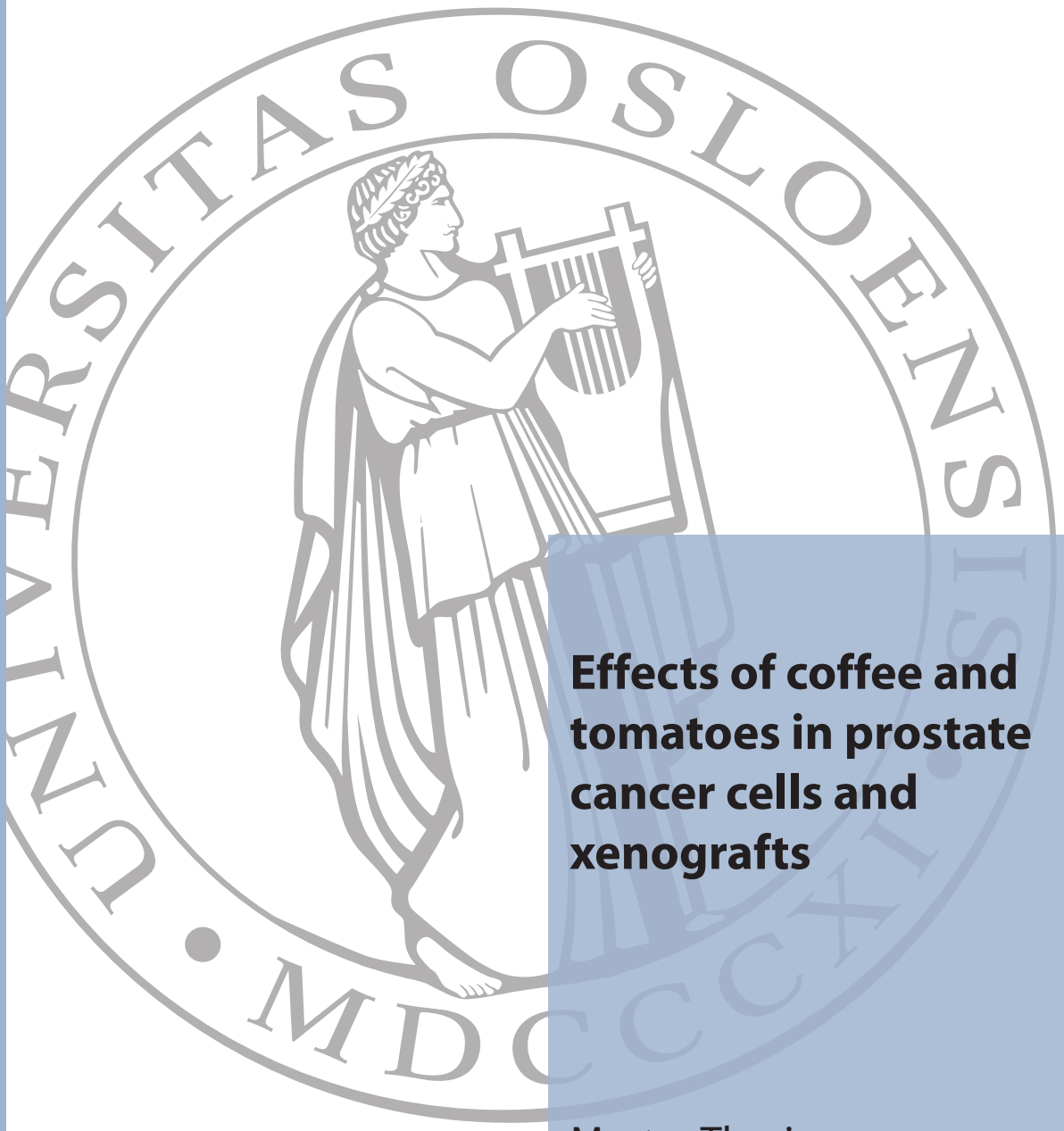

Effects of coffee and tomatoes in prostate cancer cells and xenografts

Master Thesis

by

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May 2011

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Oslo, May 2011

Sigrid Pedersen

Summary

Background and aims: Prostate cancer is the second most common cancer among men worldwide and the number of new cases is increasing with the increasing life expectancy. The disease is associated with an aberrant upregulation of the transcription factor NF-κB. In previous studies, tomatoes have shown promising results in terms of prevention of prostate cancer, and coffee has been reported to be among the most potent inhibitors of NF-κB. Therefore, this thesis examined the effect of coffee and tomatoes on NF-κB and inflammation- and cancer related genes in prostate cancer cells both *in vitro* and in xenografts, as well as studying the tissue accumulation of tomato constituents.

Methods: The ability of coffee and tomatoes to modulate NF-κB activity was investigated using a prostate cancer (PC3) cell line stably transduced with a luciferase reporter gene coupled to a promoter with NF-κB binding sites. Q-RT-PCR was used to analyse the effect of coffee and tomatoes on expression of genes related to immunity, inflammation and cancer in PC3 cells in culture and in xenografts. Analysis of accumulation of carotenoids in tissue of mice fed a diet enriched with tomato paste was performed using HPLC.

Results: Coffee induced basal NF-κB activity, but inhibited TNF-α induced NF-κB activity in PC3 cells *in vitro*. In general, the mRNA levels of genes involved in cytoprotection were upregulated, wheras a number of genes related to inflammation, apoptosis and tumor suppression were downregulated in cultured PC3 cells treated with TNF-α and extract of coffee as compared to controls treated with TNF-α only. In xenograft of PC3 cells, genes related to inflammation, apoptosis, cytoprotection and cell proliferation and differentiation were downregulated in mice fed a coffee containing diet. Tomatoes had a strong inhibitory effect on TNF-α induced NF-κB activity in PC3 cells. In TNF-α stimulated PC3 cells, genes related to inflammation and tumor suppression were downregulated by tomato extract, whereas genes related to apoptosis and cancer were upregulated. The ability of tomato to modulate the gene expression was less prominent in PC3 xenografts, however, ICAM-1 was upregulated, while the TGFβ1 gene was downregulated. Furthermore, carotenoids accumulated in several tissues, including liver, heart, spleen and seemingly also prostate, in mice fed a diet supplemented with tomato paste.

Conclusions: We found that coffee and tomatoes are strong inhibitors of TNF-α induced NF-κB activity in PC3 cells, and have the ability to modulate a number of genes related to cancer. Furthermore, we found that carotenoids accumulate in tissues of mice fed a diet rich in tomato paste. Our results may support a preventive role of coffee and tomatoes in prostate cancer, and further research should focus on elucidating the mechanisms behind the effects of coffee and tomatoes on NF-κB activity and expression of genes related to prostate cancer.

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List of Abbrevations

Ank	Ankyrin
ATP	Adeninetriphosphate
AUC	Area under the curve
BAD	BCL2-associated X protein
BHT	Butylated hydroxytoluene
BMI	Body mass index
CASP3	Caspase 3, apoptosis-related cystein peptidase
CAT	Catalase
CD40	Cluster of differentiation 40
CK2	Casein kinase 2
CO	Carbon monoxide
COX-2	Cyclooxygenase-2
C_t	Cycle treshold
CXCL11	Chemokine ligand 11
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulphoxide
DTT	Dithiothreitol
EDTA	Diethylenediamine tetraacetic acid
EDN1	Endothelin 1
EpRE	Electrophile respons element
FAS	Fas (CD95)
FBS	Fetal bovine serum
FRET	Fluorescence resonance energy transfer
GCLC	Glutamate-cysteine ligase, catalytic subunit
GPx	Glutathione peroxidase
GR	Glutatathione reductase
GSH	Glutathione (reduced form)
GSSH	Glutathione disulfide (oxidized form)
Gusb	Glucoronidase-β
HBV	Hepatitis B virus
HCC	Hepatocellular carcionoma
HCV	Hepatitis C virus
HIF1A	Hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)
HO	Heme oxygenase
H_2O_2	Hydrogen peroxide
HPV	Human papilloma virus
GPx	Glutathione peroxidase
ICAM1	Intercellular adhesion molecule 1 (CD54)
IκB	Inhibitor kappa B
IKBKB	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta /IKKβ
IKK	Inhibitor kappa B kinase
IGF-1	Insulin-like growth factor 1
IL	Interleukin
5-LOX	5-Lipoxygenase
LPS	Lipopolysaccharide
Luc	Luciferase
MeOH	Methanol
$MgSO_4$	Magnesiumsulfat-heptahydrat

MMP	Matrix Metalloproteinase
mRNA	messenger RNA
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NEMO	NF-κB Essential Modulator
NFE2L2	Nuclear factor (erythroid-derived 2)-like 2 (Nrf2)
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p50/p105))
NFKBIB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor beta(NF-kappa-B inhibitor beta/ IκBβ)
NF-κB	Nuclear Factor kappa B
NIK	NF-κB Inducing Kinase
NIST	National Institute of Standards and Technology
NLS	Nuclear Localization Sequence
NO	Nitric Oxide
$NO_2^{\cdot}$	Nitrogen dioxide
Nrf2	Nuclear Factor-erythoid 2-related factor 2
NQO1	NAD(P)H dehydrogenase, quinone 1
$^1O_2$	Singlet oxygen
O_2^-	Superoxide anion
$OH^{\cdot}$	Hydroxyl Radical
Q-RT-PCR	Quantitative Reverse-Transcription Polymerase Chain Reaction
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PSA	Prostate Specific Antigene
RelA	v-rel reticuloendotheliosis viral oncogene homolog A, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3, p65 (avian)
RelB	v-rel reticuloendotheliosis viral oncogene homolog B, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3 (avian)
RHD	Rel Homology Domain
RIN	RNA Integrity Number
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species (also used collectively for reactive oxygen, iron and nitrogen species in this thesis
SOD	Superoxide Dismutase
TBP	TATA Box binding protein
TD	Transactivation Domain
TGFB1	Transforming growth factor, beta 1
TNF	Tumor Necrosis Factor
TNFR	TNF receptor
TP53	Tumor protein 53 (p53)
UV	Ultraviolet
VEGF	Vascular Endothelial Growth Factor
WCRF	World Cancer Research Fund

1 Introduction

1.1 Dietary plants and health

1.1.1 Beneficial health effects of dietary plants

High consumption of dietary plants is widely believed to be beneficial for health. Data from both epidemiological studies and experimental studies have consistently shown that a diet rich in plant based foods can reduce the risk of a variety of chronic diseases such as certain cancers, cardiovascular disease and type 2-diabetes (1-5). In accordance with the increasing amount of data for the potential public health benefits, the government in Norway and in many other countries, recommend a diet rich in dietary plants, such as fruits, berries, vegetables, whole grain cereals, and legumes (6;7). Even though there is convincing evidence for the beneficial health effects of a diet rich in dietary plants, the mechanisms behind are not fully established.

Dietary plants have a high content of both micro- and macro nutrients, such as vitamins, minerals and dietary fiber. The physiological effects of these nutrients are reasonably well established. Plant foods generally have a low energy density. Therefore a high intake might reduce the risk of overweight and obesity, and related chronic diseases. Importantly, a high intake of dietary plants can also replace less favorable foods in the diet (6;8).

Still, the micro- and macro nutrients and the low energy density do not seem to fully explain the beneficial effects of a diet rich in dietary plants. A number of different mechanisms behind the protective effects have been suggested, including modification of detoxifying enzymes, dampening of inflammation and oxidative stress, stimulation of the immune system and changes in whole-body metabolism (3;9). Dietary plants contain a vast number of compounds commonly referred to as phytochemicals, and many of these may possibly modify these me chanisms.

1.1.2 Phytochemicals

Phytochemicals is a diverse group of chemical compounds that occur naturally in plants. "Phyto" is the Greek word for plants, and phytochemicals are therefore plant chemicals. Dietary plants contain a vast number of phytochemicals, which are classified according to their chemical structure and functional characteristics. Phytochemicals include carotenoids, phenolics, alkaloids, nitrogen-containg compounds and organosulfur compounds (**Figure 1.1**) (10). All of theses subgroups have been shown to have potentially beneficial health effects, either in humans or in laboratory experiments (3). There are estimations of approximately 100 000 different phytochemicals (3;8). Eating a plant based meal would lead to an intake of many different phytochemicals (8).

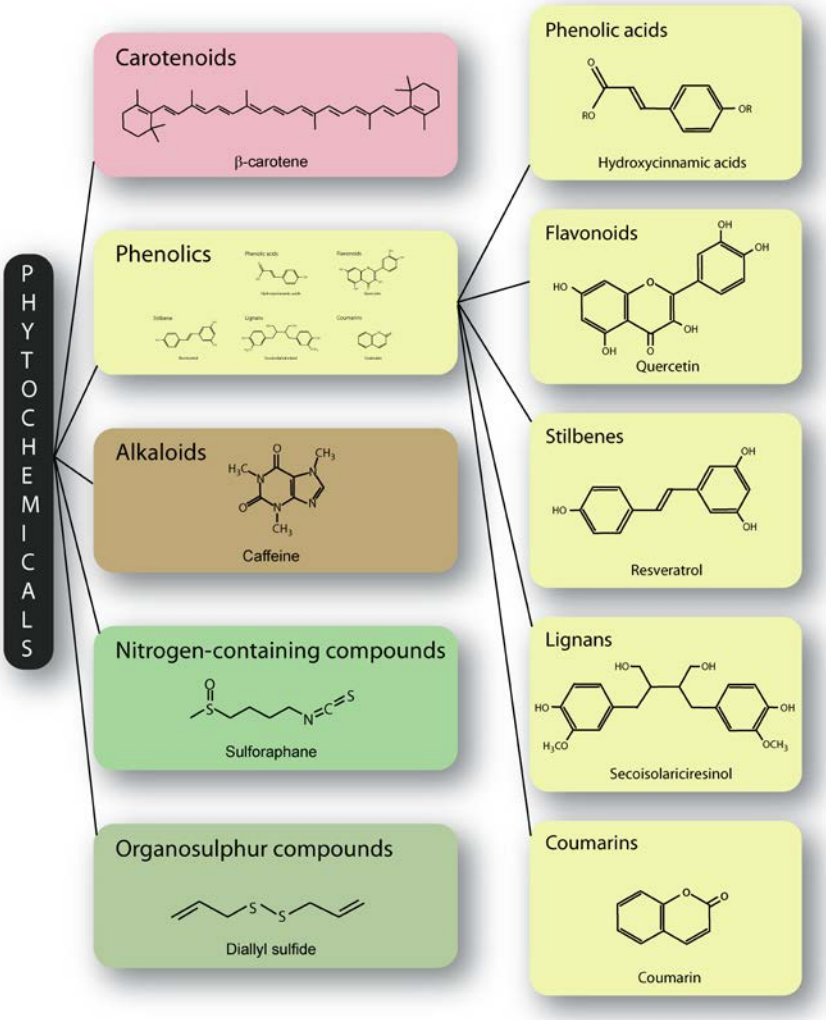


Figure 1.1: Classification of dietary phytochemicals. Phytochemicals can be classified as carotenoids, phenolics, alkaloids, nitrogen-containg compounds, and organosulfur compouns. This classes are again divided into several subgroups (10). By courtesy of I.Paur.

Phytochemicals perform important protective functions in the plants. Even though they are not established as essential nutrients for humans they are generally believed to have a positive impact on human health. Most phytochemicals have antioxidant properties *in vitro*, and one commonly suggested hypothesis is that the antioxidant properties of the phytochemicals have the potential of attenuating oxidative stress. However, it is still largely unclear whether phytochemicals in general exert antioxidant properties *in vivo* (3).

Over the last decades, chemoprevention through dietary phytochemicals has become an attractive area of research, and many phytochemicals have been shown to exhibit anti-carcinogenic properties. Phytochemicals have been shown to affect cellular processes like cell cycling, DNA repair, proliferation, differentiation and apoptosis. Furthermore, phytochemicals can affect the expression of oncogenes and tumor-suppressor genes, angiogenesis and metastasis, and hormonal activity (11). However, the effect varies with the different types of cancers and phytochemicals (3).

In terms of dietary intake of phytochemicals, coffee is the single larges contributor to phytochemicals in the Norwegian diet, followed by tea, fruits, and cereals (12;13).

1.2 Oxidative stress and antioxidant defense

1.2.1 Oxidative stress

Oxidative damage is involved in a large number of diseases, including cardiovascular diseases, cancer, type 2 diabetes, osteoporosis and neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease (8;14).

Free radicals are formed as byproducts of normal cellular metabolism (9;15). Examples of free radicals are reactive oxygen species, reactive nitrogen species and reactive iron species, hereafter collectively abbreviated ROS. They can also be formed as a consequence of diseases, tobacco smoke, environmental pollutants, drugs, natural food components and radiation (9).

Reactive species are involved reactions called redox (reduction-oxidation) reactions. In redox reactions there is a transfer of electrons from one substance to another substance. Oxidation is the loss of electrons from a substance, whereas reduction is the addition of electrons to a substance (9). Reactive species include superoxide anion radical ($O_2^{\cdot -}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$), nitric oxide (NO) and nitrogen dioxide ($NO_2^{\cdot}$) (15)

ROS are highly reactive molecules which can react with cellular components like proteins, lipids, carbohydrates, RNA and DNA, causing altered structure and function. A high level of reactive species seems to play an important role in the development of cancer. The primary mechanism is thought to be direct DNA damage, which is necessary, although not sufficient, for cancer development (16).

Normally, ROS exist in all aerobic cells in balance with antioxidants. However, if there is an imbalance because of excess ROS, depletion of antioxidants, or both, oxidative stress occurs. Antioxidants have the ability to limit oxidative stress by reacting with reactive oxidants. When an antioxidant reacts with ROS, the antioxidant itself is often transformed to an antioxidant radical. This radical has a reduced ability to react with cellular components, but can still cause damage. In order to further lower the reactivity, the radical needs to react with another antioxidant. These redox reactions can continue in many steps, until the antioxidant radical no longer has the capacity to damage the cellular components (9;16).

Different antioxidants have different biochemical properties and work together to attenuate oxidative stress. This has been called “the antioxidant network hypothesis”(9). Accordingly, this might be an explanation as to why high doses of one or a few antioxidants in supplements have generally not produced beneficial effects in human randomized, controlled trials (9;17;18).

Since oxygen is absolutely necessary, even though its metabolism produce ROS, all aerobe plants and animals have evolved comprehensive defense mechanisms against oxidative damage, including DNA repair and an endogenous antioxidant defense (19).

1.2.2 The endogenous antioxidant defense and cytoprotective proteins

The endogenous antioxidant defense includes both enzymatic and non-enzymatic antioxidants, which eliminate or prevent ROS from making damage (20).

Important antioxidant enzymes include superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPx), catalase (CAT) and heme oxygenase (HO) (20).

HO catalyzes the first step in the degradation of heme, producing carbon monoxide, biliverdin, and free ferrous iron (21). Biliverdin is subsequently degraded to bilirubin. Heme is pro-oxidative, whereas both biliverdin and bilirubin may act as physiological antioxidants (22;23).

SOD has a key role and catalyses the dismutation of superoxide anion ($O_2^{\bullet -}$) into oxygen (O_2) and hydrogen peroxide (H_2O_2). The hydrogen peroxide formed by SOD is less reactive than $O_2^{\bullet -}$, but can still be harmful. Further degradation of H_2O_2 to H_2O can be achieved by catalase, GPx or through conversion of reduced glutathione (GSH) to oxidized glutathione (GSSG) (20;24).

Glutathione is one of the key non-enzymatic antioxidants. Other important compounds in the non-enzymatic antioxidant system include vitamin C, vitamin E, carotenoids, flavonoids, thioredoxins, glutaredoxins and metal binding proteins. The thioredoxins have been identified as important for the defense system acting both directly and indirectly as an antioxidant and by affecting regulation of signal transduction (20;24). Fruits and vegetables may contain compounds that enhance the endogenous antioxidant defense (9). However, antioxidant

supplements have not been proved to prevent chronic diseases by interfering with oxidative damage (25).

The body removes xenobiotics by metabolism through phase I and phase II enzymes. Phase I enzymes convert toxic substances into more polar substances by oxidation, reduction or hydrolysis. These substances are subsequently converted into more hydrophilic forms by conjugation through hepatic phase II enzymes. In this way, the substances can be excreted through urine, feces, breath or sweat. Thus, the induction of phase I and II enzymes represent a crucial protection against carcinogens (26).

The endogenous antioxidant proteins have often been referred to as phase II enzymes, but this is however imprecise, and a more appropriate term would be cytoprotective proteins. The cytoprotective proteins include antioxidant enzymes, such as the enzymes described above, non-enzymatic proteins, phase II enzymes and enzymes acting on substrates which not are derived from phase I metabolism. The genes coding for many of the cytoprotective proteins have an electrophile response element (EpRE) (also called antioxidant response element) in their promoter which is regulated by the transcription factor Nuclear factor-erythoid 2-related factor 2 (abbreviated Nrf2 or NFE2L2, in this thesis Nrf2 is used) (27).

The transcription factor Nrf2 (encoded by the NFE2L2 gene) is a key regulator of the endogenous antioxidant defense through its ability to induce genes encoding for cytoprotective proteins. Upon activation Nrf2 translocates to the nucleus were it can bind to EpRE, thereby regulating a large number of genes. Experiments with Nrf2-knockout mice have demonstrated that Nrf2-dependent proteins are protective against carcinogens and important for the tumor cells reponse to chemotherapy (28-30). NQO1 is an inducible enzyme regulated by the Nrf2/EpRE pathway. It exerts its cytoprotective function through several mechanisms. NQO1 has a direct antioxidant function; it catalyses the two-electron reduction of quinones to hydroquinones, thereby preventing formation of ROS by other enzymes. In addition to its antioxidant role, NQO1 binds to and stabilizes p53 and other tumor suppressor proteins. Also, NQO1 has superoxide scavenging activity, which might be important in tissues with low SOD expression (31). GCLC is the rate limiting enzyme in the synthesis of GSH and plays an essential role in the endogenous antioxidant defense, maintaining the redox homeostasis (32).

1.3 Regulation of gene expression

Appropriate regulation of the gene expression is crucial for normal life. However, in a variety of diseases, including cancer, the normal regulation is disrupted.

The genome contains the entire genetic information of an organism. The human genome contains both coding sequences, and non-coding sequences, called introns and exons, respectively. The coding sequences contain all the information needed to make the RNA and proteins. Usually, a cell only express a fraction of its genes, so although most cells of multicellular organisms contains the same genome, differences in the gene expression ensures cell differentiation.

Transcription, translation and post-translational modifications are the three main steps of the process of converting the information stored in DNA into mature proteins (33).

Transcription is the process where one of the DNA strands is copied into a complementary RNA sequence by the enzyme RNA polymerase. The RNA polymerase binds in the promoter region to begin the transcription. The promoter is a nucleotid sequence in the DNA that facilitates the transcription of a specific gene. The RNA polymerase can not recognize the promoter and initiate the transcription itself. A wide range of regulatory proteins are required, including transcription factors, activators and repressors (33;34). Transcription factors regulate the gene transcription through binding of specific sites in the promoter.

In addition to the promoter, there are other regulatory regions in the DNA sequence, called enhancers or silencers, which can be thousands of base pairs away from the site of transcription start. Transcriptional activators and repressors respond to environmental stimuli, and bind to DNA. Repressors bound to silencers prevent the RNA polymerase to bind to DNA, whereas activators bound to enhancer regions attract the polymerase and increase its affinity for the promoter (35). Even though the activators and repressors are in long distance from the promoter in terms of the DNA sequence, looping of the DNA allows physical contact between the activators or repressors and the transcription factors bound to the promoter.

Depending of which RNA that is produced, the primary transcript undergoes different additional processing before it is functional. Transcripts assigned to become messenger RNAs (mRNAs) are capped, or added, a guanin nucleotide at the 5’end. Another processing

step required, is polyadenylation, that it the addition of a repeated adenine nucleotide sequence (33). The entire length of the gene, introns as well as exons, are transcribed into RNA. Introns are removed and the exons stitched together in a process called RNA splicing. The RNA can be spliced in many ways, thus allowing different proteins to be produced from the same gene (33;36).

Translation is the process by which the nucleotid sequence in the mRNA molecule directs the incorporation of amino acids into a protein. This process occurs on ribosomes (37). When the protein synthesis is completed, the activity of the protein can be regulated by post translational modifications, such as phosphorylation or methylation, by binding of other proteins or by degradation (33). The gene expression can be controlled at any of the steps in the pathway from DNA to protein as illustrated in **Figure 1.2**.

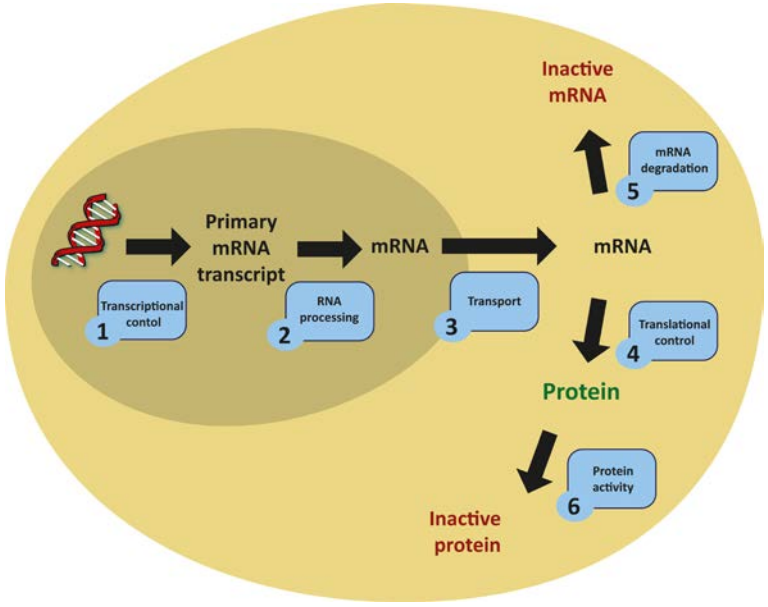


Figure 1.2: Control of the gene expression can occur at all of the steps in the pathway from DNA to protein. Modified from Alberts, by courtesy of I.Paur.

Another form of transcriptional regulation occurs through the structure of DNA. To fit into the nucleus the DNA has to be packaged into an extremely compact structure. This packaging is performed by special proteins that bind to and fold the DNA. This highly compact structure represents a barrier for transcription, ensuring the inactivity of thousands of genes. Only upon gene activation, the DNA remodels, allowing the transcriptional machinery to interact with the promoter DNA (34).

1.3.1 Regulation of gene expression by foods

The effect of nutrition on health and disease can not be understood without understanding how nutrients act at the molecular level. The study of how food or food components can influence gene and protein expression is referred to as nutrigenomics. The long-term goal of nutrigenomic studies is to provide evidence based dietary recommendations that can prevent diseases related to diet. Furthermore, nutrigenomics research seeks to understand the mechanisms underlying genetic predispositions and to identify genes that influence the risk of diseases related to diet.

New technologies have given us exiting opportunities to examine and understand how nutrients can modulate gene and protein expression, which eventually influence metabolic pathways. Both micro- and macronutrients can modulate metabolic pathways. For example, fatty acids can modulate the transcription factors liver X receptor (LXR), peroxisome proliferator-activated receptors (PPARs) and sterol-responsive-element binding proteins (SREBPs). Other food components, like flavonoids and xenobiotics have also been shown to regulate transcription factors pathways (38). One of the transcription factors that can be modulated by nutritional factors is Nuclear Factor-kappa B (NF-κB) (38-42).

1.4 Nuclear Factor-κB

1.4.1 Regulation of gene expression by NF-κB

Nuclear Factor-kappa B (NF-κB) is a family of transcription factors expressed in all cells in the body. The transcription factor is necessary for life, but is shown to be dysregulated in a variety of diseases, including chronic inflammatory conditions and cancers (43). The genes regulated by NF-κB can be divided into four major functional categories: immunoregulatory and inflammatory genes, anti-apoptotic genes, genes that positively regulate cell proliferation and genes that encode negative regulators of NF-κB (**Figure 1.3**) (44).

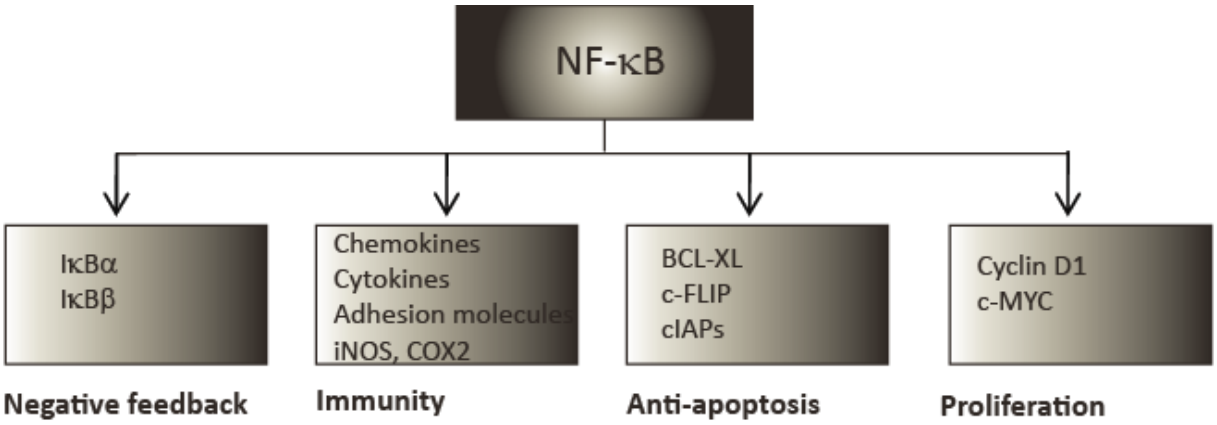


Figure 1.3: Classification of the NF-κB target genes. The NF-κB target genes can be divided into four major functional classes as genes involved in negative-feedback control of NF-κB activity, immunity, anti-apoptosis and proliferation (44). Modified from Karin (44).

1.4.2 NF-κB proteins and their regulators

NF-κB is not a single protein, but a group of closely related protein dimers that can bind to sequence motifs known as κB-sites. In mammals, the NF-κB family consists of five proteins, NF-κB1 (p105/p50), NF-κB2 (p100/p52), RelA (p65), RelB and c-Rel (**Figure 1.4 A**). All NF-κB proteins contain a well conserved N-terminal Rel-homology domain (RHD). The RHD works as the dimerization, DNA binding and regulatory domain. In addition it contains a nuclear localization sequence (NLS) in the C terminal end which is the site for binding of specific NF-κB inhibitors, known as the inhibitor of κB proteins (IκBs) (44-46). RelA, RelB and c-Rel also contain a transactivation (TA) domain, while NF-κB1 and NF-κB2 contain a glycine-rich "hinge" region followed by C-terminal ankyrin (Ank) repeats. An ankyrin repeat is a repeating sequence of 30-33 amino acids that mediates protein-protein interactions. While RelA, RelB and c-Rel are synthesized as mature proteins, the NF-κB1 and NF-κB2 are synthesized as large precursors (p105 and p100, respectively). Ubiquitin-dependent proteolytic processing removes the C-terminal ankyrin domain which results in production of the mature DNA-binding proteins (p50 and p52, respectively) (44;45).

These proteins form various NF-κB homo- and heterodimers that are associated with different biological responses through their differential ability to regulate target gene transcription. Formation of dimers is necessary for DNA binding, because each subunit binds to one half site of the κB-site (44;46).

The inhibitor of κB (IκB) proteins include IκBα, IκBβ, IκBγ, IκB-ε, Bcl-3, p105 (precursors of p50) and p100 (precursors of p52) (**Figure 1.4 B**). The IκB proteins contain five to seven conserved ankyrin repeat motifs that are required for their inhibitory activity and binding to the RHD. The IκBs associate with NF-κB dimers in the cytoplasm, which renders the dimers inactive (44).

The inhibitor of κB kinase (IKK) complex consists of three IKK polypeptides. Two of the polypeptides, IKKα and IKKβ are catalytic subunits, whereas the third polypeptide, IKKγ (also known as NF-κB Essential Modulator, NEMO) is a regulatory subunit (**Figure 1.4 C**) (45;47).

The primary structures of IKKα and IKKβ are similar, with 52 % sequence identity. The subunits have a N-terminal protein kinase domain and a C-terminal helix-loop-helix domain separated by leucine zipper motifs. The interactions between the subunits are mediated by the leucine motifs, whereas the helix-loop-helix domain interacts with the kinase domain and regulatory subunits. IKKγ consists of three large α-helical regions, including a leucine zipper (47).

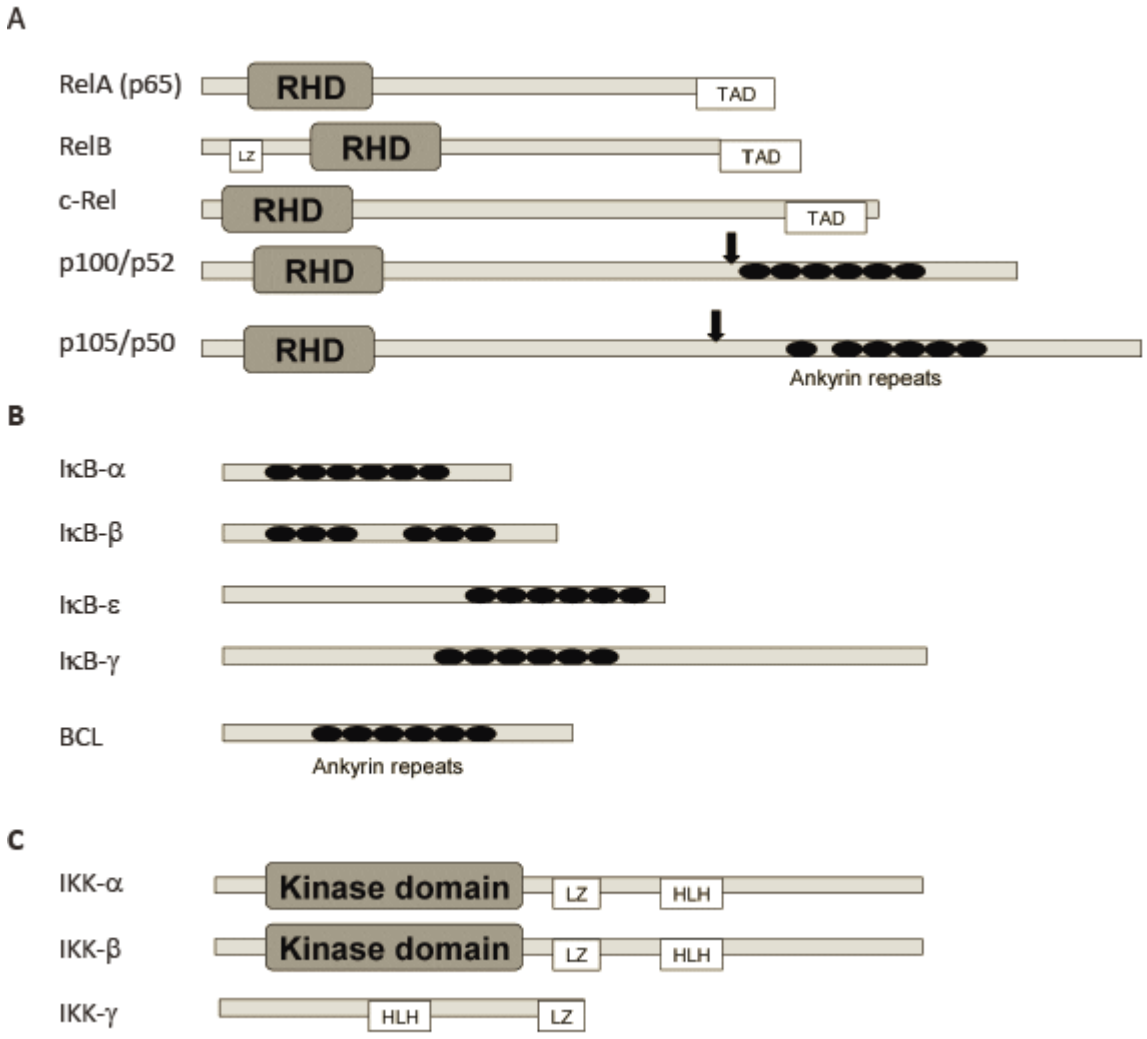


Figure 1.4: Schematic structure of the NF-κB proteins, IκB proteins and IKK complex. A) All NF-κB proteins contain a N-terminal Rel homology domain (RHD). In addition, the precursors p105 and p100 contains an ankyrin domain (black beads) which is removed through proteolytic processing, reulting in the production of the mature NF-κB proteins p52 and p50, respectively. B) The IκB proteins have six to seven ankyrin repeats (black beads). C) Ikkα and IKKβ contain a N-terminal protein kinase domain (KD), leucine zipper motifs (LZ) and a C-terminal helix-loop-helix domain (HLH), whereas IKKγ consists of three α-helical regions and leucin zipper motifs (45;47). TAD = Trans Activation Domain. By courtesy of M. Kolberg.

1.4.3 NF-κB signaling pathways

The activity of the transcription factors are regulated by two main pathways, the classical pathway (canonical) (**Figure 1.5**) and the alternative (non-canonical) pathway. In addition there are atypical pathways (44;46;48).

The classical pathway is mainly activated in response to microbial and viral infections or by inflammatory cytokines. These stimuli leads to activation of the IKK complex (44). In the

classical pathway all three IKK subunits are required, although the complex predominantly acts through IKKβ in an IKKγ dependent manner. The targets for the IKK complex in this pathway are homo-and heterodimers composed of RelA, c-Rel og p50 (44). Before cell stimulation, most dimers of NF-κB are kept in the cytoplasm in an inactive form through interactions with IκBs. Upon appropriate stimulation the NF-κB bound IκBs are phosphorylated at two conserved serines (Ser-32 and Ser-36) in the N-terminal regulatory domain by the IKK complex. Phosphorylation of the IκBs target them for rapid polyubiqutination and subsequent degradation by the 26S proteasome. The liberated NF-κB dimers can then translocate to the nucleus where they bind to κB sites in the DNA and regulate transcription of several hundred target genes (44). The classical pathway is essential for innate immunity and increases the transcription of genes required for migration of inflammatory and phagocytic cells to sites of infection or injury (46).

Certain members of the TNF cytokine family, but not TNF-α itself, activate the alternative pathway. The pathway affects NF-κB2, which preferentially dimerizes with RelB (44). Unlike the classical pathway, this pathway is entirely dependent on IKKα and independent of IKKβ and IKKγ (45). A upstream kinase NF-κB-inducing kinase (NIK) activates IKKα homodimers (49). These activated IKKα homodimers targets NF-κB2/p100 and phosphorylates it at two C-terminal sites. The phosphorylation leeds to polyubiqutination and proteasomal degradation of the inhibitory C-terminal half, consequently the N-terminal p52 polypeptide is released (44). The alternative pathway is not directly involved in innate immunity, but is required for adaptive immunity (46;49).

NF-κB can also be activated through other atypical pathways. These pathways are classified as atypical because they are independent of IKK. However, like the classical and alternative pathway the activation requires IκBα degradation via proteasomes. First, in response to DNA damage by ultraviolet (UV) radiation p38-activated casein kinase 2 can phosphorylate serine residues located in a C-terminal cluster (47;48). Second, anoxia can stimulate phosphorylation of IκBα at the N-terminal tyrosine 42. These phosphorylations leads to rapid proteolysis of IκBα (47).

The NF-κB signaling pathways are regulated by negative feedback control (44;50). IκBα is a target gene for NF-κB and binding of NF-κB to DNA lead to the transcription of many genes, including those encoding for IκBα. IκBα contains both nuclear localization and export sequences, enabling it to shuttle between the nucleus and cytoplasm. A high level of newly

synthesized IκBα causes the binding of IκBα to nuclear NF-κB, which leads to export of the complex from nucleus to the cytoplasm (50;51).

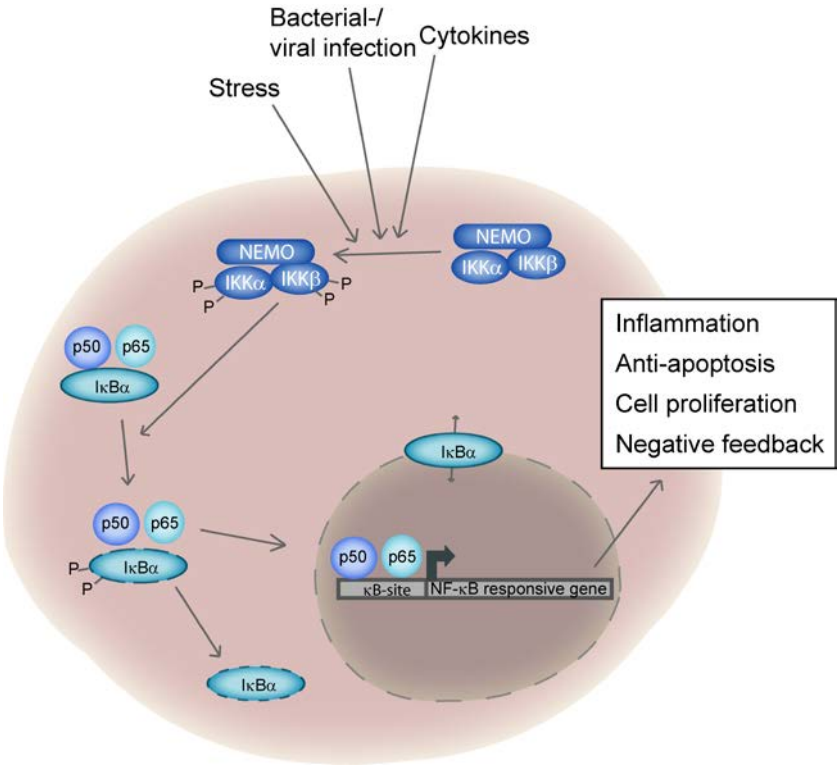


Figure 1.5: The classical NF-κB signalling pathway. The classical pathway is activated by stress, bacterial and viral infections, and inflammatory cytokines, such as TNF-α and LPS. Activation of this pathway depends on the IKK complex consisting of IKKα, IKKβ and IKKγ (NEMO), which phosphorylates IκBs. This results in ubiqutination and proteosomal degradation of the IκBs, and subsequent liberation of NF-κB dimers that can translocate to the nucleus. The classical pathway is essential for activation of innate immunity and inflammation, and for cell survival. TNF-α = Tumor Necrosis Factor- α, LPS = Lipopolysaccharide, IKK = Inhibitor κB kinase, p50 = NF-κB1, p52 = NF-κB2, κB-sites = NF-κB binding elements (45). By courtesy of I.Paur.

1.4.4 Activators and inhibitors

NF-κB is an important regulator of genes involved in immunity, anti-apoptosis and proliferation, and is shown to be overexpressed in a variety of cancers. Therefore, NF-κB represents a potential molecular target for cancer treatment, and a lot of research has focused on inhibitors of this pathway. To date, more than 750 NF-κB inhibitors have been identified, both natural and synthetic compounds. These compounds include phytochemicals, peptides, viral and bacterial proteins, small RNA, DNA and metals (41;52).

Activators of NF-κB

A wide range of stimuli which activate NF-κB have been identified. These stimuli include inflammatory cytokines, viral and bacterial infections, bacterial products, such as lipopolysaccharide (LPS), physiologcal stress, UV radiation, environmental pollution, several therapeutic drugs, apoptotic mediators, growth factors, and oxidative stress TNF is among the most studied activator of the NF-κB pathway (52).

TNF-α is a member of the TNF/TNF receptor (TNFR) cytokine superfamily which consist of 19 ligands and 29 receptors. TNF-α, often just referred to as TNF, is involved in immune reactions, inflammation and tissue homeostasis. TNF-α is a type II transmembrane protein with an intracellular amino terminus. It exists both as a membrane-integrated protein and as a soluble cytokine liberated after proteolytic cleavage. TNF itself is usually induced by pathogenic stimuli, often through Toll-like receptors. TNF has two receptors, TNFRI (also called p55) and TNFRII. The former is expressed in almost all cells, while TNFRII is primarily found on hematopoietic cells. TNFRI is activated upon binding of the soluble ligand, whereas TNFRII is mainly activated by the membrane-integrated protein. Activation of the TNFR results in recruitment of intracellular adaptor proteins that activate numerous signal transduction pathways (53;54).

Binding of ligand to TNFR1 can have two different outcomes, depending on the cellular context. The standard pathway is induction of genes involved in inflammation and cell survival. The activated TNFR1 recruits a protein called TNF receptor-associated death domain (TRADD), which activate the MAPK- and NF-κB pathways, leading to activation of AP-1 and NF-κB transcription factors. Together, the activation of these transcription factors induces genes involved in inflammation and survival (52-54). Seemingly in contrast to this, TNF can also promote apoptosis through activation of caspases. Activation of TNFRII also leads to activation of NF-κB and a variety of other signaling pathways, but the biological role of this receptor is still not clear (53;54).

Inhibition of NF-κB by Foods and Phytochemicals

Several natural compounds, such as phytochemicals, have been demonstrated to have the ability to modulate NF-κB activity both *in vitro* and *in vivo* (39-41;55). Most of the research so far has been done on purified compounds, even though some studies have investigated the effect of whole food items (39-41;55).

Paur *et al.* found that a number of extracts of dietary plants could attenuate LPS-stimulated NF-κB activity in monocytes. Of the 35 dietary plant extracts screened, the most potent inhibitors of NF-κB were oregano, thyme, clove, turmeric, and coffee (39). In a follow-up study, combined extracts of oregano, thyme, clove, walnuts, and coffee inhibited LPS induced NF-κB activity in transgenic NF- κB reporter mice. This combination extract also exerted synergistic effect on NF-κB activity in a monocytic cell line (40).

Mechanisms for inhibition of the NF-κB pathway

The inhibition can occur at several levels of the NF-κB activation, including blockage of the upstream incoming signal, interference with the recruitment of adaptors and activation of the IKK complex and inhibition of the NF-κB nuclear activity (41).

First, the NF-κB pathway can be inhibited by blockage of the signals upstream of the IKK complex. A variety of cytokines, for example TNF-α, can induce NF-κB activation, therefore, anti-TNF antibodies or agents that block the TNF receptor can inhibit the NF-κB activation (41). This kind of therapy has been used in the treatment of inflammatory diseases, such as Crohn's disease and arthritis (56-59).

Secondly, the IKK complex can be inhibited, and this has been the primary target in the development of NF-κB inhibitors. More than 150 agents have been identified which inhibit the IKK complex (41;60;61).

Furthermore, NF-κB activity can be hampered by a high level of inhibitors in the cytoplasm, either achieved by increased synthesis (62;63) of IκBs, or by inhibition of the degradation of IκBs through blocking the IκB ubiquitination (64) and proteosomal degradation (65). Inhibitors of the nuclear translocation of NF-κB have also been described. Cell permeable peptids that contain the nuclear localization sequence of p50 can inhibit the nuclear translocation of dimers containing p50 by saturating the machinery responsible for the nuclear import of NF-κB dimers consisting of p50 (66-68).

Inhibitors of NF-κB DNA binding represents another mechanism for inhibiton of the NF-κB pathway (69). Since the early 1990's, ROS have been implicated in NF-κB activation (70). Both cytoplasmic and nuclear steps in the NF-κB activation are proposed to be redox regulated, hence, it is believed that antioxidants can inhibit NF-κB activation (71). However, the exact role of redox regulation is not fully understood (72).

In theory, agents that disrupt the dimerization of NF-κB could be an effective way to block the NF-κB activity, however, no such molecule has yet been identified (41;52). Finally inhibition of the NF-κB transactivation may be a mechanism for blocking the NF-κB signaling. This could potentially be a mechanism for interference of specific NF-κB target genes (41).

1.5 Inflammation

The NF-κB transcription factor family is essential for orchestrating an appropriate acute and chronic inflammatory response. Acute inflammation is a part of the immune systems defense response, and is well established as a local, protective response to injury or infection. Usually, inflammation is self-limiting, however, sometimes the response moves into a chronic condition which can contribute to the development of cancer, diabetes, cardiovascular-, pulmonary- or neurological diseases (73). Chronic inflammation might be due to persistence of pro-inflammatory factors or failure of the mechanisms required to attenuate the inflammatory response (74).

In response to infections, tissue injury or other stimuli that initiate inflammation, a multi factorial network of chemical signals regulates a host response designed to remove the inflammatory trigger. Macrophages that have encountered microbes produce cytokines (e.g. TNF) activate endothelial cells. The activated endothelial cells produce selectines, integrin ligands, and chemokines, which coordinately recruit inflammatory cells to sites of infection. Activated neutrophils and macrophages produce free radicals and nitric oxide (NO). Usually, this microbicidal substances only act on the ingested microbes inside the lysosomes and are efficiently removed, thus they do not damage the phagocytic cells. However, in strong or persistent inflammatory reactions, free-radicals and NO can be liberated into the extracellular space and injure the host tissue. Therefore, this normally protective response to infection or injury, may cause increased production of reactive oxygen species and damage to the host (75).

1.5.1 Inflammation and cancer

Inflammation is crucial in order to manage pathogenic and damaging agents (76). Even so, a causal connection between inflammation, infection and cancer has been suspected for many years. Almost all solid tumors contain inflammatory infiltrates. These immune cells have a major impact on tumor initiation, growth and progression where proinflammatory cytokines mediate many of the effects. There are numerous pro-inflammatory gene products that mediate important roles in anti-apoptosis, proliferation, angiogenesis, invasion and metastasis. Among these are TNF and members of its superfamiliy, interleukins and several chemokines.

Already in the 19th century Virchow suggested that chronic inflammation could lead to malignancy (43;77). Today it is estimated that underlying infections and chronic inflammation are linked to 15-20 % of all deaths from cancer worldwide (78).

Hanahan *et al.* summarized the six main characteristics of cancer as self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, unlimited replicative potential, tissue invasion and metastasis and sustained angiogenesis (79). NF-κB can influence nearly all of these processes and is therefore a link between inflammation and cancer (**Figure 1.6**).

Epidemiological studies have shown that chronic inflammation predisposes to a variety of cancers (78). Infections with hepatitis B and C virus, human papilloma viruses and Helicobacter pylori are associated with hepatocellular carcinoma, cervical cancer and gastric cancer, respectively (49;78). Other triggers of chronic inflammationcan also increase the risk of developing cancer, examples are inflammatory bowel disease which is strongly associated with colon cancer (67) and prostatitis which is associated with prostate cancer (80).

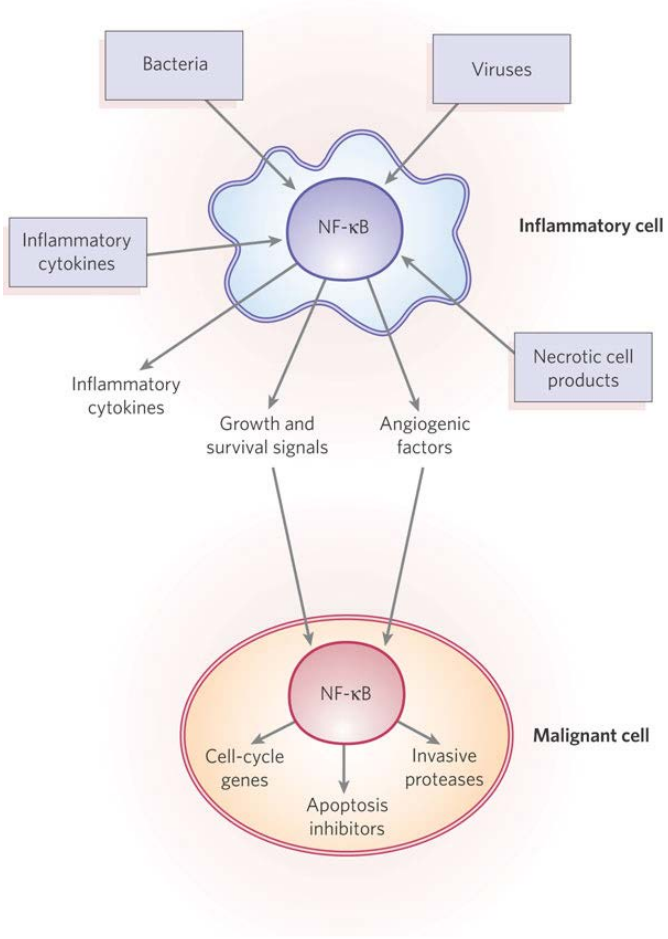


Figure 1.6: NF-κB provides a link between inflammation and cancer. A variety of stimuli, like infectious agents, inflammatory cytokines and proteins, can activate NF-κB in inflammatory cells. The activation of NF-κB can then lead to production and secretion of factors that enhance growth, survival and angiogenesis of malignant cells. Activation of NF-κB in malignant cells results in increased expression of cell cycle genes, inhibitors of apoptosis, and proteases that promote invasion (43). From Karin (43).

1.6 Prostate cancer

1.6.1 The prostate gland

The prostate is a walnut-sized gland that surrounds the urethra (**Figure 1.7**). Its primary function is to secrete a slightly alkaline fluid which constitutes 10-30 % of the seminal fluid. Within the prostate, the urethra coming from the bladder merges with the two seminal ducts. During ejaculation, the muscular compartment of the prostate help to propel the prostate fluid, in addition to sperm produced in the testicles, into the urethra (81;82).

Normally the prostate reaches mature size at the ages of 10-14 years. Around the age of 50, both the size of the prostate and the amount of its secretions often decrease. Increase in size after midlife may be a result of inflammation or malignancy (83).

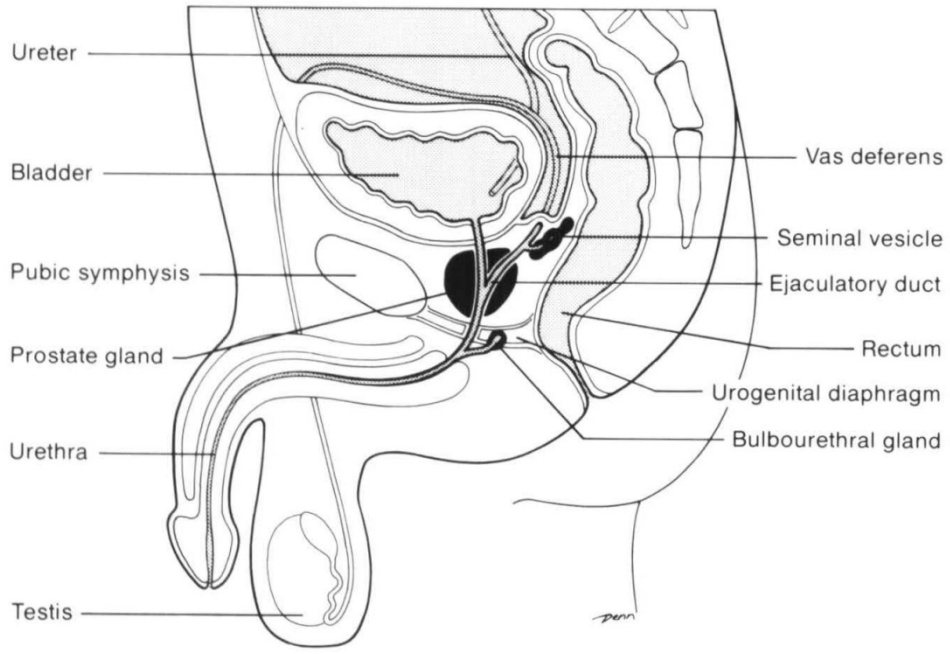


Figure 1.7: The male reproductive organs. From Cunha (82).

1.6.2 Incidence, prevalence, mortality and survival

Prostate cancer is the second most common cancer among men worldwide, accounting for about 12 % of all new cases of cancer in men (3). In Norway and many other high income countries prostate cancer accounts for an even greater share, and is the most frequent cancer in men (3;84;85). There were 4168 new cases of prostate cancer in 2008 in Norway, and the

incidence of prostate cancer is increasing in all age groups (84). This is largely because of longer lifespan and earlier diagnosis of microscopic, asymptomatic prostate cancers by screening for prostate specific antigen (PSA) (84). PSA is a protein secreted by the prostate and is normally present at low concentrations in the blood. Elevated levels of PSA in blood can be a sign of prostate cancer, but can also be a sign of other non-cancerous conditions, such as benign prostatic hyperplasia or prostatitis (86). Screening for PSA leads to detection of many prostate cancers that otherwise might have remained unrecognized. Interestingly, the age-adjusted rates of prostate cancers were already increasing before the availability of PSA testing. This suggests that the increase in prostate cancer is influenced also by environmental factors (3).

At the end of 2008 the total number of prostate cancer survivors in Norway were 13 795 (84).

The rate of mortality from prostate cancer in Norway is among the highest in the world, even though the mortality rate has declined slightly the last 10-15 years. There are great differences in long-term survival with respect to age at diagnosis. Long-term survival among patients diagnosed aged under 50 is actually lower than for patients diagnosed at age 50-59. This can in part be explained by more aggressive tumors in the younger age group, and may be the impact of screening in the older group (84;87).

1.6.3 Pathogenesis

Prostate cancer often remains asymptomatic during the entire lifespan and most prostate cancers are diagnosed before symptoms develop through PSA screening or a digital rectal exam (86). Early prostate cancer usually has no symptoms, while in more advanced disease, individuals might experience problems with urinating, such as weak or interrupted urine flow, need to urinate frequently, blood in the urine or discomfort or burning when urinating. Noteworthy, these symptoms also occur frequently as a result of non-cancerous conditions. Other symptoms are anemia, fatigue, weight loss and increased levels of PSA in serum (88). Advanced prostate cancer often metastasizes to the bones, causing back and skeletal pain.

Age, race/ethnicity and family history are well-established risk factors for prostate cancer (3;86;89). In addition many putative risk factors, including androgens, lifestyle and diet, have been implicated, though the strength of the evidence differs (3;85;86;89). The risk increases with age and over 80 % of prostate cancers in the U.S. are diagnosed in men over age 65

(3;85). Family history of prostate cancer is positively related to prostate cancer risk. If a man has one first-degree relative (a father or a brother) with prostate cancer he has a two to three times higher risk of developing prostate cancer. A man that has more than one relative with the disease has a three to five times higher prostate cancer risk (86).

Androgens influence the maturation of the prostate and are believed to contribute to the development of prostate cancer. However, the precise role of androgens in the etiology of prostate cancer is uncertain (85;86). Castrated men do not develop prostate cancer and animal studies have shown that androgens promote cell proliferation and inhibit apoptosis. Yet, data from epidemiological studies are inconclusive and there are few prospective studies that have found a significantly higher risk of prostate cancer among men with higher serum testosterone concentrations (85).

Ethnicity is a consistently reported, but not well understood risk factor (85). There are striking variations in incidence and mortality within the US. This might in part reflect different genetic factors in populations originating from different parts of the world. Incidence and death rates for prostate cancer are higher among Caucasian and African-American men, as compared to men of other racial and ethnic groups, including men of Asian heritage. However, migration studies have shown that men of Asian heritage living in the US have a higher risk than men of Asian heritage living in Asia. This suggests that not only the ethnicity, but also lifestyle has an impact on prostate cancer risk (86).

There are plausible mechanisms for how physical activity may be protective against prostate cancer. Physical activity may decrease the level of testosterone, prevent obesity, and enhance the function of the immune system, all of which may lead to reduced risk of prostate cancer. However, the results from studies of prostate cancer and physical activity are not completely consistent (85;86).

The role of obesity in prostate cancer is not clearly established. In the American Cancer Society Cancer Prevention Study-II (CPS-II) Nutrition Cohort they found that higher body mass index (BMI) was associated with lower risk of non-metastatic low-grade prostate cancer, but higher risk of high-grade, metastatic and fatal prostate cancer (86). Obesity is linked to many putative risk factors, including high meat and fat intake, and hormone metabolism. Also, there is a correlation between the prevalence of obesity and prostate cancer

risk across populations (85). Some studies have shown associations between sexually transmitted diseases and prostate cancers (85;86).

1.6.4 Nutrition and prostate cancer

The World Cancer Research Fund (WCRF) reported in 2007 that *foods containing lycopene or selenium, as well as selenium supplements, probably decrease the risk for prostate cancer, while diets high in calcium are a probable risk factor of this cancer.* Tomatoes are the main contributor to dietary lycopene and there is a substantial amount of evidence for the protective effects on prostate cancer by tomato products in particular. There is limited evidence indicating that pulses, foods containing vitamin E and alpha-tocopherol supplements are protective. It is also limited evidence suggesting that processed meat, and milk and dairy products increase the risk of this cancer (3). The possible association between meat consumption, particularly red meat and processed meat, and increased risk of prostate cancer, may be due to the high-fat content, mutagens induced during high-temperature cooking or other unidentified factors (85).

In ecological studies there has been shown a strong correlation between the incidence of prostate cancer and dietary fat intake. A high-fat diet can increase the production of both androgens and estrogens. The majority of epidemiological studies suggest a potential positive association between prostate cancers and monounsaturated and saturated fats, and an inverse association with omega-3 fatty acids. The role of polyunsaturated fat as a risk factor is less clear (85).

Consumption of fruits and vegetables is associated with a lower risk of several cancers, but their role in prostate cancer is less clear, except for foods containing lycopene (85).

1.6.5 NF-κB, inflammation and prostate cancer

Epidemiological studies have shown an increased prostate cancer risk in men with a history of prostatitis, and an increased level of pro-inflammatory cytokines from prostatic fluid collected from prostatectomies as been shown (89;90). Several preclinical and clinical studies have shown that NF-κB has a potential role in prostate cancer, andNF-κB has been demonstrated to be constitutionally active in androgen independent prostate cancer cell lines, as well as in prostate cancer patient samples (44;49;91).

An inflammatory microenvironment is an essential component of most tumors and a upregulation of NF-κB can increase the level of cytokines and growth factors that eventually can cause tumor growth (43).

Normally, TNF is usually not detected in blood of healthy individuals, but has been detected in many cancer patients, usually in those with an advanced disease. One example is prostate cancer, where an elevated level of TNF is associated with advanced, cachectic disease and poor prognosis (53;54). The role of TNF in malignant diseases is paradoxical. It has been shown that local administration of supra-physiological concentrations of TNF selectively can necrotize tumor blood vessels, hence the name tumor necrosis factor. However, endogenous produced TNF may act as a tumor promoter.

Blockade of NF-κB activity in human prostate cancer cells have been shown to suppress angiogenesis, invasion and metastasis (92).

Taken together, significant amounts of data suggest that NF-κB inhibitors are a promising target for prevention of prostate cancers or as a part of the therapy.

2 Aims of the thesis

Prostate cancer is second most common cancer among men worldwide and the number of new cases is increasing rapidly with the increasing life expectancy. Prostate cancer is associated with an aberrant upregulation and constitutive activation of the transcription factor NF-κB. Therefore, it is of great interest to identify lifestyle factors that can modulate the NF-κB activity and possibly contribute to preventing prostate cancer.

Previous work in the research group of Rune Blomhoff has identified several dietary plants with the capacity to inhibit LPS induced NF-κB activity. In this previous work, coffee was found to be among the most potent inhibitors of NF-κB. This observation, in combination with the widespread consumption of this beverage, rendered a special interest in coffee. With the promising results summarized by the World Cancer Research Fund, tomatoes are also of special interest in combination with prostate cancer. TNF-α, an activator of NF-κB, is normally not detected in the blood of healthy subjects, but are found in blood samples from prostate cancer patients, and in the microenvironment of the tumors. Therefore, this thesis examines the effect of coffee and tomatoes on prostate cancer cells both *in vitro* and in xenografts, as well as studying the tissue accumulation of tomato constituents.

The specific aims of this thesis were:

- To study the ability of coffee and tomato to modulate basal and TNF-α induced NF-κB activity in a prostate cancer (PC3) cell line.
- To elucidate whether coffee and tomato can influence the expression of genes coding for proteins involved in inflammation, cytoprotection and cancer development in a PC3 cells and PC3 xenografts.
- To identify tissues in which lycopene and other carotenoids accumulate in mice fed tomato paste.

3 Materials

Cell line

Cell line	Distributor	Location
PC3-κB-luc	The American Type Culture Collection	Manassas, VA, USA

Cell culture equipment

Chemical/compound/equipment	Manufacturer	Location
Cell culture flask, 75 cm^3	BD Biosciences	Franklin Lakes, NJ, USA
Cell culture plates, 6 wells	BD Biosciences	Franklin Lakes, NJ, USA
Cell culture plates, 24 wells, white	Perkin Elmer	Shelton, CT, USA
Countess® chamber slide	Invitrogen	Carlsbad, CA, USA
Fetal Bovine Serum	Sigma-Aldrich	St.Louis, MO, USA
F12K-Nutrient Mixture	Invitrogen	Carlsbad, CA, USA
Penicillin/Streptomycin	Sigma-Aldrich	St.Louis, MO, USA
TNF-α	R&D Systems	Minneapolis, MN, USA

Extracts of dietary plants

Name	Latin name	Producer	Location
Cielito Lindo coffee	Coffea Arabica	Naciemento	Oslo, Norway
Löfbergs Lila, Black Mystery	Coffea Arabica	Löfbergs Lila Kaffe AS	Sandefjord, Norway
Tomato paste	Lycopersicon	Eldorado	Oslo, Norway

Chemicals

Chemical/compound	Manufacturer	Location
Aceton	Merck	Darmstadt, Germany
α-carotene	Sigma-Aldrich	St.Louis, MO, USA
Argon	AGA	Oslo, Norway
ATP	Roche Diagnostics	Ottweiler, Germany
Astaxanthin	Sigma-Aldrich	St.Louis, MO, USA
Benzene	Merck	Darmstadt, Germany
β-cryptoxanthin	Sigma-Aldrich	St.Louis, MO, USA
BHT	Sigma-Aldrich	St.Louis, MO, USA
Coenzym A	Roche Diagnostics	Ottweiler, Germany
D-Luciferin	Biosynth AG	Staad, Switzerland
DMSO	Sigma-Aldrich	St.Louis, MO, USA
DTT	Sigma-Aldrich	St.Louis, MO, USA
EDTA 0.5 M	Merck	Darmstadt, Germany
Ethanol	Arcus	Oslo, Norway
Isopropanol	Arcus	Oslo, Norway
Lutein	Sigma-Aldrich	St.Louis, MO, USA
Lycopene	Sigma-Aldrich	St.Louis, MO, USA
Methanol	Merck	Darmstadt, Germany
$MgSO_4$ x 7 H_2O	Merck	Darmstadt, Germany
MilliQ water	Millipore	Bedford, MA, USA
NIST 968c SRM	NIST	Gaithersburg, MD, USA
PBS	Sigma-Aldrich	St.Louis, MO, USA
RNaseZap	Ambion, Inc	Austin, TX, USA
TaqMan gene expression	Applied Biosystems	Foster City, CA, USA
Tricine	Sigma-Aldrich	St.Louis, MO, USA
Trypan blue	Invitrogen	Carlsbad, CA, USA
Zeaxanthin	Sigma-Aldrich	St.Louis, MO, USA

Kits

Name	Manufacturer	Location
Agilent RNA 6000 Nano Kit	Agilent Technologies	Palo Alto, CA, USA
Bio-Rad Protein Assay	Bio-Rad Laboratories, Inc	Hercules, CA, USA
High capacity cDNA reverse transcription kit	Applied Biosystems	Foster City, CA, USA
RNase-free DNase set	Qiagen	Valencia, CA, USA
RNeasy Mini Kit	Qiagen	Valencia, CA, USA

Equipment

Equipment	Manufacturer	Location
Bulk beads 1.4 mm, zirconium oxida beads	Bertin Technologies	Montigny, France
Centrifuge tubes, 15 and 50 ml	BD Biosciences	Franklin Lakes, NJ, USA
Micro tubes, 2 ml	Sarstedt	Nümbrecht, Germany

Instruments

Instrument	Manufacturer	Location
7900 HT Fast Real-Time	Applied Biosystems	Foster City, CA, USA
Agilent 2100 Bioanalyzer	Agilent Technologies	Palo Alto, CA, USA
Agilent Technologies 1100 HPLC System	Agilent Technologies	Palo Alto, CA, USA
Biofuge Fresco	Heraeus Instruments	Osterode, Germany
Chip priming station	Agilent Technologies	Palo Alto, CA, USA
Countess™ automated cell counter	Invitrogen	Carlsbad, CA, USA
Coulter counter ZM	Beckman Coulter, Inc	Fullerton, CA, USA
Labofuge 400e	Heraeus Instruments	Osterode, Germany
Nanodrop™ ND-1000	Thermo Scientific	Wilmington, DE, USA
Sonicator, 2510 Branson	Branson Ultrasonics Corp.	Dansbury, CT
Synergy 2	BioTek® Instruments, Inc	Winooski, VT
TaqMan® Array Micro Fluidic	Applied Biosystems	Foster City, CA, USA
Titertek Multiskan Plus	ELFAB	Finland

Software

Software	Manufacturer	Location
2100 Expert Software	Agilent Technologies	Palo Alto, CA, USA
Adobe Illustrator CS4	Adobe Systems Incorporated	San Jose, CA
Gen5 Data Analysis Software	BioTek® Instruments, Inc	Winooski, VT, USA
Microsoft Office XP	Microsoft Corporation	Redmont, WA
PASW Statistics 18	SPSS Statistics	Chicago, IL
Reference Manager 12	ISI Research Soft	Carlsbad, CA
RQ Manager	Applied Biosystems	Foster City, CA, USA
SDS 2.3	Applied Biosystems	Foster City, CA, USA
ND-1000 v.3.2.1	Thermo Scientific	Wilmington, DE, USA

4 Methods

4.1 Background studies

This master thesis is a part of a prostate cancer research project in professor Rune Blomhoffs research group. Prior to the start of this thesis work, two mice experiments were performed by PhD student Marit Kolberg, and these experiments provide the background for this thesis. Both experiments were intervention studies using the same diet with tomato and coffee as the intervention. Several tissues from these mice studies were used and analyzed in the present master thesis, andsome background information about these studies is therefore included here.

4.1.1 Experimental diets

Three different diets were used in the two animal studies. All diets were based on a feed high in fat and sugar, and low in phytochemicals (**Table 4.1**). The animals assigned to the control group were given diet without any supplementation, whereas diet of the intervention groups was supplemented with either 10 % wet weight (w/w) tomato paste (Eldorado) or 5 % w/w brewed espresso coffee (Löfbergs lila, Black mystery). The calorie content was the same in all feeds.

Table 4.1: Nutrient content in the control diet.

Nutrient	kcal /g diet	Energy % (kcal)
Protein	0.98	20
Carbohydrate	1.67	34
Fat	2.25	46
Total	**4.9**	**100**

4.1.2 Pilot study

The first of the two studies was conducted as a pilot study in order to test if the mice consumed the diets, and to examine carotenoid accumulation in various tissues.

Wild type mice (C57/BL6) were bred and housed at Section for Comparative Medicine (IMB, UiO) in accordance with the guidelines of the Federation of European Laboratory Animal Science Association (FELASA). The mice were randomized into three groups, one control

group (n = 4), one tomato group (n = 4) and one coffee group (n = 4). The mice were fed their assigned diet for two weeks before they were euthanized and the organs snap frozen. Only organs from the control and the tomato group were used in the further analysis, as the set up conditions for analyzing coffee compounds was not ready.

4.1.3 Prostate cancer xenograft

Xenografts

The second experiment was conducted to study the effect of tomato and coffee on tumor growth, mRNA expression and NF-κB activity in prostate cancer xenografts. Naval Medical Research Institute (NMRI) nude mice were purchased from Harland Laboratories Inc. The main characteristics of these mice are immunodeficiency because they lack thymus, rapid growth, high reproduction rate, high capacity for learning, aggressiveness from 8 weeks of age (male) and renal disease in aged animals.

Male NMRI nude mice (6 weeks old) were injected with PC3-κB-luc cells (see description of the cell line in section 4.3.1) subcutaneously in both the right and left flank. Prior to the injection, PC3-κB-luc cells were prepared in 50 % matrigel (BD Bioscience, Bedford, MA) and 50 % F-12K Nutrient mixture medium. A suspention of 10^6 cells/0.1 ml medium:matrigel was injected into each flank.

Experimental outline

The experimental outline is schematically presented in **Figure 4.1**.

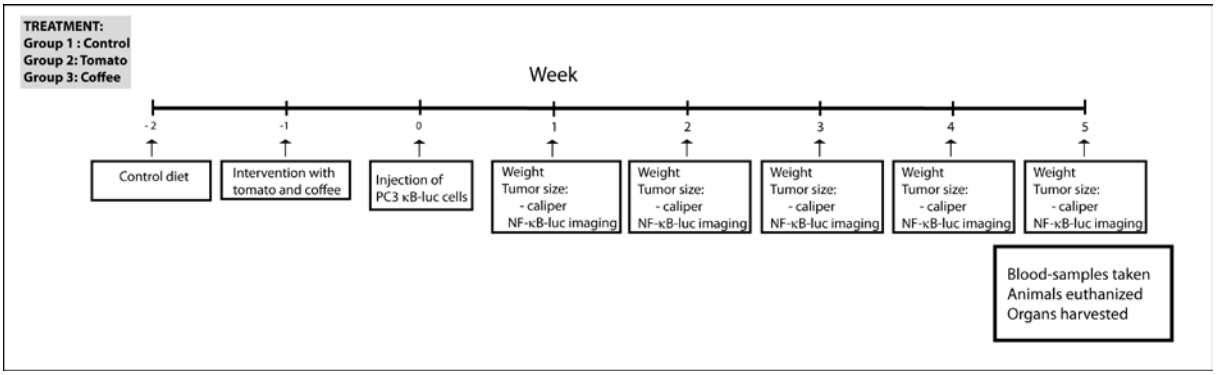


Figure 4.1 Experimental outline intervention study in NMRI mice with PC3-κB-luc xenografts. By courtesy of M. Kolberg.

All mice were fed the control diet for one week before they started on their intervention diets. The mice were randomized into three groups, which received the diets described above; one group received control diet (n = 15), one group received control diet mixed with 10% w/w tomato paste (n = 15) and one group received control diet mixed with 5 % w/w coffee (n = 15). After one week on their respective diets, PC3-κB-luc cells were injected. The intervention lasted for five weeks, and every week the NF-κB activity in xenografts was measured by *in vivo* imaging. The animals were anesthetized using 2-3 % isoflurane. D-luciferin in PBS was injected intraperitoneally (110 mg/kg), and the mice were imaged 10 minutes after injection using an IVIS spectrum instrument (Caliper Life Sciences, USA). In addition, the mice were weighted twice a week and tumor size measured with a caliper. At the end of week 5 the animals were euthanized and organs harvested for further analysis.

Imaging results of the xenograft study

In vivo imaging was performed at week 1, 2, 3, 4, 5 and there was a trend towards lower NF-κB luciferase activity in the xenografts of the tomato and coffee group compared to control. This was not statistically significant, except at week 3 where the coffee group had significantly lower NF-κB activity. Based on area under the curve (AUC), tomato and coffee had a non-significant lower NF-κB activity than the control group. There was no difference in tumor size between the groups. The results from the xenograft experiment are summarized in **Figure 4.2**.

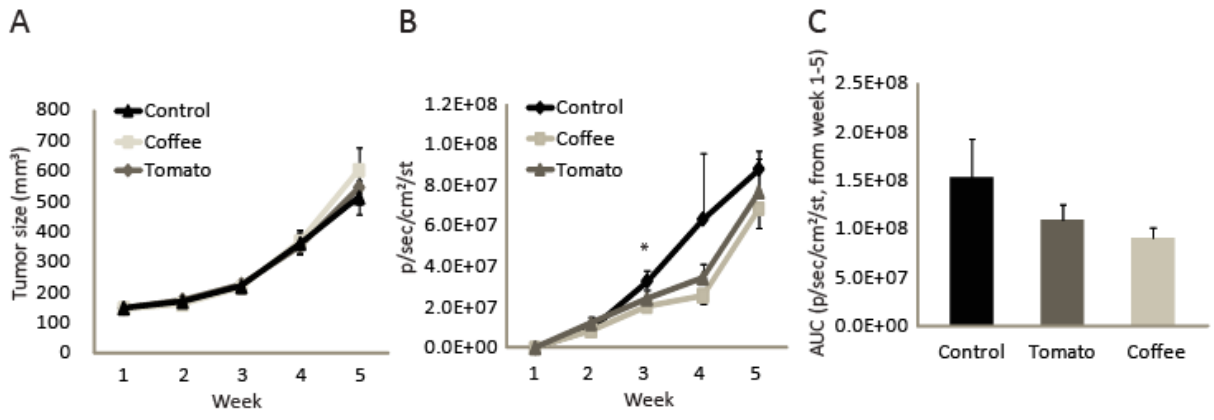


Figure 4.2: Effect of coffee and tomatoes in prostate cancer (PC3) xenograft model. Mice were randomized into three groups which received control diet (n = 15), control diet mixed with 5 % wet weight coffee (n = 15) or control diet mixed with 10 % wet weight tomato paste (n = 15). Every week for five weeks, tumor size and NF-κB activity were measured by caliper and in vivo imaging, respectively. A) Tumor size (mm3). B) NF-κB activity (p/sec/cm2/st) in xenografts. C) Area under curve (AUC) NF-κB activity in xenografts (based on p/sec/cm2/st). By courtesy of M Kolberg.

4.2 Extracts of coffee and tomato paste

Tomato paste and coffee were obtained from local stores in Oslo, Norway. The coffee beans were pulverized, and 20 ml boiling MilliQ water was added to 10 g of the sample. The mixture was cooled down before 20 ml methanol was added. Tomato paste (70 % water content) was added 3 ml MilliQ water and 10 ml methanol. The samples were vortex mixed and subsequently treated with an ultrasonic water bath for 30 min at 0^{o}C. The mixture was centrifuged at 4000 rpm at 4^{o}C for 15 min and transferred to small Erlenmeyer flasks. The liquid phase was concentrated to a viscous fluid (≤ 5ml) under nitrogen gas. The concentrated coffee and tomato extracts were diluted in dimethyl sulphoxide (DMSO) and phosphate buffered saline (PBS) and PBS only, respectively, to a final concentration of 2 g per milliliter of extract. The extracts were sterile filtered and stored under argon gas in airtight tubes at –70^{o}C.

4.3 Cell line and culturing

4.3.1 PC3

The human prostate cell line PC3 was originally isolated from a bone metastasis of a grade IV prostatic adenocarcinoma from a male Caucasian. PC3 cells do not express PSA and androgen receptors, thus they are androgen insensitive. The cell line is an aggressive form of prostate cancer and have the characteristics of late-stage prostate cancer (93).

The PC3-κB-luc cell line is a subclone of the PC3 cell line, and is stably transduced with a reporter construct containing tandem repeats of κB-sites (NF-κB binding elements), coupled to the luciferase gene (**Figure 4.3**). Luciferase activity in these cells is thus a measurement of the NF-κB activity.

Figure 4.3: Schematic view of the NF-κB luciferase reporter construct in PC3-κB-luc cells.

The PC3-κB-luc cell line also expresses a puromycin resistance gene (puromycin N-acetyl transferase gene). Puromycin is an antibiotic used as a selection agent. It prevents growth of a wide range of eukaryotic and prokaryotic cells by interfering with protein synthesis. Thus, the presence of puromycin in the growth medium secures that only cells with the puromycin resistance gene, that is the stably transduced cells, are selected.

The cells were cultured in F-12K Nutrient mixture medium supplemented with 10 % heat inactivated fetal bovine serum (FBS), penicillin (50 U/ml), streptomycin (50 mg/ml) and puromycin (1 μg/ml) at 37^{o}C, 5 % $CO_{2.}$ When the cells reached about 80 % confluence, medium was removed and the cells treated with 0.25 % trypsin to disperse the cells. After five to ten minutes, complete growth medium were added to inactivate the trypsin. Appropriate aliquots of the cell suspension were added to new culture vessels containing complete growth medium.

4.3.2 Storage and thawing

The cells were stored in liquid nitrogen vapor phase in growth medium supplemented with 5 % DMSO to prevent crystal formation during storage. The cells were quickly thawed in a 37^{o}C water bath and medium was changed the subsequent day to remove the DMSO. The cells were kept in culture for at least one week after thawing, before they were used in experiments.

4.3.3 Cell counting

The cells were counted using a Beckman Coulter counter® apparatus based on the Coulter principle. The aperature has two electrodes, one inside an aperture tube and one in a beaker containing a suspension of the cells to be counted in a low concentration electrolyte solution. Since cells are non-conducting particles, the electrical conductance is altered when the electrolyte is drawn through the aperature, and the impedance between the electrodes can be measured (94).

Before cell counting the adherent PC3-κB-luc cells were detached from the cell culture flask surface using trypsin.

4.3.4 Cell viability

To examine potential cytotoxity of the plant extracts trypan blue exclusion and morphological observation in light microscope were performed.

The cell membrane of live cells are not permeable for the trypan blue dye, however, in dead cells the dye is readily absorbed. Hence, the non-viable cells are dyed blue and can be distinguished from the viable cells (95). The total number of cells, and the percent of viable and non-viable cells was counted using a Countess® automated cell counter. All extracts were tested at all concentrations used in order to investigate cytotoxity.

The cells were seeded in 6 well plates and treated as in the corresponding experiments investigating the NF-κB-luciferase activity. Cytotoxity was examined in both basal and TNF-α stimulated cells after 6.5 hours incubation with the extracts. First, the cells were observed under the microscope to investigate if there were any visual differences in the morphology in cells treated with extract compared to control. Secondly, the cell medium was removed and the cells trypsinated to make a cell suspension which was used to perform the trypan blue exclusion. Cell suspension (10 µl) was mixed with 0.4 % trypan blue (10 µl), and 10 µl of sample were pipetted into a Countess® chamber slide. The slide was immediately inserted into the cell counter and analyzed. The cell viability test was performed in duplicates. Cut-off was determined as the lowest concentration that caused a morphology change and/or 10 % non-viable cells compared to controls.

4.3.5 Experimental outline of *in vitro* experiments

PC3-κB-luc cell experiments - luciferase activity

One day prior to the experiments, the PC3-κB-luc cells were diluted to 0.15 million cells per ml in order to be in an exponential growth phase at the time of the experiment. The cells were seeded in 24 well plates, 0.15 million cells per well, and cultured over night. On the day of the experiment, the medium supplemented with 10 % FBS was carefully replaced with medium containing 2 % FBS. The controls cells were added the same amount of DMSO (<0,1 % DMSO) as the cells treated with the highest concentration of extract. The extracts (coffee or tomato paste) were diluted to desired concentration using medium containing 2 % FBS. The cells were incubated with extract or vehicle for 6.5 hours. Both basal and TNF-α induced NF-κB activity was measured. If the cells were treated with TNF-α (10 ng/ml), it was

added 30 minutes after addition of the plant extracts. At the end of the experiment the medium containing extracts and TNF-α was replaced with new medium. D-luciferin (0.2 mg/ml) was added to each well and incubated for 5 minutes before luminescence were detected by a Synergy 2 instrument (see section 4.4). All experiments were repeated at least three times and each treatment was always performed in triplicates.

PC3-κB-luc cell experiments – mRNA levels

One day prior to the experiments, the cells were diluted to 0.15 million cells per ml in order to be in an exponential growth phase. Cell suspension were seeded in 6 well plates, 2.5 ml in each, and cultured over night. The day of experiment, medium supplemented with 10 % FBS were carefully replaced with medium containing 2 % FBS. The cells were incubated with extract (10 mg/ml tomato paste and 6 mg/ml coffee) or vehicle for 0.5 hours, added TNF-α (10 ng/ml), and incubated for additionally six hours before the cells were harvested and used for further analysis of mRNA levels. Triplicates of each treatment (basal vehicle, TNF-α control and coffee/tomato + TNF-α) were pooled together at each experiment. All experiments were repeated three times. The further analysis is described in section 4.5.1.

4.4 Luciferase reporter

Bioluminescence is the production and emission of light by a living organism. The luciferase gene occurs naturally in several organisms, including bacteria, beetles (e.g. firefly) and sea pansy (Renilla). The luciferase gene has been successfully cloned from several of these, and is widely used as reporter genes. The most commonly used bioluminescent reporter is firefly luciferase, which is used for the experiments in this thesis. Binding of NF-κB to the κB sites in the promoter leads to production of the enzyme luciferase. In the presence of the co-factors ATP, Mg^{2+} and 0_2, the luciferase catalyzes the oxidative decarboxylation of luciferin to oxyluciferin and CO_2, producing a yellow-green light (560 nm) that can be detected using an instrument that measures the luminescence (**Figure 4.4**) (96).

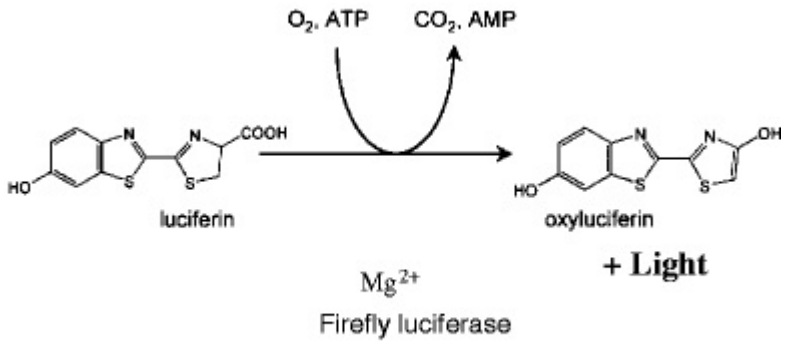


Figure 4.4: Chemical reaction catalyzed by firefly luciferase. The enzyme firefly luciferase catalyzes the oxidative decarboxylation of D-luciferin to oxyluciferin in the presence of cofactors, including ATP, O_2, Mg^{2+}, producing yellow-green light. Modified from Berger *et.al* (96).

4.4.1 Measuring luciferase activity *in vitro*

At the end of the PC3-κB-luc experiments, luciferin in excess (0.2 mg/ml) was added to the cell culture medium and luminescence was measured using Synergy 2 from BioTek® Instruments. The instrument was controlled using BioTec's Gen 5 TM PC software. Synergy 2 measures the relative luminescence units (RLU) in each well for the duration of integration time (10 seconds), and Gen 5 reports the average of these data points.

4.4.2 Measuring luciferase activity in PC3 Xenografts

Luciferase assay

Luciferase activity in PC3 xenografts was analyzed by the luciferase assay. By lysis and homogenization of the tissues, the luciferase synthesized within the cells is released, thus making the enzymes available to the D-luciferin in the assay substrate solution.

The frozen PC3 xenografts were transferred to 2 ml Sarstedt tubes with ceramic beads and added 1ml reporter lysis buffer. The samples were homogenized by shaking at 5000 rpm in 2 x 20 seconds with the Precellys24 lysis and homogenization instrument. After homogenization, cell debris was removed by microcentrifugation for 15 minutes at 4^{o}C. The supernatant was used for the luciferase and protein measurements. For luciferase measurements, 20 µl of the sample was added to 96 well plates in duplicates. Subsequently 100 µl luciferase assay substrate (**Table 4.1**) was automatically injected directly to the lysates and luciferase activity was measured using a Synergy 2 instrument. The luciferase activity

was standardized to total protein concentration to correlate for differences in sample size and homogenization efficiency.

Table 4.1: Luciferase assay substrate solution. The reagents were mixed, the pH adjusted to 7.4 and ddH2O added to a total volume of 100 ml. The addition of Coenzyme A leads to a sustained light reaction and increased sensitivity of the assay (97).

Reagent	Amount
ATP	52.1 mg
Coenzyme A	20.7 mg
Tricine	358.4 mg
$MgSO_4$ x 7 H_2O	92.2 mg
DTT	513.5 mg
EDTA	15 µl
ddH2O	80 ml + adjustment volume
D-luciferin	650 µl

Protein assay

The protein content in the samples was measured by Bio Rad protein assay. The Bio Rad Protein assay is based on the method of Bradford (98). The unbound form of Coomassie Brilliant Blue G-250 dye is red. Upon binding to proteins, primarily to basic and aromatic amino acid residues, the dye is converted into a blue color. This color shift results in a higher absorbance, and protein concentration can therefore be estimated by measuring the absorbance. The absorbance maximum shifts from 465 nm to 595 nm when binding to protein occurs (99).

The samples were diluted 40 to 60 times in MilliQ water to ensure that the protein concentration was within the standard curve. Protein standards, 0.041, 0.061, 0.091, 0.273 and 0.410 mg albumin/ml, and triplicates of the samples were added to a 96 well plate, 10 µl in each well. In addition a blank of 10 µl MilliQ water was included. BioRad assay (200 µl) was added to each well, and absorbance measured at 590 nm in a Titertek microplate reader.

4.5 Quantitative reverse-transcription PCR

To further examine the effects of coffee and tomatoes in prostate cancer, we studied the mRNA levels of genes involved in cancer, endogenous antioxidant defense, inflammation and immunity. This was performed using specially designed TaqMan® Array Micro fluidic Cards with 384 wells. The sample preparation included, homogenization of the cells or tissues, RNA isolation, evaluation of the RNA quality, cDNA synthesis and loading and running the micro fluidic card. Finally, the data were analyzed and statistical analysis performed.

Polymerase chain reaction

Polymerase chain reaction (PCR) is a method that selectively and rapidly amplifies a given DNA sequence. The PCR starts with a double-stranded DNA, for example cDNA synthesized from mRNA isolated from cells or tissues. In the first step of the reaction, the two strands are separated by a brief heat treatment (step 1). These single-stranded DNA sequences serve as templates for a thermally stable DNA polymerase. The DNA is cooled in the presence of a large excess of two DNA oligonucleotids, called primers. These primers are specially designed for the experiment, and allow us to only replicate the target sequences. The two primers hybridize to the complementary sequences of the two DNA strands (step 2). The PCR mixture contains four deoxyribonucleoside triphosphates (ATP, GTP, CTP, TTP) which the DNA polymerase uses to synthesized DNA, starting from the two primers (step 3). The cycle is then begun again, by a heat treatment to separate the newly synthesized DNA strands. For each repeated cycle, the amount of DNA from the previous cycle is doubled by using the newly synthesized DNA as templates. A schematic view of the PCR amplification of a DNA sequence is shown in **Figure 4.5**.

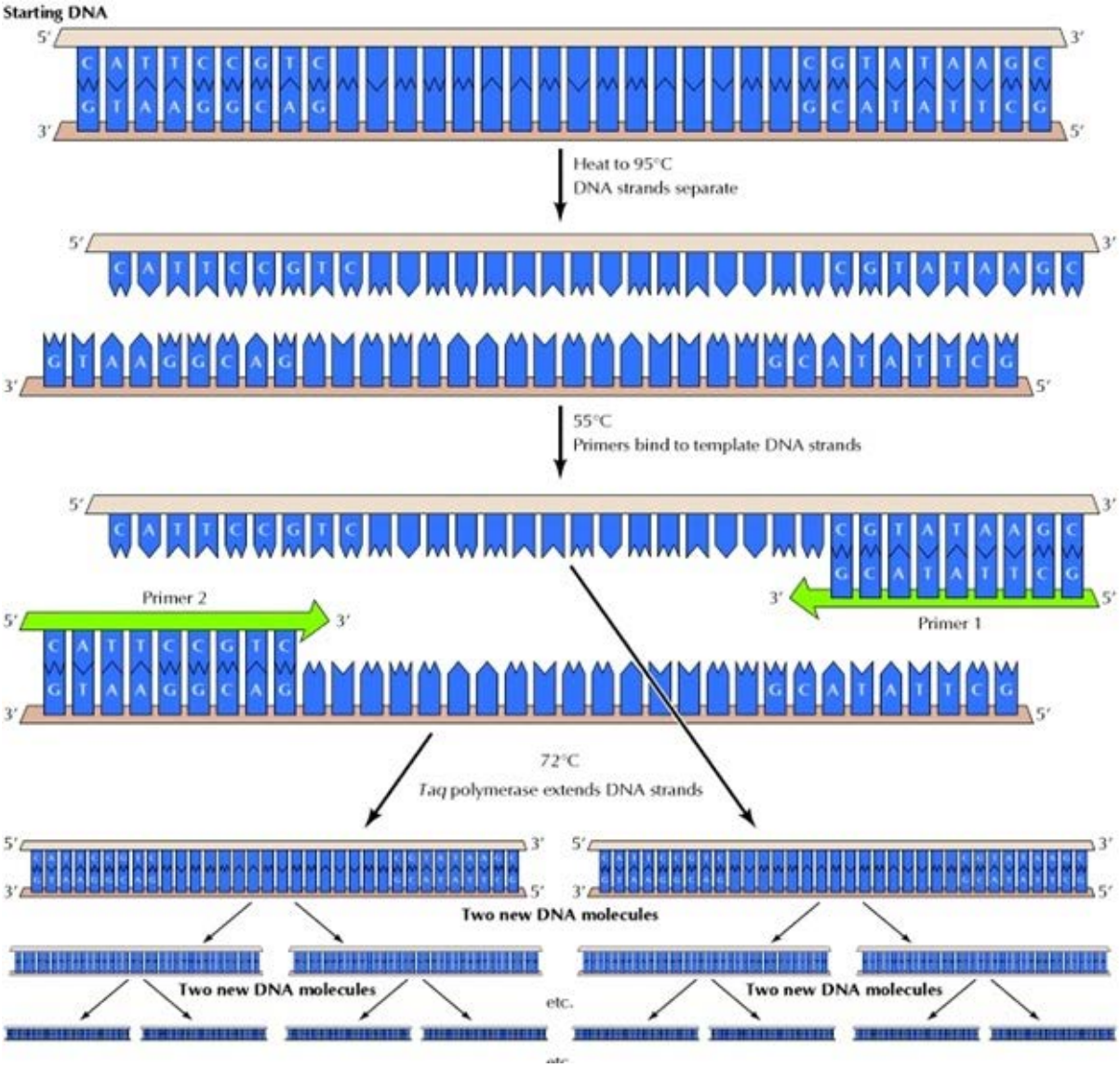


Figure 4.5: The polymerase chain reaction amplifies a DNA sequence. The PCR starts with a double stranded DNA (cDNA). First, a brief heat treatment separates the two strands (step 1). Second, the DNA is cooled in the presence of a large excess of the two primers, this makes it possible for these primers to hybridize to complementary sequences in the two DNA strands (step 2). Third, incubation of the mixture with DNA polymerase and dNTP (ATP, GTP, CTP, TTP) so that DNA is synthesized (step 3).The cycle is then repeated, and in each cycle the amount of DNA from the previous cycle is doubled. Only the DNA sequences which the primers binds to is amplified (100). From Cooper (101).

Quantitative reverse-transcription PCR

Quantitative reverse-transcription PCR (Q-RT-PCR) is a method to determine, characterize and quantify gene expression. Unlike standard PCR, Q-RT-PCR detect the amplification of target genes as the reaction progress, rather than after a fixed number of cycles (102).

The TaqMan assay used in this thesis consist of two primers and a fluorogenic probe for each gene to be studied **(Figure 4.6)**. The probes are sequence specific to the target sequences and

contain a fluorescent reporter dye at the 5’ end and a quencher dye at the 3’ end. Although not completely, the quencher reduce the fluorescent emitted from the reporter by fluorescence resonance energy transfer (FRET), and only a low background flourescence is observed. If the target sequence is present, the probe anneals to it downstream of one of the primers.. As the primer is extended by the Taq DNA polymerase, 5’ exonuclease activity causes cleavage of the probe, releasing the reporter molecule. This shifts the reporter molecule away from the quencher, and as a result, the fluorescence intensity increases. The reporter dye signal measured is thus proportional to the amount of amplicon produced. The probe is removed from the target strand, permitting the extension of the nascent strand to continue to the end of the template strand, thus the probe does not interfere with the overall PCR process.

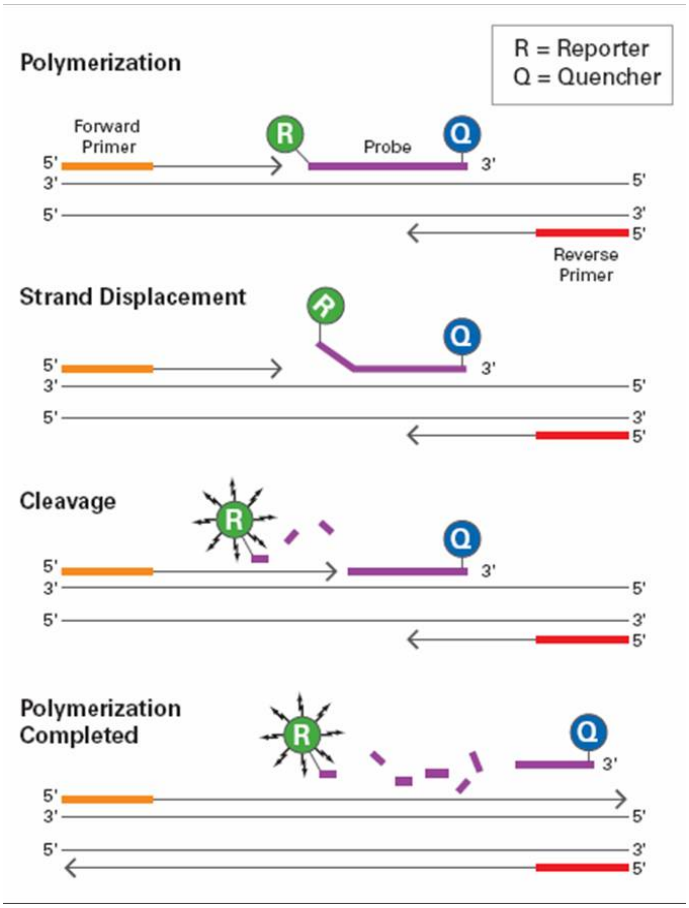


Figure 4.6: How the TaqMan® probes workes. The TaqMan® probe consist of a fluorogenic reporter at the 5’ end and a quencher dye at the 3’ end. The proximity of the reporter and the quencher, reduce the fluorescence of the reporter dye by fluorescence resonance energy transfer (FRET) through the space. The TaqMan probe anneals to an internal region of the DNA sequence, between the forward and reverse primer. The polymerase synthesize a new strand by extending the primers, and its 5’ to 3’ exonuclease activity cause cleavage of the bound probe. This release the reporter molecule away from the quencher, and as a result the fluorescence intensity increases. The probe is removed from the target strand and allows the primer extension to continue to the end of the template. Importantly, the probe does not interfere with the overall PCR process. The fluorescence intensity is proportional to the amount of amplicon produced (103). From Asuragen (104).

4.5.1 Disruption and homogenization of the samples

Complete disruption and homogenization of the samples is crucial for successfully RNA isolation. Incomplete disruption and homogenization leads to poor RNA yields and RNA quality.

PC3 Xenografts

Xenografts frozen in RNAlater RNA Stabilization Reagent were thawed in room temperature, and 15-20 mg tissue was used. The samples were transferred to 2 ml Sarstedt tubes with ceramic beads and the plasma membranes were disrupted by addition of 600 µl RLT-buffer containing dithiothreitol (DTT). The samples were then homogenized at 5000 rpm for 2 x 20 seconds in a Precellys24 instrument. After homogenization, the samples were microcentrifuged at 14 000 rpm for 3 minutes at room temperature, and the supernatant was used for further analysis.

PC3 Cells

In addition to analysing the xenografts, the PC3-κB-luc cells from the experiments described in section 4.3.6, were harvested in order to examine mRNA levels. At the end of the experiment the medium was removed and the cells washed with PBS. The cells were incubated with trypsin for approximately five minutes, and subsequently added medium with 10 % FBS to inactivated the trypsin. The cell suspension was transferred to RNase free tubes and microcentrifuged at 300 *G* in five minutes. The supernatant was carefully removed and 120 µl RLT buffer was immediately added to each sample in order to lyse the cells. To complete the cell lysis, the cells were vortex mixed for 2 minutes. The experiment was performed in triplicates and repeated three times. The triplicates from each experiment were pooled and the lysate stored at -70^{o}C until it was used for further analysis.

4.5.2 RNA isolation

Total RNA from cells and xenografts was isolated using the RNeasy-kit from Qiagen. This technology uses a silica-based membrane which selectively binds RNA longer than 200 nucleotides. mRNA are usually longer than 200 nucleotides, while other types of RNA, such as rRNA, 5S rRNA and tRNA, are mostly less than 200 nucleotids in length, and are therefore excluded.

The lysates were thawed and ethanol added to create conditions that promote selective binding of RNA to the membrane in the spin column. The samples were then applied to an RNeasy spin column. Total RNA bound to the membrane, while contaminants were washed away and RNA was finally eluted in RNase-free water. The RNeasy Mini procedure is shown in **Figure 4.7**. To minimize repeated freeze-thaw cycles of the RNA, the samples were aliquoted and stored at – 80^{o}C.

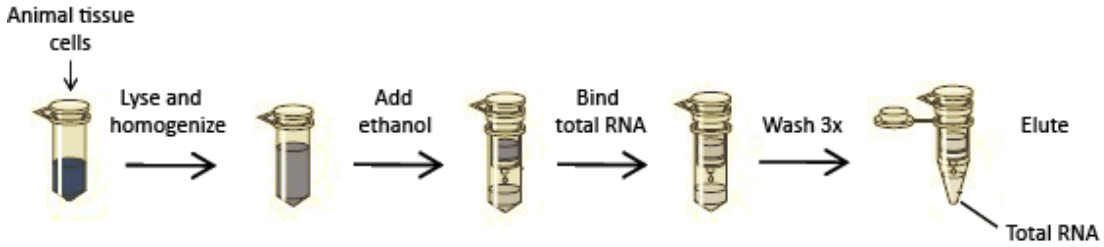


Figure 4.7: Workflow using the RNeasy Mini Kit to isolate total RNA. The samples were lysed and homogenized. Ethanol was added to promote selctive binding of RNA to the spin columns membrane. The samples were applied to an RNeasy spin column, washed three times to remove contaminants, and RNA eluted in RNase-free water. All steps were performed by microcentrifugation. Modified from Qiagen (105).

4.5.3 Quantification and quality check of RNA

A high quality of RNA is crucial for successful gene expression analysis. RNA is easily degraded in the presence of RNase enzymes, and the short fragments in a degraded RNA sample can lead to unreliable results in Q-RT-PCR.

The NanoDrop® ND-1000 Spectrophotometer is a full spectrum (220-750 nm) spectrophotometer that have several applications, including measuring the concentration and purity of nucleic acids. Briefly, fiber optic technology is combined with surface tension to hold and measure small amounts of samples. Sample (1.2 µl) is loaded onto the end of a fiber optic cable and a second fiber cable is then brought into contact with the sample droplet. Because of the surface tension, this results in a "bridge" between the gap of the two fiber optic ends. A light is sent through the sample and the absorbance at 260 and 280 nm (106). A ratio of A_{260}:A_{280} greater than 1.8 is typically considered as an adequate indicator of RNA purity.

In addition to analysing the samples using the spectrophotometer, the quality was evaluated using the Agilent 2100 Bioanalyzer combined with the Agilent RNA 6000 Nano Kit.

The Agilent 2100 bioanalyzer provides an automated electrophoretic separation of DNA, RNA, and protein samples. Small amounts of RNA samples can be separated according to their molecular weight in gel-filled channels of the micro fabricated chips. Next, the RNA subunits are detected via laser-induced fluorescence detection. This is visualized as an electropherogram and gel-like image (**Figure 4.8**) (107;108). Degradation of RNA is a gradual process and with increasing degradation of the sample there is a decrease in the ratio between the 18S to 28S ribosomal band. There is also an increase in the baseline signal between the two ribosomal peaks. A RNA integrity number (RIN) is calculated based on an algorithm using the 18S and 28S ratio. The RIN scale reaches from 1 to 10, with 1 being the most degraded, and 10 being the least degraded, and thus the highest quality (108). In this way the evaluation of the RNA quality is standardized and makes it possible to compare samples.

The samples were thawed on ice and diluted to a concentration of 150 ng/µl in RNase free water. All samples and the ladder were heat denaturated at 70^{o}C for 2 minutes on a heating block. The samples were immediately cooled down and kept on ice until they were applied on the RNA microchip.

RNA Nano gel matrix was pipetted into a spin filter and centrifuged at 4000 rpm for 10 minutes at room temperature. The gel was added dye, vortex mixed and centrifuged at 13 000 rpm for 10 minutes at room temperature before it was loaded into the chip as described in the manufacturers protocol.

Samples and ladder were applied to the chip in accordance with manufacturer's protocol, and the chip was then immediately run in the Agilent 2100 bioanalyzer.

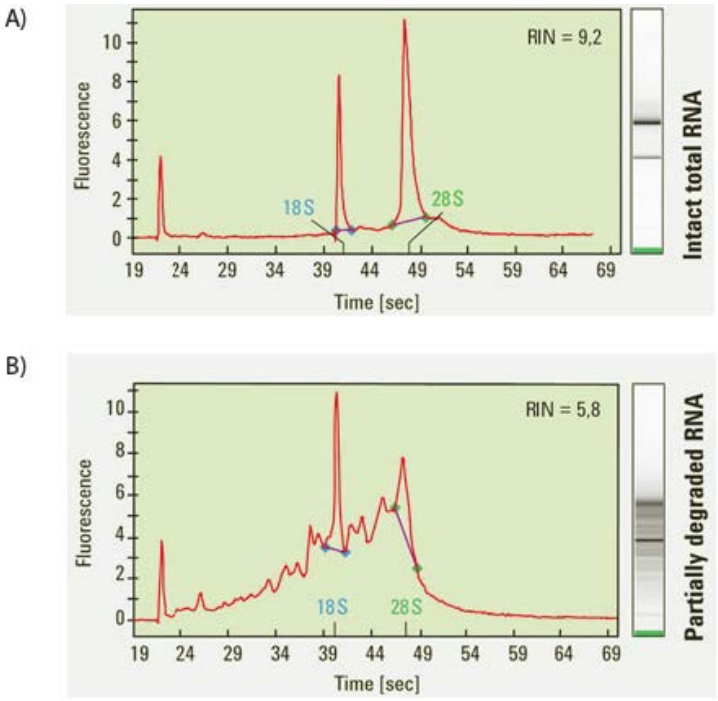


Figure 4.8: Comparison of intact and degraded RNA with corresponding RNA Integrity Number. A) Intact total RNA with a RIN 9.2. B) Partially degraded RNA with RIN 5.8. RNA =Ribonucleic Acid, 18S = subunit of ribosomal RNA, 28S = subunit of ribosomal RNA, RIN= RNA Integrity Number. From Agilent Technologies (109).

4.5.4 cDNA-synthesis

The cDNA synthesis was performed using the High Capacity cDNA Reverse Transcription Kit from Applied Biosystems.

The RNA samples and the kit components were thawed on ice, and the samples diluted in RNase free water to a concentration of 0.08 µg/µl. The 2X RT master mix was prepared as described in **Table 4.2** in accordance with the number of reactions required.

Table 4.2: 2X RT Master mix.

Component	Volum/Reaction (µL)
10X RT buffer	2.0
25X dNTP mix (100mM)	0.8
10X RT Random primers	2.0
Multiscribe Reverse Transcriptase	1.0
Nuclease-free water	4.2
Total per reaction	10.0

2X RT mastermix and samples, 20 µl of each, were pipetted into RNase free eppendorf tubes. The tubes were briefly centrifuged to eliminate any air bubbles, and kept on ice until the reverse transcription was run in a heating block. First, the samples were incubated at 25^{o}C in 10 minutes. The temperature was then increased to 37^{o}C and the samples incubated in additionally 120 minutes. Finally, to terminate the reactions, the samples were incubated at 85^{o}C for 5 minutes. The samples were cooled down on ice and stored at -20^{o}C.

4.5.5 Preparing and Running the Micro Fluidic Card

The TaqMan® Array Micro fluidic Cards contains 384 wells preloaded with the TaqMan® Gene Expression Assays. These are customizable cards where you choose which primers to include. The relative expression of 48 genes, 43 target genes and 5 endogenous controls, was analyzed. The endogenous controls were included to normalize possible variations in the amount of sample DNA added to each reaction.

The cDNA was thawed on ice, briefly vortex mixed and spun down. RNase free water was used to dilute the samples to a concentration of 2 ng/µl. Diluted samples (60 µl) were added 60 µl of TaqMan Gene Expression Master mix. The samples were vortex mixed, before they were centrifuged to eliminate any air bubbles.

Each reservoir of the TaqMan Array card was loaded with 100 µl of the sample PCR reaction mix (200 ng cDNA). The card was subsequently centrifuged and sealed. The quantitative reverse transcription PCR was performed in a 7900 HT Fast Real-Time PCR system combined with the SDS Software version 2.3.

4.5.6 Analysis of data

RQ Manager version 1.2 was used for the data analysis. Average of Gusb (glucuronidase-β) and TBP (TATA box binding protein) was used as endogenous control. Automatic cycle threshold (C_t) was selected, that is the baseline values for each well and the threshold values for each detector were set automatically. Thereafter, we verified that the baseline and threshold were set correctly for each target gene. If not, the threshold and baseline were set manually. The expression of target gene was quantified using the cycle threshold (C_t) normalized against the average of the endogenous controls. Hence, ΔC_t represent the C_t value of the target gene minus the average C_t value of the endogenous controls. This was used to calculate $2^{-\Delta Ct}$.

4.6 Analysis of Carotenoids by Chromatography

Chromatography is a technique for separation of molecules in a sample. Chromatography can be used for either purification of components (preparative chromatography) or for measuring the relative proportions of the components (analytical chromatography). There are several numbers of chromatographic techniques, such as liquid chromatography and gas chromatography. The principle is the same in all of them; a sample dissolved in a "mobile phase" passes through a "stationary phase", which separates the analyte to be measured from other molecules in the mixture. Separation of analytes are achieved by introducing the sample to the mobile phase. The mobile phase may be liquid or gas, depending on the type of chromatography, and the stationary phase may be solid or gel. Interactions between the mobile and the stationary phase leads to separation of the sample (110).

In column chromatography (**Figure 4.9**), the mobile phase is pumped through a column with a bed of particles, the "stationary phase". In front of the stationary phase, the sample is introduced into the mobile phase by an injector. The molecules in the sample move differently through the stationary phase, in this way the molecules becomes separated. After passing through the column, the mobile phase (with the sample) enters into a detector that detects the different molecules that passes through it, and these results are presented graphically as a chromatograph (**Figure 4.10**). The different peaks in the chromatograph correspond to the different molecules in the separated sample (110).

Today, liquid chromatography often utilize very small particles and a relatively high inlet pressure and are referred to as high performance (or high pressure) liquid chromatography (HPLC) (110).

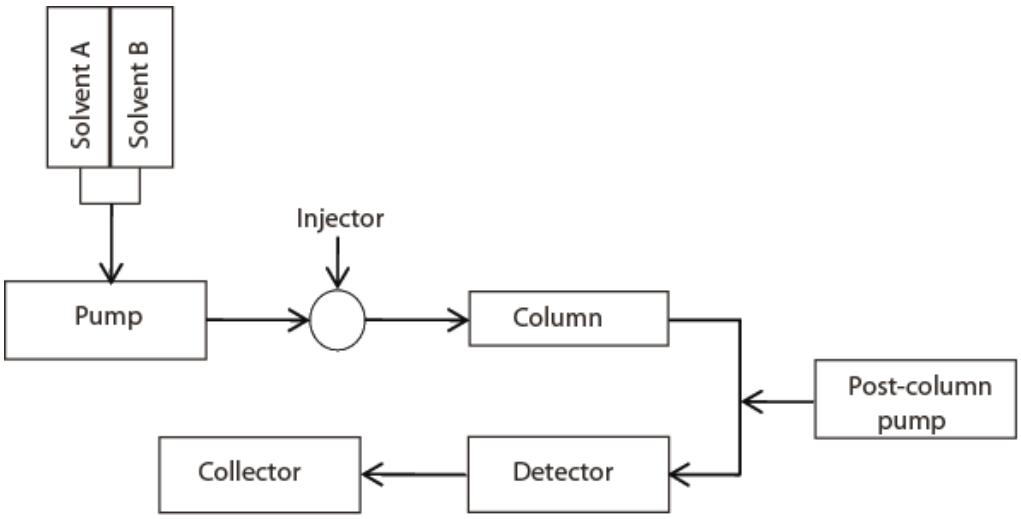


Figure 4.9: Schematic view of the principle of liquid chromatography. A liquid called the mobile phase, is pumped through a column with a bed of particles, the stationary phase. A injector introduce the sample with the molecules to become separated into the mobile phase in front of the stationary phase. The sample is then transported by the mobile phase through the column (the stationary phase). The molecules in the sample adsorb differently to the stationary phase, and the molecules become separated. After passing through the column the mobile phase with the sample enters into a detector which detects the different molecules that passes through it. Finally, the mobile phase finally enters a collector. Signals are sent from the detector into a computer which presents the results graphically as a chromatograph. The different peaks in the chromatograph represents the different molecules in the separated sample (110).

Eleven different tissues from mice included in the pilot study were analyzed by HPLC with UV detection to examine in which tissues carotenoids accumulates. Tissues were weighed (20-25 mg), and added 4.5 µl precipitating solution per mg tissue. The precipitating solution consisted of astaxanthin (internal standard) in isopropanol. The samples were homogenized at 6500 rpm for 2 x 20 seconds in a Precellys24 instrument. The samples were then treated in an ultrasonic ice cold water bath for 20 min and centrifuged at 13 000 rpm at 4^{o}C for 15 min. The supernatant were transferred to dark vials and stored at -20^{o}C.

The instrument used for the analysis was an Agilent Technologies 1100 HPLC system. The chromatographic separation was performed on an analytical column (YMC C-30, 2.1 x 150 mm) with 3-µm particles thermostated at 45^{o}C. The mobile phases were A) 20 % MilliQ

water, 56 % ethanol and 24 % aceton, and B) 100 % aceton. The flow rate was 0.3 ml per minute.

The separation was accomplished with a linear gradient from 2 % to 100 % of the mobile phase B over 25 minutes followed by a linear gradient back to 2 % over 9 minutes. 25 µl of the samples were injected into the HPLC system and the temperature set to 9^{o}C. Plasma standards which quantified against the NIST (National Institute of Standards and Technology) 968c SRM were used for calibration. The detection was performed at 453 nm.

The sample preparation, and partly the data analysis, was performed as part of this master project, while the HPLC was kindly performed by Amrit Kaur Sakhi and Nasser Ezzatkhah Bastani.

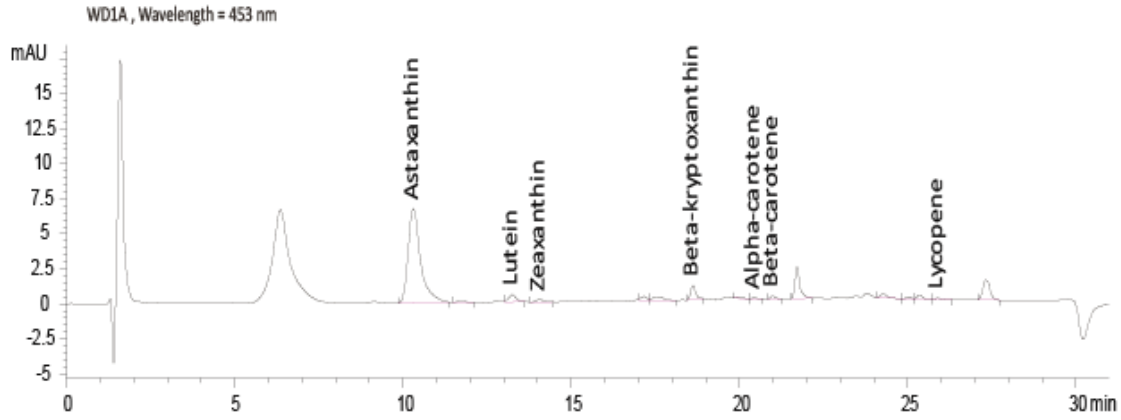


Figure 4.10: Chromatograph of plasma standards used for calibration in the HPLC analysis.

4.7 Statistical analysis

One-Way ANOVA was used to examine possible effects of extracts on NF-κB activity in PC3-κB-luc cells. Differences were identified using Dunnet's comparison. Possible differences in the effect of green and dark roasted coffee were examined using Student's T-test.

Data from the animal experiments were not normally distributed, thus non-parametric tests were used. Differences in relative gene expression in xenograft and cells were examined using Kruskal-Wallis test and identified using the Mann-Whitney U test. Possible effects of coffee or tomato paste on luciferase activity in homogenates of xenografts were also identified using the Kruskal-Wallis test. Since the H_0 was retained no further analysis were performed.

Differences in the accumulation of carotenoids in tissues were identified using Mann-Whitney U test, as there were only two groups and two variables.

Data are presented as mean ± SD when normally distributed or median (min-max) if not normally distributed. Statistical significant difference was set to $p < 0.05$ for all analysis. All statistical analysis was performed using PASW Statistics 18.

5 Results

5.1 Effect of TNF-α on NF-κB activity in PC3-κB-luc cells

The ability of TNF-α to induce NF-κB-reporting luciferase activity in stably transduced PC3 cells (PC3-κB-luc cells) was tested over a time period of 24 hours. TNF-α dramatically increased the NF-κB activity to more than 1100 % of control at 6 hours incubation **(Figure 5.1 A)**, followed by gradually decreasing activity from 6 to 24 hours. Based on these results, further experiments were performed with 6 hours incubations. **Figure 5.1 B** shows the average basal and TNF-α induced NF-κB-reporting luciferase activity after 6 hours incubation in nine experiments performed in this thesis. The ability of TNF-α to induce NF-κB-reporting luciferase activity in these experiments were in accordance with the results from the 24 hours experiment.

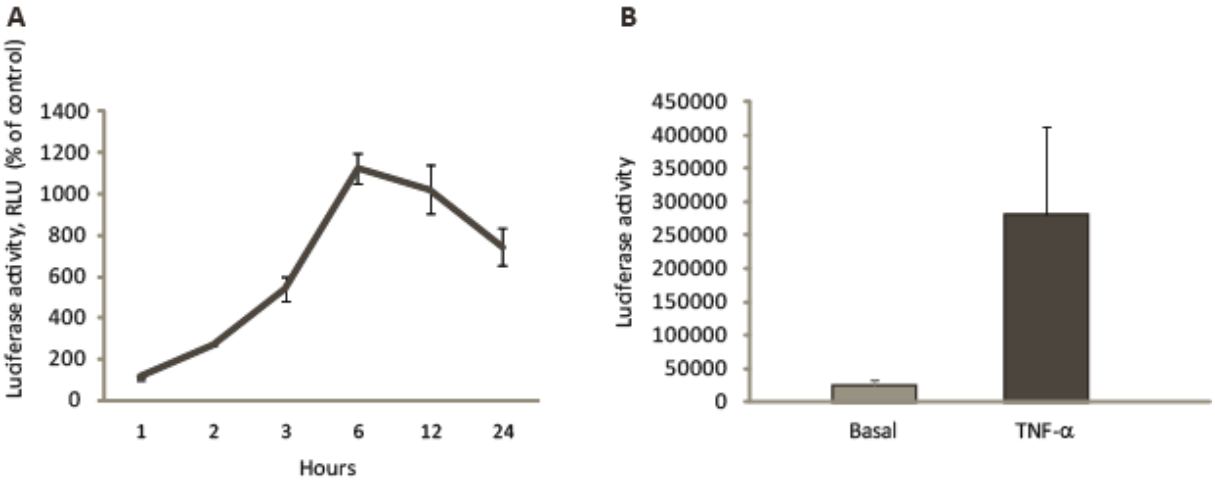


Figure 5.1: TNF-α induced NF-κB activity in PC3-κB-luc cells. PC3-κB-luc cells were incubated with vehicle or TNF-α (10 ng/ml). At the end of the experiment luciferin (0.2 mg/ml) was added to each well and luciferase activity measured by a Synergy 2 instrument. A) PC3-κB-luc cells incubated with TNF-α for the indicated time points. The experiment was performed one time in triplicates. B) PC3-κB-luc cells incubated with vehicle or TNF-α for 6 hours. The experiment was performed nine times in triplicates. Results are shown as mean ±SD.

5.2 Effect of coffee and tomatoes in PC3-κB-luc cells

Next, we tested whether extracts of coffee or tomatoes could modify basal or TNF-α induced NF-κB activity in PC3-κB-luc cells. The extracts were tested in different concentrations and compared to vehicle control.

5.2.1 Effect of tomato paste on NF-κB activity

Extract of tomato paste was tested at five different concentrations, 1.5, 3.0, 6.0, 10.0 and 15.0 mg/ml, in order to examine the effect of tomato paste on basal or TNF-α induced NF-κB activity in PC3-κB-luc cells. The cytotoxity of the extract were tested, and no cell death was detected at these concentrations.

Tomato paste did not statistically significant modulate basal NF-κB activity at any concentration (**Figure 5.2 A**). A significant inhibition of TNF-α induced NF-κB activity was found in PC3-κB-luc cells incubated with tomato paste at a concentration of 6.0 mg/ml and higher (**Figure 5.2 B**), with a maximum inhibition of 59 % compared to controls at 15 mg/ml ($p = 0.001$).

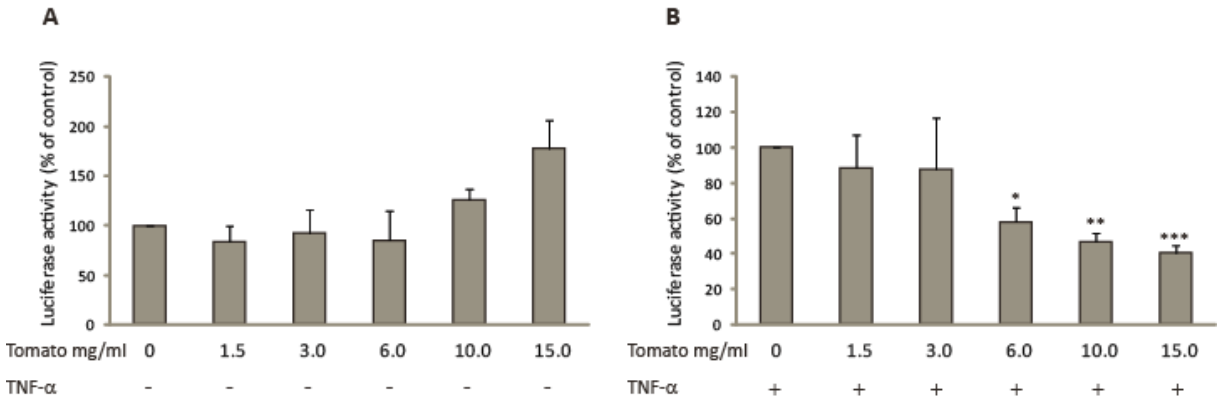


Figure 5.2: Effect of tomato paste extract on basal and TNF-α induced NF-κB activity in PC3-κB-luc cells. PC3-κB-luc cells were incubated with vehicle or extracts of tomato paste with the indicated concentrations for 6.5 hours. Cells stimulated with TNF-α were pre-incubated with the extract for 0.5 hour, added TNF-α (10 ng/ml) and incubated for additional 6 hours. At the end of the experiments luciferin (0.2 mg/ml) was added to each well and luciferase activity measured by a Synergy 2 instrument. A) Basal NF-κB activity. B) TNF-α induced NF-κB activity. Bars show mean ±SD of three experiments, each performed in triplicates. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

5.2.2 Effects of dark roasted coffee on NF-κB activity

A dark roasted coffee was tested at five different concentrations, 0.5, 1.0, 1.5, 3.0 and 6.0 mg/ml, to investigate if there was an effect on basal or TNF-α induced NF-κB activity. The cytotoxity of the extract were tested (see section 4.3.4), and no cell death was detected at these concentrations. Basal NF-κB activity was significantly increased at 6.0 mg/ml ($p < 0.001$) (**Figure 5.3 A**). Dark roasted coffee significantly inhibited NF-κB activity at all concentrations ≥ 1 mg/ml (**Figure 5.3 B**). At the highest concentration (6.0 mg/ml) the NF-κB activity was reduced by 66 % compared to control ($p < 0.001$).

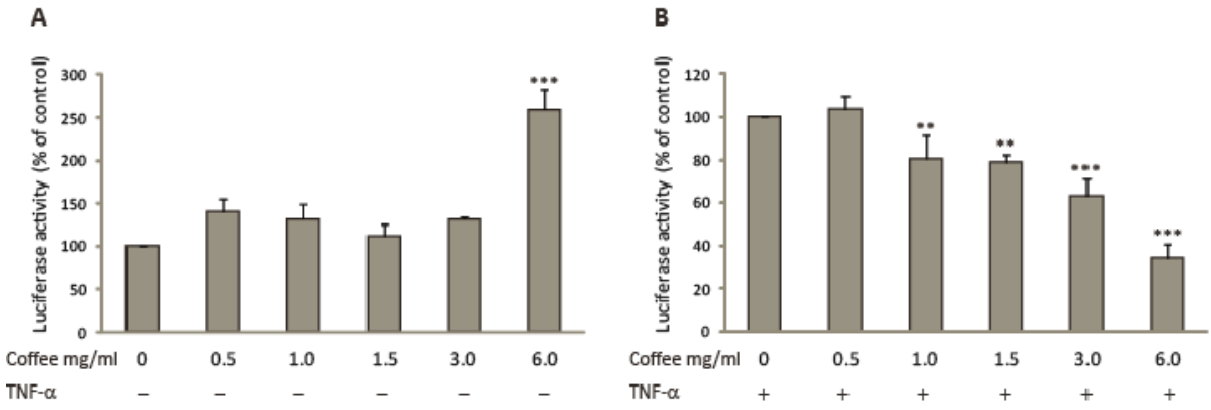


Figure 5.3: Effect of dark roasted coffee on basal and TNF-α induced NF-κB activity in PC3-κB-luc cells. The PC3-κB-luc cells were incubated with vehicle or extracts of coffee with the indicated concentrations for 6.5 hours. Cells stimulated with TNF-α were pre-incubated with the extract for 0.5 hour, added TNF-α (10 ng/ml) and incubated additionally 6 hours. At the end of the experiment luciferin (0.2 mg/ml) was added and luciferase activity measured by a Synergy 2 instrument. A) Basal NF-κB activity. B) TNF-α induced NF-κB activity. Bars show mean ±SD of three experiments each performed in triplicates. ** = $p < 0.01$, *** = $p < 0.001$

5.2.3 Effects of green and dark roasted coffee on NF-κB activity

Previous studies in the Rune Blomhoff group have shown that the degree of roasting is the main determinant of the effects of coffee on NF-κB activity in U937 monocytes (111). To test whether the same type of coffee beans with different degrees of roasting could modulate NF-κB activity also in the PC3 cell line, green and dark roasted Arabica coffee were tested in two different concentrations, 1.0 mg/ml and 5.0 mg/ml, and compared to vehicle control.

Dark roasted coffee did not modulate basal NF-κB activity significantly in this experiment, whereas green coffee induced NF-κB activity at 5.0 mg/ml ($p = 0.005$) (**Figure 5.4 A**). Also,

the difference in NF-κB luciferase activity in the cells treated with dark roasted coffee compared to cells treated with green coffee was statistically significant (p = 0.015).

TNF-α induced NF-κB activity was inhibited at 5 mg/ml for both green and dark roasted coffee (**Figure 5.4 B**). At 5 mg/ml green coffee reduced the NF-κB activity by 42 % (p = 0.0002), whereas dark roasted coffee decreased the NF-κB activity by 51 % (p = 0.0003). Green and dark roasted coffee did not show any statistically significant differences in the ability to inhibit TNF-α induced NF-κB luciferase activity in PC3 cells.

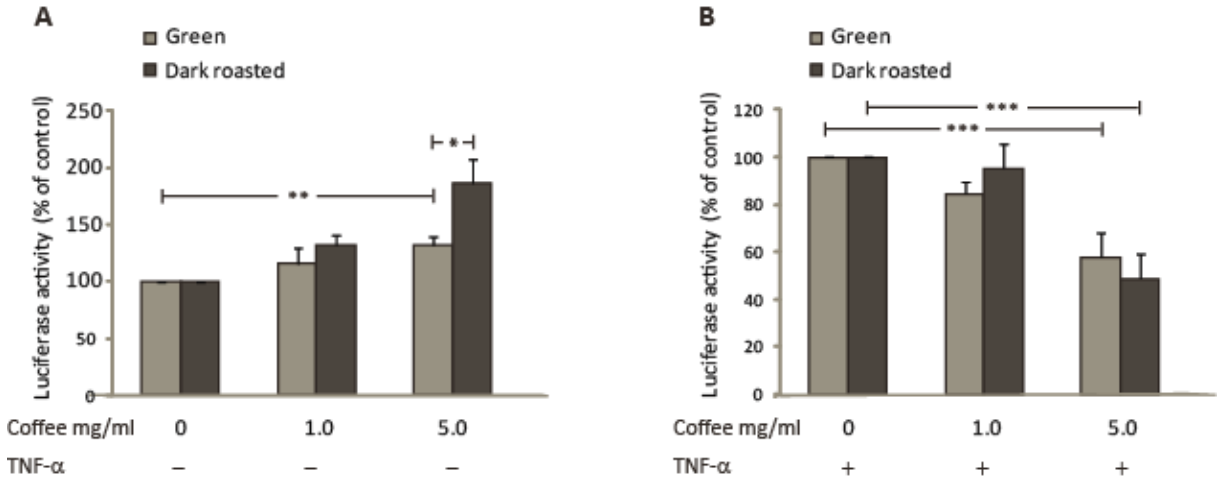


Figure 5.4: Effect of green and dark rosted coffee on basal and TNF-α induced NF-κB activity in PC3-κB-luc cells. The PC3-κB-luc cells were incubated with vehicle or extracts of coffee (green or dark roasted) with the indicated concentrations for 6.5 hours. Cells stimulated with TNF-α were pre-incubated with the extract for 0.5 hour, added TNF-α (10 ng/ml) and incubated additionally 6 hours. At the end of the experiment, luciferin (0.2 mg/ml) was added before luciferase activity was measured by a Synergy 2 instrument. A) Basal NF-κB activity. B) TNF-α induced NF-κB activity. Bars show mean ±SD of three experiments each performed in triplicates. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

5.2.4 Effect of coffee and tomatoes on mRNA levels PC3-κB-luc cells

Furthermore, we measured the mRNA expression level of 43 genes related to NF-κB, inflammation, immunity, endogenous antioxidant defense and cancer development in PC3-κB-luc cells.

In TNF-α-treated PC3-κB-luc cells, the mRNA expression level of the tumor suppressor gene TP53 (tumor protein 53) (**Figure 5.5 H**) was significantly lower in the cells treated with coffee compared to the control cells ($p = 0.0495$). The apoptosis related genes Casp3 (Caspase 3) (**Figure 5.5 A**) and FAS (also called CD95) (**Figure 5.5 C**) was also down regulated by coffee ($p = 0.0459$). Also, CD40 (Cluster of differentiation 40) (**Figure 5.5 B**) which is involved in immune and inflammatory responses was downregulated in the coffee group compared to the control cells only treated with TNF-α ($p = 0.0459$). The mRNA level of Nrf2 (Nuclear factor-erythroid 2-related factor 2) (**Figure 5.5 I**), GCLC (γ-glutamylcysteine synthetase) (**Figure 5.5 J**) and NQO1 (NAD(P)H-quinone oxidoreductase 1) (**Figure 5.5 N**), all of which are a part of the endogenous antioxidant defense were significantly upregulated in the cells treated with coffee as compared to control ($p = 0.459$ for all).

Since the NF-κB pathway is a focus in this work, we looked specifically into a range of genes coding for proteins in this pathway. NF-κB2 (**Figure 5.5 L**) had a significant lower expression in the cells treated with coffee compared to control cells only treated with TNF-α ($p = 0.0495$). Also, the expression of the NF-κB related genes RelB (v-rel reticuloendotheliosis viral oncogene homolog B), IκBκB (inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta), NF-κB1 (Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)) and RelA (v-rel reticuloendotheliosis viral oncogene homolog A) were lower, whereas NFKBIB (Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta/ IKKβ) were higher, in the coffee group compared to control, but this was not significant (for further details see **Table A1** in Appendix).

As for the cells treated with coffee, the TNF-α stimulated cells treated with tomato paste, had a significantly lower mRNA level of CD40 ($p = 0.0495$) (**Figure 5.5 B**) and TP53 ($p = 0.0495$) (**Figure 5.5 H**). Also the adhesion molecule ICAM-1(Intercellular adhesion molecule 1) (**Figure 5.5 O**) were downregulated, whereas FAS ($p = 0.0495$) (**Figure 5.5 C**) were

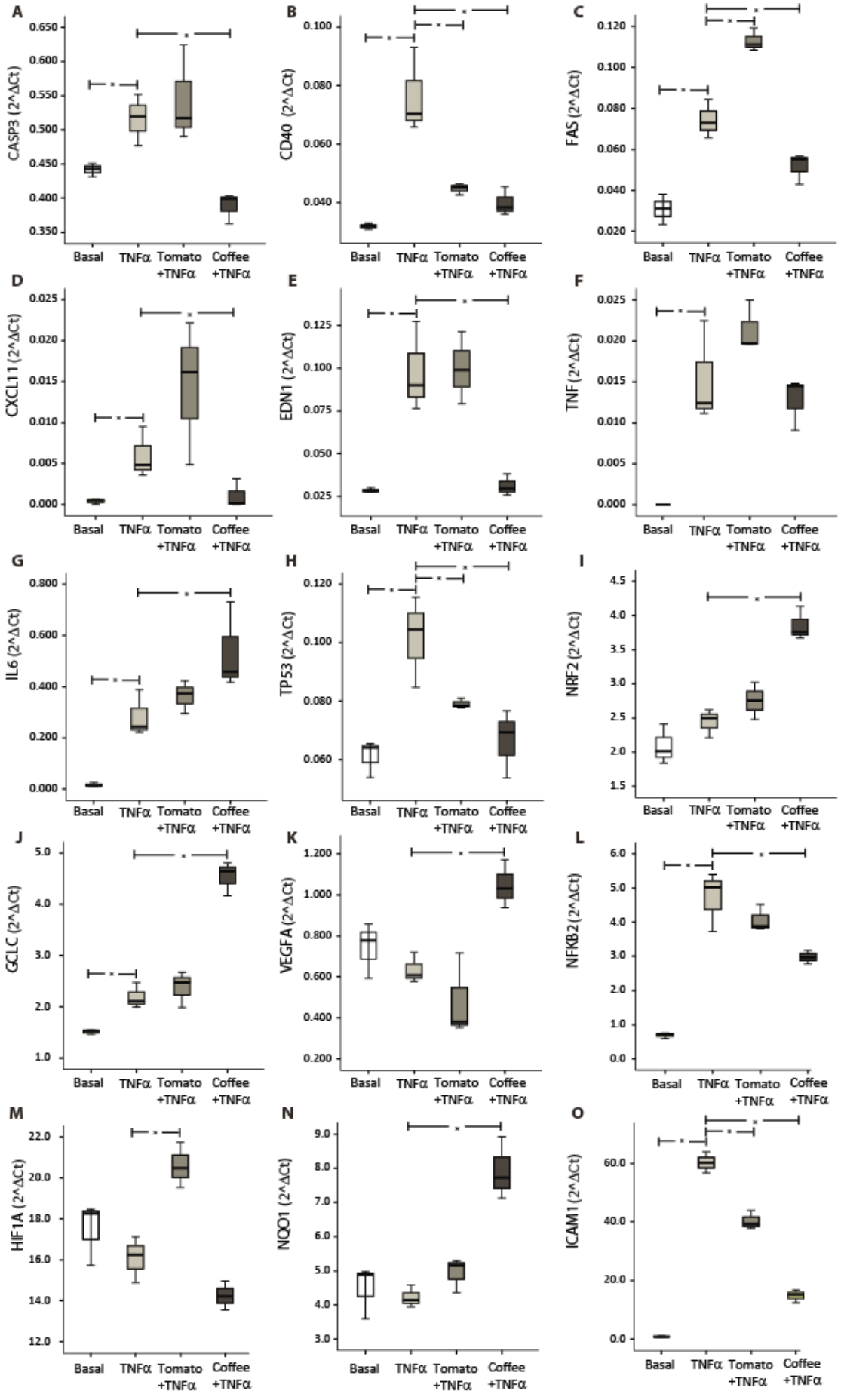
significantly higher in both intervention groups compared to control cells only treated with TNF-α ($p = 0.0495$).

The modulation of gene expression of the NF-κB genes RelA, NFKBIB, IκBκB, and NF-κB2 were non-significantly lower, and RelB and NF-κB1 higher, in the cells treated with tomato paste as compared to control cells (for further details see **Table A1** in Appendix).

At the basal level, the mRNA expression level of 11 genes were significantly lower compared to cells incubated with TNF-α ($p = 0.0495$ for all), and 2 genes were upregulated (**Figure 5.5**). All the NF-κB related genes analyzed had a lower expression level in the basal cells compared to the TNF-α treated cells, of which this was statistically significant for NFKBIB, RelB, NF-κB1 and NF-κB2 ($p = 0.0495$ for all), and non-significant for RelA ($p = 0.827$) and IκBκB ($p = 0.513$) (for further details see **Table A1** in Appendix).

The expression of the other genes analyzed was not significantly modulated by either coffee or tomatoes (for further details see **Table A1** in Appendix).

Figure 5.5: Coffee and tomato extracts change the gene expression in prostate cancer (PC3) cells. The PC3-κB-luc cells were incubated with vehicle or extracts of dark roasted coffee (6 mg/ml) or tomato paste (10 mg/ml) for 6.5 hours. Cells stimulated with TNF-α were pre-incubated with vehicle or extract for 0,5 hour, added TNF-α (10 ng/ml) and incubated additionally 6 hours. At the end of the experiment the cell were harvested and RNA isolated. For determination of relative mRNA expression, the expression level was normalized against the average Ct of the endogenous controls β-glucuronidase (Gusb) and TATA box binding protein (TBP). The box plots represent $2^{-\Delta Ct}$. Differences were identified using Mann-Whitney tests. * = $p < 0.05$. CASP3 = Caspase 3, CD40 = Cluster of differentiation 40, FAS = Fas/CD95 , CXCL 11 = Chemokine ligand 11, EDN1 = Endothelin 1 , TNF = Tumor necrosis factor, IL-6 = Interleukin 6, TP53 = Tumor protein 53 (p53), Nrf2 = Nuclear factor- erythroid 2- related factor 2, Gclc = γ-glutamylcysteine synthetase, VEGFA = Vascualar endothelial growth factor A, NFκB2 = Nuclear factor of κB 2/p52/p100, HIF1A = Hypoxia-inducible factor 1 alpha, NQO1 = NAD(P)H-quinone oxidoreductase 1, ICAM-1 = Intercellular adhesion molecule 1.


A
CASP3 (2^ΔCt)
0.650
0.600
0.550
0.500
0.450
0.400
0.350
B
CD40 (2^ΔCt)
0.100
0.080
0.060
0.040
C
FAS (2^ΔCt)
0.120
0.100
0.080
0.060
0.040
0.020
D
CXCL11 (2^ΔCt)
0.025
0.020
0.015
0.010
0.005
0.000
E
EDN1 (2^ΔCt)
0.125
0.100
0.075
0.050
0.025
F
TNF (2^ΔCt)
0.025
0.020
0.015
0.010
0.005
0.000
G
IL6 (2^ΔCt)
0.800
0.600
0.400
0.200
0.000
H
TP53 (2^ΔCt)
0.120
0.100
0.080
0.060
I
NRF2 (2^ΔCt)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
J
GCLC (2^ΔCt)
5.0
4.0
3.0
2.0
1.0
K
VEGFA (2^ΔCt)
1.200
1.000
0.800
0.600
0.400
0.200
L
NFKB2 (2^ΔCt)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
M
HIF1A (2^ΔCt)
22.0
20.0
18.0
16.0
14.0
12.0
N
NQO1 (2^ΔCt)
9.0
8.0
7.0
6.0
5.0
4.0
3.0
O
ICAM1 (2^ΔCt)
60.0
40.0
20.0
0.0
Basal
TNFα
Tomato +TNFα
Coffee +TNFα

5.3 Effect of coffee and tomatoes in PC3 xenografts

To test for possible effects of coffee and tomato *in vivo*, NMRI mice with PC3-κB-luc xenografts were randomized to receive either control, tomato- or coffee-diet. After injection of PC3-κB-luc cells, the mice received their assigned diet for 5 weeks before the xenografts were excised and stored for further analysis (see details in Method section for the xenograft mice experiment).

5.3.1 NF-κB luciferase activity in xenografts

The NF-κB luciferase activity in homogenates of xenografts was measured and normalized to total protein content.

There were no statistically significant differences between the NF-κB activity in the xenografts of mice fed either a tomato or a coffee diet, compared to the control diet. There was, however, a non-significant trend for an increase in NF-κB activity in the xenografts from mice fed a diet containing 5 % coffee (p = 0.324) (**Figure 5.6**).

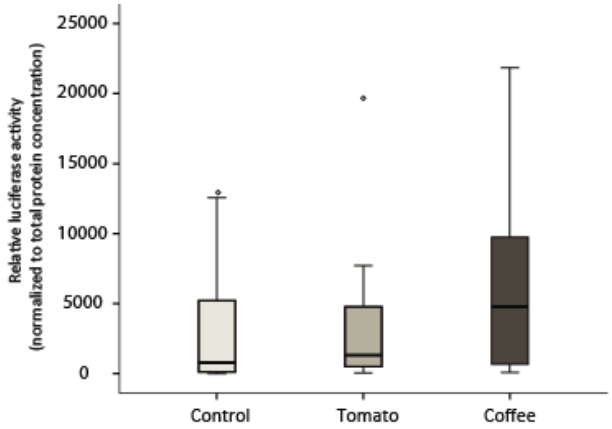


Figure 5.6: NF-κB activity in homogenates of xenografts. Luciferase activity was measured in homogenates of PC3 xenografts from mice fed with control diet (n = 15), a diet supplemented with 5 % coffee (n = 15) or 10 % tomato paste (n = 15). Luciferase activity was normalized to the total protein content. Possible effects of coffee or tomato paste on luciferase activity in homogenates of xenografts were also identified using the Kruskal-Wallis test.

5.3.2 Effect of coffee and tomatoes on mRNA levels in PC3 xenografts

The mRNA expression level of 43 genes related to NF-κB, inflammation, immunity, endogenous antioxidant defense, cytoprotection and cancer development were also measured in PC3 xenografts. Of these, the expression levels of RelA ($p = 0.0046$), TGFβ1 ($p = 0.0207$), TXN ($p = 0.0129$), and BAD ($p = 0.0362$) were significantly lower in the group that received a diet with 5 % coffee, compared to the control group. TGFβ1 expression level was significantly lower ($p = 0.0401$), while the expression of ICAM-1 was significantly increased ($p = 0.0401$) in the group that received a diet with 10 % tomato paste, as compared to controls (**Figure 5.7**).

In addition to RelA, the mRNA level of NFKBIB showed a non-significant trend towards lower expression in the coffee group compared to control ($p = 0.101$). The mRNA level of other NF-κB related genes analyzed, including RelB, IkBkB, IKBKB, NF-κB1 and NF-κB2, were not modulated by either coffee or tomato (for further details see **Table A1** in Appendix).

The expression of the other genes analyzed was not significantly modulated by either coffee or tomatoes (for further details see **Table A1** in Appendix).

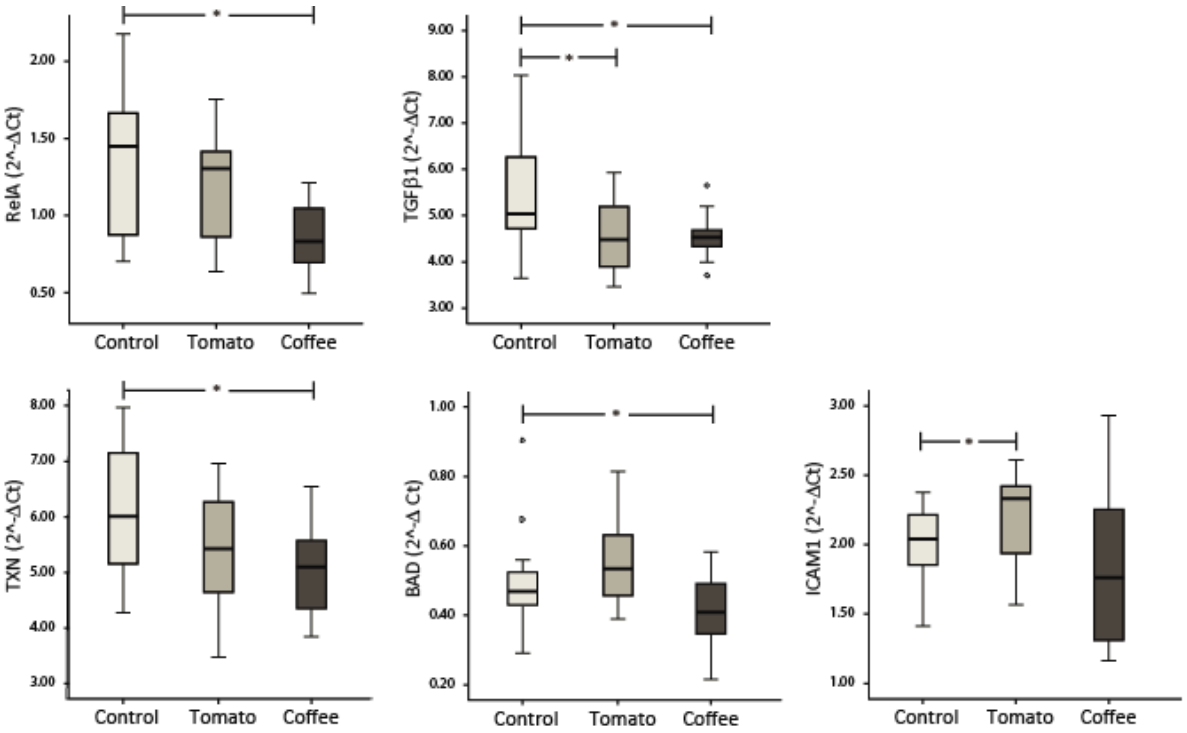


Figure 5.7: Coffee and tomato modulate the gene expression of NF-κB-, inflammatory-, antioxidant, apoptosis- and adhesion related genes in prostate cancer (PC3) xenograft model. The mice were fed control diet (n = 15) diet with 5 % coffee (n = 14) or diet with 10 % tomato paste (n = 15). PC3-κB-luc xenografts were injected in both flanks. The mice continued the intervention for five weeks before they were euthanized and xenografts stored for further analysis. For determination of relative mRNA expression, the expression level was normalized against the average C_t of the endogenous controls β-glucuronidase (Gusb) and TATA box binding protein (TBP). The box plots represent $2^{-\Delta Ct}$. Differences were identified using Mann-Whitney tests. * = $p < 0.05$ for control compared to intervention groups. Rel A = v-rel reticuloendotheliosis viral oncogene homolog A, TGFβ1 = Tumor Growth Factor β1, TXN = thioredoxin, BAD = BCL2-antagonist of cell death, ICAM-1 = Intercellular adhesion molecule-1.

5.4 Differences in gene expression between PC3 cells in culture and in xenografts

To examine the effect of the microenvironment on gene expression, the mRNA levels were compared in PC3 xenografts versus PC3 cells in culture (**Table 5.1**).

The mRNA expression level of the NF-κB related genes Rel B and IKBKB were significantly lower in the PC3 cells in culture than in the PC3 xenografts. SOD1, NQO1, GCLC and TXN, all of which are involved in the endogenous antioxidant defense, had significantly higher expression levels in cells than in xenografts. The anti apoptotic gene BCL2 had a lower expression level, whereas BAX, which promotes apoptosis by competing with BCL2, had a higher expression level in cells compared to xenografts. Also, the NF-κB target genes CXCL11, IL6 and IL1A had lower expression level in cells than in xenografts.

Table 5.1: Differential expression of mRNA levels in PC3 xenografts versus PC3 cells in culture. For determination of relative mRNA expression, the expression level was normalized against the average C_t of the endogenous controls β-glucuronidase (Gusb) and TATA box binding protein (TBP). Data are presented as median (min-max) $2^{-\Delta Ct}$. Differences were identified using Mann-Whitney tests.

	PC3 cells (n = 3) $2^{-\Delta Ct}$		**PC3 xenograft** (n = 15) $2^{-\Delta Ct}$		
Gene	Median	(min-max)	Median	(min-max)	**p-**
Inflammation					
RelB	0.043	(0.039-0.058)	0.087	(0.004-0.066)	0.038
IKBKB	0.302	(0.296-0.310)	0.763	(0.555-1.449)	0.008
CXCL11	0.001	(0.001-0.001)	0.004	(0.001-0.008)	0.008
EDN1	0.028	(0.028-0.030)	0.118	(0.095-0.194)	0.008
IL6	0.013	(0.012-0.026)	0.036	(0.018-0.074)	0.015
CSF1	0.096	(0.076-0.170)	0.033	(0.013-0.062)	0.008
IL1A	0.002	(0.002-0.003)	0.004	(0.002-0.007)	0.028
ICAM1	0.779	(0.777-1.178)	2.040	(1.412-2.375)	0.008
Cytoprotection					
SOD1	12.784	(12.065-15.459)	5.582	(2.801-24.277)	0.028
NQO1	4.884	(3.596-4.976)	1.918	(1.635-2.590)	0.008
GCLC	1.525	(1.466-1.546)	1.944	(1.405-2.423)	0.021
TXN	8.953	(8.150-10.342)	6.010	(4.270-7.971)	0.008
Cancer					
HIF1A	18.268	(15.726-18.473)	9.923	(6.723-12.526)	0.008
VEGFA	0.778	(0.593-0.859)	1.256	(0.707-1.694)	0.015
FAS	0.031	(0.023-0.038)	0.122	(0.075-0.163)	0.008
BCL2	0.017	(0.015-0.020)	0.049	(0.030-0.110)	0.008
BAX	1.751	(1.697-1.843)	1.056	(0.749-1.220)	0.008
CASP9	0.246	(0.212-0.263)	0.335	(0.278-0.398)	0.008
TP53	0.064	(0.054-0.066)	0.114	(0.095-0.154)	0.008

n indicates number of samples

RelB = v-rel reticuloendotheliosis viral oncogene homolog B, IKBKB = Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta, CXCL 10 = Chemokine ligand 10, CXCL 11 = Chemokine ligand 11, EDN1 = Endothelin 1, IL-6 = Interleukin 6, TNF = Tumor necrosis factor, CSF1 = colony stimulating factor 1, IL1A = Interleukin 1A, ICAM1 = Intercellular adhesion molecule 1, SOD1 = Superoxide dismutase 1, NQO1 = NAD(P)H-quinone oxidoreductase 1, Gclc = γ-glutamylcysteine synthetase , TXN = thioredoxin, HIF1A = Hypoxia-inducible factor 1 alpha, VEGFA = Vascular endothelial growth factor A, FAS = Fas/CD95, BCL2 = B-cell lymphoma 2, BAX = BCL2-associated X protein, CASP9 = Caspase 9, TP53 = Tumor protein 53 (p53).

5.5 Accumulation of carotenoids in tissues

To investigate whether caroteniods from the tomato diet are absorbed and accumulated in various tissues, eleven different tissues from mice fed a control diet or a diet supplemented with 10 % tomato paste were analyzed by HPLC. Since tomatoes are especially rich in lycopene and due to the special interest in lycopene, the results are given as concentrations of lycopene and total carotenoids.

Carotenoids, including lycopene, accumulated in several tissues in the mice fed a diet supplemented with 10 % tomato paste. Lycopene was detected in spleen, thymus, lung, heart, prostate, testis and liver from the mice in the tomato group with the highest concentration found in liver, while no lycopene was found in any tissues of the control mice (**Table 5.2**). These differences were statistically significant for lycopene in spleen ($p = 0.014$), heart ($p = 0.028$), testis ($p = 0.014$) and liver ($p = 0.014$). To few samples were available in order to do statistics on the results from the prostate, however lycopene was detected in the prostate of one mouse fed tomato, and not in any of the two control mice tested.

Total carotenoids were detected in all of the tissues analyzed in the tomato group, and also in most of the tissues in the control group, except prostate, testis and liver. There was a significantly higher concentration of total carotenoids in the spleen ($p = 0.021$), thymus ($p = 0.021$), lung ($p = 0.021$), heart ($p = 0.034$), testis ($p = 0.014$) and liver ($p = 0.014$) in the tomato group as compared to the control group.

Table 5.2: Carotenoids accumulates in several tissues in mice when fed a diet rich in tomato paste. Mice were given either control feed or feed with 10 % tomato paste for two weeks. Carotenoids were analyzed by HLPC in tissue homogenates.

Tissue	Group	Lycopene (nmol/g tissue)	p-value	Total carotenoids (nmol/g tissue)	p-value	n
Spleen	Tomato	0.139	0.014	0.161	0.021	4
	Control	0.000		0.014		4
Epididymis	Tomato	0.000	1.000	0.014	1.000	4
	Control	0.000		0.014		2
Thymus	Tomato	0.023	0.686	0.069	0.021	4
	Control	0.000		0.033		4
Epidermal fat	Tomato	0.000	1.000	0.012	1.000	4
	Control	0.000		0.011		4
Lung	Tomato	0.051	0.131	0.077	0.021	4
	Control	0.000		0.019		4
Kidney	Tomato	0.000	1.000	0.100	0.564	4
	Control	0.000		0.089		4
Heart	Tomato	0.091	0.028	0.190	0.034	4
	Control	0.000		0.093		3
Brain	Tomato	0.000	1.000	0.028	1.000	4
	Control	0.000		0.029		4
Prostate	Tomato	0.029	N.A.	0.037	N.A	1
	Control	0.000		0.000		2
Testis	Tomato	0.032	0.014	0.039	0.014	4
	Control	0.000		0.000		4
Liver	Tomato	0.867	0.014	0.879	0.014	4
	Control	0.000		0.000		4

N.A. = Not applicable

6 Discussion

In this thesis, we studied the ability of coffee and tomato to modulate basal and TNF-α induced NF-κB activity in the prostate cancer cell line PC3. Furthermore, we investigated whether coffee and tomato could influence the expression of genes coding for proteins involved in inflammation, cytoprotection and cancer development in cultured PC3 cells and in PC3 xenografts in mice. In addition, we identified tissue accumulation of lycopene and other carotenoids in mice fed a diet rich in tomato paste. This section starts with a discussion of the methods used in this work, followed by further discussion of the results.

6.1 Discussion of methods.

6.1.1 The luciferase reporter

The enzyme luciferase catalyzes the oxidative decarboxylation of luciferin to oxyluciferin and CO_2, producing light that can be measured. In the work presented in this thesis the luciferase gene is coupled to a promoter with binding sites for NF-κB, thus the luciferase activity reflects the level of NF-κB activity.

The luciferase reporter system represents a rapid, easy, sensitive and affordable method for measuring transcriptional activity. It has a wide range of applications and can be used in both *in vitro* and *in vivo* experiments (97;112). The water-soluble substrate luciferin is not toxic to mammalian cells or organisms, and is easily transported into cells if added to growth medium or injected into organisms. The firefly luciferase does not require any posttranslational modifications, but is a mature enzyme with catalytic competence after release from the ribosome. These properties, combined with a short half-life in cells (approximately 3 hours), makes luciferase a very useful reporter. Another advantage is the absence of luciferase in the target cells, thus making the system practically background free. In addition, the expression of luciferase have no adverse effects on the metabolism when stably introduced into cells and organisms (113;114).

Other commonly used reporter genes include chloramphenicol acetyltransferase (CAT), β-galactosidase (β-Gal) and fluorescent proteins, such as green fluorescent protein (GFP). CAT and β-Gal are more stable, with a longer half-life than luciferase, thus luciferase are superior to them in terms of responsiveness (115;116). One disadvantage of fluorescent proteins is the

high background level and therefore fluorescence can be more difficult to measure in an *in vivo* situation as compared to bioluminescence (116).

Even though the luciferase reporter system has a broad range of valuable features, as in all methods, there are some disadvantages as well. The NF-κB activity is not measured directly, but indirectly through assessments of the luciferase activity.

Changes in bioluminescence intensity is a measure of the level of NF-κB activity, but the method does not provide information about which NF- κB target gene that is up- or downregulated. Therefore, we also measured the mRNA level of NF-κB target genes involved in immunity and inflammation using RT-PCR.

Luciferase assay

Luciferase activity in PC3-kB-luc xenografts from mice were measured and normalized to total protein concentration using luciferase assay and Bio Rad protein assay.

Immune cells and blood vessels infiltrate tumors and contribute to a varying percentage of the tumor weight. In our analysis of luciferase activity in xenografts, approximately one fourth of the tumor was measured. It is possible that the concentration of PC3-κB-luc cells vary in different parts of the tumor, so despite that the luciferase activity were normalized to total protein concentration, we do not know if the ratio between PC3-κB-luc cells to infiltrating cells derived from the host were the same in all samples. If a sample have a high content of cells without the reporter construct it could lead to a lower level of luciferase activity compared to samples with low or modest infiltration, thereby under estimating the luciferase activity. To conclude, these luciferase measurements are possibly not as well suited for these xenografts studies as compared to measurements in organs of transgenic reporter mice.

6.1.2 Cell viability

In this work cell viability was determined using trypan blue exclusion and morphologic changes examined by light microscope.

Trypan blue exclusion takes the advantage of the ability of healthy cells to exclude the trypan blue dye, whereas dead cells will be stained. In this way the healthy cells can be counted directly. Trypan blue exclusion is a simple, rapid and convenient method to assess the total amount of cells in a sample, as well as the ratio of live and dead cells.

Other commonly used methods for cell viability analysis are often based on metabolic activity or loss of membrane integrity. Metabolic activity can be measured by incubation with a tetrazolium salt, for example MTT. The tetrazolium salt is cleaved into a formazan product, changing color from yellow to blue, thereby increasing the absorption (117). Cell death causes loss of membrane integrity, allowing intracellular enzymes, such as lactate dehydrogenase (LDH), to leak into the culture medium wherein its catalytic activities can be measured. LDH can catalyzes the reduction of a tetrazolium salt to a highly colored formazan product, and the absorption is readily measured (118;119). However, these assays have a major shortcoming with respect to usage in the experiments presented in this thesis. Both these assays are largely based on redox reactions, thus the redox capacities of the extracts may interfere with these methods.

Another well suited method for measuring cell viability, apoptosis and necrosis, is flow cytometry, however, this method was too complex and time consuming to be set-up within the scope and time limits of this thesis.

6.1.3 Prostate Cancer Models

To increase our understanding of the biology of prostate cancer and to develop new therapies, model systems of prostate cancer is crucial. The complexity of prostate cancer makes it difficult to develop suitable models system, and an ideal model which reflects the whole cancer development and progression has not yet been developed. However, thoughtful use and interpretation of the available model systems today can increase our knowledge and our chances to handle the disease (120).

Currently, more than 200 different cell lines and subclones are used as models in human prostate cancer research. These have been isolated from normal prostate tissue, primary tumors, ascites or different metastasis, all having various characteristics and reflecting different stages of the disease (121). Xenograft models and transgenic mice models are also extensively used in cancer research (120).

The PC3 cell line is isolated from a bone metastasis and represents a model of a very late stage of prostate cancer. The cell line does not express PSA or androgen receptor, hence, it is androgen independent and non-responsive to androgen withdrawal (93).

The transcription factor NF-κB is demonstrated to be abnormally upregulated in many prostate cancer cells, and to have a critical role in inflammation and cancer (91;122;123), thus, it is interesting to investigate if there are any dietary plants that can prevent dysregulation, or restore normal regulation of NF-κB. Previous studies have revealed dietary plants can modulate LPS induced NF-κB activity *in vitro*, and also NF-κB activity *in vivo* (39;40). Another important activator of NF-κB is TNF-α, a cytokine that has been detected in prostatic tumors (124). The PC3-κB-luc cells express receptors for TNF and respond rapidly to TNF-α stimulation. The microenvironment of the tumor has an important role in the development of prostate cancer. Unfortunately, it is difficult to mimic the actual microenvironment, however, adding TNF-α to the cell culture is a way to activate NF-κB, thus enabling investigation of possible inhibitory effects on NF-κB activity by different dietary plants.

Cell lines are simple model systems and to further investigate results from *in vitro* experiments, mice represents a model system that allows us to study carcinogenesis in a biologically complex environment (120).

In this thesis a xenograft model of prostate cancer was used to study the effect of coffee and tomatoes on carcinogenesis. Human prostate cancer cells were injected into mice with a mutation in the transcription factor $Foxn1^{nu}$ which leads to lack of thymus development (125). Because the mice lack thymus, the model has some limitations regarding studying the link between dietary plants, inflammation and cancer. However, as previously mentioned, there are no perfect mechanistically model, therefore, one should use feasible models and optimize these. Even though the mice lack thymus, and consequently T lymphocytes, they

have an immune system, and it is possible that they have higher numbers of macrophages and natural killer cells to compensate for the lack of T lymphocytes.

6.1.4 Extracts

Methanol and MilliQ water was used for the extraction of coffee and tomatoes. The use of methanol ensures that not only water soluble, but also fat soluble substances are extracted. Heat treatment and DMSO, as well as storage, can affect the antioxidant capacity of the extracts. As little as possible of DMSO was used, and the extracts were stored under argon gas to prevent oxidation. Although the method allows extraction of many or most water and fat soluble substances, there are certainly also some compounds which will not be efficiently extracted from tomatoes and coffee. The composition of coffee and tomato extracts could be analyzed by HPLC, however, it would be a time consuming task. Analysis of extracts is not within the scope of this study.

6.1.5 Q-RT-PCR

Analysis of the gene expression in PC3 cells and PC3 xenografts were performed using real-time Q-RT-PCR. In order to get reliable results, several factors have to be controlled, such as variation in initial sample amount, RNA integrity, RNA purity, efficiency of cDNA synthesis and differences in the overall transcriptional activity of cells or tissues analyzed (126).

Low quality of the RNA can cause false positive or negative differential expression, hence it is crucial to ensure that all samples have a high quality. In addition to the quality, the quantitative detection of the targets depends on the template abundance, therefore it is important to evaluate both the quality and quantity of the RNA before performing the Q-RT-PCR (127).

The RNA quantity was analyzed using the Nanodrop ND-1000, which is a simple, rapid and affordable method. The quality of the RNA was analyzed by the Agilent 2100 Bioanalyzer. This is a widely used method which provides a RIN independent of the user and subjective assessments. Only a small amount of RNA is required and the measurements seems to be unaffected by contaminants (107;127).

Q-RT-PCR has several advantages over traditional PCR. There are two main differences between traditional PCR and Q-RT-PCR. While traditional PCR detects the end-point, that is

the quantity in the plateau phase, the detection in Q-RT-PCR occurs while the reaction take place in the exponential phase and the PCR product is directly proportional to the amount of template nucleic acid. Due to different reaction kinetics, samples that started out with the same amount in the beginning of the reaction, can have amplified to different quantities at the plateau phase. Therefore, traditional PCR are less accurate than Q-RT-PCR. Further, traditional PCR is more time consuming and requires post PCR processing. Also, Q-RT-PCR is very sensitive, able to detect as little as a two-fold change, unlike gel detection for traditional methods which can not detect changes less than about ten-fold.

In this thesis, TaqMan chemistry (also called fluorogenic 5' nuclease chemistry) was used in the Q-RT-PCR. An alternate method is SYBR Green based detection, which is non-specific. SYBR Green binds to any double stranded DNA, while the TaqMan probes are specific to an already determined target. However, one disadvantage of the TaqMan chemistry is that it can be expensive since a different probe has to be synthesized for each unique target sequence (128).

When the results were calculated, relative quantification using the comparative method was chosen and the C_t value of the target genes were normalized to the average of the two housekeeping genes Gusb and TBP. Choosing appropriate endogenous controls is a critical point in Q-RT-PCR, reducing possible internal errors. The reference genes chosen should be stably expressed, independent of the experimental conditions, and their abundance strongly correlated to the total amount of mRNA in each sample. As there is no universal reference gene, the appropriate reference genes must be experimentally determined (129;130). Glyceraldehyd-3-phosphate dehydrogenase (GAPDH), hypoxanthine-guanine phosphoribosyl transferase (HPRT), and 18S ribosomal RNA are commonly used reference genes. They are appropriate for certain experimental conditions, however they are often not suitable as reference genes in cancer research (131;132). This was also confirmed in our analysis.

It is important to remember that the transcriptional activity is dynamic and constantly chancing, therefore, the Q-RT-PCR results only provides a snapshot of the mRNA level. Also, the method measures the mRNA level, but does not provide any information about the level of proteins that these genes encode.

The interpretation of the results from the micro fluidic cards is complicated, since the various genes analyzed might have different functions in different cell types and under various

conditions. Also, a certain mRNA level can be positive, whereas a higher or lower gene expression can be harmful. The up-or downregulation of a particular gene may have positive effects in one cell type or tissue, and adverse effects in another,, thereby making it difficult to interpret if the total change in gene expression in relation to the net effect on inflammation and cancer.

6.1.6 HPLC

HPLC was used to identify in which tissues carotenoids accumulates. The method has several advantages, such as a high sensitivity, speed, accuracy, automation, high resolution and reproducibility. However, if two compounds escape the column at the same time, it is difficult to detect, leading to inaccurate results. Also, the method is complex, requiring a trained technician to operate the system and the equipment needed is expensive.

The small size of the prostate in mice made it difficult to analyze these samples. The sample size was under the required level for analysis for five of eight prostate samples in this thesis. Perhaps a better way to perform these analyses would be to pool the prostate samples from each group together in order to increase the sample volume. However, both lycopene and other carotenoids were detected in the one prostate sample from the tomato group analyzed, and not in any of the two control samples analyzed. This might be an indication that lycopene and carotenoids accumulate in prostate when mice are fed a diet rich in carotenoids.

6.2 General discussion

In the work presented in this thesis, we studied possible effect of tomatoes or coffee on prostate cancer cells *in vitro* and in xenografts. In the following section, the findings will be discussed separately for the two items.

6.2.1 Coffee and prostate cancer

Extracts of coffee were investigated for their ability to modulate NF-κB activity and mRNA levels of genes relevant for prostate cancer development. We found that coffee induced basal NF-κB activity at the highest concentration, whereas it inhibited TNF-α induced NF-κB activity already at a low concentration in PC3 cells *in vitro*. In general, the mRNA level of genes involved in cytoprotection were upregulated, whereas a number of genes related to inflammation, apoptosis and tumor suppression were downregulated in cultured PC3 cells treated with TNF-α and extract of coffee as compared to controls treated with TNF-α only. In xenograft of PC3 cells, genes related to inflammation, apoptosis, cytoprotection and control of cell proliferation and differentiation were downregulated in mice fed a coffee containing diet compared to controls.

Studies have previously shown that coffee is a strong inhibitor of NF-κB activity both *in vitro* and *in vivo* (39;40;111). Coffee is one of the most commonly consumed beverages worldwide and consist of a complex mixture of substances, including vitamins, minerals, lipids, carbohydrates, caffeine and phytochemicals (3). Because of the high consumption, coffee is a major contributor to the total intake of phytochemicals (13).

Coffee consumption has been associated with a reduced risk of several chronic diseases, including Parkinson disease (133), cardiovascular disease (134), chronic liver disease (135), and type 2 diabetes mellitus (136). On the other hand, consumption of coffee is also associated with increases in several risk factors for cardiovascular disease, such as blood pressure and plasma homocysteine (137). Unfiltered coffee has a high content of diterpenes, such as cafestol and kahweol, which can increase the level of total- and LDL cholesterol, and WHO concluded that unfiltered coffee probably increase the risk of cardiovascular disease (2) The Norwegian Health Ministry stated that there are limited evidence for adverse effects on health in adults having a moderate intake of coffee (8).

Regarding cancer, WCRF concluded in 2007 that it is unlikely that intake of coffee have an impact on cancer in pancreas and kidney, but the evidence for other types of cancers are to limited to conclude (3). Recently, a prospective analysis of about 50 000 men in the Health Professionals Follow-up Study found that there was a strong inverse association between coffee consumption and risk of lethal prostate cancer (138). Hypotheses for this reduced risk of prostate cancer are improved glucose metabolism and regulation of the sex hormone level (138).

We found that dark roasted coffee significantly inhibited TNF-α induced NF-κB activity *in vitro* at a concentration as low as 1 mg/ml. This corresponds to an intake by an adult of 5 g coffee or about half a cup of regular coffee. The highest concentrations of coffee extract tested *in vitro* (6 mg/ml) corresponds to about 30 g coffee or about 3 cups of regular coffee.

Unlike results from previous studies in the research group, the degree of roasting was not a main determinant of the effect on NF-κB activity (111). Both green and dark roasted coffee decreased the NF-κB activity in TNF-α treated PC3 cells, of which dark roasted coffee had a slightly, non-significantly, stronger inhibition of NF-κB. Reasons for the different results in our experiments could be that the extracts were made of other coffee beans, and/or that the extracts were tested in a monocytic cell line (U937) treated with LPS in the previous study (111).

Extracts made of both green and dark roasted coffee had a dual effect on NF-κB activity *in vitro*. Basal NF-κB activity was increased at the highest concentration, whereas TNF-α induced NF-κB activity was inhibited. However, it is important to notice that these inductions are small compared to the huge NF-κB activation in cells stimulated with the very potent activator TNF-α.

In PC3 cells in culture, the inflammation related genes CD40, CXCL11, NF-κB2 and ICAM-1 were downregulated, whereas IL-6 and EDN1 were upregulated by coffee. The cancer related genes CASP3, FAS and TP53 were down regulated, whereas VEGFA was upregulated by coffee. In addition, Nrf2, GCLC and NQO1, genes involved in cytoprotection, were upregulated by coffee. In xenografts, coffee reduced the mRNA level of RelA, TXN, BAD and TGFβ1.

Activation of NF-κB in tumor cells can result in upregulation of genes involved in inhibiting apoptosis and promoting angiogenesis and metastasis (44). Apoptosis is important in the

control of malignant diseases, and an imbalance in the rates of apoptosis have been proposed to be necessary, as well as sufficient, for tumor growth. Both FAS receptor and caspase 3 (CASP3) protein initiate apoptosis when activated (139;140). FAS receptor is expressed in a variety of non-malignant cells, whereas FAS loss-of-function is a common characteristic in malignant cells. Loss-of-function of FAS is associated with aggressive disease and poor prognosis (139).

p53 is a tumor suppressor protein (encoded by the TP53 gene) and its central role in cell cyclus control and cancer prevention is well-known. p53 has the ability to activate DNA repair proteins if DNA is damaged, or initiate apoptosis if the DNA repair fails. Somatic mutations of the TP53 gene occurs in nearly all types of cance and more than 50 % of human tumors contain a mutation or deletion of the TP53 gene. In prostate cancer approximately one fifth of all tumors contain a somatic TP53 mutation (141).

In accordance with our findings, Paur *et al.* observed an increased mRNA level of GCLC, NQO1 and Nrf2 by dark roasted coffee in HepG2 cells (111). Also, Higgins *et al.* found that coffee induces cytoprotective proteins regulated by Nrf2 in both liver and intestine in mice fed coffee (142). Furthermore, the coffee constituents 5-0-caffeoylquinic acid and N-methylpyridinium were reported to be potent activators of Nrf2, leading to induction of cytoprotective proteins both *in vitro* and *in vivo* (143). In an intervention study with healthy participants, a daily consumption of 3-4 cups of coffee reduced oxidative damage, body fat mass and energy intake (144). Lopez-Garcia *et al.* found that intake of coffee was associated with decrease levels of the inflammatory markers E-selectin and CRP in humans (145).

6.2.2 Tomatoes and prostate cancer

Furthermore, extracts of tomatoes were examined for their ability to modulate NF-κB activity and mRNA levels of genes related to inflammation and cancer in PC3-κB-luc cells. NF-κB activity and mRNA levels of the same genes, were also investigated in xenografts from mice fed with a diet rich in tomato paste. Also, accumulation of carotenoids in mice fed a diet supplemented with tomato paste was investigated. We found that extracts from tomato paste had no effect on basal NF-κB activity, while it had a strong inhibitory effect on TNF-α induced NF-κB activity in PC3 cells. In TNF-α stimulated PC3 cells in culture, genes related to inflammation and tumor suppression were downregulated by tomato extract, whereas genes related to apoptosis and cancer were upregulated. The ability of tomato extract to

modulate the gene expression was less prominent in PC3 xenografts. However, the inflammation related gene ICAM-1 was upregulated, while the TGFβ1 gene coding for proteins involved in proliferation, differentiation and cell survival was downregulated. Carotenoids accumulated in several tissues, including liver, heart, spleen and probably also prostate (see discussion above), in mice with tomato paste in their diet.

Tomatoes are one of the most consumed vegetables worldwide, and the consumption seems to increase (146). During the past decades much research has focused on the relationship between tomatoes and/or lycopene and cancer prevention. Epidemiological studies have suggested that consumption of tomatoes and tomato-based products reduce the risk of prostate cancer (147). WCRF concluded in their last report that both foods containing lycopene naturally and foods supplemented with lycopene probably decrease the risk of prostate cancer. The main sources for lycopene are tomatoes and tomato products. In addition, fruits such as grapefruit, watermelon, guava, and apricot contains lycopene naturally (3).

The carotenoid lycopene is believed to be one of the major active components in tomatoes and tomato products responsible for the reduced risk of prostate cancer. One possible mechanisms of action of lycopene is its antioxidant function and the potential to attenuate oxidative damage. Other suggested mechanisms include gene regulation, gap-junction communication, modulation of hormone and immune activity, and metabolism of carcinogens (148). **Figure 6.1** summarizes the main suggested mechanisms of lycopene in preventing chronic diseases.

The extract of tomato paste in this study contains lycopene and a wide variety of other compounds found in tomatoes. We are not sure if lycopene is the most prominent compound in the extract and the inhibition of NF-κB *in vitro* observed in our experiment might be attributed to not only lycopene, but also other compounds in the extract.

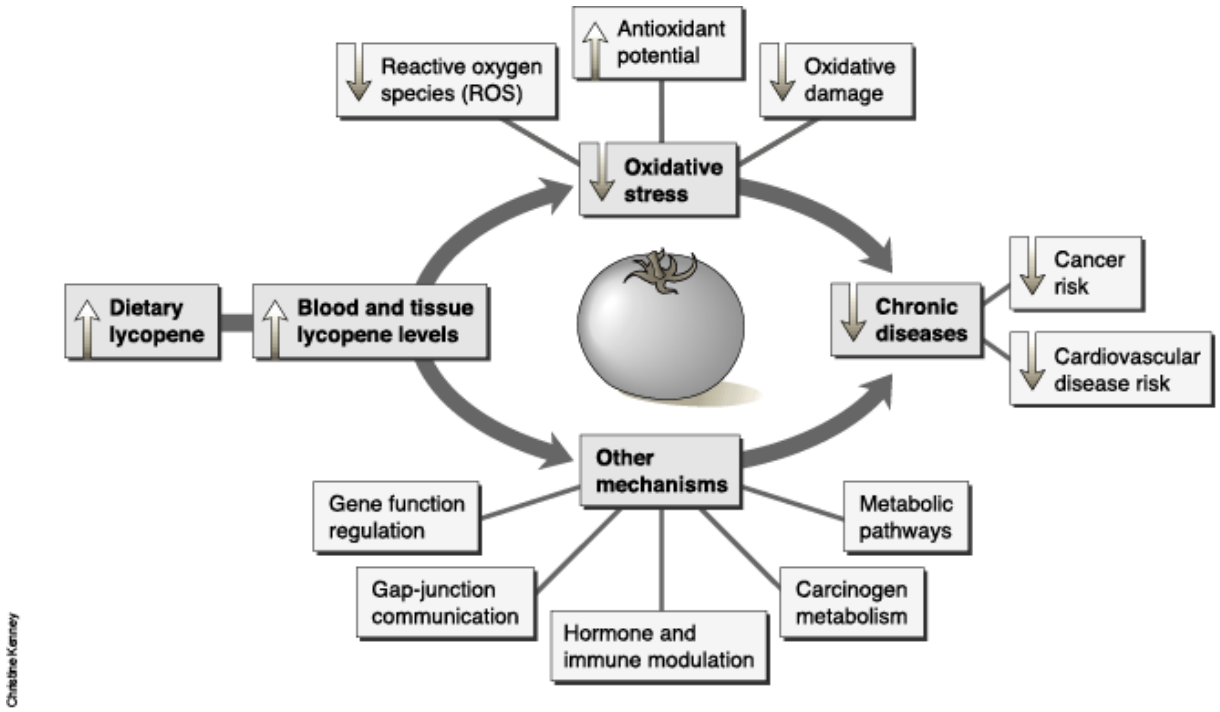


Figure 6.1: Suggested mechanisms for the effects of lycopene on reduced risk of chronic diseases. Intake of dietary lycopene might increase the level of lycopene in blood and tissue. By acting as an antioxidant, lycopene can reduce oxidative stress which in turn might lead to reduced risk of cancer and cardiovascular disease. Other mechanisms proposed mechanisms include regulation of gene functions, improved cell-cell communication, modulated hormone and immune response, improved metabolism of carcinogenes and regulation of metabolic pathways, thereby reducing the risk of chronic diseases (149). From Agarwal S (149).

Most cell experiments have investigated the effect of single compounds, such as lycopene, rather than whole tomato products, on prostate cancer. It has been reported that treatment of the prostate cancer cell lines PC3, LNCaP and DU145 with lycopene results in G_0/G_1 cell cycle arrest. Also, exposure to lycopene induced apoptosis in the LNCaP and DU145 cells (150). Palozza *et al.* showed that lycopene inhibited cell growth by reducing Ras-dependent activation of NF-κB in LNCaP (151).

Lee *et al.* found that alpha-tomatine (a compound in tomatoes) induced apoptosis and inhibited NF-κB activation in prostate cancer cells, suggesting that this compound might be useful in protection against prostate cancer development and progression (152).

Animal experiments have previously shown that lycopene downregulates several genes related to inflammation, as well as genes of IGF-1 and 5α-reductase in prostate tissue (153). Pannellini *et.al* studied the effect of a diet enriched with processed whole tomatoes in a mice model for progressive prostate cancer. In accordance with our results they showed that tomato paste had an impact on several genes. They found that a diet rich in tomatoes

modulated the concentration of several angiogenic and inflammatory chemokines in serum, and efficiently prevented prostate cancer (154). Another study using the same model investigated the effect of tomato paste and pure lycopene provided in beadlets. They reported a greater chemopreventive effect against prostate cancer of pure lycopene as compared to lycopene provided from tomato paste (155). However, other studies have suggested that tomato products contain components in addition to lycopene that might have a cancer preventive effect (156).

In one study investigating prostate cancer development in transgenic mice, a combination of vitamin E, selenium and lycopene was found to reduce prostate cancer. A combination of vitamin E and selenium did, however, not have the same protective effect, indicating that lycopene was the active component (150).

By using HPLC we analyzed eleven different tissues from mice fed a control diet or a diet supplemented with tomato paste in order to examine the bioavailability of tomato carotenoids and in which tissues carotenoids accumulate. We found that lycopene accumulated in seven different tissues, including possibly prostate, with the highest concentration in liver and spleen. The fact that lycopene accumulates in the prostate is an interesting observation, supporting the hypothesis that lycopene reaches the prostate gland and might reduce the risk of prostate cancer (3).

In humans, consumption of processed tomato products has been shown to increase the serum lycopene level with accumulation in several tissues, of which the most concentrated amounts were found in the adrenal gland, testes, liver and prostate gland (157;158). Lycopene is one of the most potent antioxidants in laboratory experiments and one of the main carotenoids in human plasma (149). In natural plant sources lycopene mainly exists in an all-*trans* configuration, but in serum and prostate tissue lycopene primarily exist in its *cis* form (159). During mechanical and heat-related processing of the plant foods, the bioavailability of lycopene increases, which might be due to isomerization from the *trans* form to the *cis* form.

In addition to lycopene, other carotenoids were detected in all tissues analyzed of mice fed a diet supplemented with tomato paste, and also in lower amounts in many of the tissues of mice fed a control diet. The control diet was very low in carotenoids, thus, the carotenoids detected in the control mice is probably residues of carotenoids provided from the regular diet.

It is generally agreed that carotenoid-containing fruits and vegetables have beneficial health effects. It is also suggested that carotenoids contribute to the favorable effects found in epidemiological studies. However, clinical trials using single compounds, such as vitamin E or β-carotene have failed to prove a beneficial effect (160). This indicates that it might be the complex network of a large number of compounds that exerts the valuable effects of fruits and vegetables.

Even though there are data supporting beneficial effects of lycopene, we do not know the long term consequences of a high intake of lycopene supplements. Also, tomato products probably contain other compounds besides lycopene that contribute to the positive effects on prostate cancer, therefore, eating natural tomato products would be the preferably way to consume lycopene.

6.2.3 Differences in mRNA level between PC3 cells in culture and in xenografts

The fact that there were differences in the gene expression of PC3 cells in culture and PC3 xenografts may underlines the importance of the microenvironment in the gene regulation. There is a growing amount of data suggesting that an inflammatory microenvironment is a crucial component of tumors. Solid tumors are composed of malignant cells, as well as non-malignant cells, such as immune cells (161). The role of inflammation in cancer development and progression is generally accepted, and overexpression of inflammatory cytokines is thought to promote the carcinogenesis (54;161). Cancer therapy has traditionally targeted the tumor cells exclusively, however, with the new awareness of the great impact of the microenvironment on carcinogenesis, this is changing (162). In the mice, the microenvironment is highly complex and the PC3 cells are influenced by the environment (e.g. bloodflow, cytokines, and oxygen supply) over a 5 week period. This could have a great impact on the gene expression, which again might alter the growth rate of the tumor cells.

Another plausible explanation for the differential gene expression in cultured PC3 cells versus PC3 xenografts could be infiltration of immune cells into the tumor. The probes used in the micro fluidic card are specific for human genes, however, one can not exclude that there can be some overlapping between mouse and human genes, so that also genes expressed in cells from mice (not the PC3 cells) were amplified in the PCR, thus resulting in differences between mRNA level observed in PC3 cells in culture and PC3 cells in xenografts.

Assuming that the infiltration of immune cells are the same in the xenografts in the control group as in the intervention groups, it is possible to study the effect of dietary plants by comparing the mRNA level of the control group with the intervention groups. However, comparing samples consisting of only PC3 cells with samples where the proportion of PC3 cells are unknown, could lead to misinterpretation of the result. Cross-reactivity between human and mice transcripts included in the low density array card will be tested in future experiments.

6.2.4 NF-κB luciferase activity in homogenates of xenografts

The results from the analysis of NF-κB luciferase activity in homogenates of xenografts showed a trend different from the one observed by the *in vivo* imaging. Based on AUC, the *in vivo* imaging had a non-significant lower NF-κB luciferase activity in both the tomato and coffee group, of which the coffee group was lowest, compared to the control group at week five. On the other side, the measurements of NF-κB luciferase activity in homogenates of xenografts revealed a non-significant higher activity in the coffee group compared to the control group. However, the standard deviations of both *in vivo* imaging and the *ex vivo* analysis were high, making it difficult to conclude.

There are several possible explanations for this high variance. First, the NMRI mice in this study is an outbred mice model, which leads to greater differences between the mice (e.g. weight, size). It is conceivable that effects of coffee or tomato on NF-κB luciferase activity could have been detected if the mice were more similar at the beginning of the intervention. Also, the injection of the PC3-κB luc cells in mice can be difficult, potentially resulting in differences in the amount injected into each mouse.

As are in our experiments, other studies have shown differential luciferase activity in organs measured by *in vivo* imaging and subsequently by luciferase assays (163). Compared to *in vivo* imaging, measurements of *ex vivo* NF-κB luciferase activity have some limitations. The *ex vivo* analysis include several steps, and it is possible that changes occurs in the sample after harvesting, thus modulating the NF-κB activity. *In vivo* imaging allows the researcher to measure the NF-κB luciferase activity at several time points, thus providing information about the NF-κB activity over time, whereas the *ex vivo* analysis only gives information about the NF-κB activity at one time point. To provide reliable results of the imaging, the organs of interest should be in close proximity to the surface. The xenografts in this study

were injected subcutanously, thereby making *in vivo* imaging a suitable method for measuring the NF-κB activity (163).

6.2.5 Possible mechanisms of regulation of NF-κB activity

Inhibition of NF-κB can occur at every step in the NF-κB activation pathways, including blockage of the incoming signal (e.g. binding of ligand to receptor), inhibition of the cytoplasmic steps in the activation pathway (e.g. activation of the IKK complex or degradation of IκB) or inhibition of the nuclear activity of NF-κB (e.g. translocation of NF-κB from cytoplasma to nucleus or its binding to DNA). A large number of inhibitors of NF-κB have been identified, but a shortcome of these is that they are not cell specific, potentially leading to serious adverse effects.

Plausible mechanisms for the inhibition of NF-κB observed in the *in vitro* experiments by extracts of coffee and tomato paste could be redox regulation. DNA is packaged into a very compact structure, and remodeling of the DNA is necessary in order to begin transcription. Also, recruitment of co-activators and binding of NF-κB to the DNA is needed to facilitate transcription. All of these step are believed to be redox regulated (71). The extracts used in the experiments in this thesis contains phytochemicals, of which many of them might exert antioxidant properties, hence it is possible that the extracts inhibit NF-κB activation by attenuating oxidative stress in the cells. However, a growing amount of data propose that ROS might not play such a critical role in the activation of NF-κB as previously assumed (72;164). Also, even though many of the compounds that can inhibit NF-κB activation are antioxidants, these compounds might inhibit the NF-κB activation independent of their antioxidant function. For example, Hayakawa *et al.* showed that N-acetyl-L-cystein inhibit TNF-induced NF-κB activation by lowering the affinity of the TNF receptor to ligand (72).

7 Future perspectives

The present thesis has provided new information in the area of prostate cancer research, and demonstrated potential cancer preventive effects of coffee and tomatoes. However, more research is required to fully understand the role of coffee and tomatoes in the development and progression of prostate cancer.

In order to understand the mechanisms behind the observed effects of coffee and tomatoes on NF-κB activity *in vitro*, as well as the effects on mRNA level of genes related to immunity, inflammation and cancer, isolation and characterization of the bioactive compounds in the extracts are required. Also, identification of the effect of various coffee and tomatoes compounds on the different steps in the NF-κB signaling pathway is needed.

The observed differences in gene expression in prostate cancer cells in culture and in xenografts, generates new questions about the role of the inflammatory microenvironment and infiltration of immune cells. Detection of the cytokines present in prostatic tumors and in the serum of the mice would be valuable in order to further investigate the relationship between diet, inflammation and cancer development.

8 Conclusion

In this thesis we have shown that extracts made of coffee and tomato paste inhibited TNF-α induced NF-κB activity in the prostate cancer cell line PC3-κB-luc. Basal NF-κB activity was not modulated by extracts of tomato paste, but was upregulated by the highest concentration of coffee. There were no significant differences between green and dark roasted coffee in the ability to inhibit TNF-α induced NF-κB activity, however, dark roasted coffee increased basal NF-κB activity more than green coffee.

The expression of several genes related to inflammation, immune responses, cytoprotection and carcinogenesis were modulated by extracts of coffee or tomato paste in PC3 cells in culture and in xenografts.

In PC3 cells in culture, the inflammation related genes CD40, CXCL11, NF-κB2 and ICAM-1 was down regulated, whereas IL-6 was upregulated by coffee. The cancer related genes CASP3, FAS and TP53 was downregulated, whereas VEGFA was upregulated by coffee. In addition, Nrf2, GCLC and NQO1, genes involved in cytoprotection, were upregulated by coffee. Tomato paste downregulated the inflammation related genes CD40 and ICAM-1, as well as the tumor supressor gene TP53. FAS and HIF1A, both related to cancer, were upregulated.

The modulation of gene expression in PC3 cells in xenografts were less prominent than in PC3 cells in culture. However, coffee downregulated four genes involved in inflammation, cytoprotection and apoptosis, whereas tomatoes modulated the expression of two genes related to inflammation and cancer.

Analysis of tissues from mice fed a diet enriched with tomato paste revealed that lycopene and other carotenoids accumulated in various amounts in several tissues, including liver, heart, spleen and seemingly also prostate. No lycopene was detected in any tissue of mice fed a control diet.

In conclusion, our results show that both coffee and tomatoes are potent inhibitors of NF-κB *in vitro. In vivo*, the results are more complex with both up- and downregulation of several genes involved in immunity, inflammation and cancer. Coffee and tomatoes might reduce the risk of chronic diseases through induction of genes encoding for cytoprotective proteins, as well as downregulation of expression of genes related to inflammation. However, there was a simultaneous downregulation of pro-apoptotic genes, which might contribute to tumor promotion.

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Appendix

Additional list of abbrevations in the appendix.

BAD	BCL2-antagonist of cell death
BAX	BCL2-associated X protein
BCL2	B-cell lymphoma 2
CASP3	Caspase 3, apoptosis-related cysteine peptidase
CASP9	Caspase 9, apoptosis-related cysteine peptidase
CCL3	Chemokine (C-C motif) ligand 3
CD40	CD40 molecule, TNF receptor superfamily member 5
CD80	CD80 molecule
CSF1	Colony stimulating factor 1 (macrophage)
CXCL10	Chemokine (C-X-C motif) ligand 10
CXCL11	Chemokine (C-X-C motif) ligand 11
CYCS	Cytochrome c, somatic
EDN1	Endothelin 1
FAS	Fas/CD95 (TNF receptor superfamily, member 6)
GCLC	Glutamate-cysteine ligase, catalytic subunit
GSTA1	Glutathione S-transferase alpha 1
HIF1A	Hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)
HMOX1	Heme oxygenase (decycling) 1
ICAM1	Intercellular adhesion molecule 1 (CD54), human rhinovirus receptor
IKBKB	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta
IL12B	Interleukin 12B (natural killer cell stimulatory factor 2, cytotoxic lymphocyte maturation factor 2, p40)
IL1A	Interleukin 1, alpha
IL2RA	Interleukin 2 receptor, alpha
IL6	Interleukin 6 (interferon, beta 2)
MMP9	Matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)
NFKB2	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)
NFKBIB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta
NOS2	Nitric oxide synthase 2
NQO1	NAD(P)H dehydrogenase, quinone 1
NRF2	Nuclear factor (erythroid-derived 2)-like 2
PTPRC	Protein tyrosine phosphatase, receptor type, C
RELA	v-rel reticuloendotheliosis viral oncogene homolog A, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3, p65 (avian)
RELB	v-rel reticuloendotheliosis viral oncogene homolog B, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3 (avian)
SOD1	Superoxide dismutase 1
TGFB1	Transforming growth factor, beta 1
TLR4	Toll-like receptor 4
TNF	Tumor necrosis factor (TNF superfamily, member 2)
TP53	Tumor protein p53 (p53)
TXN	Thioredoxin
VCAM1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor

Table A1: mRNA level in PC3 cells in culture. The mice fed control diet (n =15) with 5 % coffee (n = 14) or diet with 10 % tomato paste (n = 15). PC3-κB-luc xenografts were injected in both flanks. The mice continued with the intervention for five weeks before they were euthanized and xenografts stored for further analysis. For determination of relative mRNA expression, the expression level was normalized against the average Ct of the endogenous controls β-glucoronidase (Gusb) and TATA box binding protein (TBP). Data are presented as median (min-max) $2^{-\Delta Ct}$. Differences were identified using Mann-Whitney U test.

	Group											
	Basal		**TNF-α** (n= 3)		**TNF-α+ Tomato** (n = 3)		**Coffee** (n = 3)					
	$2^{-\Delta Ct}$		$2^{-\Delta Ct}$		$2^{-\Delta Ct}$		$2^{-\Delta Ct}$					
Gene	median	(min-max)	median	(min-max)	median	(min-max)	median	(min-max)	P^{KW}	P^{MW1}	P^{MW2}	P^{MW3}
BAD	0.70	(0.51 – 0.85)	0.74	(0.67 – 0.75)	0.71	(0.67 – 0.77)	0.51	(0.46 – 0.67)	0.192			
BAX	1.71	(1.70 – 1.84)	1.74	(1.68 – 2.01)	1.74	(1.69 – 1.87)	1.63	(1.46 – 1.66)	0.099			
BCL2	0.02	(0.01 – 0.02)	0.01	(0.01 – 0.01)	0.01	(0.01 – 0.01)	0.01	(0.01 – 0.02)	0.055			
CASP3	0.44	(0.43 – 0.45)	0.52	(0.48 – 0.55)	0.52	(0.49 – 0.62)	0.40	(0.36 – 0.40)	0.025	0.0495		0.0495
CASP9	0.25	(0.21 – 0.26)	0.22	(0.20 – 0.25)	0.34	(0.33 – 0.43)	0.25	(0.18 – 0.26)	0.086			
CCL3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.				
CD40	0.03	(0.03 – 0.03)	0.07	(0.07 – 0.09)	0.05	(0.04 – 0.05)	0.04	(0.04 – 0.05)	0.022	0.0495	0.0495	0.0495
CD80	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.				
CSF1	0.10	(0.08 – 0.17)	0.93	(0.69 – 0.95)	0.86	(0.72 – 1.15)	0.73	(0.66 – 0.86)	0.066			
CXCL10	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.				
CXCL11	n.d.	n.d.	< 0.01	(< 0.01 – 0.01)	0.02	(<0.01 – 0.02)	n.d.	n.d.				
CYCS	0.15	(0.15 – 0.25)	0.15	(0.14 – 0.16)	0.15	(0.13 – 0.16)	0.16	(0.13 – 0.17)	0.622			
EDN1	0.03	(0.03 – 0.03)	0.09	(0.08 – 0.13)	0.10	(0.08 – 0.12)	0.03	(0.03 – 0.04)	0.040	0.0495		0.0495
FAS	0.03	(0.02 – 0.04)	0.07	(0.07 – 0.08)	0.11	(0.11 – 0.12)	0.06	(0.04 – 0.06)	0.016	0.0495	0.0495	0.0495
GCLC	1.52	(1.47 – 1.55)	2.11	(2.00 – 2.47)	2.47	(1.98 – 2.67)	4.64	(4.16 – 4.80)	0.024			
GSTA1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.				
HIF1A	18.27	(15.73 18.47)	16.25	(14.89 – 17.12)	20.48	(19.54 – 21.73)	14.21	(13.55 – 14.97)	0.027		0.0495	0.0495
HMOX1	0.20	(0.14 – 0.49)	0.22	(0.17 – 0.73)	0.27	(0.21 – 0.51)	28.90	(26.09 – 31.90)	0.075			
ICAM1	0.78	(0.78 – 1.18)	60.19	(56.69 – 64.02)	39.32	(37.88 – 44.01)	15.28	(12.40 – 16.86)	0.016	0.0495	0.0495	0.0495
IKBKB	0.30	(0.30 – 0.31)	0.41	(0.28 – 0.42)	0.27	(0.24 – 0.37)	0.25	(0.16 – 0.35)	0.347			
IL12B	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.				

IL1A	n.d.	n.d.	0.01	(0.01 – 0.02)	0.02	(0.01 – 0.03)	0.01	(0.01 – 0.03)	0.863			
IL2RA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.				
IL6	0.01	(0.01 – 0.03)	0.24	(0.22 – 0.39)	0.37	(0.30 – 0.42)	0.46	(0.42 – 0.73)	0.027	0.0495		0.0495
MMP9	0.01	(0.01 – 0.02)	0.03	(0.02 – 0.03)	0.02	(0.02 – 0.02)	0.01	(0.01 – 0.01)	0.057			
MYC	3.70	(2.73 – 3.95)	3.52	(2.45 – 3.66)	3.08	(2.12 – 3.58)	4.60	(2.84 – 5.10)	0.392			
NRF2	2.02	(1.84 – 2.41)	2.50	(2.21 – 2.62)	2.75	(2.48 – 3.02)	3.76	(3.67 – 4.13)	0.027			0.0495
NFKB1	0.89	(0.70 – 1.00)	1.51	(1.42 – 2.85)	1.78	(1.64 – 2.03)	1.48	(1.35 – 2.01)	0.070			
NFKB2	0.72	(0.58-0.75)	5.02	(3.73 – 5.39)	3.88	(3.80 – 4.52)	2.97	(2.79 – 3.17)	0.024	0.0495		0.0495
NFKBIB	0.35	(0.34 - 0.38)	0.83	(0.59 - 0.88)	0.77	(0.70 - 0.78)	0.35	(0.34 – 0.38)	0.075			
NOS2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.				
NQO1	4.88	(3.60 – 4.98)	4.14	(3.94 – 4.58)	5.15	(4.36 – 5.29)	7.72	(7.12 – 8.93)	0.599			
PTPRC	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.				
RELA	1.61	(0.97 - 1.75)	1.67	(0.93 - 2.14)	1.19	(0.98 - 1.83)	1.12	(0.84 - 1.42)	0.679			
RELB	0.04	(0.04 – 0.06)	0.95	(0.69 – 1.40)	0.96	(0.91 – 1.49)	0.70	(0.65 – 1.33)	0.070			
SOD1	12.78	(12.07 -15.50)	12.06	(11.37 – 13.92)	11.73	(8.92 – 11.86)	7.47	(7.24 – 11.47)	0.060			
TGFB1	6.68	(4.88 – 8.56)	5.11	(4.69 – 8.58)	6.19	(5.78 – 6.67)	5.05	(4.68 – 5.35)	0.408			
TLR4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.				
TNF	n.d.	n.d.	0.01	(0.01 – 0.02)	0.02	(0.02 – 0.02)	0.01	(0.01 – 0.01)	0.041	0.0495		0.0495
TP53	0.06	(0.05 – 0.07)	0.10	(0.08 – 0.12)	0.08	(0.08 – 0.08)	0.07	(0.05 – 0.08)	0.024	0.0495	0.0495	0.0495
TXN	8.95	(8.15 – 10.34)	8.05	(7.66 – 8.89)	7.30	(7.24 – 8.53)	9.50	(9.16 – 11.20)	0.057			
VCAM1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.				
VEGF	0.78	(0.59 – 0.86)	0.61	(0.58 – 0.72)	0.38	(0.35 – 0.72)	1.03	(0.94 – 1.17)	0.044			0.0495

[KW] Kruskal Walis test, between basal, TNF-a, tomato and coffee
[MW1] Mann Whitney U test, between basal and TNF-α
[MW2] Mann Whitney U test, between TNF-α and tomato
[MW3] Mann Whitney U test, between TNF-α and coffee

Table A2: mRNA levels in PC3 xenografts. The mice were fed control diet (n = 15) diet with 5 % coffee (n = 14) or diet with 10 % tomato paste (n = 15). PC3-κB-luc xenografts were injected in both flanks. The mice continued the intervention for five weeks before they were euthanized and xenografts stored for further analysis. For determination of relative mRNA expression, the expression level was normalized against the average C_t of the endogenous controls β-glucuronidase (Gusb) and TATA box binding protein (TBP). Data are presented as median (min-max) $2^{-\Delta Ct}$. Differences were identified using Mann-Whitney tests

	Group								
	Control (n= 15)		Tomato (n = 15)		Coffee (n = 15)				
	$2^{-\Delta Ct}$		$2^{-\Delta Ct}$		$2^{-\Delta Ct}$				
Gene	media	(min-max)	median	(min-max)	media	(min-max)	P^{KW}	P^{MW1}	P^{MW2}
BAD	0.468	(0.291-0.905)	0.534	(0.389-0.814)	0.409	(0.215-0.583)	0.006		0.036
BAX	1.056	(0.747-1.220)	1.035	(0.804-1-419)	1.063	(0.678-1.346)	0.918		
BCL2	0.049	(0.030-0.110)	0.050	(0.024-0.093)	0.044	(0.019-0.276)	0.573		
CASP3	0.407	(0.320-0.514)	0.402	(0.313-0.504)	0.374	(0.318-0.492)	0.096		
CASP9	0.335	(0.278-0.398)	0.355	(0.296-0.411)	0.336	(0.268-0.410)	0.309		
CCL3	n.d	n.d.	n.d.	n.d.	n.d.	n.d.			
CD40	0.025	(0.006-0.065)	0.031	(0.015-0.090)	0.031	(0.018-0.063)	0.363		
CD80	n.d	n.d.	n.d.	n.d.	n.d.	n.d.	0.578		
CSF1	0.033	(0.013-0.062)	0.035	(0.015-0.076)	0.040	(0.018-0.073)	0.693		
CXCL1	n.d	n.d.	n.d.	n.d.	n.d.	n.d.			
CXCL1	0.004	(0.001-0.009)	0.003	(0.001-0.105)	0.003	(0.001-0.019)	0.965		
CYCS	0.186	(0.142-0.301)	0.197	(0.163-0.229)	0.169	(0.125-0.221)	0.083		
EDN1	0.118	(0.095-0.194)	0.117	(0.091-0.188)	0.120	(0.091-0.227)	0.985		
FAS	0.122	(0.075-0.163)	0.099	(0.057-0.136)	0.103	(0.062-0.128)	0.151		
GCLC	1.944	(1.405-2.423)	1.826	(1.623-2.207)	1.770	(1.424-2.351)	0.280		
GSTA1	n.d	n.d.	n.d.	n.d.	n.d.	n.d.			
HIF1A	9.923	(6.723-12.53)	10.029	(7.210-12.30)	9.323	(6.622-12.16)	0.571		
HMOX1	0.353	(0.239-0.707)	0.378	(0.226-0.879)	0.457	(0.255-1.268)	0.520		
ICAM1	2.040	(1.412-2.375)	2.331	(1.567-2.609)	1.763	(1.163-2.929)	0.031	0.040	
IKBKB	0.763	(0.555-1.449)	0.854	(0.384-1.336)	0.738	(0.519-0.871)	0.450		
IL12B	n.d	n.d.	n.d.	n.d.	n.d.	n.d.			
IL1A	0.004	(0.002-0.007)	0.004	(0.002-0.010)	0.004	(0.002-0.008)	0.938		
IL2RA	n.d	n.d.	n.d.	n.d.	n.d.	n.d.			
IL6	0.036	(0.018-0.074)	0.053	(0.021-0.075)	0.042	(0.020-0.088)	0.275		
MMP9	0.006	(0.002-0.015)	0.008	(0.003-0.017)	0.008	(0.003-0.017)	0.284		
MYC	3.490	(2.585-4.095)	3.370	(2.435-4.714)	3.152	(2.604-4.105)	0.471		
NRF2	1.727	(1.187-2.340)	1.802	(1.541-2.112)	1.716	(1.6569-	0.833		
NFKB1	0.467	(0.366-1.068)	0.492	(0.347-0.692)	0.512	(0.354-0.654)	0.578		
NFKB2	0.636	(0.488-0.924)	0.596	(0.478-0.828)	0.648	(0.525-0.849)	0.278		
NFKBIB	0.380	(0.257-0.485)	0.370	(0.280-0.642)	0.738	(0.519-0.871)	0.101		
NOS2	n.d	n.d.	n.d.	n.d.	n.d.	n.d.			
NQO1	1.918	(1.635-2.590)	1.954	(1.390-2.911)	1.808	(1.424-2.466)	0.473		
PTPRC	n.d	n.d.	n.d.	n.d.	n.d.	n.d.			
RELA	1.448	(0.705-2.179)	1.304	(0.637-1.755)	0.833	(0.497-1.214)	0.005		0.005
RELB	0.087	(0.041-0.233)	0.097	(0.067-0.197)	0.081	(0.046-0.191)	0.590		
SOD1	5.582	(2.801-24.28)	8.429	(3.614-12.85)	4.472	(3.914-12.26)	0.291		
TGFB1	5.038	(3.643-8.032)	4.485	(3.462-5.935)	4.527	(3.713-5.651)	0.040	0.040	0.021
TLR4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.			
TNF	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.			
TP53	0.114	(0.095-0.154)	0.110	(0.075-0.163)	0.103	(0.082-0.131)	0.142		0.040
TXN	6.010	(4.270-7.971)	5.423	(3.474-6.959)	5.098	(3.842-6.542)	0.041		0.013
VCAM1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.			
VEGF	1.256	(0.707-1.694)	1.075	(0.723-2.308)	1.081	(0.744-1.568)	0.251		

^{KW} Kruskal Walis Test, between tomato, coffee and control group
^{MW1} Mann Whitney Test, between tomato and control group
^{MW2} Mann Whitney Test, between coffee and control group
n.d. = Not detected