pegah Johansson** and **Karin Starnberg** for creating such an open, friendly and very "scientific" environment. It has been a pleasure working with you!

Aida and **Susanne** – without you guys I would still be lost somewhere in the corridors! **Aida**, what can I say – the lab is simply not the same without you. I can only hope that we will be colleagues again in the future. Until then, I am more than happy to have you as "just" my friend.

Sherin, thank you for working so hard for me to be able to finish this thesis on time. Good luck with your own. You have already come a long way!

Malin Berggren and **Åsa Nilsson**; My time at Klinkem is split into two eras – before and after "Malin och Åsa". The before-era was a lot more fun! It is a good thing that I still get to see you sometimes for lunch and I hope we can increase the frequency of those meetings from now on.

Heléne Andersson, we really did some long hours together back in the days!

Everyone at Genanalys; thank you for taking care of me when I was new in the lab and for putting up with me continuously occupying your instruments. Thank you **Mona Seibt Palmér**; without you and your robot-like PCR skills, this thesis would likely have taken a few more years to complete!

Lars Enochson, **Johan Stenberg** and **Joakim Sandstedt**, my present and former room mates; it was fun having you around, to discuss experimental methods and to play "scientific table tennis".

All my friends on the outside of the hospital walls; Thank you for reminding me of the importance of sometimes discussing other things than science. The question "what is it that you do, really?" has helped to keep me at least a bit connected to reality.

My large and wonderful family; Thank you for simply being the best family in the world!

Martin and **Kim**; You two have changed my life forever! See you soon!

Mormor, jag saknar dig.

REFERENCES

1.   Halliwell, B. and J.M.C. Gutteridge, *Free Radicals in Biology and Medicine. 2007. **4th edition**.*

2.   Church, D.F. and W.A. Pryor, *Free-radical chemistry of cigarette smoke and its toxicological implications. Environ Health Perspect, 1985. **64**: p. 111-26.*

3.   Griffiths, H.R., et al., *Molecular and cellular effects of ultraviolet light-induced genotoxicity. Crit Rev Clin Lab Sci, 1998. **35**(3): p. 189-237.*

4.   Innamorato, N.G., I. Lastres-Becker, and A. Cuadrado, *Role of microglial redox balance in modulation of neuroinflammation. Curr Opin Neurol, 2009. **22**(3): p. 308-14.*

5.   Davies, K.J., *Oxidative stress: the paradox of aerobic life. Biochem Soc Symp, 1995. **61**: p. 1-31.*

6.   Federico, A., et al., *Mitochondria, oxidative stress and neurodegeneration. J Neurol Sci, 2012. **322**(1-2): p. 254-62.*

7.   Link, E.M. and P.A. Riley, *Role of hydrogen peroxide in the cytotoxicity of the xanthine/xanthine oxidase system. Biochem J, 1988. **249**(2): p. 391-9.*

8.   Chance, B., H. Sies, and A. Boveris, *Hydroperoxide metabolism in mammalian organs. Physiol Rev, 1979. **59**(3): p. 527-605.*

9.   Mann, B.E. and R. Motterlini, *CO and NO in medicine. Chem Commun (Camb), 2007(41): p. 4197-208.*

10.   McCord, J.M. and I. Fridovich, *Superoxide dismutase: the first twenty years (1968-1988). Free Radic Biol Med, 1988. **5**(5-6): p. 363-9.*

11.   Gomez-Cabrera, M.C., et al., *Mitochondria as sources and targets of damage in cellular aging. Clin Chem Lab Med, 2012. **50**(8): p. 1287-95.*

12.   Harman, D., *Aging: a theory based on free radical and radiation chemistry. J Gerontol, 1956. **11**(3): p. 298-300.*

13.   Shaw, P.J. and C.J. Eggett, *Molecular factors underlying selective vulnerability of motor neurons to neurodegeneration in amyotrophic lateral sclerosis. J Neurol, 2000. **247 Suppl 1**: p. I17-27.*

14.   Andersen, J.K., *Oxidative stress in neurodegeneration: cause or consequence? Nat Med, 2004. **10 Suppl**: p. S18-25.*

15.   Nagahara, N., *Intermolecular disulfide bond to modulate protein function as a redox-sensing switch. Amino Acids, 2011. **41**(1): p. 59-72.*

16.   Go, Y.M. and D.P. Jones, *Thiol/disulfide redox states in signaling and sensing. Crit Rev Biochem Mol Biol, 2013. **48**(2): p. 173-81.*

17.   Bjelakovic, G., et al., *Antioxidant supplements for prevention of mortality in healthy participants and patients with various diseases. Cochrane Database Syst Rev, 2008(2): p. CD007176.*

18.   Morris, C.D. and S. Carson, *Routine vitamin supplementation to prevent cardiovascular disease: a summary of the evidence for the U.S. Preventive Services Task Force. Ann Intern Med, 2003.* **139**(1): p. 56-70.

19.   Vivekananthan, D.P., et al., *Use of antioxidant vitamins for the prevention of cardiovascular disease: meta-analysis of randomised trials. Lancet, 2003.* **361**(9374): p. 2017-23.

20.   Trewavas, A. and D. Stewart, *Paradoxical effects of chemicals in the diet on health. Curr Opin Plant Biol, 2003.* **6**(2): p. 185-90.

21.   Birringer, M., *Hormetics: dietary triggers of an adaptive stress response. Pharm Res, 2011.* **28**(11): p. 2680-94.

22.   Watson, J.D. and F.H. Crick, *The structure of DNA. Cold Spring Harb Symp Quant Biol, 1953.* **18**: p. 123-31.

23.   Paabo, S., *The mosaic that is our genome. Nature, 2003.* **421**(6921): p. 409-12.

24.   Brosius, J., *The fragmented gene. Ann N Y Acad Sci, 2009.* **1178**: p. 186-93.

25.   Moi, P., et al., *Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region. Proc Natl Acad Sci U S A, 1994.* **91**(21): p. 9926-30.

26.   Nioi, P., et al., *Identification of a novel Nrf2-regulated antioxidant response element (ARE) in the mouse NAD(P)H:quinone oxidoreductase 1 gene: reassessment of the ARE consensus sequence. Biochem J, 2003.* **374**(Pt 2): p. 337-48.

27.   Rushmore, T.H., M.R. Morton, and C.B. Pickett, *The antioxidant responsive element. Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. J Biol Chem, 1991.* **266**(18): p. 11632-9.

28.   Wasserman, W.W. and W.E. Fahl, *Functional antioxidant responsive elements. Proc Natl Acad Sci U S A, 1997.* **94**(10): p. 5361-6.

29.   Itoh, K., et al., *An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem Biophys Res Commun, 1997.* **236**(2): p. 313-22.

30.   Kwak, M.K., et al., *Enhanced expression of the transcription factor Nrf2 by cancer chemopreventive agents: role of antioxidant response element-like sequences in the nrf2 promoter. Mol Cell Biol, 2002.* **22**(9): p. 2883-92.

31.   Friling, R.S., S. Bergelson, and V. Daniel, *Two adjacent AP-1-like binding sites form the electrophile-responsive element of the murine*

glutathione S-transferase Ya subunit gene. Proc Natl Acad Sci U S A, 1992. **89**(2): p. 668-72.

32. Inamdar, N.M., Y.I. Ahn, and J. Alam, The heme-responsive element of the mouse heme oxygenase-1 gene is an extended AP-1 binding site that resembles the recognition sequences for MAF and NF-E2 transcription factors. Biochem Biophys Res Commun, 1996. **221**(3): p. 570-6.

33. Rushmore, T.H. and C.B. Pickett, Transcriptional regulation of the rat glutathione S-transferase Ya subunit gene. Characterization of a xenobiotic-responsive element controlling inducible expression by phenolic antioxidants. J Biol Chem, 1990. **265**(24): p. 14648-53.

34. Favreau, L.V. and C.B. Pickett, Transcriptional regulation of the rat NAD(P)H:quinone reductase gene. Identification of regulatory elements controlling basal level expression and inducible expression by planar aromatic compounds and phenolic antioxidants. J Biol Chem, 1991. **266**(7): p. 4556-61.

35. Copple, I.M., et al., The Nrf2-Keap1 defence pathway: role in protection against drug-induced toxicity. Toxicology, 2008. **246**(1): p. 24-33.

36. Ishii, T., et al., Transcription factor Nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages. J Biol Chem, 2000. **275**(21): p. 16023-9.

37. McMahon, M., et al., Keap1-dependent proteasomal degradation of transcription factor Nrf2 contributes to the negative regulation of antioxidant response element-driven gene expression. J Biol Chem, 2003. **278**(24): p. 21592-600.

38. Itoh, K., et al., Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. Genes Dev, 1999. **13**(1): p. 76-86.

39. Hong, F., M.L. Freeman, and D.C. Liebler, Identification of sensor cysteines in human Keap1 modified by the cancer chemopreventive agent sulforaphane. Chem Res Toxicol, 2005. **18**(12): p. 1917-26.

40. McMahon, M., et al., Keap1 perceives stress via three sensors for the endogenous signaling molecules nitric oxide, zinc, and alkenals. Proc Natl Acad Sci U S A, 2010. **107**(44): p. 18838-43.

41. Yamamoto, T., et al., Physiological significance of reactive cysteine residues of Keap1 in determining Nrf2 activity. Mol Cell Biol, 2008. **28**(8): p. 2758-70.

42. Kobayashi, M., et al., The antioxidant defense system Keap1-Nrf2 comprises a multiple sensing mechanism for responding to a wide range of chemical compounds. Mol Cell Biol, 2009. **29**(2): p. 493-502.

43. Tong, K.I., et al., Two-site substrate recognition model for the Keap1-Nrf2 system: a hinge and latch mechanism. Biol Chem, 2006. **387**(10-11): p. 1311-20.

44.   Kobayashi, A., et al., *Oxidative and electrophilic stresses activate Nrf2 through inhibition of ubiquitination activity of Keap1.* Mol Cell Biol, 2006. **26**(1): p. 221-9.

45.   Eggler, A.L., et al., *Modifying specific cysteines of the electrophile-sensing human Keap1 protein is insufficient to disrupt binding to the Nrf2 domain Neh2.* Proc Natl Acad Sci U S A, 2005. **102**(29): p. 10070-5.

46.   McMahon, M., et al., *The Cap'n'Collar basic leucine zipper transcription factor Nrf2 (NF-E2 p45-related factor 2) controls both constitutive and inducible expression of intestinal detoxification and glutathione biosynthetic enzymes.* Cancer Res, 2001. **61**(8): p. 3299-307.

47.   Wild, A.C., H.R. Moinova, and R.T. Mulcahy, *Regulation of gamma-glutamylcysteine synthetase subunit gene expression by the transcription factor Nrf2.* J Biol Chem, 1999. **274**(47): p. 33627-36.

48.   Chanas, S.A., et al., *Loss of the Nrf2 transcription factor causes a marked reduction in constitutive and inducible expression of the glutathione S-transferase Gsta1, Gsta2, Gstm1, Gstm2, Gstm3 and Gstm4 genes in the livers of male and female mice.* Biochem J, 2002. **365**(Pt 2): p. 405-16.

49.   van Muiswinkel, F.L., et al., *L-Dopa stimulates expression of the antioxidant enzyme NAD(P)H:quinone oxidoreductase (NQO) in cultured astroglial cells.* Free Radic Biol Med, 2000. **29**(5): p. 442-53.

50.   Dinkova-Kostova, A.T. and P. Talalay, *Persuasive evidence that quinone reductase type 1 (DT diaphorase) protects cells against the toxicity of electrophiles and reactive forms of oxygen.* Free Radic Biol Med, 2000. **29**(3-4): p. 231-40.

51.   Ross, D., et al., *NAD(P)H:quinone oxidoreductase 1 (NQO1): chemoprotection, bioactivation, gene regulation and genetic polymorphisms.* Chem Biol Interact, 2000. **129**(1-2): p. 77-97.

52.   Nioi, P. and J.D. Hayes, *Contribution of NAD(P)H:quinone oxidoreductase 1 to protection against carcinogenesis, and regulation of its gene by the Nrf2 basic-region leucine zipper and the arylhydrocarbon receptor basic helix-loop-helix transcription factors.* Mutat Res, 2004. **555**(1-2): p. 149-71.

53.   Venugopal, R. and A.K. Jaiswal, *Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H:quinone oxidoreductase1 gene.* Proc Natl Acad Sci U S A, 1996. **93**(25): p. 14960-5.

54.   McWalter, G.K., et al., *Transcription factor Nrf2 is essential for induction of NAD(P)H:quinone oxidoreductase 1, glutathione S-transferases, and glutamate cysteine ligase by broccoli seeds and isothiocyanates.* J Nutr, 2004. **134**(12 Suppl): p. 3499S-3506S.

55.   Alam, J., et al., Nrf2, a Cap'n'Collar transcription factor, regulates induction of the heme oxygenase-1 gene. J Biol Chem, 1999. **274**(37): p. 26071-8.

56.   Chen, K., K. Gunter, and M.D. Maines, Neurons overexpressing heme oxygenase-1 resist oxidative stress-mediated cell death. J Neurochem, 2000. **75**(1): p. 304-13.

57.   Vile, G.F., et al., Heme oxygenase 1 mediates an adaptive response to oxidative stress in human skin fibroblasts. Proc Natl Acad Sci U S A, 1994. **91**(7): p. 2607-10.

58.   Keum, Y.S., et al., Mechanism of action of sulforaphane: inhibition of p38 mitogen-activated protein kinase isoforms contributing to the induction of antioxidant response element-mediated heme oxygenase-1 in human hepatoma HepG2 cells. Cancer Res, 2006. **66**(17): p. 8804-13.

59.   Yeh, C.T., H.F. Chiu, and G.C. Yen, Protective effect of sulforaphane on indomethacin-induced cytotoxicity via heme oxygenase-1 expression in human intestinal Int 407 cells. Mol Nutr Food Res, 2009. **53**(9): p. 1166-76.

60.   Magesh, S., Y. Chen, and L. Hu, Small molecule modulators of Keap1-Nrf2-ARE pathway as potential preventive and therapeutic agents. Med Res Rev, 2012. **32**(4): p. 687-726.

61.   Fahey, J.W., Y. Zhang, and P. Talalay, Broccoli sprouts: an exceptionally rich source of inducers of enzymes that protect against chemical carcinogens. Proc Natl Acad Sci U S A, 1997. **94**(19): p. 10367-72.

62.   Brooks, J.D., V.G. Paton, and G. Vidanes, Potent induction of phase 2 enzymes in human prostate cells by sulforaphane. Cancer Epidemiol Biomarkers Prev, 2001. **10**(9): p. 949-54.

63.   Danilov, C.A., et al., Sulforaphane protects astrocytes against oxidative stress and delayed death caused by oxygen and glucose deprivation. Glia, 2009. **57**(6): p. 645-56.

64.   Dinkova-Kostova, A.T., et al., Induction of the phase 2 response in mouse and human skin by sulforaphane-containing broccoli sprout extracts. Cancer Epidemiol Biomarkers Prev, 2007. **16**(4): p. 847-51.

65.   Fahey, J.W., et al., Sulforaphane inhibits extracellular, intracellular, and antibiotic-resistant strains of Helicobacter pylori and prevents benzo[a]pyrene-induced stomach tumors. Proc Natl Acad Sci U S A, 2002. **99**(11): p. 7610-5.

66.   Fimognari, C. and P. Hrelia, Sulforaphane as a promising molecule for fighting cancer. Mutat Res, 2007. **635**(2-3): p. 90-104.

67.   Han, J.M., et al., Protective effect of sulforaphane against dopaminergic cell death. J Pharmacol Exp Ther, 2007. **321**(1): p. 249-56.

68.   Juge, N., R.F. Mithen, and M. Traka, *Molecular basis for chemoprevention by sulforaphane: a comprehensive review. Cell Mol Life Sci, 2007. **64**(9): p. 1105-27.*

69.   Pergola, P.E., et al., *Bardoxolone methyl and kidney function in CKD with type 2 diabetes. N Engl J Med, 2011. **365**(4): p. 327-36.*

70.   Gold, R., et al., *Placebo-controlled phase 3 study of oral BG-12 for relapsing multiple sclerosis. N Engl J Med, 2012. **367**(12): p. 1098-107.*

71.   Calabrese, E.J. and L.A. Baldwin, *U-shaped dose-responses in biology, toxicology, and public health. Annu Rev Public Health, 2001. **22**: p. 15-33.*

72.   Calabrese, E.J. and L.A. Baldwin, *Hormesis: U-shaped dose responses and their centrality in toxicology. Trends Pharmacol Sci, 2001. **22**(6): p. 285-91.*

73.   Calabrese, E.J. and L.A. Baldwin, *Applications of hormesis in toxicology, risk assessment and chemotherapeutics. Trends Pharmacol Sci, 2002. **23**(7): p. 331-7.*

74.   Mattson, M.P., *Hormesis defined. Ageing Res Rev, 2008. **7**(1): p. 1-7.*

75.   Fahey, J.W., A.T. Zalcmann, and P. Talalay, *The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. Phytochemistry, 2001. **56**(1): p. 5-51.*

76.   van Poppel, G., et al., *Brassica vegetables and cancer prevention. Epidemiology and mechanisms. Adv Exp Med Biol, 1999. **472**: p. 159-68.*

77.   Innamorato, N.G., et al., *The transcription factor Nrf2 is a therapeutic target against brain inflammation. J Immunol, 2008. **181**(1): p. 680-9.*

78.   *The International HapMap Project. Nature, 2003. **426**(6968): p. 789-96.*

79.   Lawrence, R., et al., *Genetically indistinguishable SNPs and their influence on inferring the location of disease-associated variants. Genome Res, 2005. **15**(11): p. 1503-10.*

80.   Gabriel, S.B., et al., *The structure of haplotype blocks in the human genome. Science, 2002. **296**(5576): p. 2225-9.*

81.   Rice, T.K., N.J. Schork, and D.C. Rao, *Methods for handling multiple testing. Adv Genet, 2008. **60**: p. 293-308.*

82.   Yamamoto, T., et al., *Identification of polymorphisms in the promoter region of the human NRF2 gene. Biochem Biophys Res Commun, 2004. **321**(1): p. 72-9.*

83.   Marczak, E.D., et al., *Polymorphisms in the transcription factor NRF2 and forearm vasodilator responses in humans. Pharmacogenet Genomics, 2012. **22**(8): p. 620-8.*

84.   Marzec, J.M., et al., *Functional polymorphisms in the transcription factor NRF2 in humans increase the risk of acute lung injury. Faseb J, 2007. **21**(9): p. 2237-46.*

85.   *Arisawa, T., et al., The relationship between Helicobacter pylori infection and promoter polymorphism of the Nrf2 gene in chronic gastritis. Int J Mol Med, 2007. **19**(1): p. 143-8.*

86.   *Arisawa, T., et al., Nrf2 gene promoter polymorphism is associated with ulcerative colitis in a Japanese population. Hepatogastroenterology, 2008. **55**(82-83): p. 394-7.*

87.   *Guan, C.P., et al., The susceptibility to vitiligo is associated with NF-E2-related factor2 (Nrf2) gene polymorphisms: a study on Chinese Han population. Exp Dermatol, 2008. **17**(12): p. 1059-62.*

88.   *Hua, C.C., et al., Functional haplotypes in the promoter region of transcription factor Nrf2 in chronic obstructive pulmonary disease. Dis Markers, 2010. **28**(3): p. 185-93.*

89.   *Siedlinski, M., et al., Level and course of FEV1 in relation to polymorphisms in NFE2L2 and KEAP1 in the general population. Respir Res, 2009. **10**: p. 73.*

90.   *Suzuki, T., et al., Regulatory Nexus of Synthesis and Degradation Deciphers Cellular Nrf2 Expression Levels. Mol Cell Biol, 2013.*

91.   *von Otter, M., et al., Association of Nrf2-encoding NFE2L2 haplotypes with Parkinson's disease. BMC Med Genet, 2010. **11**: p. 36.*

92.   *Burton, N.C., T.W. Kensler, and T.R. Guilarte, In vivo modulation of the Parkinsonian phenotype by Nrf2. Neurotoxicology, 2006. **27**(6): p. 1094-100.*

93.   *Hirota, A., et al., Ultraviolet A irradiation induces NF-E2-related factor 2 activation in dermal fibroblasts: protective role in UVA-induced apoptosis. J Invest Dermatol, 2005. **124**(4): p. 825-32.*

94.   *Shih, A.Y., P. Li, and T.H. Murphy, A small-molecule-inducible Nrf2-mediated antioxidant response provides effective prophylaxis against cerebral ischemia in vivo. J Neurosci, 2005. **25**(44): p. 10321-35.*

95.   *Rangasamy, T., et al., Disruption of Nrf2 enhances susceptibility to severe airway inflammation and asthma in mice. J Exp Med, 2005. **202**(1): p. 47-59.*

96.   *Rangasamy, T., et al., Cigarette smoke-induced emphysema in A/J mice is associated with pulmonary oxidative stress, apoptosis of lung cells, and global alterations in gene expression. Am J Physiol Lung Cell Mol Physiol, 2009. **296**(6): p. L888-900.*

97.   *Wakabayashi, N., et al., Keap1-null mutation leads to postnatal lethality due to constitutive Nrf2 activation. Nat Genet, 2003. **35**(3): p. 238-45.*

98.   *Lees, A.J., J. Hardy, and T. Revesz, Parkinson's disease. Lancet, 2009. **373**(9680): p. 2055-66.*

99.   *Schapira, A.H., Neurobiology and treatment of Parkinson's disease. Trends Pharmacol Sci, 2009. **30**(1): p. 41-7.*

100.   *Chinta, S.J. and J.K. Andersen, Redox imbalance in Parkinson's disease. Biochim Biophys Acta, 2008. **1780**(11): p. 1362-7.*

101. Kumar, H., et al., *The role of free radicals in the aging brain and Parkinson's disease: convergence and parallelism. Int J Mol Sci, 2012.* **13**(8): p. 10478-504.

102. Barone, M.C., G.P. Sykiotis, and D. Bohmann, *Genetic activation of Nrf2 signaling is sufficient to ameliorate neurodegenerative phenotypes in a Drosophila model of Parkinson's disease. Dis Model Mech, 2011.* **4**(5): p. 701-7.

103. Clements, C.M., et al., *DJ-1, a cancer- and Parkinson's disease-associated protein, stabilizes the antioxidant transcriptional master regulator Nrf2. Proc Natl Acad Sci U S A, 2006.* **103**(41): p. 15091-6.

104. Costa, J., et al., *Caffeine exposure and the risk of Parkinson's disease: a systematic review and meta-analysis of observational studies. J Alzheimers Dis, 2010.* **20 Suppl 1**: p. S221-38.

105. Hernan, M.A., et al., *A meta-analysis of coffee drinking, cigarette smoking, and the risk of Parkinson's disease. Ann Neurol, 2002.* **52**(3): p. 276-84.

106. Trinh, K., et al., *Decaffeinated coffee and nicotine-free tobacco provide neuroprotection in Drosophila models of Parkinson's disease through an NRF2-dependent mechanism. J Neurosci, 2010.* **30**(16): p. 5525-32.

107. Jazwa, A., et al., *Pharmacological targeting of the transcription factor Nrf2 at the basal ganglia provides disease modifying therapy for experimental parkinsonism. Antioxid Redox Signal, 2011.* **14**(12): p. 2347-60.

108. Matigian, N., et al., *Disease-specific, neurosphere-derived cells as models for brain disorders. Dis Model Mech, 2010.* **3**(11-12): p. 785-98.

109. Cook, A.L., et al., *NRF2 activation restores disease related metabolic deficiencies in olfactory neurosphere-derived cells from patients with sporadic Parkinson's disease. PLoS ONE, 2011.* **6**(7): p. e21907.

110. Andersen, P.M. and A. Al-Chalabi, *Clinical genetics of amyotrophic lateral sclerosis: what do we really know? Nat Rev Neurol, 2011.* **7**(11): p. 603-15.

111. Miller, R.G., J.D. Mitchell, and D.H. Moore, *Riluzole for amyotrophic lateral sclerosis (ALS)/motor neuron disease (MND). Cochrane Database Syst Rev, 2012.* **3**: p. CD001447.

112. Sojka, P., P.M. Andersen, and L. Forsgren, *Effects of riluzole on symptom progression in amyotrophic lateral sclerosis. Lancet, 1997.* **349**(9046): p. 176-7.

113. Sarlette, A., et al., *Nuclear erythroid 2-related factor 2-antioxidative response element signaling pathway in motor cortex and spinal cord in amyotrophic lateral sclerosis. J Neuropathol Exp Neurol, 2008.* **67**(11): p. 1055-62.

114.   Vargas, M.R., et al., *Nrf2 activation in astrocytes protects against neurodegeneration in mouse models of familial amyotrophic lateral sclerosis. J Neurosci*, 2008. **28**(50): p. 13574-81.

115.   Daniel, S.E. and A.J. Lees, *Parkinson's Disease Society Brain Bank, London: overview and research. J Neural Transm Suppl*, 1993. **39**: p. 165-72.

116.   Bergman, O., et al., *PITX3 polymorphism is associated with early onset Parkinson's disease. Neurobiol Aging*, 2008.

117.   Westerlund, M., et al., *Cerebellar alpha-synuclein levels are decreased in Parkinson's disease and do not correlate with SNCA polymorphisms associated with disease in a Swedish material. Faseb J*, 2008. **22**(10): p. 3509-14.

118.   Andersen, P.M., et al., *EFNS task force on management of amyotrophic lateral sclerosis: guidelines for diagnosing and clinical care of patients and relatives. Eur J Neurol*, 2005. **12**(12): p. 921-38.

119.   Araque, A., G. Carmignoto, and P.G. Haydon, *Dynamic signaling between astrocytes and neurons. Annu Rev Physiol*, 2001. **63**: p. 795-813.

120.   Ben Achour, S. and O. Pascual, *Astrocyte-neuron communication: functional consequences. Neurochem Res*, 2012. **37**(11): p. 2464-73.

121.   Dringen, R., B. Pfeiffer, and B. Hamprecht, *Synthesis of the antioxidant glutathione in neurons: supply by astrocytes of CysGly as precursor for neuronal glutathione. J Neurosci*, 1999. **19**(2): p. 562-9.

122.   Jakel, R.J., et al., *Nrf2-mediated protection against 6-hydroxydopamine. Brain Res*, 2007. **1144**: p. 192-201.

123.   Ward, J.F., *The yield of DNA double-strand breaks produced intracellularly by ionizing radiation: a review. Int J Radiat Biol*, 1990. **57**(6): p. 1141-50.

124.   Cullinan, S.B., et al., *The Keap1-BTB protein is an adaptor that bridges Nrf2 to a Cul3-based E3 ligase: oxidative stress sensing by a Cul3-Keap1 ligase. Mol Cell Biol*, 2004. **24**(19): p. 8477-86.

125.   Ishmael, F.T. and C. Stellato, *Principles and applications of polymerase chain reaction: basic science for the practicing physician. Ann Allergy Asthma Immunol*, 2008. **101**(4): p. 437-43.

126.   Livak, K.J., et al., *Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. PCR Methods Appl*, 1995. **4**(6): p. 357-62.

127.   Holland, P.M., et al., *Detection of specific polymerase chain reaction product by utilizing the 5'----3' exonuclease activity of Thermus aquaticus DNA polymerase. Proc Natl Acad Sci U S A*, 1991. **88**(16): p. 7276-80.

128.   Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods, 2001. **25**(4): p. 402-8.*

129.   Rana, T.M., *Illuminating the silence: understanding the structure and function of small RNAs. Nat Rev Mol Cell Biol, 2007. **8**(1): p. 23-36.*

130.   Cook, J.A., S.N. Iype, and J.B. Mitchell, *Differential specificity of monochlorobimane for isozymes of human and rodent glutathione S-transferases. Cancer Res, 1991. **51**(6): p. 1606-12.*

131.   Cathcart, R., E. Schwiers, and B.N. Ames, *Detection of picomole levels of hydroperoxides using a fluorescent dichlorofluorescein assay. Anal Biochem, 1983. **134**(1): p. 111-6.*

132.   Rogakou, E.P., et al., *Megabase chromatin domains involved in DNA double-strand breaks in vivo. J Cell Biol, 1999. **146**(5): p. 905-16.*

133.   Rogakou, E.P., et al., *DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. J Biol Chem, 1998. **273**(10): p. 5858-68.*

134.   Kuo, L.J. and L.X. Yang, *Gamma-H2AX - a novel biomarker for DNA double-strand breaks. In Vivo, 2008. **22**(3): p. 305-9.*

135.   Buck, S.B., et al., *Detection of S-phase cell cycle progression using 5-ethynyl-2'-deoxyuridine incorporation with click chemistry, an alternative to using 5-bromo-2'-deoxyuridine antibodies. Biotechniques, 2008. **44**(7): p. 927-9.*

136.   Sanger, F., S. Nicklen, and A.R. Coulson, *DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci U S A, 1977. **74**(12): p. 5463-7.*

137.   Shendure, J.A., et al., *Overview of DNA sequencing strategies. Curr Protoc Mol Biol, 2011. **Chapter 7**: p. Unit7 1.*

138.   Livak, K.J., *Allelic discrimination using fluorogenic probes and the 5' nuclease assay. Genet Anal, 1999. **14**(5-6): p. 143-9.*

139.   Lee, L.G., C.R. Connell, and W. Bloch, *Allelic discrimination by nick-translation PCR with fluorogenic probes. Nucleic Acids Res, 1993. **21**(16): p. 3761-6.*

140.   Chen, P.C., et al., *Nrf2-mediated neuroprotection in the MPTP mouse model of Parkinson's disease: Critical role for the astrocyte. Proc Natl Acad Sci U S A, 2009. **106**(8): p. 2933-8.*

141.   Wilson, J.X., *Antioxidant defense of the brain: a role for astrocytes. Can J Physiol Pharmacol, 1997. **75**(10-11): p. 1149-63.*

142.   Tenhunen, R., H.S. Marver, and R. Schmid, *The enzymatic conversion of heme to bilirubin by microsomal heme oxygenase. Proc Natl Acad Sci U S A, 1968. **61**(2): p. 748-55.*

143.   Otterbein, L.E., et al., *Heme oxygenase-1: unleashing the protective properties of heme. Trends Immunol, 2003. **24**(8): p. 449-55.*

144.   Kim, H.J., et al., *CO/HO-1 Induces NQO-1 Expression via Nrf2 Activation. Immune Netw, 2011. **11**(6): p. 376-82.*

145. *Bergstrom, P., et al., Repeated transient sulforaphane stimulation in astrocytes leads to prolonged Nrf2-mediated gene expression and protection from superoxide-induced damage. Neuropharmacology, 2011. **60**(2-3): p. 343-53.*

146. *Hu, R., et al., In vivo pharmacokinetics and regulation of gene expression profiles by isothiocyanate sulforaphane in the rat. J Pharmacol Exp Ther, 2004. **310**(1): p. 263-71.*

147. *Ye, L., et al., Quantitative determination of dithiocarbamates in human plasma, serum, erythrocytes and urine: pharmacokinetics of broccoli sprout isothiocyanates in humans. Clin Chim Acta, 2002. **316**(1-2): p. 43-53.*

148. *Tounekti, O., et al., The ratio of single- to double-strand DNA breaks and their absolute values determine cell death pathway. Br J Cancer, 2001. **84**(9): p. 1272-9.*

149. *Barnett, G.C., et al., Normal tissue reactions to radiotherapy: towards tailoring treatment dose by genotype. Nat Rev Cancer, 2009. **9**(2): p. 134-42.*

150. *Hayes, J.D. and M. McMahon, NRF2 and KEAP1 mutations: permanent activation of an adaptive response in cancer. Trends Biochem Sci, 2009. **34**(4): p. 176-88.*

151. *Yu, S., et al., Nrf2 expression is regulated by epigenetic mechanisms in prostate cancer of TRAMP mice. PLoS ONE, 2010. **5**(1): p. e8579.*

152. *Yu, D., et al., Chemopreventive agent sulforaphane enhances radiosensitity in human tumor cells. Int J Cancer, 2009. **125**(5): p. 1205-11.*

153. *Zhang, C., et al., Sulforaphane Enhances Nrf2 Expression in Prostate Cancer TRAMP C1 Cells through Epigenetic Regulation. Biochem Pharmacol, 2013.*

154. *Block, G., B. Patterson, and A. Subar, Fruit, vegetables, and cancer prevention: a review of the epidemiological evidence. Nutr Cancer, 1992. **18**(1): p. 1-29.*

155. *Bjelakovic, G., et al., Mortality in randomized trials of antioxidant supplements for primary and secondary prevention: systematic review and meta-analysis. Jama, 2007. **297**(8): p. 842-57.*

156. *Miller, E.R., 3rd, et al., Meta-analysis: high-dosage vitamin E supplementation may increase all-cause mortality. Ann Intern Med, 2005. **142**(1): p. 37-46.*

157. *Ristow, M., et al., Antioxidants prevent health-promoting effects of physical exercise in humans. Proc Natl Acad Sci U S A, 2009. **106**(21): p. 8665-70.*

158. *Mattson, M.P., Dietary factors, hormesis and health. Ageing Res Rev, 2008. **7**(1): p. 43-8.*

159. *Lisi, M., et al., Acute (but not chronic) smoking paradoxically protects the endothelium from ischemia and reperfusion: insight into the "smoking paradox". Clin Res Cardiol, 2013. **102**(5): p. 387-9.*

160.   Higgins, L.G. and J.D. Hayes, The cap'n'collar transcription factor Nrf2 mediates both intrinsic resistance to environmental stressors and an adaptive response elicited by chemopreventive agents that determines susceptibility to electrophilic xenobiotics. Chem Biol Interact, 2011. **192**(1-2): p. 37-45.

161.   Hwang, O., Role of oxidative stress in Parkinson's disease. Exp Neurobiol, 2013. **22**(1): p. 11-7.

162.   Mizuta, I., et al., Multiple candidate gene analysis identifies alpha-synuclein as a susceptibility gene for sporadic Parkinson's disease. Hum Mol Genet, 2006. **15**(7): p. 1151-8.

163.   Ferrante, R.J., et al., Evidence of increased oxidative damage in both sporadic and familial amyotrophic lateral sclerosis. J Neurochem, 1997. **69**(5): p. 2064-74.

164.   Nagai, M., et al., Astrocytes expressing ALS-linked mutated SOD1 release factors selectively toxic to motor neurons. Nat Neurosci, 2007. **10**(5): p. 615-22.

165.   Pehar, M., et al., Mitochondrial superoxide production and nuclear factor erythroid 2-related factor 2 activation in p75 neurotrophin receptor-induced motor neuron apoptosis. J Neurosci, 2007. **27**(29): p. 7777-85.

166.   Vargas, M.R., et al., Fibroblast growth factor-1 induces heme oxygenase-1 via nuclear factor erythroid 2-related factor 2 (Nrf2) in spinal cord astrocytes: consequences for motor neuron survival. J Biol Chem, 2005. **280**(27): p. 25571-9.

167.   Davenport, R.J., et al., Avoiding false positive diagnoses of motor neuron disease: lessons from the Scottish Motor Neuron Disease Register. J Neurol Neurosurg Psychiatry, 1996. **60**(2): p. 147-51.

168.   Andersen, P.M., Amyotrophic lateral sclerosis associated with mutations in the CuZn superoxide dismutase gene. Curr Neurol Neurosci Rep, 2006. **6**(1): p. 37-46.

169.   Al-Chalabi, A., et al., An estimate of amyotrophic lateral sclerosis heritability using twin data. J Neurol Neurosurg Psychiatry, 2010. **81**(12): p. 1324-6.

170.   Wirdefeldt, K., et al., Complete ascertainment of Parkinson disease in the Swedish Twin Registry. Neurobiol Aging, 2008. **29**(12): p. 1765-73.

171.   Baranzini, S.E., et al., Genome, epigenome and RNA sequences of monozygotic twins discordant for multiple sclerosis. Nature, 2010. **464**(7293): p. 1351-6.

172.   Smith, R.G., et al., Presence of 4-hydroxynonenal in cerebrospinal fluid of patients with sporadic amyotrophic lateral sclerosis. Ann Neurol, 1998. **44**(4): p. 696-9.

173.   Tohgi, H., et al., Increase in oxidized NO products and reduction in oxidized glutathione in cerebrospinal fluid from patients with

sporadic form of amyotrophic lateral sclerosis. Neurosci Lett, 1999. **260**(3): p. 204-6.

174. *Suh, J.H., et al., Decline in transcriptional activity of Nrf2 causes age-related loss of glutathione synthesis, which is reversible with lipoic acid. Proc Natl Acad Sci U S A, 2004.* **101**(10): p. 3381-6.

175. *Ungvari, Z., et al., Vascular oxidative stress in aging: a homeostatic failure due to dysregulation of NRF2-mediated antioxidant response. Am J Physiol Heart Circ Physiol, 2011.* **301**(2): p. H363-72.