The interplay between environmental contaminants, genes and diet in obesity and intestinal cancer

Ha Thi Ngo

2014

Dissertation for the degree of Philosophiae Doctor (Ph.D.)

Department of Biosciences, Faculty of Mathematics and Natural Sciences, University of Oslo

Norwegian Institute of Public Health

Department of Food, Water and Cosmetics, Division of Environmental Medicine, Norwegian Institute of Public Health

Series of dissertations submitted to the
Faculty of Mathematics and Natural Sciences, University of Oslo
No. 1517

ISSN 1501-7710

Cover: Inger Sandved Anfinsen.
Printed in Norway: AIT Oslo AS.

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ACKNOWLEDGEMENTS

This thesis was carried out during the period 2010-2014 at the Norwegian Institute of Public Health, Oslo, Norway. The research project was financially supported by the Research Council of Norway (NFR), under the MILPAAHEL (Miljøpåvirkning og helse) programme, former MILGENHEL (Miljø, gener og helse).

During my time as a PhD student, I have been blessed with the opportunity to meet and become acquainted with so many amazing people. I will always be grateful for the chance I got to complete this PhD degree. It has been tough and challenging, but yet such a rewarding time. I am honoured and grateful to be a part of such as extensive and exciting study.

I wish to thank my main supervisor, Inger-Lise Steffensen for giving me great guidance and help throughout the doctoral time. Your rich knowledge and hardworking spirit has been a huge inspiration for me. I also want to give a special thank to my co-supervisor Ragna Bogen Hetland, for being a great support and always checking up on how I am doing. Your bubbly personality and kind heart has been precious to me during the time working at the institute.

My thank also go to the division director Toril Attramadal for providing additional funding for me and a good working environment at the Division of Environmental Medicine (MI). Thanks to the department director, Hubert Dirven at the Department of Food, Water and Cosmetics (MIVM) for the guidance and for showing much interest in my progress writing this thesis.

I wish to thank Hege Hjertholm and Tone Rasmussen for good technical laboratory work. A warm thanks to Hildegunn Dahl for being a good friend and colleague. Thanks to Victor Labay Ong for your help in the animal facility.

I also wish to thank my international collaborators Dr. Janice E. Drew and Andrew J. Farquharson at the Rowett Institute of Nutrition and Health, University of Aberdeen. The time in Scotland has been a great growing experience for me.

Other people I wish to thank are Line Småstuen Haug, Azemira Sabaredzovic, Unni Cecilie Nygaard, Åse Eikeset and Else-Carin Groeng for your contributions in this project.

I want to thank Wenche Jacobsen for showing me strategies regarding literature search, and Anders Aak for helping me with technical questions.

I wish to thank my former and present internal supervisor Steinar Øvrebø and Ketil Hylland at the University of Oslo, and my colleagues at the Norwegian Institute of Public Health, for being part of the good working place during these past years. Thanks to Camilla Svendsen for sharing tips and tricks on Excel and Anja Hortemo Sæther for helping out in the animal facility when needed.

I am uttermost grateful for my friends and family, for their endless support, love and encouragement. Thank you for always believing in me, giving me advice and cheering on me. It has truly meant the world to me.

Oslo, March 2014

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ABBREVIATIONS

ACF	abberant crypt foci
ACTH	adrenocorticotropic hormone
AgRP	agouti-related peptide
APC	adenomatous polyposis coli
BMI	body mass index
BPA	bisphenol A
CART	cocaine and amphetamine regulated transcript
CD-1	cluster of differentiation 1
CRP	C - reactive protein
CXCL1	(C-X-C) motif ligand 1
DES	diethylstilbestrol
DIO	diet-induced obesity
DOHaD	developmental origins of health and disease
Dsh	dishevelled
EDC	endocrine disrupting chemical
FAP	familial adenomatous polyposis
GSK3β	glycogen synthase kinase 3 β
HNPCC	hereditary non-polyposis colorectal cancer
IGF1	insulin-like growth factor 1
IGF1R	insulin-like growth factor 1 receptor
IL-1β	interleukin 1β
IL-6	interleukin 6
ITT	insulin tolerance test
LEF	lymphocyte enhancer binding factor

Lep	leptin
LOH	loss of heterozygosity
MCP-1	monocyte chemoattractant protein-1
MC4R	melanocortin 4 receptor
Min	multiple intestinal neoplasia
MSH	melanocyte-stimulating hormone
NK	natural killer
NPY	neuropeptide Y
Ob	obese
PAR	predictive adaptive response
PFAA	perfluoroalkyl acids
PFOA	perfluorooctanoate
PFOS	perfluorooctane sulfonate
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
POMC	pro-opiomelanocortin
SHBG	sex hormone binding globulin
TCF	T-cell factor
TNF-α	tumor necrosis factor alpha
WC	waist circumference
WAT	white adipose tissue
WHO	world health organization
WHR	waist-to-hip ratio
Wnt	Wingless-related integration site
Wt	wild-type

SUMMARY

The prevalence of overweight and obesity has escalated over the past several decades, in parallel with increasing use of chemicals. Obesity is a major risk factor for the development of diabetes, insulin resistance, metabolic syndrome, cardiovascular diseases, and several cancers, including colon cancer. Consumption of a high fat diet can over time lead to accumulation of excess body fat that is associated with adipose tissue dysfunction. Obesity is considered a state of low-grade inflammation, characterized by increased circulating levels of pro-inflammatory cytokines, tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6) and interleukin 1β (IL-1β). Both nutritional factors and environmental chemicals act during specific sensitive windows of development.

The first objective of the present thesis was to investigate whether genetical or diet-induced obesity affects 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP)-induced or spontaneous intestinal tumorigenesis in adult mice. In order to assess this, we studied the relationship between genetically or diet-induced obesity on intestinal tumorigenesis in a double mutant mouse model obtained by crossing the well-established mouse model for intestinal tumorigenesis, the C57BL/6J-*ApcMin* (multiple intestinal neoplasia)/+ mouse with a mouse which becomes obese, the C57BL/6J-*Lepob* (obese)/+ mouse. The *Min*/+ mouse is a good model for the inherited disorder familial adenomatous polyposis (FAP), as well as for sporadic colorectal cancer in humans. The germline mutation in the *adenomatous polyposis coli (Apc)* gene causes spontaneous development of tumors, mainly in the small intestine, but also in the colon. The *Min*/+ mouse model, with one inherited mutated or lost *Apc* allele is particularly susceptible to intestinal carcinogens that affect the remaining wild-type allele of the *Apc* gene. The *ob/ob* mouse model is an excellent model for study of obesity because it has similar genetics and phenotype as humans. In addition, we investigated the expression of pro-inflammatory genes and other selected genes relevant for development of colon cancer in a diet-induced obesity model in wild-type C57BL/6J mice.

As our second objective, we wanted to find out if obesogenic conditions *in utero* affected the body weight development and susceptibility to environmental contaminants in the offspring as adults. We identified the most susceptible period in life that affected body weight and intestinal tumorigenesis in adult mice, by exposing *Min*/+ mice to a high fat

diet (45% fat) during various periods of life; *in utero,* during nursing, during both *in utero* and nursing, as adults and throughout life.

Lastly, we investigated whether *in utero* exposure to the environmental contaminants perfluorooctanoate (PFOA) or perfluorooctane sulfonate (PFOS) affected the body weight development and susceptibility to environmental contaminants in the offspring.

In experiment 1, the F1 mice were given a 45% fat diet from weaning to termination at 11 weeks of age, and terminal body weight and number of small intestinal tumors were compared with a 10% fat diet. We found that the mice with *ob/ob* genotype had increased body weight, evaluated in three different ways, and increased number of small intestinal tumors. We tested two hypotheses for the association between obesity and intestinal tumorigenesis. The first one comprises disturbed blood glucose regulation. Blood glucose levels (non-fasted) and area under the curve (AUC) in a glucose tolerance test (GTT) (fasted), as well as plasma insulin levels, were higher in *ob/ob* mice, compared with *ob/wt* and *wt/wt*, implicating insulin resistance and hyperinsulinemia. The second hypothesis implicates inflammation, where obesity is considered as a low-grade inflammatory state releasing several pro-inflammatory cytokines affecting cancer. The pro-inflammatory cytokine TNF-α was increased in *ob/ob* mice on a 45% fat diet compared with *ob/wt* and *wt/wt* mice.

In experiment 2, mechanistic studies showed that a high fat diet increased the expression of the inflammatory markers IL-6, IL-1β, the chemokine CXCL1, IGF1 and its receptor IGF1R and haptoglobin in the liver. The increased expression of the pro-inflammatory cytokines in the liver could possibly affect inflammation also in the intestines, and this could be linked to increased colorectal cancer. Increased expression of IGF1 and IGF1R in the liver may indicate disrupted blood sugar regulation, which is associated with increased colon cancer risk.

In experiment 3, the body weight expressed as AUC from day 3 to termination was significantly increased after a 45% fat diet during nursing, and during both *in utero* and nursing, as well as throughout life, compared with the control group given a 10% fat diet throughout life. In GTT, early life exposure to a 45% fat diet did not affect blood glucose, whereas exposure to a 45% fat diet as adults or throughout life significantly increased AUC. Exposure to a 45% fat diet during nursing, and during both *in utero* and nursing, also increased the number of small intestinal tumors compared with the control group. The

result showed that early life exposure, in the intrauterine and nursing period, is a critical window for exposure to a high fat diet both for increased body weight and intestinal tumorigenesis as adults.

In experiment 4, PFOA or PFOS did not increase the body weight of the mice in any of the doses tested (0.01, 0.1 or 3.0 mg/kg bw/day), at least not up to 20 weeks of age. PFOA or PFOS did not increase the number or size of small intestinal or colonic tumors in the *Min/+* mice, nor affect the location of the tumors. The lack of tumorigenic effect in the intestines of PFOA and PFOS could be explained by their apparent lack of genotoxicity.

In conclusion, we have used mouse models to study the relationship between obesity and cancer. The interaction between the two genes *Apc* and *ob* was examined in a double mutant mouse model, and also the influences of environmental factors (a high fat diet and the food mutagen PhIP) on these genetic conditions were examined. The results show that there is an association between obesity and cancer, and that insulin resistance and inflammation may be involved. Early life exposure *in utero* and during the nursing period to a high fat diet is a critical window of susceptibility for development of both obesity and intestinal tumorigenesis later in life. We have also contributed with new knowledge about the environmental contaminants PFOA and PFOS, and found that neither of these two chemicals were obesogenic or increased intestinal tumorigenesis. The results obtained in these experiments support and increase our understanding of the association between obesity and cancer. Such knowledge is also needed for the interventional and preventive strategies against obesity-related diseases, including cancer.

AIM OF THE STUDY

The aim of this thesis was to examine several aspects of the relationship between obesity and intestinal cancer by answering the following questions:

1) Can genetical or diet-induced obesity affect carcinogen (PhIP)-induced or spontaneous intestinal tumorigenesis in adult mice?

To answer this question, we studied the relationship between genetically or diet-induced obesity on intestinal tumorigenesis in a double mutant mouse model obtained by crossing the well-established mouse model for intestinal tumorigenesis, the C57BL/6J-*ApcMin*/+ mouse with a mouse which becomes obese, the C57BL/6J-*Lepob*/+ mouse (**Paper I**).

In addition, as part of the research visit at the Rowett Institute of Nutrition and Health, in Aberdeen, Scotland, we investigated the expression of pro-inflammatory genes and some other selected genes relevant for the development of colon cancer in a diet-induced obesity model (**Paper II**).

2) Can obesogenic conditions *in utero* affect the body weight development and susceptibility to environmental contaminants in the offspring?

We identified the most susceptible period in life that affected intestinal tumorigenesis and body weight in adult mice, by exposing *ApcMin*/+ mice to a high fat diet (45% fat) during various periods of life; *in utero,* during nursing, during both *in utero* and nursing, as adults and throughout life (**Paper III**).

3) Can exposure to environmental contaminants *in utero* affect the body weight development (i.e. according to the obesogen hypothesis) and susceptibility to environmental contaminants in the offspring?

We investigated whether exposure of the dams during pregnancy to the environmental contaminants perfluorooctanoate (PFOA) or perfluorooctane sulfonate (PFOS) had obesogenic effect and if they increased intestinal tumorigenesis, in the wild-type and *ApcMin*/+ offspring, respectively (**Paper IV**).

1 GENERAL INTRODUCTION

1.1 Colon cancer

Colon cancer is the third most common cancer type, and the fourth most common cause of death worldwide, accounting for roughly 1.2 million new cases and more than 600 000 deaths per year [1, 2] . The incidence is low at ages younger than 50 years, but increases with age. The median age at diagnosis is about 70 years in developed countries [3]. In Norway, colon cancer was the third most common type of cancer in men (after prostate and lung cancer), and the second most common type (after breast cancer) in women in 2011 [4]. New cases of colon cancer registered in 2011 were 1220 cases among men and 1386 cases among women in Norway. This cancer is the most common cancer in women above 70 years. The incidence and mortality rates seem to have stabilised, but the colon cancer incidence rates among both men and women in Norway remain highest among the Nordic countries, which they have been for the past 20 years [4].

There are great geographical differences worldwide, and the rates of this cancer increase with industrialization and urbanization. With a history of being considerable more common in high-income countries, it is now increasing in middle- and low-income countries [1]. The highest incidence rates of colorectal cancer is being estimated in Australia/New Zealand and Western Europe, the lowest in Africa (except Southern Africa) and South-Central Asia, and are intermediate in Latin America [5]. The incidence rates are considerably higher in men than in women worldwide [1, 5]. Five-year survival estimates in men have been reported to be 65% in North America and 54% in Western Europe, 34% in Eastern Europe and 30% in India [6].

There is convincing evidence that high consumption of red meat, processed meat, excessive alcohol consumption, body fatness and abdominal fatness are among the causes of colorectal cancer [1, 6]. A family history of colorectal cancer, inflammatory bowel disease and smoking are other factors contributing to this cancer. All the mentioned risk factors can co-occur and interact, thus increasing the risk of colorectal cancer [2], while dairy products, calcium, dietary fibre and garlic probably protects against this cancer [1, 6]. There is also sufficient evidence in humans that physical activity has a cancer preventive effect on the colon. The preventive effects increase with increasing duration and intensity of physical activity [6].

1.1.1 Inherited and sporadic colorectal cancer

Inherited syndromes account for about 3-5% of all colorectal cancer cases [2]. The two most common forms of hereditary colorectal cancers are familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC) or Lynch syndrome [7]. Both syndromes are autosomal dominant disorders predisposing to colorectal cancer [2]. In this thesis, the focus will be on a mouse model for FAP, as well as sporadic colorectal cancer.

Patients affected with FAP develop hundreds to thousands of adenomas throughout the colon during their second and third decades of life [8]. Some of these adenomas will progress to carcinoma if they are left untreated [9]. FAP affects 1 in 10 000 individuals [10] and is nearly 100% penetrant, which means that almost every individual who inherits the genetic defect will develop the disease [11].

The majority of cases of FAP are due to germline mutations in the tumor suppressor gene *adenomatous polyposis coli* (*APC*), identified by Groden *et al.* (1991) and Nishisho *et al.* (1991) [12, 13]. Mutations in the *APC* gene are not only responsible for FAP, but have also been observed in >80% of tumors from patients with sporadic colorectal cancers [14]. Sporadic colorectal cancer is mainly observed in individuals over 50 years of age, probably as a result of dietary and environmental factors in addition to normal aging [15]. The frequency of somatic *APC* mutations is similar in adenomas and advanced carcinomas. These observations suggest that mutation of *APC* is an early, if not the initiating event in colorectal cancer [16, 17].

The multiple intestinal neoplasia (*Min*) mouse (see section 1.3) has a germline non-sense mutation in the *Apc* gene similar to in human FAP, and thus functions as an excellent model for both inherited and sporadic forms of human colorectal cancer [9]. In both FAP and *Min* mice, inactivation of both alleles of the *APC* gene seems to be required for tumor development [18-20]. The inactivation of both alleles is often caused by loss of heterozygosity (LOH), but can also happen by two mutations [21]. This “two hit” model was first suggested by Knudson (1971), explaining inherited and non-hereditary incidences of retinoblastoma [22].

1.1.2 Adenoma-carcinoma sequence

Colorectal tumors arise through a series of histopathological changes, also known as the "adenoma-carcinoma" sequence, each accompanied by a genetic alteration in a specific oncogene or tumor suppressor gene [23] (see Figure 1). At least four sequential genetic changes need to occur to ensure colorectal cancer evolution. One oncogene (*KRAS)*, and three tumor suppressor genes (*APC*, *SMAD4* and *TP53)*, are the main targets for these genetic changes [17]. The dominant recessive nature of these genes predicts that at least seven mutations are required: one oncogenic mutation in *KRAS* and six additional ones to inactivate both alleles of the mentioned tumor suppressor genes [17].

APC mutations in chromosome 5q21 are found in the very early stages of the adenoma-carcinoma sequence, and seem to underlie both tumor initiation and promotion in the large bowel [17]. The earliest manifestations of colorectal neoplasia are the aberrant crypt foci (ACF). ACF are visible by methylene blue staining and microscopy and can be composed of either cells with normal morphology (non-dysplastic), or dysplastic cells (Figure 1) [17]. The adenoma-carcinoma sequence is further promoted by activation of the *KRAS* oncogene [2]. *KRAS* mutations are found in approximately 50% of sporadic colorectal adenomas and carcinomas, which indicates that mutations in other unknown oncogenes might be involved in a considerable proportion of colorectal cancer cases [17]. Further progression towards the carcinoma stage is followed by LOH of the long arm of chromosome 18q [24]. Two of the tumor suppressor genes identified in this region are *SMAD4* and *SMAD2*. *SMAD4* encodes a key signalling molecule within the growth-suppressing transforming growth factor β (TGF-β) pathway. *SMAD2* encodes another member of the TGF-β pathway that is specifically mutated in a subset of colorectal carcinomas [17]. Allelic deletions of the short arm of chromosome 17p have been shown in over 75% of colorectal carcinomas [25]. The *TP53* gene, encoding for the tumor suppressor p53, has been localized to this chromosome region and is involved in the control of the cell cycle and apoptosis [24]. Chromosome 17p deletions are often late events associated with the transition from adenoma to carcinoma [25].

Furthermore, nuclear β-catenin staining and increased chromosomal instability occurs along the adenoma-carcinoma sequence.

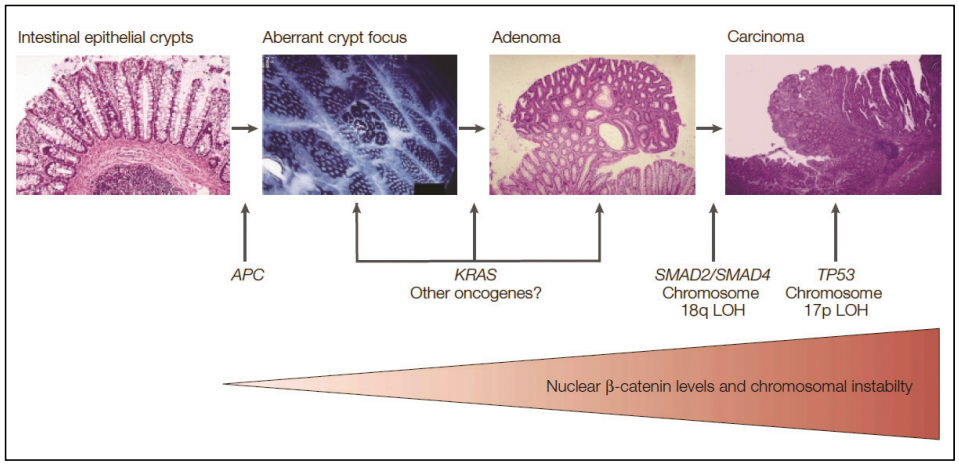

Figure 1. *The adenoma-carcinoma sequence. At least four sequential genetic changes are required to ensure colorectal cancer evolution [17].*

1.2 APC and its role in the development of intestinal tumors

APC is a large multidomain protein (2843 amino acids) located on chromosome 5q, and function as a negative regulator of the Wnt (Wingless-related integration site) signal transduction pathway [26-29]. The Wnt sigalling is essential for tumorigenesis, development and homeostasis of several cell types, such as epithelial and lymphoid cells [30]. A member of the Wnt signaling pathway is β-catenin, a protein which APC binds to [30], and has an important role in the formation of tumors, including in colon cancer [31].

In the absence of the extracellular Wnt signal, free β-catenin is bound to and phosphorylated (p) by a destruction complex (Figure 2), consisting of the scaffolding protein axin and conductin, glycogen synthase kinase 3 β (GSK3β) and APC. Phosphorylation of β-catenin by this complex earmarks it for ubiquitination and subsequent proteolytic degradation [23].

In the presence of the Wnt signal, the Wnt proteins bind to the Frizzled receptors that subsequently inactivate GSK3β through the intracellular protein Dishevelled (Dsh) (Figure 2). There is a decrease in the phosphorylation of β–catenin and an increase in its stability [32]. Failure to phosphorylate β–catenin results in its accumulation. It eventually enters the nucleus and binds to the T-cell factor/lymphocyte enhancer binding factor (TCF/LEF)

complex and activates transcription of Wnt target genes, thus leading to cancer development. Activation of the canonical Wnt pathway in the colonic epithelium seems to be one of the key events in the adenoma initiation process [33].

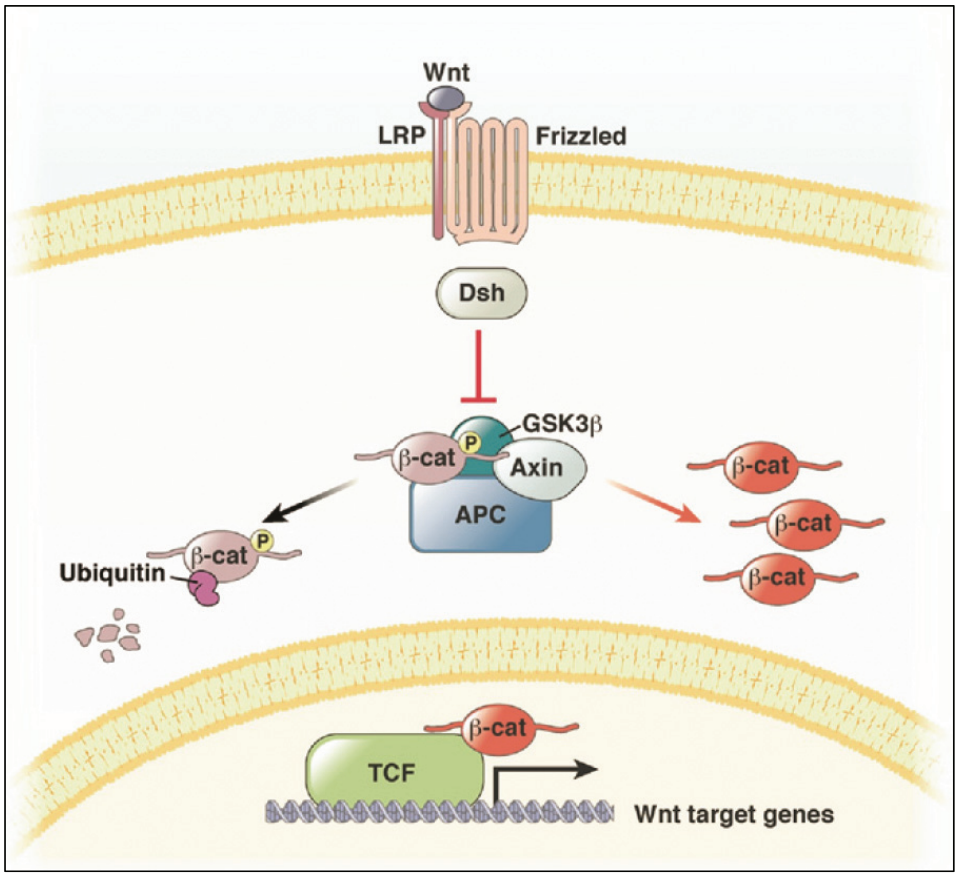

Figure 2. *Overview of the role of APC and β-catenin in the Wnt signaling pathway. In the absence of the Wnt signal (left), the destruction complex phosphorylates and targets β-catenin for degradation by the proteasome. In the presence of the Wnt signal (right), the destruction complex is inactive and β-catenin accumulates and enters the nucleus where it acts as a co-activator for transcription of Wnt target genes [23, 27], affecting proliferation, apoptosis and differentiation leading to cancer development. (Illustration from [33]).*

The Wnt pathway was first linked to cancer formation when it was found to be permanently activated in both FAP and spontaneous colon cancer [34, 35]. Mutation in the *APC* gene results in chronic activation of the Wnt pathway and accumulation of nuclear β-catenin [30], due to a non-functional APC protein which is not able to form the destruction complex. The chronic activation of the Wnt pathway in intestinal epithelial cells drives their expansion into benign adenomas, which frequently progress into invasive colon carcinoma, following additional genetic mutations later in life [36]. The *ApcMin*/+ mice have mutations in *Apc* similar to FAP patients and develop multiple intestinal adenomas soon after birth [36].

In addition to its role in the regulation of β-catenin, APC also regulates cytoskeletal proteins and is involved in cell adhesion, cell migration, mitosis, and chromosome segregation and stability [27, 37]. Despite the fact that each of these roles is potentially linked to cancer, the main tumor suppressing function of the APC protein lies in its ability to down-regulate intracellular β-catenin levels through the Wnt signal transduction pathway [17, 29].

In some cases of colorectal cancer in which *APC* is not mutated, regulation of β-catenin also fails when β-catenin itself contains mutations that prevent it from being marked for destruction [38]. β-catenin is mutated in around 10% of colorectal carcinoma, and have mainly been detected in microsatellite instabile colorectal tumors [39].

1.3 The Min mouse model

The multiple intestinal neoplasia (*Min*) mouse was identified after random chemical mutagenesis with ethylnitrosurea [40]. These mice are heterozygous for a germline non-sense mutation in codon 850 of the *Apc* gene, changing a leucine (TTG) to a stop (TAG) codon which leads to a truncated non-functional APC protein [9, 40]. As mentioned earlier (section 1.2), the *Min* mouse is a good model for the inherited disorder FAP, as well as for sporadic colorectal cancer in humans [9]. The germline mutation in the *Apc* gene causes spontaneous development of tumors mainly in the small intestine, but also in the colon, whereas in human hereditary and sporadic cancer caused by *Apc* mutations the tumors are mainly located in the colon [31]. LOH or mutation of the wild-type *Apc* allele is required in order for tumorigenesis to take place [37]. The *Min*/+ mouse model, with already one

lost *A*pc allele, are particularly susceptible to intestinal carcinogens that affect the remaining wild-type allele of the *Apc* gene [41].

These mice can develop up to 100 spontaneous adenomas in the small intestine depending on the genetic background [42]. *Min*/+ mice on the C57BL/6J background develop an average total number of 29 tumors in the small intestine and colon, while the progeny of these mice crossed to mice on another background such as AKR had only an average of 6 tumors [43]. Several *Apc* mutations have been constructed using gene knockout technology in embryonic stem cells [33]. $Apc^{\Delta 716}$ harbor a truncation mutation in codon 716 and develops ~300 adenomas in the small intestine, whereas Apc^{1638N} contains a truncation mutation in codon 1638 and forms ~3 adenomas in the small intestine. Even though these have the same background C57BL/6J, the adenoma numbers are very different, but similar to $Apc^{Min/+}$ mice mutated in codon 850, both knockout mutants also develop adenomas mainly in the small intestine [33].

The C57BL/6 is the most widely used inbred mouse strain as a genetic background for congenic and mutant mice [44]. C57BL/6J-*Min*/+ heterozygotes mice are born normally and have an average lifespan of 120 days [40, 43], whereas, homozygosity for mutant *Apc* leads to early embryonically lethality [45]. The *Min* mouse can also develop anemia by 60 days of age and die due to chronic blood loss from the gastrointestinal tract [40]. In the *Min*/+ mice, mostly adenomas are found, since death because of anemia occurs before they develop carcinomas. In order to obtain offspring from a crossing, the *Min* mutation is propagated through the males, since intestinal adenomas and anemia interfere with pregnancy [40].

The *Apc Min*/+ mice have proven to be useful in studies of colorectal carcinogenesis related to environmental factors, e.g. high fat diet or process-induced carcinogens or environmental contaminants, but also for the establishment of intersecting pathways or several effects together through crossbreeding with other mouse models [46] (see section 1.7). This model has also been the most widely used mouse model for cancer prevention studies that involve the gastro-intestinal tract [31].

1.4 The food mutagen and carcinogen PhIP

The diet is one of the major contributing factors to human cancer [47], and the frequent consumption of red meat and processed meat, especially cooked well done [48], has been positively associated with colorectal cancer in numerous epidemiological studies [1]. The cooking of meat is known to generate chemical carcinogens with genotoxic potency, such as the heterocyclic amines [49]. The most abundant of the heterocyclic amines is 2-amino-1-methyl-6-phenylimidazo [4,5-*b*] pyridine (PhIP), which was first isolated from fried ground beef [50]. PhIP has been shown to induce tumors in the colon, small intestine, cecum, breast and prostate in rats [51-53]. In mice, PhIP induces lymphomas but no intestinal tumors [54], although PhIP-induced ACF have been reported [55]. Cynomolgus monkeys treated with PhIP (10 and 20 mg/kg bw/day, 5 days/week) showed no indication of tumorigenesis on the 5th year of exposure. However, PhIP have been shown to form DNA adducts in tissues such as lung, heart, salivary gland, liver and pancreas of one monkey chronically exposed to PhIP (about 72 g cumulative dose of PhIP) [56].

In C57BL/6J-*Min*/+ mice, PhIP increased intestinal tumors by truncation mutations and LOH [57]. The predominating types of PhIP-induced truncation mutations were found to be G to T transversions and G deletions [58]. In order to be mutagenic, genotoxic and carcinogenic, PhIP must be metabolized to a reactive electrophilic arylnitrenium ion, which is able to bind covalently to DNA, forming adducts that may cause mutations and lead to induction of cancer [59, 60]. In *Min*/+ mice, a higher level of PhIP-DNA adducts was found after exposure to PhIP on day 12 after birth, compared with day 36 after birth [61]. This observation was found in all parts of the small intestine, which was in accordance with the numbers of tumors present. The levels of PhIP-DNA adducts along the intestines were highest in the middle and distal part of the small intestine, where the number of tumors also was highest [61]. The exposure to heterocyclic amines is widespread, and the most important factors affecting the yield of these mutagens in cooked meat products are the temperature, duration and method of cooking [62].

1.5 Overweight and obesity

The prevalence of overweight and obesity has escalated over the past several decades [63], and these conditions are the fifth leading risk for global deaths, causing at least 2.8 million adult deaths each year [64]. In 2008, more than 1.4 billion adults were overweight worldwide, and of these over 200 million men and nearly 300 million women were obese [64]. Obesity is a major risk factor for the development of diabetes, insulin resistance, metabolic syndrome, cardiovascular diseases and several cancers, including colon cancer [63, 65]. The metabolic syndrome is a multifactorial disease which comprises features of impaired glucose tolerance, hypertension, central obesity and dyslipidemia [66].

Overweight and obesity in adults are commonly defined by body mass index (BMI), where the weight in kilograms is divided by the square of the height in metres (kg/m^2). The World Health Organization (WHO) classifies a BMI greater than or equal to 25 as overweight, and a BMI greater than or equal to 30 as obese. The BMI provides a useful population-level measure of overweight and obesity, however, it should be considered as a rough guide because it may not correspond to the same degree of fatness in different individuals or ethnic groups [64]. Measures of the central adiposity, the waist-to-hip ratio (WHR) and waist circumference (WC) have also been commonly used in epidemiological studies [67]. The Nord-Trøndelag Health Study (The Hunt Study), followed BMI and WC in a large adult population in Norway over a 22-year period. The study reported that an increase in obesity prevalence defined by BMI was greater in men, in contrast, the obesity prevalence defined by WC was greater in women [68].

The ultimate cause of overweight and obesity is a positive energy balance, in which more calories are consumed than expended over a prolonged time. The resulting accumulation of subcutaneous and visceral fat contributes to an increase in body mass [69]. Subcutaneous adipose tissue is generally defined as fat tissue between the skin and muscle, whereas visceral adipose tissue is found within the main cavities of the body, mainly in the abdominal cavity [67]. Energy-dense diets with refined carbohydrates and saturated fat, and a sedentary lifestyle is also associated with the development of obesity [70].

1.5.1 Single gene mutations causing obesity

In addition to environmental factors, several single gene defects that cause severe human obesity have been identified, such as the genes encoding leptin and its receptors, pro-opiomelanocortin (POMC) and melanocortin 4 receptor (MC4R) [71]. Leptin is a cytokine-like peptide mainly expressed by adipocytes and believed to play a key role in the regulation of fat metabolism and energy intake. The mouse has a homologue obese gene, whose homozygous mutation causes hereditary obesity in mice (ob mice, see section 1.6). Individuals with mutation in the leptin receptor have normal birth weight, but exhibit rapid weight gain in the first few months of life, with severe hyperphagia and aggressive behaviour when food is denied [72].

POMC produce an array of smaller biologically active products including adrenocorticotropic hormone (ACTH) and α, β and γ melanocyte stimulating hormones (MSH) [73]. Homozygous and heterozygous individuals for mutations in POMC gene have been detected [74]. In neonatal life these children showed adrenocorticotropic hormone (ACTH) deficiency [75]. They are hyperphagic and develop early onset obesity, display red hair and pale skin due to the lack of α-MSH action at the melanocortin-1 receptors (MC1R) in the skin and hair follicles [75].

MC4R deficiency is the most common monogenic form of obesity [76]. Individuals with MC4R deficiency exhibit early onset obesity, hyperphagia, increase in lean body mass and marked increase in bone mineral density. The frequency of MC4R mutations in obese humans is rather high compared with mutations in leptin and leptin receptor. This is probably due to the fact that the partial loss-of-function in the heterozygous form does not appear to interfere with reproductive function and fertility [75]. Even though most of the MC4R mutations have been found in the heterozygous state, there are a few groups that have reported a number of patients with homozygous MC4R mutations. For all cases, the persons carrying homozygous mutations had a more severe phenotype than the heterozygous carriers [76].

Thus, both genetic and environmental factors are contributing to obesity. However, it is generally believed that obesity in most people is not caused by a single gene defect, but rather by multiple genetic changes as well as environmental factors.

1.5.2 Obesity and colorectal cancer

Around 11% of colorectal cancer cases have been attributed to overweight and obesity in Europe [77]. Obesity is associated with a 30-70% increased risk of colon cancer in men, whereas the association is less consistent in women. The risk seems to be lower for colorectal adenoma, although the trend is similar [77].

There are many hypotheses that try to explain why and how obesity is a risk factor for colorectal cancer. The classical view to see how overweight and obesity is related to cancer is to consider the adipose tissue as an active and metabolic "organ", which acts through endocrine, autocrine and paracrine processes [78]. White adipose tissue (WAT) is the largest energy storage organ with an important lipid storage capacity when energy input exceeds energy expenditure and with lipolytic function during energy deprivation [69]. In addition to its primary function as storage of energy, WAT has also been recognized as a major endocrine organ, with the capacity to synthesize and secrete an array of sex steroids, and bioactive peptides termed "adipokines", involved in the physiological regulation of fat storage, energy metabolism, food intake, insulin sensitivity and immune function among others [69].

Thus, it is has been hypothesised that cancer may be caused by different biological factors related to obesity and acting through diverse mechanisms, including release of growth factors and/or pro-inflammatory cytokines and insulin resistance [78].

1.5.3 Obesity as a state of low-grade inflammation

A biological factor that may support the link between obesity and colorectal cancer is inflammation [77]. Obesity is considered as a state of low-grade inflammation due to its association with increased circulating levels of pro-inflammatory cytokines [79], such as tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6) and interleukin 1β (IL-1β) [80]. Enlarged adipocytes and expanded adipose cell mass also contribute to increased circulating factors of leptin and other adipokines, but decreased levels of adiponectin [81].

TNF-α is a pro-inflammatory cytokine secreted by both immune cells and adipocytes [82]. In adipose tissue, TNF-α has been proposed to play a role in the development of insulin resistance [83], through inhibition of the insulin receptor signaling pathway [84]. It has been shown that TNF-α is over-expressed in WAT in different animal models of obesity, and the molecule is considered to be a link between inflammation and obesity [85].

IL-6 is another cytokine that has increased expression in obese adipose tissue [86]. IL-6 is produced by several cells such as fibroblasts, endothelial cells, monocytes and by adipose tissue [87]. This cytokine is important for regulating the growth and differentiation of immune cells [85]. One of the major actions of IL-6 is the control of the hepatic production of inflammatory proteins, such as C-reactive protein (CRP) [87]. It has also been demonstrated that IL-6 is able to decrease insulin action, and that the increase in circulating cytokine levels of both TNF-α and IL-6 could contribute to insulin resistance [85].

IL-1β is a pro-inflammatory cytokine [88], which exert pleiotropic effects on a variety of cells and plays a key role in acute and chronic inflammatory and autoimmune disorders [89]. The cytokine is primarily a product of monocytes, macrophages and dendritic cells, as well as B-lymphocytes and natural killer (NK) cells [88]. Inactive IL-1β precursor is cleaved by caspase-1 and converted into an active cytokine. IL-1β binds to IL-R1 to trigger a pro-inflammatory signal [88].

The adipokine leptin is secreted by adipocytes and plays an important role in the inflammatory process [90, 91]. Circulating levels and mRNA expression in adipose tissue of leptin are strongly associated with BMI and fat mass in obese individuals [85]. Plasma leptin levels were found to correlate closely with inflammatory cytokine levels (TNF-α, IL-6) and also with acute phase proteins, such as CRP [90]. Even though leptin mainly acts at the level of the central nervous system to control food intake and energy expenditure, there is a relationship between leptin and the low-grade inflammatory state in obesity, suggesting that leptin could exert peripheral biological effects as a function of its cytokine-like structure [85]. It appears that TNF-α and IL-6 are capable of stimulating adipocyte leptin production [87]. In the low-grade inflammatory milieu where the insulin function, lipid metabolism and endothelial function are adversely affected, may further result in diabetes, dyslipidemia, hypertension, metabolic syndrome and atherosclerosis [81].

Leptin has other functions than just controlling appetite and energy balance, such as playing a role in both adaptive and innate immunity [69]. Furthermore, leptin plays a critical role in regulating the reproductive system and the onset of puberty [92], where deficiency is associated with hypogonadism [93]. Leptin is also involved in glucose regulation, where it promotes insulin sensitivity [78].

1.5.4 Obesity as a determinant of hyperinsulinemia and insulin resistance

Obesity is associated with an increase in insulin release and a decrease in insulin sensitivity, which results in hyperinsulinaemia, insulin resistance and an associated increase in insulin-like growth factor (IGF) levels [77]. Hyperinsulinemia is increased blood insulin concentration, and can be caused by both genetic and environmental factors [94]. The condition is characterized by raised fasting plasma insulin levels and an exaggerated insulin response to increases in plasma glucose concentrations. Hyperinsulinemia is a compensatory response that maintains glucose homeostasis in subjects who become resistant to insulin action [94]. Insulin resistance is a state of reduced sensitivity of insulin responsive tissues to insulin [95]. With increasing insulin resistance, the pancreatic β-cells synthesize and secrete increasing amounts of insulin [94]. Chronically increased insulin levels have been associated with colon cancer pathogenesis and with cancers of the breast, pancreas and endometrium [67].

In addition to causing hyperinsulinemia in an obese state, insulin promotes the synthesis and biological activity of insulin-like growth factor 1 (IGF1) [67]. IGF1 is a peptide hormone that are structurally similar to insulin [96]. IGF1 is regulated to some extent by its six binding proteins (IGFBP1 to IGFBP6). Elevated levels of insulin may inhibit the production of IGFBP1 and IGFBP2 concentrations, resulting in increased bioavailability of IGF1.

It has been reported that IGF1 is positively correlated with body fat and WC [91]. IGF1 is a mitogen which acts as an anti-apoptotic survival factor, promotes cell migration and plays a role in glucose metabolism. Its effects are predominantly mediated by the insulin-like growth factor receptor 1 (IGF1R) [97]. A growing body of evidence suggests that the IGF1-axis might affect the development of cancer through its influence on the regulation of normal cell proliferation, differentiation and apoptosis. Inappropriate expression of IGF1 and its receptor IGF1R seems to contribute to the growth and progression of most cancers, including cancers of the colon [98].

1.6 The ob mouse model

The obese (gene symbol *ob*) mouse arose spontaneously in a colony and was discovered at the Jackson Laboratory in 1949 [99]. The *ob* mutation is located on chromosome 6, resulting in a C to T transition and changing codon for arginine 105 into a stop codon [100]. The *ob/ob* genotype results in a phenotype with marked obesity, hyperinsulinemia and insulin resistance similar to humans [101]. The mutation also cause hyperphagia and reduced energy expenditure [102]. The *ob/ob* mice are first recognizable as obese at about 4-6 weeks of age, with a shorter and rather square body. They increase rapidly in weight, and by 3 months of age they weigh about twice as much as their non-obese littermates. The weight continues to increase, but not as rapidly as up to 3 months of age. The life spans of these mice are longer than 12 months, and both male and female homozygous *ob/ob* mice are sterile [99].

Many hypotheses are proposed on why the *ob/ob* mice develop obesity, such as being a result of hyperphagia. However, the *ob/ob* mice become heavier and fatter on an identical food intake compared with their lean littermates [103]. Another hypothesis proposed by Coleman, suggested that the *ob/ob* mice were not able to produce a satiety signal to regulate its food consumption, due to the lack of a blood-borne factor [104]. This was confirmed later, when the cloning and sequencing of the mouse *ob* gene and its human homologue was done in 1994 [101]. In C57/BL6J *ob/ob* mutation, a non-sense mutation disrupts the function of a protein, which is now known as leptin (from Greek *leptos*, meaning “thin”) [105]. Leptin, a 16 kDa protein produced by WAT and plays a key role in the regulation of appetite and energy balance by inhibiting food intake and stimulating energy expenditure [69, 78]. The control of food intake is mainly mediated by the hypothalamic melanocortin pathway [73] (Figure 3). Leptin acts directly on two distinct classes of neurons, one of them co-expresses the anorexigenic peptides pro-opiomelanocortin (POMC) and cocaine and amphetamine regulated transcript (CART) which reduce food intake, while the other co-expresses the orexigenic peptides neuropeptide Y (NPY) and agouti-related protein (AgRP) which increases food intake [73]. Circulating leptin are actively transported through the blood-brain barrier and activates the hypothalamic anorexigenic neurons POMC/CART while inhibiting orexigenic NPY/AgRP neuropeptides leading to decreased appetite [69]. Circulating leptin also seems to play an important role in the regulation of puberty and reproduction [106]. Since the *ob/ob* mice are total deficient of leptin, they will exhibit infertility as mentioned earlier.

Daily intraperitoneal injections of leptin in *ob/ob* mice reduced food intake, percent body fat, body weight and serum concentrations of glucose and insulin. In addition, body temperature and metabolic rate were increased by this administration [107, 108]. Recombinant leptin is also able to restore puberty and reproductive function in the *ob/ob* mice [102, 106]. The leptin serum levels are higher in obese individuals and the concentration of leptin is positively correlated with total body fat mass also in humans, suggesting that leptin resistance develops as a result from chronic overfeeding and obesity [69]. This mouse model is an excellent model for study of obesity because it has similar genetics and phenotype as humans [102].

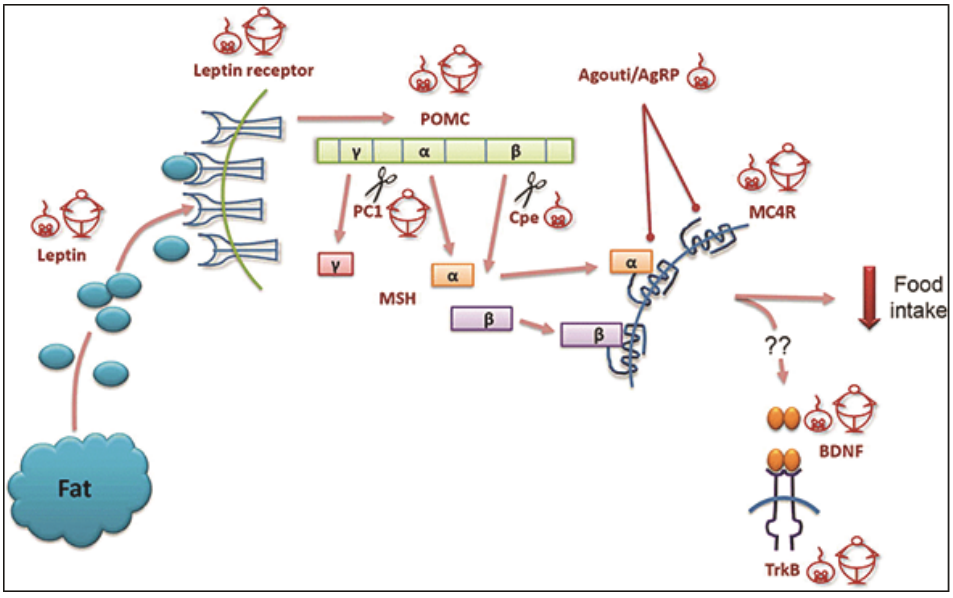

Figure 3. *The leptin melanocortin pathway and control of body weight. Disruption of this pathway results in severe obesity in mice and humans. A "fat man" or a "fat mouse" symbols represent the existence of a human monogenic obesity syndrome or an obese mouse model. PC1-prohormone convertase 1, Cpe-carboxypeptidase E, BDNF-brain-derived neurotrophic factor. Illustration from [73].*

1.6.1 Leptin mutation in humans

Mutation in the leptin gene is rare in humans, and thus not the cause of increased obesity in the general population. However, in 1997 it was reported that two children, first cousins of Pakistani origin, were homozygous for a frameshift mutation in the *ob* gene. Both children had undetectable levels of serum leptin and a syndrome of hyperphagia and severe obesity [109]. A few further affected individuals from other families in Pakistan have been identified since, with the same homozygous mutation in the leptin gene [71]. In Turkey, a family who carry a homozygous missense mutation have also been reported [72]. Treatment with recombinant leptin has been shown to have the same effects on food intake and body weight as seen in *ob/ob* mice [110].

1.6.2 Heterozygote phenotype

Heterozygote *ob/+* mice have a decreased plasma leptin concentration and are more obese than the homozygous wild-type. This observation suggest that the single normal *Lep* allele in *ob/+* mice is not sufficient to compensate for the defective allele [111]. Similar observations has been reported in heterozygous relatives of leptin-deficient humans [112]. The serum leptin levels in the heterozygous subjects were markedly lower than control subjects, especially in view of their increased mass of adipose tissue. They had also a higher prevalence of obesity compared with the control population of similar age, sex and ethnicity [112].

1.7 The combined model *Min* X *ob*

A new double mutant mouse model is obtainable by crossing the *Min*/+ mouse developing intestinal tumorigenesis with the *ob*/+ mouse developing obesity. To generate *Min* X *ob* mice, female *ob*/+ mice with a normal fertility are crossed with *Min*/+ males, both on the C57BL/6J background. Six genotype combinations are obtainable from crosses between *Min* mice and *ob* mice, via two generations (see Figure 4). First, $Apc^{+/+}$ X $Lep^{ob/+}$ females and $Apc^{Min/+}$ X $Lep^{+/+}$ males are crossed. Then, $Apc^{+/+}$ X $Lep^{ob/+}$ females and $Apc^{Min/+}$ X $Lep^{ob/+}$ males are crossed to obtain the double mutant mouse model.

The genotype combinations obtained are: $Apc^{Min/+}$ X $Lep^{ob/ob}$, $Apc^{Min/+}$ X $Lep^{ob/wt}$, $Apc^{Min/+}$ X $Lep^{wt/wt}$, $Apc^{+/+}$ X $Lep^{ob/ob}$, $Apc^{+/+}$ X $Lep^{ob/wt}$, $Apc^{+/+}$ X $Lep^{wt/wt}$. $Apc^{Min/Min}$ mice die before birth. All six genotypes are obtainable within the same litters, having similar environmental conditions, and thereby serving as each other's controls.

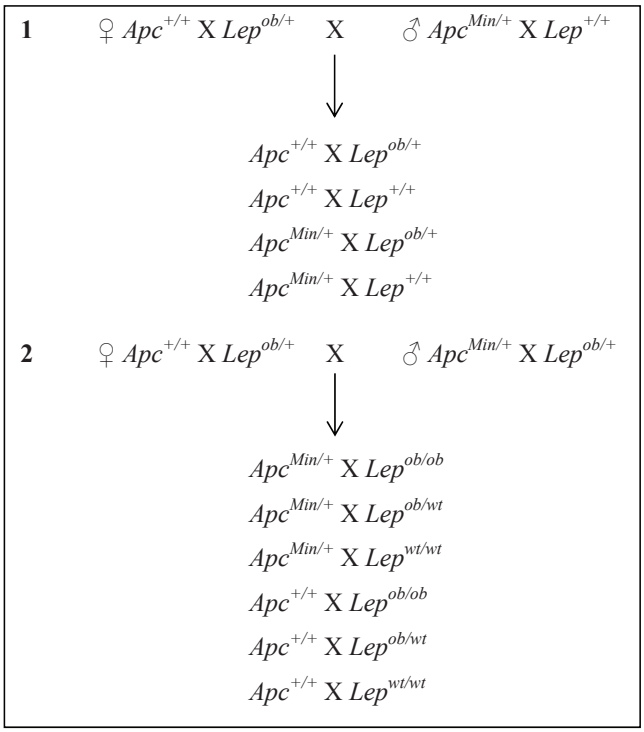

Figure 4. *Crosses between* Min *mice and* ob *mice, via two generations resulting in six different genotype combinations.*

In this new mouse model, impact of both genetic factors (the *Apc* and *ob* genes) and environmental factors (fat diet, carcinogens) on end-points such as obesity and related disorders, intestinal cancer, as well as general toxicity, can be studied in an integrated approach.

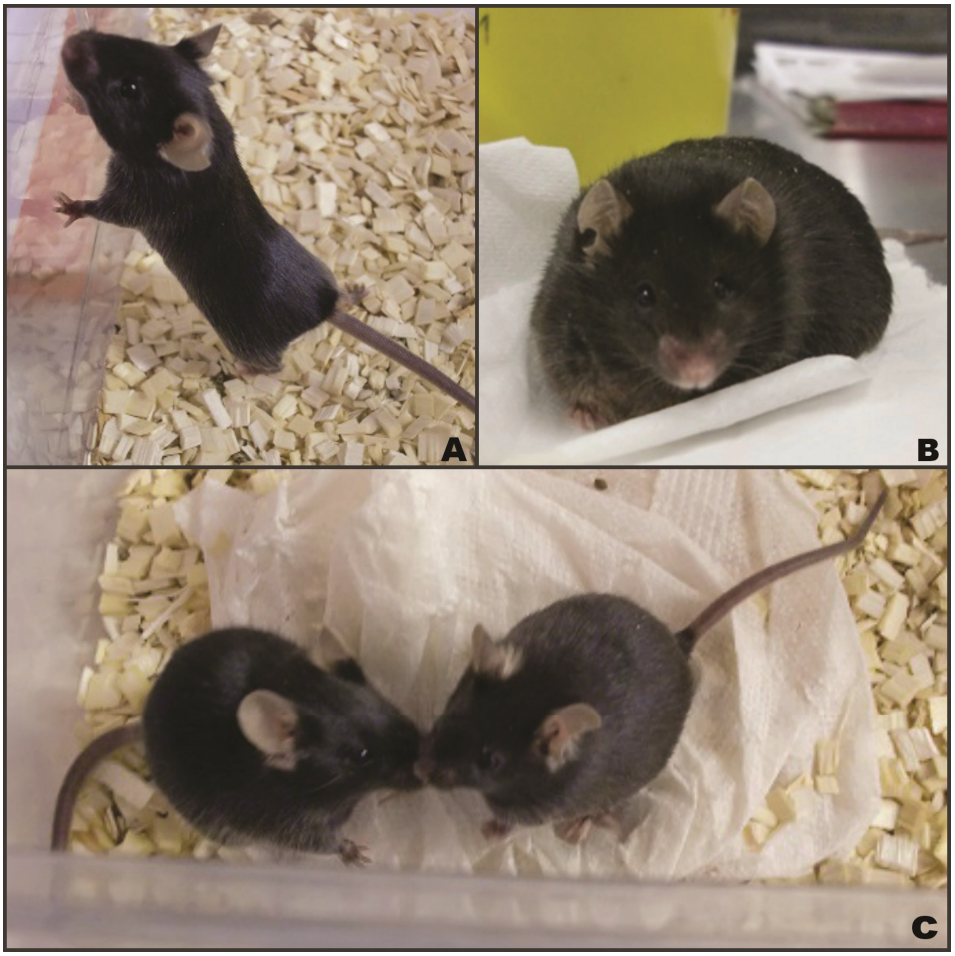

Figure 5. *A) A C57BL/6J-*Apc$^{Min/+}$ *mouse, which develops spontaneous intestinal cancer. B) A C57BL/6J-*Lep$^{ob/ob}$ *mouse, which develops obesity. C) A double mutant C57BL/6J-*Min/+ *X* ob/+ *mouse (mouse to the left), and C57BL/6J-*Min/+ *X* ob/ob *(mouse to the right) displays both obesity and intestinal tumorigenesis. (Photos: Ha Thi Ngo).*

1.8 Diet-induced obesity (DIO) model

The C57BL/6J mouse is the most widely used and a particularly good diet-induced obesity (DIO) model, because it develops a syndrome of obesity, hyperinsulinemia, hyperglycemia and hypertension when allowed *ad libitum* access to a high fat diet [113]. The obesity in the C57BL/6J mouse results from both adipocyte hypertrophy and hyperplasia [114]. However, when kept on a low fat diet, the C57BL/6J mouse remains lean and physically normal. DIO is defined as a body weight more than two standard deviations above the average body weight of mice fed a low fat diet [115].

In marked contrast to C57BL/6J, other strains such as the A/J mouse or the C57BL/KsJ are relatively resistant to this syndrome when fed a high fat diet [114, 116]. The metabolic abnormalities of C57BL/6J mice closely parallel the progression pattern of human obesity [117]. In humans, the onset of diabetes and obesity occurs gradually and often in the presence of a high fat diet. Other factors affecting DIO, in addition to mouse background, are gender and environmental factors. For instance, male mice are more affected by diabetes than female mice and therefore also used more often in DIO studies. Obese mice are sensitive to stress, and environmental factors, such as temperature, duration of light/dark periods, mice handling, food quality, cage placement etc., can all result in disturbance of the development of obesity and affect the experimental results [117].

1.9 Critical window of development

Both nutritional factors and environmental chemicals act during specific windows of sensitivity [118]. Animal studies have documented that the *in utero* and neonatal developmental periods comprise “critical windows” [119]. The effects on disease risk are likely due to multiple impacts by nutritional challenges and chemical exposures accumulated from embryonic development throughout the life-course. These sensitive periods may include early childhood, puberty, pregnancy, menopause and aging [118]. The perinatal period is a crucial time of growth, development and physiological changes in the child [120].

1.10 Early life programming

The rapid rise of obesity seen in humans during the last decades is suggested to be driven mainly by environmental factors. Although it has been much focus towards the role of current diet and lack of exercise, recent insights have also stressed the importance of nutrition during early life in the development of metabolic disorders. The phenotype of an individual can be driven by *in utero* and early postnatal environmental conditions, such as the nutritional state of the mother [121]. This has given rise to the perception of "developmental programming" or the "developmental origins of health and disease" (DOHaD). This notion proposes that environmental conditions during fetal and early postnatal development influence lifelong health and capacity through permanent effects on growth, structure and metabolism. Early life programming has been considered to be important in the etiology of obesity, type 2 diabetes, cardiovascular disease and metabolic syndrome [120].

1.10.1 The developmental origins of adult disease

The "*developmental origins of adult disease*" hypothesis, often called the "*Barker hypothesis*", originally put forward by David Barker and colleagues [122], stated that environmental factors, especially nutrition, act in early life to program the risks for the early onset of diseases like hypertension, diabetes, coronary heart disease, metabolic disorders, pulmonary, renal and mental illnesses in adult life and premature death [123-125]. Another hypothesis called the "thrifty phenotype" hypothesis proposed by Hales and Barker [126], highlighted that poor fetal and infant growth and the subsequent development of type 2 diabetes and the metabolic syndrome was a result from poor and unbalanced nutrition in early life. They further proposed that the major long-term consequences of inadequate early nutrition were impaired development of the pancreas and increased susceptibility to the development of type 2 diabetes [126].

Although the initial fetal origins hypothesis experiments dealt primarily with undernutrition and malnutrition, recent epidemiological and animals studies have studied the effects of overnutrition during critical periods of fetal development and the offspring's subsequent risk of developing the same chronic diseases associated with fetal growth restriction [127].

1.10.2 Predictive adaptive responses (PARs)

Furthermore, adaptions evolved in the fetus are processes termed predictive adaptive responses (PARs) [128], on the basis of the prenatal cues and with the aim to tune the phenotype of the offspring to meet optimally the challenges of that environment. In this way the fetus is preparing itself of an extrauterine environment with for example low food ability or high level of stress [129]. A mismatch between pre- and postnatal environment may result in inappropriate adaptions and subsequent metabolic disease [130]. This might occur if undernutrition *in utero* is followed by an abundant diet postnatally, such as a small baby born into a food-rich western society. However, there is some evidence that the converse may also occur [129]. Animals exposed to a mismatch between prenatal and postnatal environment develop obesity, leptin and insulin resistance, hypertension and vascular endothelial dysfunction. The mismatch concept may explain why effects such as obesity are now seen in developed societies even in children [128].

1.11 Obesogen hypothesis

Genetic variation contributes to an individual's propensity to develop obesity, but genetic mutations cannot account for the rapid increase in obesity rates over the last two-three decades. Environmental factors, such as nutrition and chemicals, are also being considered as contributing to the obesity epidemic [119]. In parallel with the increased prevalence of overweight and obesity, the use of chemical substances has also increased considerably. In 2002, Baillie-Hamilton postulated a role for chemical toxins in the etiology of obesity, where the exposure may have damaged many of the body's natural weight-control mechanisms [131]. The obesogen hypothesis [132] proposes that certain chemicals (natural, pharmaceutical or xenobiotic) are able to promote weight gain and obesity, especially when exposed during gestation [119]. Obesogens promote obesity by increasing the number of fat cells or the storage of fat into existing fat cells [133]. Obesogens can also act indirectly by altering the basal metabolic rate, by shifting the energy balance to favour storage of calories and alter hormonal control of appetite and satiety [133, 134]. It has been suggested that obesogen exposure during early life may modify the epigenome of multipotent stromal stem cells, biasing them to the adipocyte lineage at the expense of the bone lineage. Thus, humans exposed to obesogens during early life might have an altered stem cell compartment, which is already preprogrammed for an adipogenic outcome [133].

1.12 Endocrine disrupting chemicals (EDCs)

Many known obesogens are endocrine disrupting chemicals (EDCs) that act by disrupting the synthesis, release, transport, metabolism, binding, action or elimination of natural hormones in the body [135]. In toxicology, the classic approach is to examine high doses of environmental chemicals with the objective to determine the doses that result in negative health effects. However, EDCs are hypothesized to give effects at very low levels to which humans are usually exposed, although these exposures were previously considered "safe" because they did not cause obvious effects such as toxicity, malformations or death [119]. Both laboratory research and epidemiological studies have shown that exposure to EDCs may cause alterations in male and female reproduction and changes in neuroendocrinology, behaviour, metabolism and obesity, prostate cancer and thyroid and cardiovascular endocrinology [135].

Bisphenol A (BPA) and diethylstilbestrol (DES) are two EDCs which have been studied thoroughly, and are here described as examples of EDCs that may have obesogenic effect. Both BPA [136] and DES [137] are shown to be obesogenic in animals. BPA is an EDC produced in large quantities for the use in the manufacture of polycarbonate plastics and epoxy resins, that are ubiquitous in our environment and daily lives [138]. Low levels of BPA may be ingested by humans due to migrating from the lining of tin cans into food, from dental sealants into saliva and from plastic bottles into their content [136]. BPA is also used in electrical and electronic equipment [138]. In addition, potential environmental sources of BPA contamination are due to losses at the production site, leakage from landfill, and the presence in indoor air [139].

BPA was initially considered to be a "weak" estrogen because of its lower affinity for the estrogen receptor alpha relative to estradiol. However, research shows that BPA and estradiol are equally potent in their ability to activate responses via recently discovered estrogen receptors associated with the cell membrane [140]. Recent evidence has shown that BPA also exhibits other modes of endocrine disruption in addition to binding to estrogen receptors, such as alteration in the synthesis or metabolism of endogenous hormones. BPA exposure may also result in changes in tissue enzymes and hormone receptors, and interacts with other hormone response systems, such as the androgen and thyroid hormone receptor signaling system [139].

It has been confirmed that exposures to low doses of BPA during the perinatal period of development results in an increase in adult body weight in Sprague-Dawley rats [136]. This study by Rubin *et al.* suggests that low-dose exposure to BPA may be more detrimental than high doses, and that persistent exposure to low doses of the compound over long periods may be as effective as shorter exposures to somewhat higher levels [136]. For some EDCs such as BPA, binding to its receptor results in hormone-like effects that may occur at low doses with dose-response relationships that may be non-monotonic [118], such as U-shaped dose-response curves (also termed "hormesis"). These curves show that the effects of BPA occur at both very low and much higher concentrations and may disappear or diminish at intermediate concentrations [141]. Because of this non-traditional dose response relationship, the effects of low EDCs doses cannot be predicted by extrapolation from high dose testing results [118].

DES is a synthetic estrogen that was used for numerous purposes including by the livestock industry as a growth promoter of cattle. DES was administered to pregnant women from the late 1940s through the 1970s for the prevention of threatened miscarriages and premature births [142]. Approximately 2-8 million treated pregnancies worldwide have been estimated [143]. This treatment was stopped after links to vaginal and cervical cancers were identified in the exposed daughters [138]. It is now well recognized that prenatal exposure to DES results in a low incidence of neoplasia in the female offspring, and a high incidence of benign abnormalities in both male and female offspring [142].

Prenatal or neonatal exposure to a low dose (1 μg/kg bw/day) of DES also caused an increase in body weight of CD-1 mice as adults [143], similar to the effect of exposure to BPA. DES-exposed mice had 27.6% body fat compared to controls with 20.9% body fat at 16 weeks of age [143]. A higher dose of DES (1 mg/kg bw/day) caused a significant decrease in body weight during treatment (days 9-16 of gestation or days 1-5 of neonatal life), which was followed by a "catch-up" period around puberty that later resulted in an increase in body weight of the DES-treated mice compared with the controls at about 2 months of age [144]. The treatment time period days 9-16 of gestation is the period of major organogenesis in the mouse, while days 1-5 of neonatal life is a period of cellular differentiation of the reproductive tract, and a critical time of immune, behavioural and adipocyte differentiation [144].

1.13 Perfluoroalkyl acids PFOA and PFOS

Perfluoroalkyl acids (PFAAs) is another large group of chemical substances detected globally in the environment, wildlife and human serum as a mixture of individual members of the PFAA class [145]. The two most widely known PFAAs are perfluorooctanoate (PFOA) and perfluorooctane sulfonate (PFOS) [146]. These substances are man-made fluorinated organic compounds used in many consumer goods, such as clothing, carpeting, upholstery, food packaging materials etc., and as surfactants in industrial production, due to their grease and water-repellant properties [147].

PFAAs have carbon backbone of varying length, where hydrogen is substituted with fluorine and there is a functional group. PFOA contains a seven-carbon backbone, while PFOS have an eight-carbon backbone and the functional groups carboxylic acid and sulfonic acid, respectively. The carbon-fluorine bonds are extremely strong and stable [148], making these substances wide-spread in the environment. PFOA and PFOS have been detected in surface water, air, sludge, soils, sediments and ice caps around the world [146]. They have also been detected in blood samples in both general non-exposed and occupationally-exposed human populations [149].

In addition to being stable and persistent, PFAAs are readily absorbed, not known to be metabolized, are poorly eliminated and undergo extensive uptake from the enterohepatic circulation, with estimated half-lives in humans of 3.8 years for PFOA and 5.4 years for PFOS [146, 147, 150]. PFOA and PFOS are distributed mainly in the serum, kidney and liver, with liver concentrations being several times higher than serum concentrations [146]. The most common route of exposure is oral intake from diet and water, and for infants also via breast milk, while dermal absorption and inhalation are less important [147, 151].

Animal studies have demonstrated developmental toxicity, reproductive toxicity, hepatotoxicity, neurotoxicity, immunotoxicity and carcinogenicity of the perfluorinated compounds [152-154]. Hepatocellular adenomas, testicular Leydig cell adenomas and pancreatic acinar cell adenomas have been reported in rats after exposure to PFOA in the diet for 2 years [155, 156]. A 2-year study of PFOS given in the diet to rats reported hepatocellular and thyroid follicular cell adenomas and mammary fibroadenomas/adenomas [157]. CD-1 female mice exposed to low-dose PFOA *in utero* had increased body weight in mid-life, along with increased serum insulin and leptin. These results were not seen in mice exposed to high doses of PFOA [158]. So far, it

appears to be no reports from tumorigenicity studies on mice, and no tumorigenic effects have been reported in the intestines of rats or mice, with PFOA or PFOS. Negative results in many *in vitro* and/or *in vivo* tests at gene and/or chromosome or DNA repair levels indicate that PFOA and PFOS are not genotoxic [159].

2 GENERAL DISCUSSION

2.1 The animal models – how good are they?

Two mouse models were used in this thesis to study the effects of environmental contaminants, genes and diet in obesity and intestinal cancer; the *Apc*$^{Min/+}$ mouse in **Paper I, III,** and **IV** and the *Lep*$^{ob/ob}$ mouse in **Paper I,** both on C57BL/6J background. In **Paper II**, wild-type C57BL/6J mice were used.

The *Min/+* mouse model has one mutated or lost *Apc* allele and is particularly sensitive towards environmental influences that affects the remaining wild-type allele of the *Apc* gene, which makes this an excellent model to study the tumorigenic potential of genotoxic chemicals, for instance contaminants formed during food-processing. The *Min/+* mouse is a frequently used model to study intestinal tumorigenesis, because the mutation in *Apc* is one of the earliest events in tumor development in the intestine in both rodents and humans.

Although the *Min/+* mouse has been the most extensively studied model for intestinal tumorigenesis, the model has some limitations. Development of adenocarcinoma, which is the most common type of colorectal cancer in humans, is rare in this model. These mice spontaneously develop multiple intestinal adenomas soon after birth, but die from anemia before adenocarcinomas are developed [9, 40]. Instead adenomas are used as a surrogate end-point [31]. Another difference between this mouse model and human colorectal cancer is location of the lesions, with a predominance of tumors in the small intestine, in contrast to the human tumors, which occur mainly in the colon [31, 37]. There is still no adequate explanation for these discrepancies, but they are likely due to a combination of factors such as differences in intestinal biology, environmental influences, including diet, and genetics. The time scale of the tumorigenesis is another factor differing between this mouse model and humans. The time period for development of the disease is much longer in humans, in part reflecting the time needed to acquire additional mutations in genes causing colorectal cancer evolution [37].

The *ob* mice used in **Paper I** have a non-sense mutation in the *ob* gene, coding for the hormone leptin. This mutation disrupts the function of leptin, thereby causing obesity (see 1.6). This mouse model is an excellent model for study of obesity because it has similar

genetics and phenotype as humans. Animal models for obesity are important to increase our understanding of mechanisms in order to develop treatments for obesity.

Humans with leptin deficiency have shown the same profound effects on body weight during recombinant leptin administration as seen in the *ob* mice. However, most humans with obesity do not have leptin deficiency. Instead they have hyperleptinemia and leptin resistance, and thus generally do not respond with weight loss during recombinant leptin treatment. This discrepancy highlight that although the *ob* mouse is a valuable and useful animal model of obesity, it does not reflect perfectly the common obesity in humans, which is not often caused by monogenic mutations. This mouse model may therefore not always be predictive for the effects of pharmaceutical treatments in humans [160].

In **Paper I and III**, the mice were given one subcutaneous injection of 25 mg/kg body weight of the food mutagen PhIP on days 3-6 after birth. PhIP was used to study carcinogen induced intestinal tumorigenesis. As mentioned in the general introduction, PhIP has been shown to induce tumors in the small intestine, colon, cecum, breast and prostate in rats. In mice, PhIP induces lymphoma, but no intestinal tumors. PhIP has also been shown to form DNA adducts in organs such as the liver, lung, heart and pancreas of the cynomolgus monkey. The response to PhIP shows species differences in target organs for toxicity. However, the basic mechanism is the same, i.e. DNA adduct formation and mutation, leading to cancer development. It is common that toxic chemicals can affect different organs in different animal species.

Human studies are of great value in study of association between maternal nutritional status and offspring outcome. However, they are confounded by the influence of uncontrollable variables of genetic and environmental factors. In **Paper III** and **IV,** the mice were exposed *in utero* to a high fat diet and the environmental contaminants PFOA and PFOS, respectively. In animal models, the conditions which the offspring are exposed to can be controlled, and data collection can be performed during various stages of the exposure, providing an invaluable insight into the mechanisms of developmental programming [121].

Mice are small and easy to handle. They are also cheaper than other larger animals to propagate and maintain, hence most experimental research into etiology of human diseases uses mouse models [102].

2.2 The association between obesity and cancer

There are now sufficient evidence from both epidemiological and experimental studies to support the casual link between cancer and obesity. However, this does not apply for all types of cancer, some cancer are not associated with obesity. Cancers that have been consistently found at higher risk in overweight and obese individuals are breast cancer after menopause, cancer of the colon and rectum, as well as cancer of the endometrium [161].

However, discussions about the association between obesity and cancer raise questions about what is the cause, and what is the effect. Another hypothesis for the connection between obesity and cancer is instead of obesity causing cancer, what if obesity is a parallel consequence of another factor that also causes cancer? For instance, increased insulin may cause obesity, instead of obesity causing cancer via increased insulin (see section 2.3.1). Most researchers believe that obesity is the cause of insulin resistance, therefore to treat insulin resistance the obesity is being treated, by advising obese persons to lose weight. What if obesity is not the cause of increased insulin at all, but rather a symptom or a coping mechanism for a more serious problem going on underneath, in the cells? Maybe the perception of what is the cause and what is the effect has been wrong about obesity and insulin resistance. Is it possible that insulin resistance causes weight gain and the diseases associated with obesity, at least in most people? It is important to get the cause and the effect right, because it is better to be treating the cause rather than the effect.

Obesity is excess accumulation of fat that develops over time and do not happen spontaneously. Obesity may be the end result of nutrients in the diet, such as carbohydrate that stimulate insulin. Nutrients behind the obesity should be focused on to prevent the insulin increase. The diet can be considered as a regulator or stimulator of hormones. It has been shown in diabetes that when carbohydrate is restricted, the metabolic dysfunction is lowered, even before weight loss. The same might apply in cancer, where animal models have shown that when certain pathways involved in metabolic disturbance is inhibited, they do not develop cancer. It has also been shown in human studies i.e. in the Swedish Obese Subjects (SOS) study with more than 4000 patients who underwent bariatric surgery, that the cancer incidence was reduced in women, although not in men, but not because of weight loss [162]. This suggests that cancer is not directly associated with

obesity, and that the beneficial effects of bariatric surgery on cancer are mediated by other mechanisms than weight loss.

There have also been discussions about which approach will have the largest impact on decreasing cancer, of calorie restriction or low carbohydrate diet in the population. Calorie restriction is effective, but is difficult to continue on for longer time. However, it is difficult to know if the effect on obesity and cancer is caused by reduced calories or if it may be because of the reduced carbohydrate intake that follows.

BMI is a poor measure of obesity, and there are many relatively thin persons with normal body weight or BMI, that are metabolic obese and at risk for various illnesses. They are often hyperinsulinemic, thus these persons should be advised to lower their insulin levels, and their insulin levels should be measured regularly.

2.3 The two hypotheses for association between obesity and cancer

In this thesis, two hypotheses for the association between obesity and intestinal tumorigenesis were tested; disturbed glucose regulation (**Paper I-IV**) and increased inflammation (**Paper I and II**).

2.3.1 Disturbed glucose regulation

In **Paper I**, the *ob/ob* mice showed increased intestinal tumorigenesis and also showed a much higher blood glucose level both non-fasted and fasted compared with the *ob/wt* and *wt/wt* mice, indicating disturbed glucose regulation with hyperglucosemia and insulinemia. The situation was even worse in *ob/ob* mice given a 45% fat diet.

Recent data suggest that dietary and related factors may influence colorectal cancer risk via their effects on serum insulin concentrations and on the bioavailability of IGF1 [94]. Insulin and IGF1 are thought to activate multiple signaling pathways associated with elevated tumorigenesis (Figure 6). Insulin may down-regulate the production of insulin growth factor binding protein 1 and 2 (IGFB1 and 2), which normally bind IGF1 and inactivates it. The increase in free IGF1 leads to increased cellular proliferation and inhibition of normal apoptosis in both normal and cancer cell lines [163]. The increased bioavailability of IGF1 may over time increase risk of colorectal cancer [94]. IGF1 is also capable of promoting angiogenesis and there is a significant link between insulin/IGF1 and the ras proto-oncogene, which induces transformation from adenoma to invasive

carcinoma [163]. The expression of IGF1 was increased in the liver in mice fed a high fat diet in **Paper II**, indicating disrupted blood glucose regulation and associated increased colon cancer risk.

The cytokines TNF-α and IL-6 produced by adipose tissue can induce insulin resistance [95]. TNF-α levels were increased in plasma of *ob/ob* mice on a 45% fat diet in **Paper I,** and expression of IL-6 was increased in DIO mice **Paper II** (section 2.3.2). The increased levels of these inflammatory markers may modulate insulin resistance and contribute to the tumorigenesis.

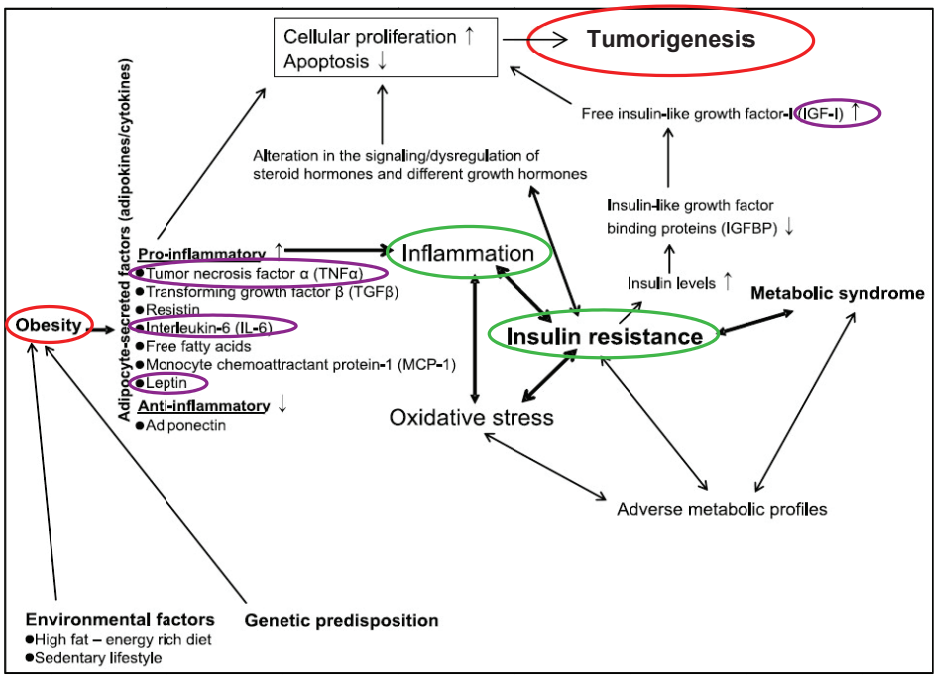

Figure 6. *Possible mechanisms for how obesity can increase cancer risk. An increased amount of adipose tissue in an overweight or obese person probably influences the development of cancer, by releasing several hormone-like factors or adipokines. The majority of adipokines are pro-inflammatory (such as IL-6, TNF-α, MCP-1 and leptin), which are involved in promotion of inflammation and pathological conditions such as insulin resistance and tumorigenesis through increased cell proliferation and decreased apoptosis. Modified from [70].*

2.3.2 Inflammation

Inflammation is thought to be a link between obesity and colorectal cancer. We measured the pro-inflammatory cytokines TNF-α, IL-6 and IL-1β in the DIO model in plasma and tissues (intestines and liver) in **Paper I and II**, respectively. In **Paper I**, increased TNF-α levels were found in *ob/ob* mice on a 45% fat diet. However, there were no significant differences in levels of IL-6 between the treatment groups. In **Paper II**, the pro-inflammatory markers IL-6 and IL-1β had up-regulated expression in the liver. In addition, the expression of the chemokine CXCL1 and the acute phase protein haptoglobin was also increased in the liver, supporting the hypothesis of obesity being a low-grade inflammatory state.

Consumption of a high fat diet along with a sedentary life style and/or genetic predisposition can lead to accumulation of excess body fat that is associated with adipose tissue dysfunction and a chronic state of low-grade inflammation. There are two types of evidence indicating obesity as a chronic inflammatory state; the increased secretion of both pro-inflammatory cytokines and acute-phase proteins, and infiltration of macrophages into the white adipose tissue. The pro-inflammatory markers include TNF-α, IL-6, IL-1β, chemokine monocyte chemoattractant protein-1 (MCP-1) and adipokines (leptin, resistin) (Figure 6), CRP and haptoglobin. There is also a reduction of anti-inflammatory adipokines (e.g. adiponectin) [69, 86]. MCP-1 is a major chemokine for macrophage recruitment [80].

Inflammation has been linked to the development of colon cancer, through several processes, including cellular transformation, promotion, survival, proliferation, invasion, angiogenesis, and metastasis. TNF-α has been shown to have controversial roles in cancer, acting as a tumor-promoting or tumor destructive factor [164]. IL-6 is another important mediator of acute inflammation [165], and a potent stimulator of colon cancer cell proliferation and tumor growth [166]. IL-1β is the master inflammatory cytokine in the IL-1 family [88] and can increase tumor invasiveness and metastasis, by promoting angiogenic factor production by stromal cells in the tumor microenvironment [167]. The mentioned inflammatory cytokines TNF-α, IL-6 and IL-1β have all been associated with poor outcome in colon cancer [80]. The precise role of these inflammatory components in carcinogenesis is not completely understood, thus continues to be an interesting topic of research [69].

2.3.3 Other mechanisms

However, there are other causes of cancer than the two discussed in this thesis. For instance, hormones such as estrogen and androgens may be involved. Adipose tissue is rich in the enzyme aromatase, which converts androgens into estrogens. In the obese, the adipose tissue produces estrogen. The higher the blood estrogen level, the higher the risk of endometrial cancer [161]. In addition, obese persons with insulin resistance have higher levels of insulin because they need more insulin to use their glucose. The high level of blood insulin causes a negative feedback on the production of sex hormone binding globulin (SHBG) in the liver. This normally binds estrogens and androgens. However, SHBG is down-regulated by insulin, leading to increased levels of estrogens and androgens. Overweight and obese people who develop insulin resistance have increased blood insulin, which result in an increased proportion of free estrogens and androgens.

2.4 Importance of critical windows of exposure for the effect

Both nutritional imbalance and environmental chemicals may act during critical windows of susceptibility. The results from **Paper III** demonstrated that the intrauterine and nursing period is a window of susceptibility for exposure to a high fat diet for development of obesity as adults. The obesogenic exposure also increased intestinal tumorigenesis in the offspring as adults.

The nursing period also appears to be a key time period in which the offspring's development can be influenced. The postnatal period is a period of continual growth and development, and has been shown to be susceptible to factors associated with a later increase in obesity [121]. Developmental programming is therefore not limited to the *in utero* environment and as physiological systems continue to develop after birth, a high fat diet may have a considerable impact also postnatally [121].

General factors that may contribute to an increased susceptibility in the fetus and/or neonate to genotoxic carcinogens compared to adults are higher rates of cell proliferation, lower rates of apoptosis or lower degree of differentiation, higher ratio of organ weight to body weight increasing the number of target cells, decreased capacity to detoxify carcinogens or lower DNA repair, lower immunological competence, or lower rates of excretion because of immature kidney function [168-171]. It was shown that the most susceptible period for tumorigenesis of PhIP in the small intestine of *Min*/+ mice was

between days 3-12 after birth, compared to much lower tumor numbers if exposed at day 36, whereas in the colon it was from day 3 before to day 3 after birth, correlating with levels of PhIP-DNA adducts [61].

However, as reported in **Paper IV**, there were no obesogenic effect in either *Min/+* or wild-type mice after exposure *in utero* to PFOA or PFOS, even when given in a putative susceptible period for development of obesity. Neither of these substances increased the number of intestinal tumors in the *Min/+* mice. Thereby, these results refute the obesogen hypothesis for these substances.

The critical period of susceptibility observed in rodents cannot be transferred directly to humans because of the obvious differences across species in development. For instance, some developmental events occur postnatally in rodents but prenatally in humans, and the "childhood" period is very short in rodents compared with that period in humans [172].

The effects of nutrient imbalance or exposure to environmental chemicals during development can cause changes that do not necessarily need to be detectable at birth. Some changes are not apparent until the appropriate stimuli are present later in life [118].

2.5 Relevance for humans

The study of intestinal tumorigenesis using the *Min/+* mice with mutation in *Apc* is relevant for both FAP and sporadic cancer in humans, because they share the same mutation. Although, the localization of tumors occurs in the small intestine of mice instead of in the colon, the mechanism is still relevant for comparison with humans.

The DIO model shows body weight responses and progressive loss of leptin sensitivity which resemble closely the human situation, making these animal models useful in the identification of contributing pathways during obesity development [66].

Even though there are only a handful of people in the world with mutation in the leptin gene, the phenotype of humans with leptin deficiency is very similar to that of *ob/ob* mice. Leptin-deficient infants are born with a normal birth weight, but soon after weaning they exhibit hyperphagia resulting in early-onset obesity [73].

Rodent models have been extensively used to investigate the developmental programming via maternal obesogenic environment. Exposure to a high fat diet during pregnancy and

lactation results in the development of a phenotype which is comparable to that of the human metabolic syndrome [66].

A maternal high fat diet has been shown to induce hyperglycaemia and hyperinsulinemia in the offspring in adulthood [173]. This is associated with altered pancreatic development and a reduction of glucose stimulated insulin secretion [66]. Changes to pancreatic structure and function are observed in fetal life, and these changes persist to adulthood [173]. The increasing knowledge gained from animal studies suggests that precautionary measures regarding dietary habits should be taken during pregnancy and postnatal development, to minimize offspring risk for enhanced susceptibility to develop metabolic disease as adults [174].

Human relevant doses, either as found in the general population or in industrially exposed populations, were used of PFOA and PFOS (0.01, 0.1, 3.0 mg/kg bw/day), thus the experiment was relevant to the human situation. A possible source of PFAA exposure is breast milk both for animals and humans. Exposure to PFOA has been associated with impaired lactation in mice, and this might also apply for humans [175].

3 FUTURE PERSPECTIVES

In this thesis, we have studied different aspects of the interplay between environmental contaminants such as PhIP, PFOA and PFOS, 45% and 10% fat diet, and the genes *Apc* and *ob* in obesity and intestinal cancer.

We tested two hypotheses for the relationship between obesity and intestinal cancer; disturbed glucose regulation and increased inflammation. In **Paper I** and **III**, a GTT was performed where the obese mice showed disturbed glucose regulation and insulinemia. Another approach would be to perform an insulin tolerance test (ITT) in addition to GTT in the mice in order to check for insulin resistance. Measurement of leptin in blood could be performed to check for leptin resistance in the obese mice.

In **Paper II,** expression of haptoglobin was measured in the liver. Another acute phase protein is CRP, which increases rapidly in response to inflammation. Thus, CRP could be measured in plasma or serum to check the inflammatory state in the obese mice. In addition, serum cholesterol is also a potential parameter that can be compared in obese and lean mice. In **Paper II**, gene expression of inflammatory markers was measured in organs such as the liver, small intestine and colon. It would also be of interest to measure gene expression of the inflammatory markers in adipose tissue of these mice.

We found that exposure to a high fat diet *in utero* and during nursing comprise a critical window both for increased body weight and intestinal tumorigenesis in **Paper III**. It would be interesting to study these effects on body weight and intestinal tumorigenesis in additional generations and see if the offspring inherit these effects, and if so, whether this may be caused by some epigenetic phenomena. Collection of additional information about the dams during pregnancy concerning maternal obesity and gestational diabetes could be of interest in relation to any long-term health effects of the offspring. Maternal obesity in humans has been associated with several long-term adverse health outcomes in the offspring, including lifelong risk of obesity and metabolic dysregulation, with increased insulin resistance, hypertension and dyslipidemia, as well as behavioural problems and risk of asthma [176].

It was investigated whether the environmental contaminants PFOA and PFOS had obesogenic effect, and if they were able to increase spontaneous tumorigenesis, with negative results in **Paper IV**. Exposure to PFOA has been associated with high cholesterol

in humans, thus measurements of cholesterol in blood could be performed in these mice. More research is needed in order to provide additional knowledge about the possible effects of these substances on other end points.

4 CONCLUSIONS

1) Obesity induced genetically or by a 45% fat diet increased spontaneous intestinal tumorigenesis. The carcinogen (PhIP)-induced intestinal tumorigenesis was not further increased with a 45% fat diet. The obesity was also associated with hyperglucosemia, disturbed glucose regulation and insulinemia, and with inflammation as seen by increased TNF-α levels (**Paper I**).

2) A high fat diet increased the expression of the inflammatory markers IL-6, IL-1β, the chemokine CXCL1, IGF1 and its receptor IGF1R and haptoglobin in the liver. Hypothetically, inflammation in the liver could possibly have an effect on inflammation also in the intestines, which could be linked to increased colorectal cancer. Increased expression of IGF1 and its receptor IGF1R in the liver may indicate disrupted blood sugar regulation, which is also associated with increased colon cancer risk (**Paper II**).

3) The intrauterine and nursing period is a window of susceptibility for exposure to a 45% fat diet for development of obesity as adults, and this obesogenic exposure has adverse health effects such as increased intestinal tumorigenesis in the *Min*/+ mice. However, this early exposure to a high fat diet did not disturb the glucose regulation, as opposed to exposure to a high fat diet as adults or throughout life (**Paper III**).

4) Exposure of C57BL/6J-*Min*/+ mice and their wild-type siblings *in utero* to PFOA or PFOA did not have obesogenic effect in either *Min*/+ or wild-type mice, at least not up to 11 or 20 weeks of age, respectively, nor did they increase the number of intestinal tumorigenesis in the *Min*/+ mice (**Paper IV**).

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6 APPENDIX: PAPERS I-IV

I

II

III

IV