Molecular mechanisms of beta-carotene action in the lung

Summary and general discussion

Chapter 10

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Summary and general discussion

Main findings

Beta-carotene (BC) was previously shown to increase lung cancer risk in smokers and asbestos exposed subjects. Both types of exposure are typically associated with an inflammatory reaction in the lung, and a prolonged inflammation has indeed been shown to play an important role in the initiation and promotion of lung cancer. A chronic inflammation in the lungs is characterized by an influx of neutrophils and involves the generation of cell damaging free radicals. Since it has been reported that BC can act as an antioxidant as well as a pro-oxidant [1,2], we aimed in the first part of this thesis to gain mechanistic knowledge on the circumstances in which BC acts as an antioxidant or pro-oxidant and how BC as such affects radical induced genotoxicity.

We demonstrate in **Chapter 2** that the origin of the radical species to be scavenged affects pro- or antioxidant properties of BC; we observed a protective role for BC against carbon centered radicals, but an enhancing effect of BC in hydroxyl radical induced damage. Indeed, BC supplemented ferrets had lower amounts of M_1dG, a DNA lesion resulting from the lipid peroxidation product MDA with carbon centered radical intermediates. However, the oxidative DNA lesion 8-oxo-dG, which directly results from DNA oxidation, was not changed in ferrets after BC supplementation, probably because BC supplementation also increased base excision repair (BER).

We subsequently focused on the effect of BC in neutrophil induced DNA damage. Smoke and asbestos both induce a chronic inflammation in the lungs [3-6] which is characterized by a neutrophilic influx [7]. Activated neutrophils generate high amounts of reactive oxygen species (ROS) which can cause oxidative DNA damage. Moreover, activated neurophils decrease cellular DNA repair capacity [8]. We demonstrated in **Chapter 3** that the BC metabolites; retinoic acid and retinal specifically increased neutrophil induced oxidative DNA lesions by decreasing MPO activity, an enzyme active in the conversion of neutrophil generated hydrogenperoxide. Moreover, BC enhanced the formation of hydroxyl radicals from hydrogenperoxide. This ultimately resulted in an increased number of oxidative DNA lesions in neutrophil exposed lung epithelial cells when exposed to retinoic acid or retinal as measured with the FPG modified comet assay. This suggests that adverse effects of BC may be encountered under conditions of chronic inflammation.

BC or its metabolites might thus alter genotoxic effects of smoke or asbestos. Besides this effect, BC may also change general gene expression thereby altering the vulnerability of lung tissue for smoke or asbestos induced carcinogenesis. Gene expression changes induced by high BC intake are difficult to assess; for obvious ethical reasons,

humans cannot be supplemented with high amounts of BC, neither is lung tissue easily accessible. Moreover, the frequently used *in vivo* models such as mice and rats highly differ in their BC metabolism compared to humans, since they almost completely convert BC into its metabolites, while humans only partly metabolize BC. Consequently, BC supplementation results in the accumulation of BC metabolites in rodents, while BC supplementation to humans results in accumulation of BC itself [9]. Other animal models that more resemble BC metabolism have been proposed for BC research. Examples are ferrets, gerbils and preruminant calves. However, these models have major disadvantages. When using ferrets or gerbils, there is not much knowledge on molecular mechanisms and a lack of commercially available molecular tools, which results in pioneering research focused on tool development, rather than resolving mechanistic questions regarding the molecular effects of BC. There is more knowledge with regard to the use of calves, however, in that case research is expensive and the calves need special housing. We therefore used a "humanized" mouse model in the second part of this thesis. In this mouse model there is no functional beta-carotene 15,15'-monooxygenase 1 (Bcmo1) due to the knock-out of this gene [10]. Consequently, these mice lack the ability to cleave BC and are like man able to accumulate intact BC. In the second part of this thesis we focused on gene expression changes induced by BC in this mouse model, to obtain more insights in functional effects of BC to contribute to the lack of knowledge of BC action in the lung. In **Chapter 4** we explain and discuss the in-house knowledge with regard to functional genomics in nutritional studies.

For the assessment of gene expression effects induced by dietary BC *in vivo*, we performed a mouse experiment in which female and male, wild type (*Bcmo1*$^{+/+}$) and *Bcmo1* knockout mice (*Bcmo1*$^{-/-}$) were given a diet containing 1500 IU vitamin A with vehicle beadlets (Co) or a diet supplemented with beadlets containing BC to generate 150 mg/kg diet BC (BC).

In **Chapter 5** we focused on the effect of high BC consumption on gene expression changes in lung tissue of female *Bcmo1*$^{-/-}$ mice. We observed a significant decrease in the expression of genes involved in the inflammatory response. After comparison of the gene expression with *Bcmo1*$^{+/+}$ mice, this decreased inflammatory response appeared to be an induced inflammatory response in the lungs of control diet fed female *Bcmo1*$^{-/-}$ mice, which was partly abolished by dietary BC. Histology of the lungs confirmed the gene expression results, showing inflammatory infiltrations in lung tissue, only in female control diet fed mice. These observations were explained by a higher dietary vitamin A demand in female *Bcmo1*$^{-/-}$ mice due to an altered expression of downstream BC metabolizing enzymes, resulting in a gene expression more focused towards vitamin A storage rather than towards retinoic acid production, which is the actual bioactive metabolite. Indeed, a deficiency in retinoic acid is known to result in an inflammations.

In **Chapter 6** we focused on gene expression changes induced in lung tissue of male *Bcmo1*$^{-/-}$ mice after BC supplementation. The higher inflammatory response in lungs of

control diet fed *Bcmo1*$^{-/-}$ mice appeared specific for female mice and was not observed in male mice. In male *Bcmo1*$^{-/-}$ mice however, *frizzled homolog 6* (*Fzd6*) and *collagen triple helix repeat containing 1* (*Cthrc1*) were significantly 2.60 and 2.99 fold down regulated after BC supplementation. Moreover, many olfactory receptors and protocadherins were up regulated. We hypothesized that these gene expression responses were related and may be involved in the detection of changes in air composition in the lung, such as hypoxia. Since pulmonary endocrine cells (PNECs) are important in the detection of hypoxia, particularly this cell type might be affected by BC supplementation. Although PNECs account for only 1 in 2500 lung cells [11], 20% of all lung cancer types originate from these cells [12], and smoking particularly increases PNEC originated lung cancer risk. These findings warrant further research on the role of *Fzd6* and *Cthrc1* in PNECs.

The CARET study reported that BC supplementation in both male and female smoking and asbestos exposed volunteers resulted in an increased lung cancer risk. Therefore, we investigated pathways that were changed in lung tissue of male and female *Bcmo1*$^{-/-}$ mice after BC supplementation in **Chapter 7**. Interestingly, the genes that were altered in their expression by BC supplementation in male and female *Bcmo1*$^{-/-}$ mice, had an opposite direction of the gene expression changes. This suggested a role for BC in the regulation of sex-hormones. These changes in lung gene expression were accompanied by a significant regulation of many enzymes involved in steroidogenesis and steroid conversion. Moreover, testosterone levels were highly variable in BC supplemented *Bcmo1*$^{-/-}$ mice, but not (as expected) in *Bcmo1*$^{+/+}$ mice. We therefore hypothesize that BC supplementation is able to alter systemic hormone production.

It is of importance for the risk-benefit assessment of BC, to know whether BC effects are specific for lung. Therefore, we compared the BC induced gene expression effects in lung tissue with those induced in white adipose tissue, an important BC storage organ [13] and liver, important in both BC metabolism and storage [14].

In **Chapter 8** we illustrate that only a few genes (~1% of the genes regulated by BC supplementation in lung), were also regulated in liver tissue and inguinal white adipose tissue (iWAT), and effects of BC on the expression of single genes is highly tissue specific. Moreover, the magnitude of changes induced by BC supplementation was much less than the differences induced by the knockout of *Bcmo1* or differences in gender (females compared with males).

Although we found in **Chapter 8** that only 1% of the genes regulated by BC in the lung were also regulated in liver and iWAT, we found that the pathways that were changed by BC in female *Bcmo1*$^{-/-}$ mice as describe in **Chapter 5**, or in male *Bcmo1*$^{-/-}$ mice as described in **Chapter 6** or the opposite change in gene expression in male and female *Bcmo1*$^{-/-}$ mice as described in **Chapter 7,** were not specific for lung tissue on pathway level which we describe in **Chapter 9.**

Altogether, we find that BC can change inflammation induced genotoxicity and gene expression in lung tissue, thereby altering processes that can be involved in lung carcinogenesis (Fig. 1).

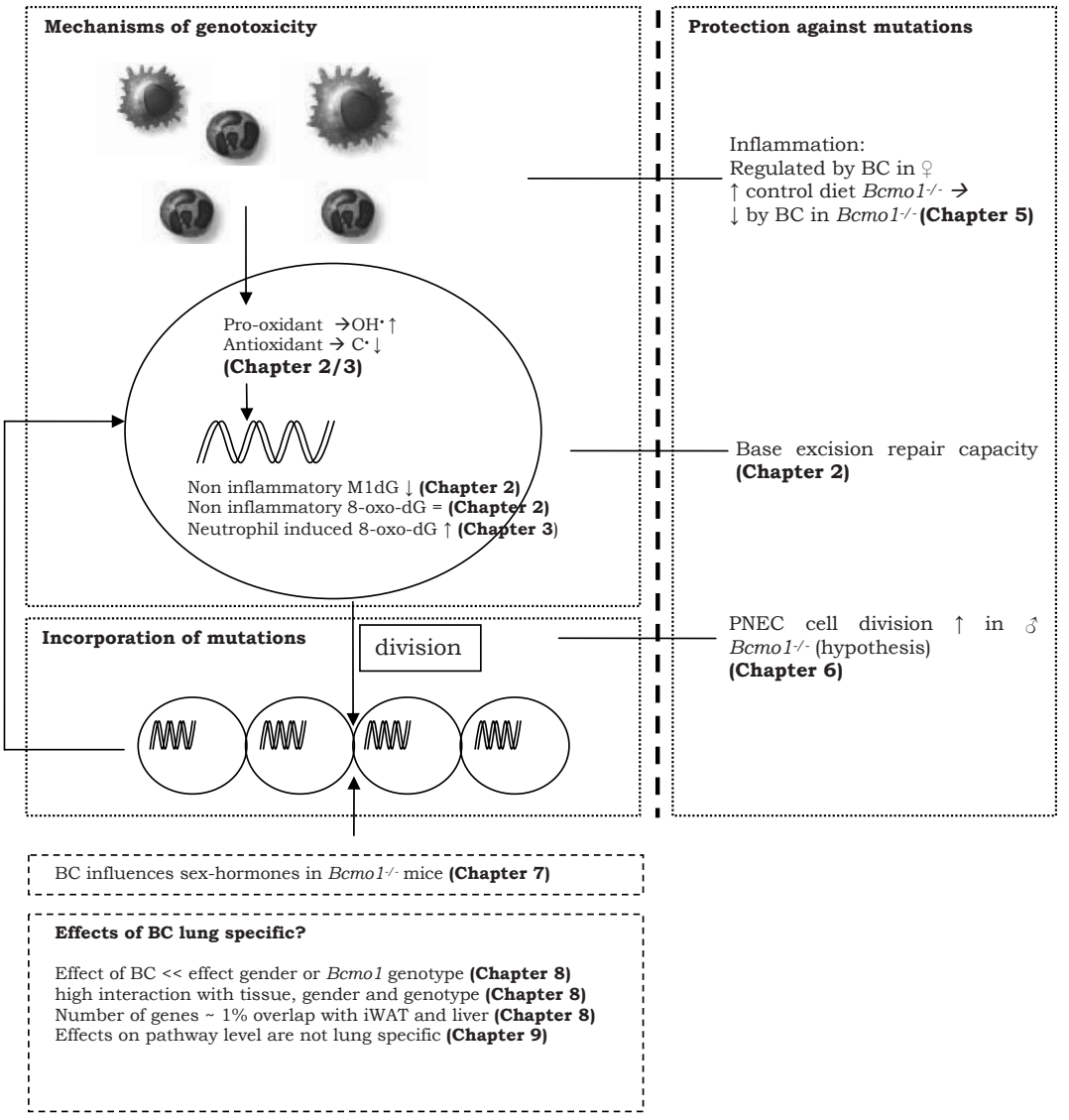


Figure 1: Summary of main findings of BC action as described in this thesis with in bold the corresponding chapters

Discussion of main findings

In vitro study

In our study, we used novel approaches to identify modes of action of BC that possibly contribute to an explanation for the previously found detrimental effects in the ATBC and CARET study. In the first part of this thesis we evaluated the role of BC in radical induced genotoxicity in cell lines. We therefore searched, within the limitations of the physiology of a cell line, good candidate cells for further *in vitro* experiments.

Human lung cancers are categorized into distinct classes, with adenocarcinoma currently being the most common type of lung cancer (also in non-smokers). It is generally believed that the tumor cells from this cancer originate from type II cells, which cover ~5% of the alveolar surface [15]. It has indeed been shown that most of the tumor cells express surfactant protein-C, which is characteristic for type II cells and do not express the Clara cell specific protein (CCSP) [16]. The presence of lamellar bodies in lung carcinoma cells has also been demonstrated, in combination with electron dense granules and mitochondria, although this is a common characteristic for Clara cells [17].

Therefore, In order to find a suitable cell line for our *in vitro* studies, we used electron microscopy to characterize several lung cell lines to choose a cell type containing lamellar bodies and mitochondria as can be seen in Fig. 2.

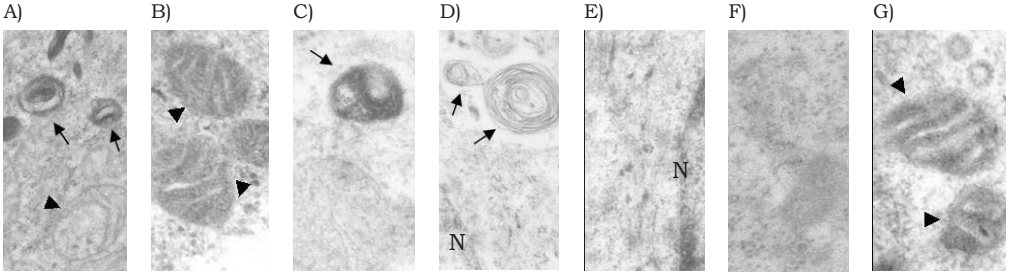


Figure 2. Electron photomicrographs of lung cell lines. A: A549 human type II cell line B: MLE-12 mouse type II epithelial cell line. C: L2 rat type II cell line D: BEAS-2B human bronchial epithelial cell line E: HBE-4 human bronchial epithelial cell line F: HBE-135, human bronchial epithelial cell line G: NL20, human bronchial epithelial cell line. Arrows indicate lamellar bodies, arrow heads indicate mitochondria, N: nucleus.

We also tried to identify the presence of CCSP and surfactant protein A and C, known markers of type II cells [18], to confirm the cell line histology. Unfortunately, we were not able to quantify these proteins due to low quality antibodies.

On basis of these results, we specifically selected Beas-2B cells and A549 cells since they are human derived, and thereby having a BC metabolism probably more similar to that of humans. Moreover, these cells contain lamellar bodies, specific for carcinoma derived from type II cells. Of course one might question the physiological relevance of A549 cell, since they are of cancer origin. Since we were specifically interested in effects of BC and BC metabolites on genotoxicity, we think that the antioxidant properties of surfactant as stored in lamellar bodies, present in BEAS-2B and A549 cells, is more important than the characteristics of the immortalization.

From our gene expression studies, we suggest that pulmonary neuroendocrine cells (PNECs) would be an interesting additional cell type to study mechanisms of BC action in lung tissue [19,20]. However, at this moment no cell line is characterized as PNEC cell using the PNEC markers CGRP, Chromogranin A, peptide YY [18]. We expect that in future experiments, a PNEC cell line is the preferred choice to study the effect of BC on smoke or asbestos induced carcinogenesis.

Besides the use of immortal cell lines, we also used primary human neutrophils derived from several healthy volunteers to investigate the effect of BC metabolites on MPO activity and on the generation of oxidative DNA lesions.

In vivo ferret studies

To assess the effect of BC supplementation on changes in human lung tissue, ferrets are considered as a suitable model [21]. BC metabolism in ferrets and humans is highly similar while BC metabolism in rodents, such as rats or mice, differs greatly from BC metabolism in humans [9]. Therefore, we also attempted to assess the effect of BC on gene expression changes in lung tissue of ferrets. For that experiment, 6 ferrets per group were fed a) a control diet, b) supplemented with 0.8 mg BC/kg body weight/day, c) supplemented with 3.2 mg BC/kg body weight/day, d) fed the control diet and supplemented with 8 mg B[a]P/kg three times a week or e) supplemented with 0.8 mg BC/kg body weight/day and supplemented with 8 mg B[a]P/kg three times a week as described in **Chapter 2**. Since no ferret arrays were (and are) commercially available, the homology of ferret RNA to hybridize against in-house made human and mouse cDNA arrays was tested and resulted in 32% spots three times above background in the human array, and 23% spots three times above background in the mouse array, and thus we concluded that ferret lung RNA had the best hybridization quality with human microarrays compared to mouse microarrays. Thereafter, we embarked on a precarious enterprise and hybridized ferret lung RNA to a human cDNA array. Of course, it was of upmost importance to confirm effects found on the human array by ferret specific Q-PCR. Unfortunately, not many ferret specific sequences are present in publically available databases. Therefore, we sequenced parts of specific genes of ferret cDNAs to be able to design ferret specific primers for ferret specific Q-PCR.

After sequencing these ferret specific cDNA fragments, the species *canis lupus familiaris* (i.e., the dog) appeared to have most sequence homology with ferrets, followed by human as can be seen in Table 1. This is in agreement with the percentage of genes above background on the human and mouse microarrays. The effects of BC observed in the ferret experiment using microarrays designated for human could not be confirmed by Q-PCR, underlining the difficulties in molecular research using ferrets.

Table 1: percentage homology of ferret RNA sequence with human, mouse, rat or dog RNA sequence.

Gene	**Abbreviation**	***Homo Sapiens***	***Mus Musculus***	***Rattus Nervoricus***	***Canis Lupus Familiaris***
Tropomyosin 1; TPM1	*TPM1a*	95%	92%	90%	**96%**
Calnexin	*CANX*	**85%**	Not found	Not found	Not found
H3 Histone Family 3A	*H3F3*	94%	86%	89%	**97%**
Histone 1, 4F	*H4F*	90%	95%	95%	**95%**
Histone deacetylase 1	*HDAC1*	94%	91%	92%	**97%**
Nuclear receptor corepressor 1	*NCOR1*	97%	94%	94%	**98%**
Retinoic Acid Receptor beta	*RARβ*	94%	88%	89%	**96%**
Thymosin beta 10	*TMSB10*	89%	Not found	88%	**94%**
Tight junction protein	*TJP*	97%	95%	96%	**98%**
Acetyl-CoA Acyltransferase 2	*ACAA2*	**87%**	85%	85%	Not found
Actin beta	*ACTB*	**94%**	90%	90%	**94%**
Creb binding protein	*CREBBP*	88%	87%	88%	**94%**
H2A histone family	*H2A*	**100%**	98%	99%	**100%**
Histone acyltransferase 1	*HAT*	96%	94%	96%	**99%**
E1A binding protein p300	*EP300*	**95%**	92%	92%	79%
P300/CBP-associated factor	*PCAF*	91%	86%	Not found	**97%**
Prothymosin alpha	*PTMA*	96%	92%	93%	**99%**
Histone deacetylase 2	*HDAC2*	92%	Not found	85%	**94%**
Histone deacetylase 3	*HDAC3*	81%	Not found	77%	**87%**
Ribosomal protein L17	*RPL17*	91%	88%	88%	**95%**

Species with the most homology with ferret sequence in bold and grey

Ferrets are also a preferred animal model in influenza research [22]. Recently, gene expression changes induced by avian influenza H5N1 in lung tissue of ferrets were analyzed using Canine arrays [23]. Canine arrays were chosen since 30 publically available ferret sequences had the highest (89%) homology with canine sequences. We found much higher homologies, which is probably caused by choosing highly conserved regions between human, mice, rats and dogs to increase the chance of a successful PCR. Overall, the results on ferret mRNA analysis on human microarrays could not be confirmed by Q-PCR, and therefore this approach was aborted. Since then the microarray quality is highly improved and continuously developed. Therefore microarray studies in ferrets may be possible in the near future.

In vivo Bcmo1$^{-/-}$ mouse model

We used *Bcmo1$^{-/-}$* mice to evaluate effects induced by BC in the second part of this thesis.

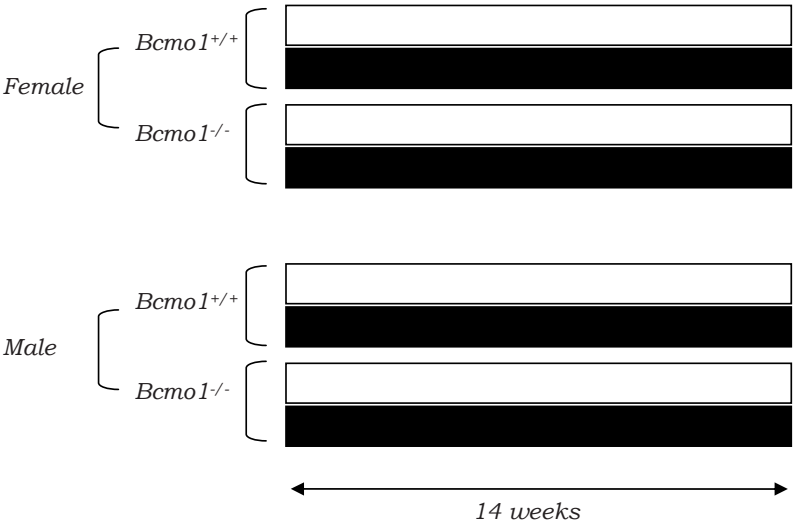


Figure 3: Experimental setup of the mouse experiment used in **Chapter 5-9** of this thesis. Six animals per arm of the experiment.

The setup of the experiment is shown in Fig. 3 and appeared highly effective to dissect effects induced by BC supplementation in the *Bcmo1$^{-/-}$* mice from effects induced in *Bcmo1$^{+/+}$*, and effects induced in female mice from effects induced in male mice. The effect of BC decreasing the inflammatory response in female *Bcmo1$^{-/-}$* mice as described in **Chapter 5**, would be interpreted as a decrease in inflammation when no *Bcmo1$^{+/+}$* mice were taken along. We now assume that female *Bcmo1$^{-/-}$* mice are more vulnerable for vitamin A deficiency and that BC is able abolish this effect. Moreover, the effects of BC on transcription were different in male and female *Bcmo1$^{-/-}$* mice as described in **Chapter 5** and **Chapter 6** and resulted in opposite direction of gene-regulation of commonly regulated genes in *Bcmo1$^{-/-}$* mice as described in **Chapter 7**.

We discussed in **Chapter 4** that a good setup of an *in vivo* mouse experiment is an important prerequisite to obtain enough power to statistically significantly assign the small effects as are often observed by nutritional interventions. In contrast to the recommended n=12, in our experiment we used only 6 animals per group. This was done

because this was the maximum capacity when taking breeding into account. Nevertheless, the differences that we have described are statistically significant, and thus enough power must have been obtained. The pathways that were significantly regulated in lung tissue after BC supplementation in *Bcmo1*$^{-/-}$ mice were also differentially regulated by BC in iWAT and liver as has been described in **Chapter 9,** which can be taken as confirmation of the power of the experiment**.**

Interpretation of main findings

The effect of BC on inflammation

We showed in **Chapter 2** that BC supplementation specifically decreased carbon centered radical formation, resulting in a decrease in the formation of M_1dG in ferrets. However, hydroxyl radical formation was increased, especially in our *in vitro* experiments. Because BC supplementation also increased DNA repair capacity (BER), BC supplementation did not result in an increase in the oxidative DNA lesion 8-oxo-dG *in vivo*. Thus, sufficient BC normally has beneficial effects on genotoxicity. Since an inflammatory response in the lungs is mainly characterized by an increase in neutrophils [7], we investigated the effect of BC or its metabolites on neutrophil induced oxidative DNA lesions. We found that retinoic acid and retinol were able to increase neutrophil induced oxidative DNA lesions as described in **Chapter 3**. This results in a lower beneficial effect of BC in neutrophil exposed (smokers or asbestos exposed subjects) humans as compared to non-exposed subjects, as is displayed in Fig. 4.

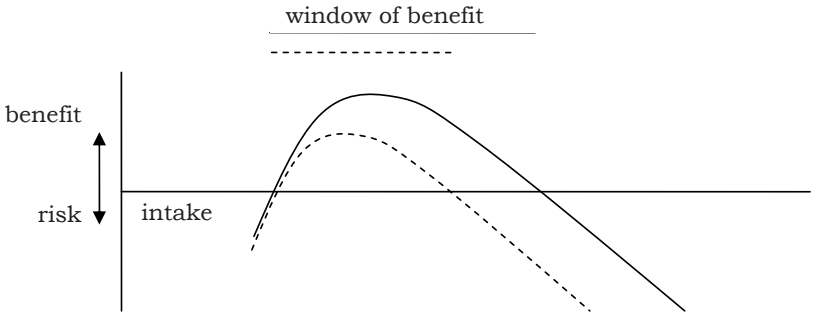


Figure 4: Hypothetical benefit-risk of BC on neutrophil induced genotoxicity in subjects that have a low neutrophilic influx in the lungs (solid line) and a high neutrophilic influx in the lungs (dashed lines). BC has beneficial effects towards radical centered radicals which results in a decrease in levels of the pro-mutagenic M_1dG. However, BC increases neutrophil induced levels of 8-oxo-dG. The presence of neutrophils is increased in smokers and asbestos exposed subjects and therefore results in a smaller window of benefit.

Although we show that these BC metabolites have the ability to increase neutrophil induced oxidative DNA lesions, it must be emphasized that these results were obtained by using cell lines. Cell lines often display altered gene expression resulting in, for example, a non-physiological poor antioxidant enzyme or repair enzyme signature [24]. Nevertheless, these data show that BC metabolites have the potential to enhance neutrophil induced oxidative stress.

In **Chapter 5** we also described that inactivation of *Bcmo1* resulted in inflammatory infiltrations in lungs of female *Bcmo1*$^{-/-}$ mice, which was abolished by dietary BC. We explained this effect by an altered downstream BC metabolism, resulting in a higher BC demand. BC supplementation resulted in a decrease of the inflammatory response, and results in a changed benefit-risk figure dependent on BCMO1 activity as is displayed in Fig. 5. A genetic polymorphism in *BCMO1* has been described in humans, which is associated with a lower BCMO1 activity. The vitamin A content in the control diet appeared, in contrast to females, physiologically sufficient for male *Bcmo1*$^{-/-}$ mice, but we don't know the mechanism at this moment.

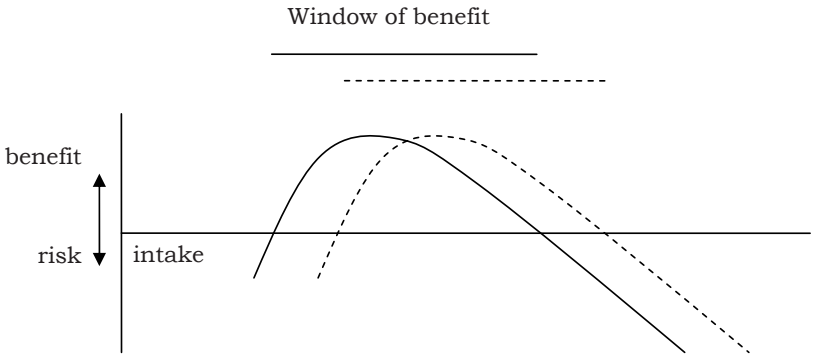


Figure 5: Hypothetical benefit-risk of BC in the female "low response" phenotype (solid line) and "low converter" phenotype. Females that have polymorphisms in the BCMO1 enzyme (associated with lower activity of BCMO1) might have a higher vitamin A demand, as was observed in female *Bcmo1*$^{-/-}$ mice (**Chapter 5**). Vitamin A deficiency results in adverse effects such as an increase in inflammation. Because of the higher Vitamin A demand, the window of benefit of the low converter phenotype shifts to the right in the figure.

Thus, significantly decreased levels of BC or BC metabolites can increase inflammation. Interestingly, smokers have both an increased inflammatory response, and decreased

vitamin A levels. An inverse correlation between the degree of inflammation and BC has been observed in several studies. For example there is an inverse correlation between white blood cell count and BC concentration in non-smokers [25] and smokers [26,27]. But, is the BC concentration modulating the inflammatory response, or does the inflammatory response alter BC levels? There is evidence for both theories. Increases in inflammation are usually associated with increased oxidative stress. BC is highly susceptible to oxidative degradation and several studies have shown that BC cleavage rapidly occurs after exposure to oxidative reagents such as hypochlorous acid [28,29]. This theory however, explains only part of the inverse correlation between BC concentration and the degree of inflammation. Studies that measured several carotenoids demonstrate that BC concentrations are decreased to a higher extent during inflammation than the more oxidative sensitive carotenoids such as lycopene and lutein [25,30]. This suggests that lower BC status increases inflammation. In agreement with this hypothesis, studies have shown that deficiency of the BC metabolite vitamin A is associated with an increased severity of infections and more deaths from infectious diseases; supplementation with vitamin A reduced these effects [31,32]. Supporting these data, vitamin A deficiency in rats resulted in inflammatory infiltrates in lungs [33]. To investigate the similarities between vitamin A deficiency and smoke induced histological changes in the lung, lungs of vitamin A deficient rats [33] were histologically compared to lungs of smoke exposed rats [34]. Remarkably, these lungs showed a similar structure, with emphysematous areas and inflammatory infiltrates [35].

The molecular mechanism underlying the vitamin A induced effects on the immune response are not well understood. As described in the introduction, RAR and RXR are transcription factors able to bind the retinoic acid responsive element in promoter regions of RAR-responsive genes. Retinoic acid binding to this receptor can consequently result in a gene expression response [36,37]. RARα and RARγ are the predominant forms expressed in immunological cells, and RARγ knockout results in a decrease in total white blood cell counts [38]. All-trans retinoic acid is, in contrast to its activating role for RAR, an effective repressor of ROR, and binding of retinoic acid has been shown to decrease immunological responses [39]. Altogether these observations lead to a hypothesis that a deficiency in the bioactive metabolite retinoic acid might lead to an increased inflammation in the lungs. In our study, supplemental BC was beneficial. Mechanistic knowledge on inflammatory responses during retinoic acid deficiency is of importance and might lead to mechanisms that explain the previously identified detrimental effects and might identify other vulnerable groups.

Retinoic acid resistance?

Thus negative effects of vitamin A deficiency on inflammation have been reported. Fig. 6 depicts that vitamin A deficiency results in an increased inflammatory response. This raises the question: “Is it possible that a chronic supplementation with BC eventually results in a decrease of the bioactive metabolite retinoic acid?” We investigated this possibility in literature.

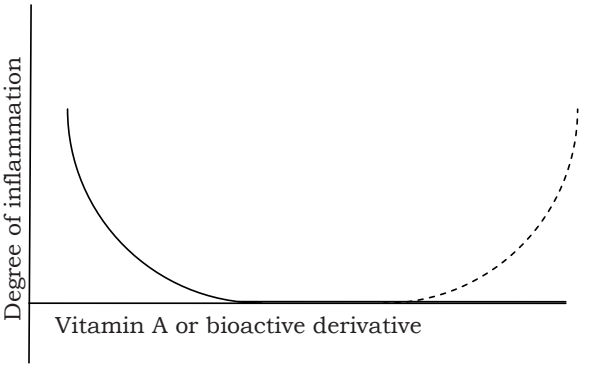


Figure 6: Vitamin A concentration can modulate the inflammatory response. During deficiency, inflammation is increased (solid line). High dose supplementation of the bioactive metabolite retinoic acid can increases inflammation in APL patients (dashed line) [40].

During illness, all-trans retinoic acid is frequently used as a therapeutic agent in patients suffering from acute promeyelocytic leukemia (APL). Without any other medication, around 25% of the patients develop the retinoic acid syndrome. Typical for the clinical manifestations of the retinoic acid syndrome are pulmonary effects (Table 2) and typical is that a large percentage of patients suffer from inflammatory infiltrates [41,42]. Although the exact mechanisms are unknown, these data indicate that an increase in retinoic acid might become detrimental under certain conditions.

Table 2: clinical manifestations of the retinoic acid syndrome [43].

Manifestation	**% of Patients**
Respiratory distress	84
Fever	81
Pulmonary edema	54
Pulmonary infiltrates	52
Pleural/pericardial effusion	36
Hypotension	18
Bone pain	14
Headache	14
Congestive heart failure	11
Acute renal failure	11

Retinoic acid levels are thought to be very well regulated [44]. Since the measurement of retinoic acid involves sophisticated equipment, the retinoic acid precursor retinol is measured in most studies that investigate effects of carotenoids. In contrast to the assumed stable retinoic acid levels, consumption of BC rich carrot juice resulted in stable retinol plasma concentrations, but almost doubled retinoic acid plasma concentrations [45]. This study was performed for the duration of only two weeks. What happens with retinoic acid levels after prolonged supplementation with beta-carotene is unknown, but for example, prolonged all trans retinoic acid treatment causes an increase in RA catabolism and can lead to retinoic acid resistance [40,46]. This is mainly caused by an increased Cytochrome P450 family 26 (CYP26) capacity. These studies indicate that RA levels may be altered after BC administration and that prolonged period of elevated RA levels induces an increase in RA catabolism. Moreover, we observed in **Chapter 5** and **Chapter 6** a positive correlation between BC concentrations in lung and retinyl ester concentrations in the lung. This indicates that there is a controlled balance between concentrations of BC and its stored form. When a more chronic BC supplementation is attenuated, BC might be stored at similar levels, while RA turnover is increased resulting in a physiological deficiency. This hypothesis is more or less strengthened by some adverse effects that have been observed in RA therapy. Administration of retinoic acid can induce RA hypercatabolism and patients cannot be actively treated with retinoic acid after a first therapy even when the dose is doubled [47,48]. Moreover, decreases in endogenous retinol stores down to 40% have been observed in patients with exogenous retinoid therapy and resulted in adverse effects on vision [49]. Moreover, the retinoic acid syndrome is characterized by weight gain (retinoic acid increase results in the opposite [41]) in association with pulmonary infiltrations.

Smokers have lower carotenoid levels, as we have described above and ferrets exposed to cigarette smoke have around half the retinoic acid concentration in their lungs compared to control ferrets [50]. Moreover smoke has been shown to induce RA catabolism in ferrets [51]. Therefore, smokers are probably already more vulnerable for a deficient state and decrease in retinoic acid might quickly result in an increase in the inflammatory response, which is similar to the observation of an increased inflammatory response in lungs of control diet fed female *Bcmo1*$^{-/-}$ mice, which was abolished by dietary BC.

BC metabolism: the important determinant in BC induced effects?

Many of our described effects are possibly the effect of changes in retinoic acid concentration, induced by BC supplementation. We explained part of these effects by differ-

ences in downstream BC metabolism (**Chapter 5 and Chapter 6)**. This raises the question whether smoke or asbestos exposed subjects might have an altered downstream BC metabolism compared to healthy volunteers. Although this question was never directly addressed in human lung tissue, technology evolved and microarray data are usually publicly accessible with which this question could be answered (at least in part).

We used GEO Datasets (http://www.ncbi.nlm.nih.gov/gds) to analyze the effect of smoking on the gene expression of BC metabolizing enzymes, using 4 studies (Table 3). Unfortunately, no data were available from lung tissue of asbestos exposed subjects. The most remarkably changed downstream BC metabolizing enzyme is ADH7, the most important enzyme in the conversion of retinol into retinal [52].

Table 3: Enzymes involved in downstream BC metabolism that are significantly changed in smokers (based on publicly available microarray data at: http://www.ncbi.nlm.nih.gov/gds).

target tissue	smokers (n)	control (n)	ADH7	ADH1a3	DHRS3	BCMO1	Aldh2	reference
large airway epithelium	26	17	3.74 (1.69e-9)	1.736 (0.0006)	n.p.	n.p.	-1.09 (0.270)	[53]
small airway epithelium	10	12	7.21 (1.35E-7)	1.368 (0.001)	1.237 (0.058)	-1.16 (0.049)	-1.099 (0.16)	[54]
small airway epithelium	6	5	6.434 (4.06e-5)	1.732 (0.0748)	1.099 (0.2136)	1.398 (0.395)	-1.177 (0.299)	[55]
general airway	33	22	2.52 (1.35e-08)	1.44 (0.0085)	1.498 (1.03e-8)	1.286 (0.234)	-1.23 (0.002)	[56]

n.p.: not present in database; p-value between brackets

We found in **Chapter 5** that *Adh7* was also significantly up regulated in female *Bcmo1*$^{-/-}$ mice after BC supplementation and non significantly in male *Bcmo1*$^{-/-}$ mice after BC supplementation. These data suggest that BC supplementation to smokers, which have already increased ADH7 activity, might lead to an additive or even synergistic effect of BC on ADH7 expression. This might have important implications of BC on smoke induced carcinogenesis, since ADH7 has also been shown to have an increased activity in many types of cancer such as esophageal cancer [57] and gastric cancer [58].

Gender effect

Interestingly, the observed effects of BC in *Bcmo1*$^{-/-}$ mice were gender specific. This resulted in an increase in inflammatory gene expression in control diet fed *Bcmo1*$^{-/-}$ mice which was specific for female mice as described in **Chapter 5**, while the decrease in

Fzd6 and *Cthrc1* in combination with the increase in olfactory receptors and protocadherins after BC supplementation to *Bcmo1*$^{-/-}$ mice were specific for male mice as described in **Chapter 6.** Moreover, the effects induced by BC supplementation in both male and female mice were regulated in opposite direction as described in **Chapter 7**. These differences have also implications for differences in the benefit-risk figure. Females seem to have a higher vitamin A demand than males, which results in a shift to the right for the start of the hypothetical window of benefit in female as can be seen in Fig. 7.

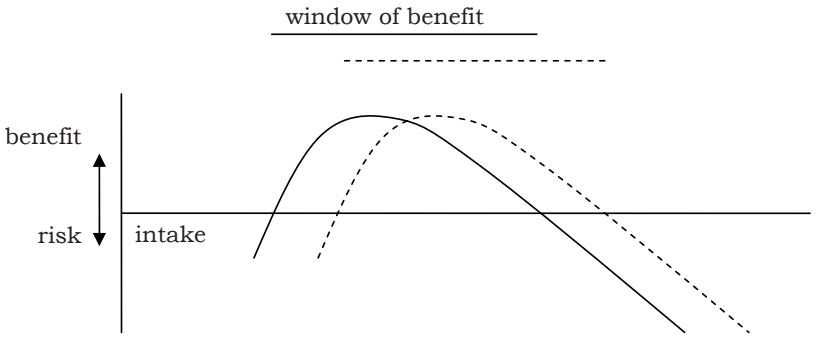


Figure 7: Hypothetical benefit-risk figure for male (solid) and female (dashed). Female Bcmo1$^{-/-}$ mice seem to have a higher vitamin A demand than male mice.

We explained the opposing gene expression differences found in lung tissue**,** by a change in hormones or steroid biosynthesis due to an evolutionary conserved overlap in the production of BC in plants and the steroid synthesis in mammals [59]. We found a significant change in the GO process steroid biosynthesis in male *Bcmo1*$^{-/-}$ mice after supplementation with BC. This process might be the cause of the opposing gene expression differences in male and female mice or its consequences. In **Chapter 9**, we focused on the effect of BC supplementation in iWAT and liver and we also found an opposite direction of gene regulation in iWAT in male and female *Bcmo1*$^{+/+}$ mice after BC supplementation. Similar to lung tissue, the GO process steroid biosynthesis was significantly regulated in one of the two genders after BC supplementation (Table 4). There were a few genes commonly regulated in the steroid biosynthesis process between male *Bcmo1*$^{-/-}$ mice and female *Bcmo1*$^{+/+}$ mice after BC supplementation, including *MvK, Hmgcs1, Fdps* and *Cyp51* There are also two genes regulated in both iWAT of female *Bcmo1*$^{+/+}$ mice and lung tissue of male *Bcmo1*$^{-/-}$ mice that play an important role in the conversion of steroids and thereby regulates the sex hormone bioactivity in the target tissue. Hsd17b12 converts estrone into the bioactive 17β-estradiol [60] and Hsd17b7 has been shown to have a dual role: activation of estrogens by converting estrone into 17β-estradiol, while Hsd17b7 inactivates dihydrotestosterone (DHT) by trans-

forming this into 5alpha -androstane-3beta,17beta-diol (3beta-diol) [61]. Changes in hormone production or activity normally results in physiological effects in adiposity. Sex steroids play a key role in the distribution of adipose tissue [62,63]. This is for example exemplified by a reduced degree of central obesity by hormone replacement therapy in postmenopausal woman and testosterone replacement therapy in older man [63]. Similarly, we found that adiposity was significantly decreased in female *Bcmo1*$^{+/+}$ mice, while not in male *Bcmo1*$^{+/+}$ mice as can be seen in Fig. 8.

Table 4: Genes present (until p=0.05) in the significantly regulated GO process steroid biosynthesis in iWAT of female *Bcmo1*$^{+/+}$ mice after BC supplementation.

Probe	Score	Symbol	Name
A_52_P67637	4.56E-04	*Sc5d*	*sterol-C5-desaturase (fungal ERG3, delta-5-desaturase) homolog (S. cerevisae)*
A_51_P153170	5.25E-04	*Cyb5r3*	*cytochrome b5 reductase 3*
A_51_P418056	9.51E-04	*Sc5d*	*sterol-C5-desaturase (fungal ERG3, delta-5-desaturase) homolog (S. cerevisae)*
A_52_P118161	0.001033	*Lss*	*lanosterol synthase*
A_52_P35064	0.001037	*Tm7sf2*	*transmembrane 7 superfamily member 2*
A_51_P482711	0.001257	*Dhcr24*	*24-dehydrocholesterol reductase*
A_51_P209372	0.002543	*Sc4mol*	*sterol-C4-methyl oxidase-like*
A_51_P329711	0.004309	*Idi1*	*isopentenyl-diphosphate delta isomerase*
A_51_P492408	0.005526	*Pmvk*	*phosphomevalonate kinase*
A_51_P290986	0.006027	*Dhcr7*	*7-dehydrocholesterol reductase*
A_51_P492410	0.006296	*Pmvk*	*phosphomevalonate kinase*
A_51_P146941	0.007138	*Hmgcs1*	*3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1*
A_51_P158922	0.009218	*Prkaa1*	*protein kinase, AMP-activated, alpha 1 catalytic subunit*
A_52_P164161	0.009666	*Cyp51*	*cytochrome P450, family 51*
A_51_P379798	0.0128	*Fdps*	*farnesyl diphosphate synthetase*
A_52_P58006	0.01367	*Acbd3*	*acyl-Coenzyme A binding domain containing 3*
A_52_P243599	0.01445	*Hsd17b12*	*hydroxysteroid (17-beta) dehydrogenase 12*
A_52_P636752	0.01622	*Cyp51*	*cytochrome P450, family 51*
A_51_P169527	0.02114	*Mvk*	*mevalonate kinase*
A_51_P355943	0.02238	*Mvd*	*mevalonate (diphospho) decarboxylase*
A_52_P1013551	0.02461	*Nsdhl*	*NAD(P) dependent steroid dehydrogenase-like*
A_52_P566605	0.02528	*Hsd17b7*	*hydroxysteroid (17-beta) dehydrogenase 7*
A_51_P485791	0.02842	*Cyp51*	*cytochrome P450, family 51*
A_51_P326942	0.03472	*Nsdhl*	*NAD(P) dependent steroid dehydrogenase-like*
A_52_P388072	0.0392	*Hmgcs1*	*3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1*
A_52_P297803	0.04138	*Nsdhl*	*NAD(P) dependent steroid dehydrogenase-like*
A_51_P296487	0.04157	*Lss*	*lanosterol synthase*

Thus the adiposity index 'perfectly' corresponds to the changes in the biosynthesis process. There is however no effect seen in adiposity in males and certainly more research is needed to identify the specific effects and mechanisms of BC induced effects on hormone synthesis or bioactivity.

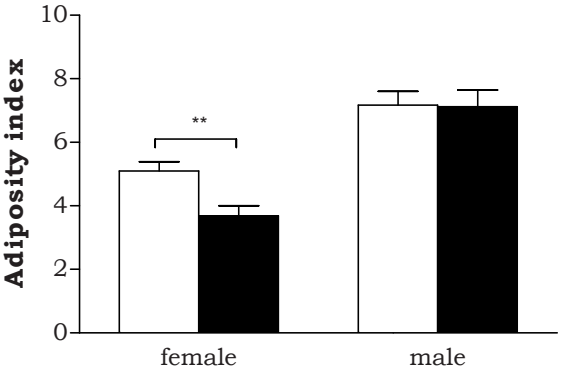


Figure 8: Adiposity index (gram adipose tissue per kg body weight) in female and male *Bcmo1*$^{+/+}$ mice in control diet fed mice (white bars) and BC supplemented mice (black bars). ** $p<0.01$ with Students' *t*-test. Error bars represent SEM.

Interaction of BC with xenobiotics?

The effect of BC resulting in an increased lung cancer risk as described in the CARET and ATBC trials was observed in subjects that were exposed to either cigarette smoke or asbestos. In this thesis we tried to obtain more insight in molecular mechanisms changed by BC in lung tissue. There are some indications that xenobiotic exposure such as in smoking, alters lung biology, thereby additionally altering effects of BC. In **Chapter 2,** ferrets were exposed to B[a]P for 10 weeks. B[a]P is one of the best studied chemical pro-carcinogens and is present in cigarette smoke. B[a]P is metabolic activated into BPDE, a chemical carcinogen able to form adducts with the DNA [64]. Fourteen weeks after the B[a]P intervention had stopped, BPDE-DNA adducts were still detectable in the ferrets' lung. More remarkably, B[a]P exposed ferrets had significantly lower 8-oxo-dG levels and had a significantly increased BER capacity. Previously a single dose of B[a]P was associated with an increased urinary excretion of 8-oxo-dG and a decrease in 8-oxo-dG in the DNA [65]. Although an exact mechanism was not tested, we found a significant increase in BER capacity after B[a]P exposure. Moreover, we found a significant negative correlation between 8-oxo-G and BPDE-DNA adducts as can be seen in

Fig. 9, indicating that the presence of these BPDE adducts was possibly sufficient to increase BER capacity.

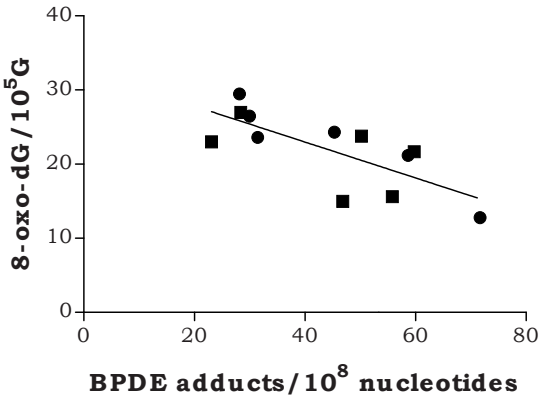


Figure 9: Correlation between BPDE-DNA adducts and 8-oxo-dG in ferrets fourteen weeks after the last B[a]P exposure in ferrets fed the control diet (circles) or fed 0.8 mg/kg BC (squares) ($R=0.74$, $p<0.01$).

Although only smokers and not asbestos exposed subjects are exposed to high concentrations of B[a]P, this example illustrates that xenobiotic exposures, are of importance in the ultimate result of the effect of BC on inflammation induced genotoxicity.

One xenobiotic that is present in both cigarette smoke as well as asbestos is iron. Cigarette smoke contains high amounts of iron (440-1150 µg/g tobacco) [66] and also asbestos fibers are associated with high concentrations of iron. The amphibole fibers have a high iron content (27-33%), while chrysotile asbestos has relatively small iron contents (6%) [67], and epidemiological studies show that asbestos fibres that contain high amounts of iron are more carcinogenic than the low or non-iron containing counterparts [68]. Moreover, iron, mainly in the Fe^{3+} form, can bind and accumulates on asbestos fibres [69]. The main mechanism described for the possible detrimental effects of iron is the Fenton reaction:

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + {}^{-}OH$

We proposed in **Chapter 3** that BC and its metabolites are able to reduce Fe^{3+} into Fe^{2+}, thereby increasing radical formation in the Fenton reaction. Besides this effect of BC on iron mediated radical formation, we actually found in **Chapter 3** that BC was also able to reduce the activity of the iron containing enzyme MPO.

Moreover, we found that BC was able to decrease the activity of the iron containing enzyme horse radish peroxidase 2-fold (unpublished data). Altogether, these data implicate that BC might alter iron biology.

Besides the investigated effect of BC and its metabolites on the generation of oxidative stress, BC or its metabolites might also have a role in the uptake of iron. Fe^{3+} has very low solubility at neutral pH and accumulates onto the particle surfaces. It is known that reducing agents present in the lung, such as ascorbate, are able to reduce Fe^{3+} into Fe^{2+}, thereby displacing the iron from the complexed surface [70]. Moreover, the uptake of iron under physiological conditions is probably also dependent on its valence. Fe^{3+} has very low solubility at neutral pH and reduction of Fe^{3+} into Fe^{2+} has been considered as essential in iron absorption [71]. There are only a few studies describing the effect of BC or BC metabolites on iron uptake, and they mainly focus on dietary iron absorption and distribution. It has been shown that vitamin A and BC were able to improve dietary nonheme iron absorption in colorectal cells of human subjects [72,73] and changes from vitamin A deficiency towards sufficiency also resulted in organ specific alterations in iron content [74,75]. Moreover, RBP4, the main transporter of retinol, has been associated with increased concentration of ferritin, the main protein that complexes with iron, which indicates a relation between BC metabolite retinol and iron biology [76,77]. These data indicate that BC or its metabolites might be involved in the physiological effects of iron in lung tissue.

How to continue to assess the risk of BC?

The aim of this thesis was to assess molecular mechanisms altered by BC action in the lung to contribute to an explanation for the previously observed adverse effects of BC on lung cancer risk. Novel observations have been made that a) contribute to explanations for the negative effects observed in the CARET and ATBC studies and at the same time agree with the many beneficial effects of BC and b) suggest novel roles of BC/RA metabolism in cell biology and c) show that large gaps remain in our knowledge of BC/RA biology.

With plenty new data, we would like to discuss questions resulting from this thesis that have to be addressed on the road towards benefit-risk assessment that will help to define a recommended daily intake and a upper limit of BC. When questions are addressed regarding the effect of BC on lung tissue in humans, we are faced with (ethical) limitations since detrimental effects have been observed and lung tissue is difficult and too invasive to obtain. *In vitro* models are perfects tools to study molecular mechanisms in detail. We successfully used this approach in **Chapter 2** and **Chapter 3.** *In vitro* studies however often fail when questions regarding the physiological response of a specific tissue to stimuli or compounds are tested in the context of the whole organism, taking absorption, distribution, metabolism and the effect on an organ due to interactions with other organs into account. We found that the response of lung tissue to oral

BC administration was different for female and male, as has been discussed in **Chapter 5** and **Chapter 6**, and that there is possibly even an interaction with other organs such as the steroid producing organs as has been described in **Chapter 7** or with the immune system as has been described in **Chapter 5**.

As we discussed, there are still many gaps in the knowledge on the action of BC. Still many scientifically and socially relevant questions need to be addressed. We will discuss some of these questions below in combination with an suggested approach towards their answer.

Are the effects found in Bcmo1$^{-/-}$ mice induced by BC or retinoic acid?
In our study we used *Bcmo1*$^{-/-}$ mice, which were described previously [78]. Although Bcmo1 is the main BC metabolizing enzyme [79], we observed that in our studies BC was able to abolish a vitamin A deficient state. We assume that Bco2, implicated in BC metabolism [80] with a low affinity for BC, was involved in this conversion of BC to correct for retinoic acid needs (**Chapter 5**).

An appropriate animal model to investigate the effects of intact BC would therefore be a mouse model with a knockout for *Bcmo1* and *Bco2*. When using this mouse model, WT controls have to be taken into account to correct for effects induced by possible alterations in downstream BC metabolism such as vitamin A deficiency, as we have observed for the female *Bcmo1*$^{-/-}$ mice in **Chapter 5**. An alternative to a *Bcmo1*/*Bco2* knockout mouse model might involve a bypass of the enterocytes. After absorption, most BC is cleaved in the enterocytes of mice and rats by *Bcmo1* [81]. There are ways to bypass the most efficient cleavage sites, for example by injecting BC or by the use of implantable pumps, such as Alzet pumps. BC concentrations and accumulation have to be tested since these experiments have not been performed before, and other organs such as liver are known to metabolize BC in mice as well [9].

To investigate the effects induced by RA, wildtype mice are perfectly suitable. These mice can be supplemented with retinoic acid although these mice might induce RA hypercatabolism. To circumvent this, it might be better to supplement mice with retinoic acid metabolism blocking agents, which specifically block CYP26 and thereby preventing RA catabolism and increase RA concentrations [51].

When performing BC or retinoic acid research in these animal models, the diet of the F1 generation as well as the dams should be considered carefully. Most experimental (chow) diets contain high concentrations of vitamin A and BC. Dams are able to store these high concentrations of vitamin A, and will also deliver vitamin A to their offspring. Although generally vitamin A stores are low at birth in humans and rodents, because of a limited transplacental vitamin A transfer [82-84], lactating periods are crucial for the vitamin A stores in the neonates. As a consequence, the amount of vitamin A in the milk, which is dependent on maternal vitamin A stores and the amount of vitamin A present in maternal feed [85,86], is an important determining factor in vitamin A stores

of the offspring. The hepatic vitamin A stores at the start of the experiment will affect general BC and vitamin A physiology and thus can result in variations in the outcome of experiments investigating effects of BC or BC metabolites. For example *Bcmo1* was shown to be not essential in the maintenance of vitamin A stores when BC is the main source of vitamin A [87]. However, in another study where dams were depleted of vitamin A, the enzyme played an important role and changed the conclusion of the role of *Bcmo1* [10]. Our experiment carefully considered vitamin A concentration in diet fed to the dams. High hepatic vitamin A stores reduce dietary BC uptake and therefore the dams were fed a diet containing 1500 IU/kg feed which is considered a vitamin A sufficient diet [correspondence with Von Lintig/Wyss] in our study. The increased inflammation in female control diet fed *Bcmo1*$^{-/-}$ mice however suggests that these mice were mildly vitamin A deficient, as discussed in **Chapter 5**.

Are there differences between a short and long BC intervention and can chronic BC supplementation result in BC or RA resistance?

The subjects in the ATBC and CARET study were supplemented with BC for medians of, respectively, 6.1 and 4 years with 20 mg or 30 mg of BC, which resulted in an increased lung cancer risk in smokers and in asbestos exposed subjects [88-90]. To evaluate changes induced by BC supplementation, the effect of a short and chronic intervention has to be assessed. This might lead to insight about a possible BC or retinoic acid resistance.

Is there a method to assay the bioactive potency induced by BC supplementation?

The bioactive potency of BC is mainly attributed to changes in RA as discussed in **Chapter 1**. Unfortunately we were not able to measure RA in the samples. At this moment it is also not completely known whether only RA has the ability to induce gene-transcription. There might be a few ways to measure the bioactive potency in tissues or plasma after BC intervention.

A first method is the measurement of RA analytically, which unfortunately requires sophisticated equipment. This approach may not be sensitive enough and has the disadvantage that bioactivity is not simultaneously tested. This may be relevant since other BC metabolites may have a bioactive antagonistic or agonistic effect.

Another method is the use of reporter assays. A reporter gene assay contains recombinant DNA containing a responsive element in combination with a reporter gene, such as luciferase. The reporter gene expression is easily detectable and quantifies the potency of the “test” substance [91]. Such assays have been developed for retinoic acid isomers and their metabolite potency. In these assays cells were cotransfected with a reporter gene containing the RARE and with vectors expressing RARα, RARβ or RARγ [92]. Plasma or extractions of tissues can be tested on their ability to induce or inhibit RAR responsive transcription. Disadvantages of these assays include difficulties with the solu-

bility or cellular uptake of the compounds of interest, or the components may be metabolized, resulting in a bias in the tested potency.

Another possibility is the (semi-) quantification of a biomarker. Biomarkers are any biological changes induced by alterations in BC or RA concentrations. In this case, a biomarker could be a gene which has a changed expression dependent on the concentration of BC or retinoic acid. A functional biomarker has often a high fold change in combination with a low p-value. As we demonstrated in **Chapter 6**, BC supplementation to male *Bcmo1*$^{-/-}$ mice resulted in a very significant down regulation of *Fzd6* and *Cthrc1*, in combination with a high absolute fold change. These genes could thus be potential biomarkers for RA or BC status. A regulation of *Fzd6* and *Cthrc1* was only significantly observed in male *Bcmo1*$^{-/-}$ mice and not in *Bcmo1*$^{+/+}$ mice or female mice. In **Chapter 9**, we observed that *Fzd6* was down regulated in lung tissue, liver tissue and in iWAT, while *Cthrc1* was only significantly down regulated in lung tissue. Although an effect of BC would be most logical, we think that retinoic acid levels in *Bcmo1*$^{-/-}$ mice were less well controlled compared to *Bcmo1*$^{+/+}$ mice after BC supplementation, which probably corresponds with the human situation where BC supplementation results in increased RA plasma levels [45]. As can be seen in Fig. 10, *Fzd6* expression was also altered in female *Bcmo1*$^{-/-}$ mice, although just not significantly. Of course these data have to be interpreted very carefully, since this figure shows again opposite results in both genders, and thus this might be an effect of changes in hormone production. Further research would be needed to evaluate the exact role and response of *Fzd6* in BC biology. to see whether it can be developed into a marker for RA or BC status.

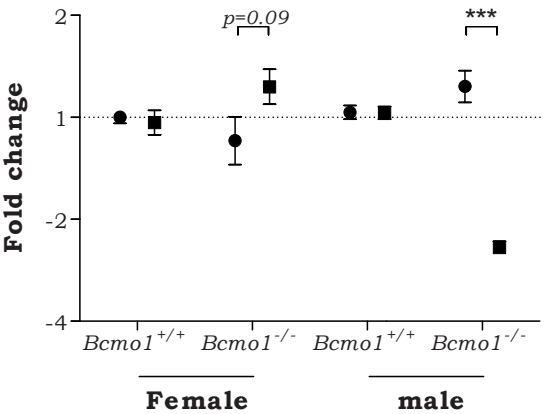


Figure 10: *Fzd6* gene expression in mice fed the control diet (circles) or that were supplemented with BC (squares) as compared to control diet fed *Bcmo1*$^{+/+}$ mice.

Do mechanisms of BC action in human lung tissue correspond to mechanisms investigated in lung tissue of animal models?

The main difficulty when performing BC research in humans is the target tissue. It is virtually impossible to obtain lung tissue from volunteers since this is too invasive. When one is primarily interested in the immune response specifically in the lung, bronchioalveolar lavages can be obtained, although this is also considered as an invasive technique. When being interested in altered molecular mechanisms of BC action in the lung, the use of surrogate tissue is probably an unavoidable fact. The concept of a surrogate tissue is that the questions addressed for a "target" tissue is determined by analyzing an accessible or "surrogate" tissue [93]. An often used easily accessible "surrogate tissue" are the human peripheral blood mononuclear cells (PBMC). Although the use of for example PBMC's has been proven to be successful in profiling nutritional changes [94] and as a diagnostic marker for certain diseases [95,96], and for other mechanistic research [97], we did not isolate PBMCs for microarray analysis. Nevertheless, to get more insights in the tissue specificity of BC induced effects we compared the gene expression changes induced by BC in lung tissue with changes induced in liver and inguinal white adipose tissue. Interestingly, white adipose tissue can be used as an accessible tissue in human research [98,99]. As we showed in **Chapter 8**, only a minor number of genes (~1%) were similarly changed in all three tissues. However, on a pathway level as described in **Chapter 9**, all of the observed altered pathways by BC in lung tissue were also observed in white adipose tissue and almost all effects were also observed in liver tissue. This indicates that the use of surrogate tissue to investigate altered mechanisms on a transcriptional level after BC supplementation may be possible. Nevertheless, the research has to be conducted in preferably at least two surrogate tissues, to dissect surrogate tissue specific effects from the more systemic effects and has to be validated in, for example, animal models. Moreover, effects have to be carefully interpreted and validated.

Are polymorphisms important in BC metabolism altering relative risks for lung cancer incidence?

As stated in **Chapter 1**, individuals have been classified as "low responders" and as "low converters", resulting in high inter-individual variations in BC or BC metabolite concentrations. polymorphisms in several genes have been demonstrated to be related to these phenotypes [100]. When material of the CARET or ATBC studies would be available, epidemiological analysis of lung cancer incidence in relation to the absence or presence of polymorphisms in the Bcmo1 gene would be relevant to study and might give answers to the questions regarding inter-individual differences in effects of BC.

Does smoking/inflammation in combination with BC supplementation result in increased genotoxicity and are there differences in BC metabolism compared to BC supplementation to non-smokers?

We showed in **Chapter 3,** that the detrimental effects of BC might be observed in smokers and asbestos exposed subjects due to an increase in inflammation induced genotox-

icity. We also showed in **Chapter 2** that the effect *in vivo* might be different from the effect *in vitro,* and therefore it would be of importance to test whether BC increases neutrophil induced genotoxicity *in vivo*. To test this, lungs of animals can be exposed to lipopolysaccharide (LPS), which is a potent endotoxin recruiting circulating PMN to the lungs [101]. The effect of BC on for example 8-oxo-dG and M_1dG can be tested. In this case ferrets might be an appropriate animal model, since BC metabolism is similar to the human situation, lung is physiologically comparable with respect to inflammation and, as we showed in **Chapter 2**, genotoxicity markers are measurable in ferrets.

Altogether, we identified many possible processes that are affected by BC. There is also still much research necessary to complete gaps in the knowledge of BC action, and also many new questions have risen, such as: Does BC or RA change hormone synthesis and is *Fzd6* involved in carcinogenic processes? Altogether this thesis contributes to our understanding of the mechanisms of action of BC knowledge and is an important lead for future BC research.

References

[1] P. Palozza Prooxidant actions of carotenoids in biologic systems, Nutr Rev 56 (1998) 257-265.

[2] P. Palozza, S. Serini, F. Di Nicuolo, E. Piccioni and G. Calviello Prooxidant effects of beta-carotene in cultured cells, Mol Aspects Med 24 (2003) 353-362.

[3] J.S. Brody and A. Spira State of the art. Chronic obstructive pulmonary disease, inflammation, and lung cancer, Proc Am Thorac Soc 3 (2006) 535-537.

[4] G. Lee, T.C. Walser and S.M. Dubinett Chronic inflammation, chronic obstructive pulmonary disease, and lung cancer, Curr Opin Pulm Med 15 (2009) 303-307.

[5] R. Robledo and B. Mossman Cellular and molecular mechanisms of asbestos-induced fibrosis, J Cell Physiol 180 (1999) 158-166.

[6] A.G. Schwartz, G.M. Prysak, C.H. Bock and M.L. Cote The molecular epidemiology of lung cancer, Carcinogenesis 28 (2007) 507-518.

[7] C.M. Doerschuk Leukocyte trafficking in alveoli and airway passages, Respir Res 1 (2000) 136-140.

[8] N. Gungor, R.W. Godschalk, D.M. Pachen, F.J. Van Schooten and A.M. Knaapen Activated neutrophils inhibit nucleotide excision repair in human pulmonary epithelial cells: role of myeloperoxidase, Faseb J 21 (2007) 2359-2367.

[9] A. Wyss Carotene oxygenases: a new family of double bond cleavage enzymes, J Nutr 134 (2004) 246S-250S.

[10] S. Hessel, A. Eichinger, A. Isken, J. Amengual, S. Hunzelmann, U. Hoeller, V. Elste, W. Hunziker, R. Goralczyk, V. Oberhauser, J. von Lintig and A. Wyss CMO1 deficiency abolishes vitamin A production from beta-carotene and alters lipid metabolism in mice, J Biol Chem 282 (2007) 33553-33561.

[11] B.I. Gustafsson, M. Kidd, A. Chan, M.V. Malfertheiner and I.M. Modlin Bronchopulmonary neuroendocrine tumors, Cancer 113 (2008) 5-21.

[12] W.D. Travis, L.B. Travis and S.S. Devesa Lung cancer, Cancer 75 (1995) 191-202.

[13] L.A. Kaplan, J.M. Lau and E.A. Stein Carotenoid composition, concentrations, and relationships in various human organs, Clin Physiol Biochem 8 (1990) 1-10.

[14] R.S. Parker Carotenoids in human blood and tissues, J Nutr 119 (1989) 101-104.

[15] R.E. Pattle and L.C. Thomas Lipoprotein composition of the film lining the lung, Nature 189 (1961) 844.

[16] E.L. Jackson, N. Willis, K. Mercer, R.T. Bronson, D. Crowley, R. Montoya, T. Jacks and D.A. Tuveson Analysis of lung tumor initiation and progression using conditional expression of oncogenic K-ras, Genes Dev 15 (2001) 3243-3248.

[17] H. Kitamura, Y. Kameda, T. Ito, H. Hayashi, N. Nakamura, Y. Nakatani, Y. Inayama and M. Kanisawa Cytodifferentiation of atypical adenomatous hyperplasia and bronchioloalveolar lung carcinoma: immunohistochemical and ultrastructural studies, Virchows Arch 431 (1997) 415-424.

[18] W.R. Otto Lung epithelial stem cells, J Pathol 197 (2002) 527-535.

[19] J.E. Muscat and E.L. Wynder Lung cancer pathology in smokers, ex-smokers and never smokers, Cancer Lett 88 (1995) 1-5.

[20] J.E. Tyczynski, F. Bray and D.M. Parkin Lung cancer in Europe in 2000: epidemiology, prevention, and early detection, Lancet Oncol 4 (2003) 45-55.

[21] C.M. Lee, A.C. Boileau, T.W. Boileau, A.W. Williams, K.S. Swanson, K.A. Heintz and J.W. Erdman, Jr. Review of animal models in carotenoid research, J Nutr 129 (1999) 2271-2277.

[22] R. Bodewes, G.F. Rimmelzwaan and A.D. Osterhaus Animal models for the preclinical evaluation of candidate influenza vaccines, Expert Rev Vaccines 9 59-72.

[23] C.M. Cameron, M.J. Cameron, J.F. Bermejo-Martin, L. Ran, L. Xu, P.V. Turner, R. Ran, A. Danesh, Y. Fang, P.K. Chan, N. Mytle, T.J. Sullivan, T.L. Collins, M.G. Johnson, J.C. Medina, T. Rowe and D.J. Kelvin Gene expression analysis of host innate immune responses during Lethal H5N1 infection in ferrets, J Virol 82 (2008) 11308-11317.

[24] H. Greim, U. Andrae, U. Forster and L. Schwarz Application, limitations and research requirements of in vitro test systems in toxicology, Arch Toxicol Suppl 9 (1986) 225-236.

[25] S.B. Kritchevsky, A.J. Bush, M. Pahor and M.D. Gross Serum carotenoids and markers of inflammation in nonsmokers, Am J Epidemiol 152 (2000) 1065-1071.

[26] T.P. Erlinger, E. Guallar, E.R. Miller, 3rd, R. Stolzenberg-Solomon and L.J. Appel Relationship between systemic markers of inflammation and serum beta-carotene levels, Arch Intern Med 161 (2001) 1903-1908.

[27] V.L. Van Antwerpen, A.J. Theron, G.A. Richards, C.A. Van der Merwe, E. Viljoen, R. Van der Walt and R. Anderson Plasma levels of beta-carotene are inversely correlated with circulating neutrophil counts in young male cigarette smokers, Inflammation 19 (1995) 405-414.

[28] W.G. Siems, O. Sommerburg and F.J. van Kuijk Lycopene and beta-carotene decompose more rapidly than lutein and zeaxanthin upon exposure to various pro-oxidants in vitro, Biofactors 10 (1999) 105-113.

[29] O. Sommerburg, C.D. Langhans, J. Arnhold, M. Leichsenring, C. Salerno, C. Crifo, G.F. Hoffmann, K.M. Debatin and W.G. Siems Beta-carotene cleavage products after oxidation mediated by hypochlorous acid--a model for neutrophil-derived degradation, Free Radic Biol Med 35 (2003) 1480-1490.

[30] T. Quasim, D.C. McMillan, D. Talwar, N. Sattar, D.S. O'Reilly and J. Kinsella Lower concentrations of carotenoids in the critically ill patient are related to a systemic inflammatory response and increased lipid peroxidation, Clin Nutr 22 (2003) 459-462.

[31] P.P. Glasziou and D.E. Mackerras Vitamin A supplementation in infectious diseases: a meta-analysis, Bmj 306 (1993) 366-370.

[32] A. Sommer Vitamin a deficiency and clinical disease: an historical overview, J Nutr 138 (2008) 1835-1839.

[33] R.C. Baybutt, L. Hu and A. Molteni Vitamin A deficiency injures lung and liver parenchyma and impairs function of rat type II pneumocytes, J Nutr 130 (2000) 1159-1165.

[34] T. Li, A. Molteni, P. Latkovich, W. Castellani and R.C. Baybutt Vitamin A depletion induced by cigarette smoke is associated with the development of emphysema in rats, J Nutr 133 (2003) 2629-2634.

[35] R.C. Baybutt and A. Molteni Vitamin A and emphysema, Vitam Horm 75 (2007) 385-401.

[36] J. Bastien and C. Rochette-Egly Nuclear retinoid receptors and the transcription of retinoid-target genes, Gene 328 (2004) 1-16.

[37] B. Desvergne RXR: from partnership to leadership in metabolic regulations, Vitam Horm 75 (2007) 1-32.

[38] L.E. Purton, S. Dworkin, G.H. Olsen, C.R. Walkley, S.A. Fabb, S.J. Collins and P. Chambon RARgamma is critical for maintaining a balance between hematopoietic stem cell self-renewal and differentiation, J Exp Med 203 (2006) 1283-1293.

[39] A.M. Jetten Retinoid-related orphan receptors (RORs): critical roles in development, immunity, circadian rhythm, and cellular metabolism, Nucl Recept Signal 7 (2009) e003.

[40] W.H. Miller, Jr. The emerging role of retinoids and retinoic acid metabolism blocking agents in the treatment of cancer, Cancer 83 (1998) 1471-1482.

[41] R.S. Larson and M.S. Tallman Retinoic acid syndrome: manifestations, pathogenesis, and treatment, Best Pract Res Clin Haematol 16 (2003) 453-461.

[42] E. Patatanian and D.F. Thompson Retinoic acid syndrome: a review, J Clin Pharm Ther 33 (2008) 331-338.

[43] M.S. Tallman, J.W. Andersen, C.A. Schiffer, F.R. Appelbaum, J.H. Feusner, A. Ogden, L. Shepherd, J.M. Rowe, C. Francois, R.S. Larson and P.H. Wiernik Clinical description of 44 patients with acute promyelocytic leukemia who developed the retinoic acid syndrome, Blood 95 (2000) 90-95.

[44] O. Loudig, C. Babichuk, J. White, S. Abu-Abed, C. Mueller and M. Petkovich Cytochrome P450RAI(CYP26) promoter: a distinct composite retinoic acid response element underlies the complex regulation of retinoic acid metabolism, Mol Endocrinol 14 (2000) 1483-1497.

[45] R. Ruhl, A. Bub and B. Watzl Modulation of plasma all-trans retinoic acid concentrations by the consumption of carotenoid-rich vegetables, Nutrition 24 (2008) 1224-1226.

[46] V.C. Njar, L. Gediya, P. Purushottamachar, P. Chopra, T.S. Vasaitis, A. Khandelwal, J. Mehta, C. Huynh, A. Belosay and J. Patel Retinoic acid metabolism blocking agents (RAMBAs) for treatment of cancer and dermatological diseases, Bioorg Med Chem 14 (2006) 4323-4340.

[47] J. Muindi, S.R. Frankel, W.H. Miller, Jr., A. Jakubowski, D.A. Scheinberg, C.W. Young, E. Dmitrovsky and R.P. Warrell, Jr. Continuous treatment with all-trans retinoic acid causes a progressive reduction in plasma drug concentrations: implications for relapse and retinoid "resistance" in patients with acute promyelocytic leukemia, Blood 79 (1992) 299-303.

[48] J.R. Muindi, C.W. Young and R.P. Warrell, Jr. Clinical pharmacology of all-trans retinoic acid, Leukemia 8 Suppl 3 (1994) S16-21.

[49] Y.M. Peng, W.S. Dalton, D.S. Alberts, M.J. Xu, H. Lim and F.L. Meyskens, Jr. Pharmacokinetics of N-4-hydroxyphenyl-retinamide and the effect of its oral administration on plasma retinol concentrations in cancer patients, Int J Cancer 43 (1989) 22-26.

[50] C. Liu, R.M. Russell and X.D. Wang Alpha-tocopherol and ascorbic acid decrease the production of beta-apo-carotenals and increase the formation of retinoids from beta-carotene in the lung tissues of cigarette smoke-exposed ferrets in vitro, J Nutr 134 (2004) 426-430.

[51] C. Liu, R.M. Russell and X.D. Wang Exposing ferrets to cigarette smoke and a pharmacological dose of beta-carotene supplementation enhance in vitro retinoic acid catabolism in lungs via induction of cytochrome P450 enzymes, J Nutr 133 (2003) 173-179.

[52] Z.N. Yang, G.J. Davis, T.D. Hurley, C.L. Stone, T.K. Li and W.F. Bosron Catalytic efficiency of human alcohol dehydrogenases for retinol oxidation and retinal reduction, Alcohol Clin Exp Res 18 (1994) 587-591.

[53] B.J. Carolan, A. Heguy, B.G. Harvey, P.L. Leopold, B. Ferris and R.G. Crystal Up-regulation of expression of the ubiquitin carboxyl-terminal hydrolase L1 gene in human airway epithelium of cigarette smokers, Cancer Res 66 (2006) 10729-10740.

[54] A.E. Tilley, B.G. Harvey, A. Heguy, N.R. Hackett, R. Wang, T.P. O'Connor and R.G. Crystal Down-regulation of the notch pathway in human airway epithelium in association with smoking and chronic obstructive pulmonary disease, Am J Respir Crit Care Med 179 (2009) 457-466.

[55] B.G. Harvey, A. Heguy, P.L. Leopold, B.J. Carolan, B. Ferris and R.G. Crystal Modification of gene expression of the small airway epithelium in response to cigarette smoking, J Mol Med 85 (2007) 39-53.

[56] A. Spira, J. Beane, V. Shah, G. Liu, F. Schembri, X. Yang, J. Palma and J.S. Brody Effects of cigarette smoke on the human airway epithelial cell transcriptome, Proc Natl Acad Sci U S A 101 (2004) 10143-10148.

[57] W. Jelski, M. Kozlowski, J. Laudanski, J. Niklinski and M. Szmitkowski Alcohol dehydrogenase isoenzymes and aldehyde dehydrogenase activity in the sera of patients with esophageal cancer, Clin Exp Med 9 (2009) 131-137.

[58] W. Jelski, L. Chrostek, B. Zalewski and M. Szmitkowski Alcohol dehydrogenase (ADH) isoenzymes and aldehyde dehydrogenase (ALDH) activity in the sera of patients with gastric cancer, Dig Dis Sci 53 (2008) 2101-2105.

[59] A.M. Gawienowski Integration of the metabolic pathways of steroids, carotenoids, and retinoids, Crit Rev Biochem Mol Biol 34 (1999) 405-410.

[60] V. Luu-The, P. Tremblay and F. Labrie Characterization of type 12 17beta-hydroxysteroid dehydrogenase, an isoform of type 3 17beta-hydroxysteroid dehydrogenase responsible for estradiol formation in women, Mol Endocrinol 20 (2006) 437-443.

[61] A. Lindqvist, Y.G. He and S. Andersson Cell type-specific expression of beta-carotene 9',10'-monooxygenase in human tissues, J Histochem Cytochem 53 (2005) 1403-1412.

[62] A. Tchernof and J.P. Despres Sex steroid hormones, sex hormone-binding globulin, and obesity in men and women, Horm Metab Res 32 (2000) 526-536.

[63] J.S. Mayes and G.H. Watson Direct effects of sex steroid hormones on adipose tissues and obesity, Obes Rev 5 (2004) 197-216.

[64] G. Boysen and S.S. Hecht Analysis of DNA and protein adducts of benzo[a]pyrene in human tissues using structure-specific methods, Mutat Res 543 (2003) 17-30.

[65] J.J. Briede, R.W. Godschalk, M.T. Emans, T.M. De Kok, E. Van Agen, J. Van Maanen, F.J. Van Schooten and J.C. Kleinjans In vitro and in vivo studies on oxygen free radical and DNA adduct formation in rat lung and liver during benzo[a]pyrene metabolism, Free Radic Res 38 (2004) 995-1002.

[66] A.B. Thompson, T. Bohling, A. Heires, J. Linder and S.I. Rennard Lower respiratory tract iron burden is increased in association with cigarette smoking, J Lab Clin Med 117 (1991) 493-499.

[67] A.J. Ghio, J. Stonehuerner, M.P. Steele and A.L. Crumbliss Phagocyte-generated superoxide reduces Fe3+ to displace it from the surface of asbestos, Arch Biochem Biophys 315 (1994) 219-225.

[68] A.D. McDonald, J.C. McDonald and F.D. Pooley Mineral fibre content of lung in mesothelial tumours in North America, Ann Occup Hyg 26 (1982) 417-422.

[69] A.J. Ghio, A. Churg and V.L. Roggli Ferruginous bodies: implications in the mechanism of fiber and particle toxicity, Toxicol Pathol 32 (2004) 643-649.

[70] E.A. Leibold and H.N. Munro Cytoplasmic protein binds in vitro to a highly conserved sequence in the 5' untranslated region of ferritin heavy- and light-subunit mRNAs, Proc Natl Acad Sci U S A 85 (1988) 2171-2175.

[71] A. Dautry-Varsat, A. Ciechanover and H.F. Lodish pH and the recycling of transferrin during receptor-mediated endocytosis, Proc Natl Acad Sci U S A 80 (1983) 2258-2262.

[72] M.N. Garcia-Casal, M. Layrisse, L. Solano, M.A. Baron, F. Arguello, D. Llovera, J. Ramirez, I. Leets and E. Tropper Vitamin A and beta-carotene can improve nonheme iron absorption from rice, wheat and corn by humans, J Nutr 128 (1998) 646-650.

[73] M.N. Garcia-Casal, I. Leets and M. Layrisse Beta-carotene and inhibitors of iron absorption modify iron uptake by Caco-2 cells, J Nutr 130 (2000) 5-9.

[74] A.J. Roodenburg, C.E. West, R. Hovenier and A.C. Beynen Evaluation of a two-generation rat model for vitamin A deficiency and the interrelationship with iron metabolism, Br J Nutr 74 (1995) 689-700.

[75] A.J. Roodenburg, C.E. West, R. Hovenier and A.C. Beynen Supplemental vitamin A enhances the recovery from iron deficiency in rats with chronic vitamin A deficiency, Br J Nutr 75 (1996) 623-636.

[76] M. Broch, J. Vendrell, W. Ricart, C. Richart and J.M. Fernandez-Real Circulating retinol-binding protein-4, insulin sensitivity, insulin secretion, and insulin disposition index in obese and nonobese subjects, Diabetes Care 30 (2007) 1802-1806.

[77] J.M. Fernandez-Real, J.M. Moreno and W. Ricart Circulating retinol-binding protein-4 concentration might reflect insulin resistance-associated iron overload, Diabetes 57 (2008) 1918-1925.

[78] W.C. Leung, S. Hessel, C. Meplan, J. Flint, V. Oberhauser, F. Tourniaire, J.E. Hesketh, J. von Lintig and G. Lietz Two common single nucleotide polymorphisms in the gene encoding beta-carotene 15,15'-monoxygenase alter beta-carotene metabolism in female volunteers, Faseb J 23 (2009) 1041-1053.

[79] C. Kiefer, S. Hessel, J.M. Lampert, K. Vogt, M.O. Lederer, D.E. Breithaupt and J. von Lintig Identification and characterization of a mammalian enzyme catalyzing the asymmetric oxidative cleavage of provitamin A, J Biol Chem 276 (2001) 14110-14116.

[80] K.Q. Hu, C. Liu, H. Ernst, N.I. Krinsky, R.M. Russell and X.D. Wang The biochemical characterization of ferret carotene-9',10'-monooxygenase catalyzing cleavage of carotenoids in vitro and in vivo, J Biol Chem 281 (2006) 19327-19338.

[81] R.S. Parker Absorption, metabolism, and transport of carotenoids, Faseb J 10 (1996) 542-551.

[82] C. Debier and Y. Larondelle Vitamins A and E: metabolism, roles and transfer to offspring, Br J Nutr 93 (2005) 153-174.

[83] J.A. Olson, D.B. Gunning and R.A. Tilton Liver concentrations of vitamin A and carotenoids, as a function of age and other parameters, of American children who died of various causes, Am J Clin Nutr 39 (1984) 903-910.

[84] J.E. Smith Preparation of vitamin A-deficient rats and mice, Methods Enzymol 190 (1990) 229-236.

[85] S.A. Akohoue, J.B. Green and M.H. Green Dietary vitamin A has both chronic and acute effects on vitamin A indices in lactating rats and their offspring, J Nutr 136 (2006) 128-132.

[86] M.H. Green, J.B. Green, S.A. Akohoue and S.K. Kelley Vitamin A intake affects the contribution of chylomicrons vs. retinol-binding protein to milk vitamin A in lactating rats, J Nutr 131 (2001) 1279-1282.

[87] Y. Fierce, M. de Morais Vieira, R. Piantedosi, A. Wyss, W.S. Blaner and J. Paik In vitro and in vivo characterization of retinoid synthesis from beta-carotene, Arch Biochem Biophys 472 (2008) 126-138.

[88] D. Albanes, O.P. Heinonen, P.R. Taylor, J. Virtamo, B.K. Edwards, M. Rautalahti, A.M. Hartman, J. Palmgren, L.S. Freedman, J. Haapakoski, M.J. Barrett, P. Pietinen, N. Malila, E. Tala, K. Liippo, E.R. Salomaa, J.A. Tangrea, L. Teppo, F.B. Askin, E. Taskinen, Y. Erozan, P. Greenwald and J.K. Huttunen Alpha-Tocopherol and beta-carotene supplements and lung cancer incidence in the alpha-tocopherol, beta-carotene cancer prevention study: effects of base-line characteristics and study compliance, J Natl Cancer Inst 88 (1996) 1560-1570.

[89] G.S. Omenn, G.E. Goodman, M.D. Thornquist, J. Balmes, M.R. Cullen, A. Glass, J.P. Keogh, F.L. Meyskens, Jr., B. Valanis, J.H. Williams, Jr., S. Barnhart, M.G. Cherniack, C.A. Brodkin and S. Hammar Risk factors for lung cancer and for intervention effects in CARET, the Beta-Carotene and Retinol Efficacy Trial, J Natl Cancer Inst 88 (1996) 1550-1559.

[90] G.S. Omenn, G.E. Goodman, M.D. Thornquist, J. Balmes, M.R. Cullen, A. Glass, J.P. Keogh, F.L. Meyskens, B. Valanis, J.H. Williams, S. Barnhart and S. Hammar Effects of a combination of beta carotene and vitamin A on lung cancer and cardiovascular disease, N Engl J Med 334 (1996) 1150-1155.

[91] A. Meager Biological assays for interferons, J Immunol Methods 261 (2002) 21-36.

[92] N. Idres, J. Marill, M.A. Flexor and G.G. Chabot Activation of retinoic acid receptor-dependent transcription by all-trans-retinoic acid metabolites and isomers, J Biol Chem 277 (2002) 31491-31498.

[93] J.C. Rockett, M.E. Burczynski, A.J. Fornace, P.C. Herrmann, S.A. Krawetz and D.J. Dix Surrogate tissue analysis: monitoring toxicant exposure and health status of inaccessible tissues through the analysis of accessible tissues and cells, Toxicol Appl Pharmacol 194 (2004) 189-199.

[94] M. Bouwens, O. van de Rest, N. Dellschaft, M.G. Bromhaar, L.C. de Groot, J.M. Geleijnse, M. Muller and L.A. Afman Fish-oil supplementation induces antiinflammatory gene expression profiles in human blood mononuclear cells, Am J Clin Nutr 90 (2009) 415-424.

[95] J. Hong, Y.C. Zang, G. Hutton, V.M. Rivera and J.Z. Zhang Gene expression profiling of relevant biomarkers for treatment evaluation in multiple sclerosis, J Neuroimmunol 152 (2004) 126-139.

[96] P.J. Valk, R.G. Verhaak, M.A. Beijen, C.A. Erpelinck, S. Barjesteh van Waalwijk van Doorn-Khosrovani, J.M. Boer, H.B. Beverloo, M.J. Moorhouse, P.J. van der Spek, B. Lowenberg and R. Delwel Prognostically useful gene expression profiles in acute myeloid leukemia, N Engl J Med 350 (2004) 1617-1628.

[97] A. Caimari, P. Oliver, J. Keijer and A. Palou Peripheral Blood Mononuclear Cells as a Model to Study the Response of Energy Homeostasis-Related Genes to Acute Changes in Feeding Conditions, Omics.

[98] E.A. van Beek, A.H. Bakker, P.M. Kruyt, M.H. Hofker, W.H. Saris and J. Keijer Intra- and interindividual variation in gene expression in human adipose tissue, Pflugers Arch 453 (2007) 851-861.

[99] E.A. van Beek, A.H. Bakker, P.M. Kruyt, C. Vink, W.H. Saris, N.L. Franssen-van Hal and J. Keijer Comparative expression analysis of isolated human adipocytes and the human adipose cell lines LiSa-2 and PAZ6, Int J Obes (Lond) 32 (2008) 912-921.

[100] F. Tourniaire, E. Gouranton, J. von Lintig, J. Keijer, M. Luisa Bonet, J. Amengual, G. Lietz and J.F. Landrier beta-Carotene conversion products and their effects on adipose tissue, Genes Nutr 4 (2009) 179-187.

[101] T.R. Martin Recognition of bacterial endotoxin in the lungs, Am J Respir Cell Mol Biol 23 (2000) 128-132.

Samenvatting

Proefschrift: Moleculaire Werkingsmechanimsen van Bètacaroteen in de Long

In het verleden is gebleken dat hoge inname van bètacaroteen (BC) het risico op het krijgen van longkanker in rokers en asbest blootgestelde mensen kan verhogen. Zowel roken als asbestblootstelling zijn geassocieerd met een ontsteking in de long, en een langdurige ontsteking lijkt een belangrijke rol te spelen in het ontstaan en uitgroeien van longkanker. Een chronische ontsteking in de longen wordt gekarakteriseerd door een toename van een aantal witte bloedcellen (onder andere neutrofielen), welke reactieve zuurstof radicalen kunnen vormen die de cel beschadigen. Omdat wetenschappelijke studies laten zien dat BC zowel zuurstofschade kan verminderen (antioxidant) als verergeren (pro-oxidant) [1,2], hadden wij ons in het eerste gedeelte van dit proefschrift als doel gesteld om te onderzoeken onder welke omstandigheden BC als een antioxidant of een pro-oxidant fungeerd. Vervolgens onderzochten wij het effect van BC op de door radicalen geïnduceerde DNA schade.

In **Hoofdstuk 2** laten we zien dat de aard van het weg te vangen radicaal bepaald of BC een pro- of een antioxidant is; we zagen een beschermende werking van BC tegen koolstof gecentreerde radicalen, terwijl BC de hydroxyl radicaal geïnduceerde schade in cellen verhoogde. In lijn met deze bevindingen zorgde BC in fretten voor een verlaging van DNA schade veroorzaakt door het lipideperoxidatie product malondialdehyde (MDA), ook bekend als M_1dG. De oxidatieve DNA laesie 8-oxo-dG, welke direct resulteert uit DNA oxidatie, was echter onveranderd in fretten na toevoeging van BC aan het dieet, waarschijnlijk omdat BC gelijktijdig de DNA herstel capaciteit verhoogde.

Vervolgens testten we het effect van BC op neutrofiel geïnduceerde DNA schade. Geactiveerde neutrofielen genereren grote hoeveelheden reactieve zuurstof radicalen (reactive oxygen species; ROS) [3-6], welke oxidatieve DNA schade kunnen veroorzaken [7]. Bovendien verlagen geactiveerde neutrofielen de cellulaire DNA herstel capaciteit [8]. In **Hoofdstuk 3** wordt beschreven dat de BC metabolieten; vitamine A zuur (retinoic acid) en retinal, neutrofiel geïnduceerd oxidatieve DNA laesies verhoogden. Dit was het resultaat van een verlaging van de activiteit van MPO, een enzym betrokken bij de omzetting van neutrofiel gegenereerd waterstofperoxide, in combinatie met een versterkte omzetting van waterstof peroxide resulterend in de toename van hydroxyl radicalen, door BC. Dit mechanisme leidde uiteindelijk tot een toename van de oxidatieve DNA schade in neutrofiel blootgestelde long epitheel cellen door retinoic acid en retinal. Dit wekt de suggestie dat negatieve effecten ten gevolge van BC inname kunnen worden verwacht tijdens een chronische ontsteking.

BC of een van zijn metabolieten kan dus de genotoxiciteit van rook of asbest veranderen. Behalve dit effect zou BC mogelijk ook de mate waarin een gen wordt afgeschreven (genexpressie) wat belangrijk is voor de regulatie van een cel, kunnen veranderen. Hier-

door kan de vatbaarheid voor rook of asbest geïnduceerde carcinogenese verder worden beïnvloed. Genexpressie veranderingen die geïnduceerd worden door BC inname in de mens zijn moeilijk te bepalen omdat aan mensen op ethische gronden geen hoge dosis BC meer kan worden gegeven door de resultaten van interventie studies in rokers en asbest blootgestelde mensen. Bovendien is long weefsel nauwelijks toegankelijk in mensen. De vaak gebruikte *in vivo* modellen, zoals ratten en muizen, verschillen echter te sterk in hun BC metabolisme van de mens; knaagdieren zetten BC bijna geheel om, terwijl de mens BC slechts deels metaboliseerd. Als gevolg hiervan resulteert BC in het dieet bij knaagdieren in de accumulatie van BC metabolieten, terwijl dit bij mensen resulteert in de accumulatie van intact BC [9]. Hoewel andere diermodellen zoals de fret, de woestijnrat en kalveren, meer gelijkenis vertonen met het humane BC metabolisme en ook vaak gebruikt zijn voor onderzoek naar de effecten van BC, hebben deze modellen grote nadelen. Voor wat betreft de fret of de woestijnrat als model voor de mens, is er weinig genetische kennis en zijn er nauwelijks commercieel verkrijgbare gereedschappen voor moleculair onderzoek. Gebruik van deze modellen voor BC onderzoek zou resulteren in pionierend onderzoek dat voornamelijk gericht is op het ontwikkelen van methoden, in plaats van onderzoek dat gericht is op het oplossen van mechanistische vragen. Er is meer kennis over kalveren, maar dit onderzoek is doorgaans duur en er is speciale huisvesting nodig. In ons onderzoek hebben we daarom gebruik gemaakt van een “gehumaniseerd” muizen model, welke beschreven wordt in het tweede gedeelte van dit proefschrift. In dit muizen model is geen functioneel beta-carotene 15,15’-monooxygenase 1 (Bcmo1), omdat dit gen uitgeschakeld is (een zogenaamd knock-out model) [10]. Als gevolg hiervan kunnen deze muizen geen BC metaboliseren en zijn ze dus evenals mensen in staat om intact BC te accumuleren. In het tweede gedeelte van dit proefschrift hebben we door BC geïnduceerde veranderingen in gen expressie onderzocht in dit “gehumaniseerde” muizen model. Met dit onderzoek kunnen we meer inzicht in de functionele effecten van BC in de long krijgen. In **Hoofdstuk 4** bediscussiëren we eerst de “in house” kennis op het gebied van functionele genomica in voeding interventie studies.

Voor de bepaling van gen expressie veranderingen *in vivo* veroorzaakt door BC hebben we een muizen experiment uitgevoerd waarbij mannetjes en vrouwtjes wildtype (*Bcmo1*$^{+/+}$) en *Bcmo1* knock-out muizen (*Bcmo1*$^{-/-}$) een dieet kregen dat 1500 IU vitamine A al dan niet met 150 mg/kg BC.

In **Hoofdstuk 5** beschrijven we het effect van BC op genexpressie veranderingen in long weefsel van vrouwelijke *Bcmo1*$^{-/-}$ muizen. In eerste instantie vonden we een statistisch betrouwbare (significante) afname in de expressie van genen betrokken bij ontstekingsreacties in *Bcmo1*$^{-/-}$ muizen door BC. Echter, na vergelijking van deze genexpressie met de genexpressie in *Bcmo1*$^{+/+}$ muizen, bleek dat de afname in de ontstekingsresponse eigenlijk een inductie van de ontstekingsresponse in de longen van controle *Bcmo1*$^{-/-}$ muizen. Deze inductie verdween wanneer er BC in het dieet aanwezig was. Histologie van de longen bevestigde de genexpressie resultaten, waarbij alleen ontstekingshaarden werden waargenomen bij controle *Bcmo1*$^{-/-}$ muizen. We konden deze bevindingen verklaren door aan te nemen dat vrouwelijke *Bcmo1*$^{-/-}$ muizen een grotere vitamine A behoefte hebben, mede doordat enzymen betrokken bij het BC metabolisme in expressie waren

veranderd. Dit resulteerde in een genexpressie die veranderd was in de richting van vitamine A opslag in plaats van de aanmaak van de bioactieve metaboliet retinoic acid. Dit is in overeenstemming met de wetenschappelijke literatuur, waarin het bekend is dat een deficiëntie in retinoic acid resulteert in ontstekingen.

In **Hoofdstuk 6** hebben we ons gericht op BC geïnduceerde genexpressie veranderingen in long weefsel van mannelijke *Bcmo1*$^{-/-}$ muizen. De toename van de ontstekingsrespons in longweefsel van controle *Bcmo1*$^{-/-}$ muizen bleek specifiek voor vrouwelijke *Bcmo1*$^{-/-}$ muizen en werd niet waargenomen in mannelijke muizen. Echter, BC suppletie aan mannelijke *Bcmo1*$^{-/-}$ muizen resulteerde in een sterk lagere expressie van *frizzled homolog 6* (*Fzd6*) (min 2.6 keer) en *collagen triple helix repeat containing 1* (*Cthrc1*) (min 3 keer). Tegelijk hadden verschillende 'olfactory' receptoren (geurreceptoren) en protocadherines een hogere genexpressie. Wij hypothetiseren dat deze BC effecten specifiek plaats vinden in cellen die betrokken zijn bij de detectie van veranderingen in de lucht samenstelling in de long. 'Pulmonary endocrine cells' (PNECs) zijn specifiek betrokken bij de detectie van luchtsamenstelling en het is dus goed mogelijk dat dit specifieke celtype door BC beïnvloed wordt. Ondanks dat slechts 1 op de 2500 long cellen een PNEC cel is [11], ontstaat 20% van alle long kanker types uit PNEC en verhoogd in het bijzonder roken long kanker die is ontstaan uit PNEC cellen [12]. Het is belangrijk om deze bevindingen verder te onderzoeken en specifiek de rol van *Fzd6* en *Cthrc1* in PNECs en in relatie tot longkanker.

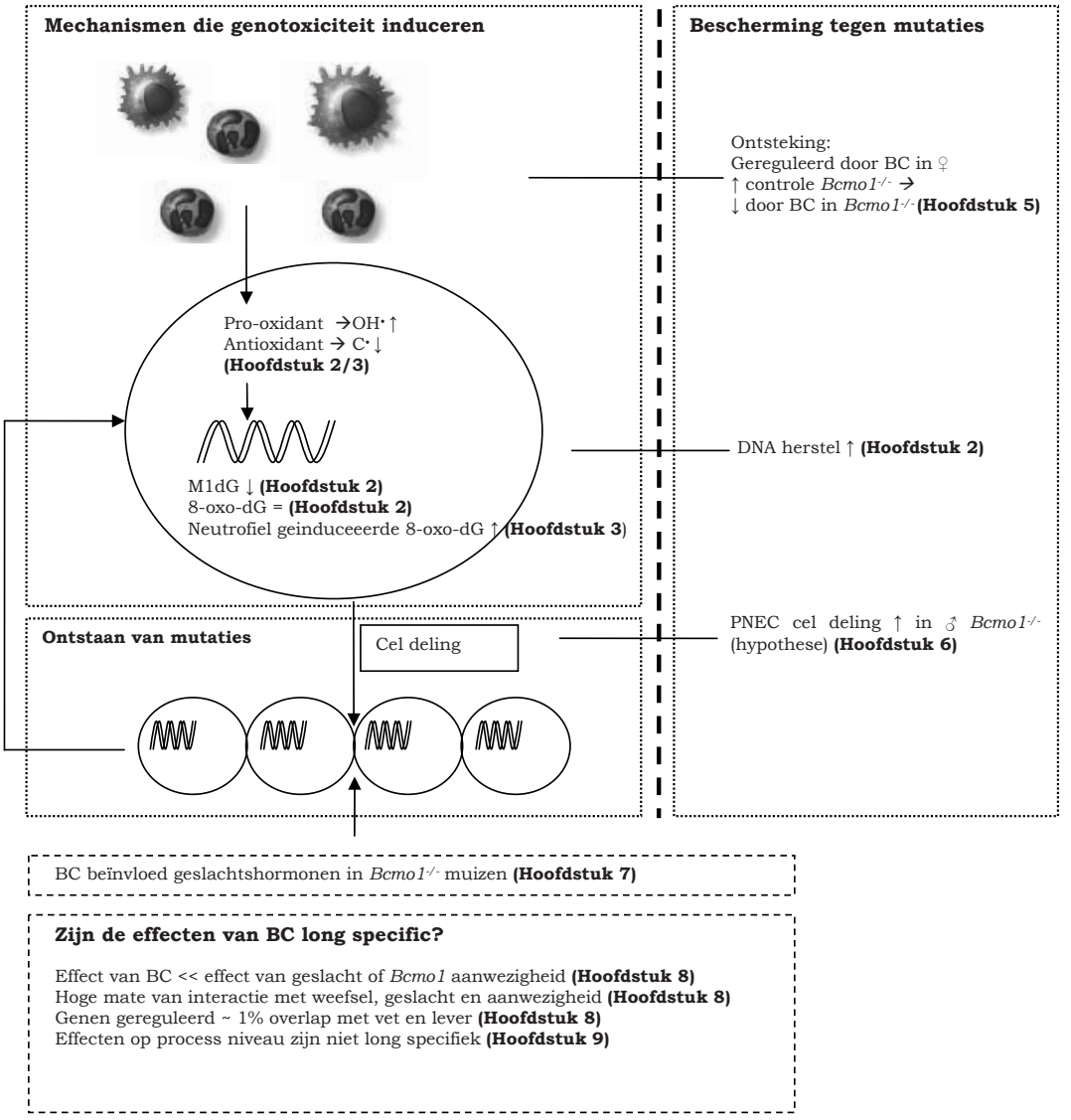
BC leidde in zowel mannelijke als vrouwelijke rokers en asbest blootgestelde mensen in de CARET studie tot een verhoging van het longkanker risico. Daarom onderzochten we de gezamenlijke processen die veranderd waren in long weefsel van zowel mannelijke als vrouwelijke *Bcmo1*$^{-/-}$ muizen door BC in **Hoofdstuk 7**. De genen die zowel in vrouwelijke als mannelijke muizen door BC veranderd tot expressie kwamen waren tegengesteld veranderd in *Bcmo1*$^{-/-}$ muizen (significante toename in het ene geslacht ging gepaard met een afname in het andere en *vice versa*). Dit suggereert een rol voor BC in de regulatie van geslachtshormonen. De tegengestelde genexpressie veranderingen in de long werden vergezeld door een significante regulatie van veel enzymen betrokken bij de steroïdenproductie en conversie maar alleen in de longen van mannelijke *Bcmo1*$^{-/-}$ muizen. Bovendien bleken testosteron niveaus zeer variabel te zijn in *Bcmo1*$^{-/-}$ muizen die BC in het dieet hadden, maar niet in de controle *Bcmo1*$^{-/-}$ muizen of de *Bcmo1*$^{+/+}$ muizen. Wij hypothetiseren dan ook dat BC in staat is om hormoon productie of conversie te veranderen.

Om een goede risico-baten analyse van BC te maken is het belangrijk om te weten of effecten van BC specifiek zijn voor de long. Daarom hebben we BC geïnduceerde genexpressie veranderingen in long weefsel vergeleken met effecten in andere organen die van belang zijn in de BC biologie. We vergeleken effecten in de long met effecten in wit vetweefsel dat belangrijk is voor de BC opslag [13], en met effecten in de lever welke belangrijk is voor zowel het BC metabolisme als de BC opslag [14].

In **Hoofdstuk 8** laten we zien dat slecht een paar genen (~1%) die gereguleerd zijn door BC in de long, ook werden gereguleerd in lever en in het zogenaamde 'inguinal' wit vetweefsel. Hieruit blijkt dat effecten van BC op de expressie van genen zeer weefsel speci-

fiek was. Bovendien was de hoeveelheid genen die van expressie veranderd waren door de BC interventie veel kleiner dan na knock-out van *Bcmo1* of verschillen in geslacht (man versus vrouw). Ondanks dat we in **Hoofdstuk 8** beschrijven dat slechts 1% van de genen die gereguleerd waren door BC in long weefsel ook gereguleerd waren in lever en vet weefsel, beschrijven we in **Hoofdstuk 9** dat de processen die door BC gereguleerd waren in longweefsel van vrouwelijke *Bcmo1*$^{-/-}$ muizen zoals beschreven in **Hoofdstuk 5** of in mannelijke *Bcmo1*$^{-/-}$ muizen zoals beschreven in **Hoofdstuk 6** of de tegengestelde verandering in gen expressie in mannelijke en vrouwelijke *Bcmo1*$^{-/-}$ muizen zoals beschreven in **Hoofdstuk 7**, niet specifiek voor longweefsel waren wanneer we de analyse op proces niveau uitvoerden.

Samenvattend beschrijven we in dit proefschrift dat BC 1) ontstekingsgeïnduceerde genotoxiciteit en 2) genexpressie in de long kan veranderen, waarbij enkele processen beinvloedt worden die betrokken kunnen zijn bij long carcinogenese (Fig. 1).



Figuur 1: Samenvatting van de belangrijkste bevindingen van de werking van BC in de long zoals beschreven in dit proefschrift, met de hoofdstukken die deze bevindingen beschrijven dik gedrukt.

Referenties

[1] P. Palozza Prooxidant actions of carotenoids in biologic systems, Nutr Rev 56 (1998) 257-265.

[2] P. Palozza, S. Serini, F. Di Nicuolo, E. Piccioni and G. Calviello Prooxidant effects of beta-carotene in cultured cells, Mol Aspects Med 24 (2003) 353-362.

[3] J.S. Brody and A. Spira State of the art. Chronic obstructive pulmonary disease, inflammation, and lung cancer, Proc Am Thorac Soc 3 (2006) 535-537.

[4] G. Lee, T.C. Walser and S.M. Dubinett Chronic inflammation, chronic obstructive pulmonary disease, and lung cancer, Curr Opin Pulm Med 15 (2009) 303-307.

[5] R. Robledo and B. Mossman Cellular and molecular mechanisms of asbestos-induced fibrosis, J Cell Physiol 180 (1999) 158-166.

[6] A.G. Schwartz, G.M. Prysak, C.H. Bock and M.L. Cote The molecular epidemiology of lung cancer, Carcinogenesis 28 (2007) 507-518.

[7] C.M. Doerschuk Leukocyte trafficking in alveoli and airway passages, Respir Res 1 (2000) 136-140.

[8] N. Gungor, R.W. Godschalk, D.M. Pachen, F.J. Van Schooten and A.M. Knaapen Activated neutrophils inhibit nucleotide excision repair in human pulmonary epithelial cells: role of myeloperoxidase, Faseb J 21 (2007) 2359-2367.

[9] A. Wyss Carotene oxygenases: a new family of double bond cleavage enzymes, J Nutr 134 (2004) 246S-250S.

[10] S. Hessel, A. Eichinger, A. Isken, J. Amengual, S. Hunzelmann, U. Hoeller, V. Elste, W. Hunziker, R. Goralczyk, V. Oberhauser, J. von Lintig and A. Wyss CMO1 deficiency abolishes vitamin A production from beta-carotene and alters lipid metabolism in mice, J Biol Chem 282 (2007) 33553-33561.

[11] B.I. Gustafsson, M. Kidd, A. Chan, M.V. Malfertheiner and I.M. Modlin Bronchopulmonary neuroendocrine tumors, Cancer 113 (2008) 5-21.

[12] W.D. Travis, L.B. Travis and S.S. Devesa Lung cancer, Cancer 75 (1995) 191-202.

[13] L.A. Kaplan, J.M. Lau and E.A. Stein Carotenoid composition, concentrations, and relationships in various human organs, Clin Physiol Biochem 8 (1990) 1-10.

[14] R.S. Parker Carotenoids in human blood and tissues, J Nutr 119 (1989) 101-104.