Effects of meat and meat components on intestinal carcinogenesis in the A/J Min/+ mouse model

Philosophiae Doctor (PhD) Thesis

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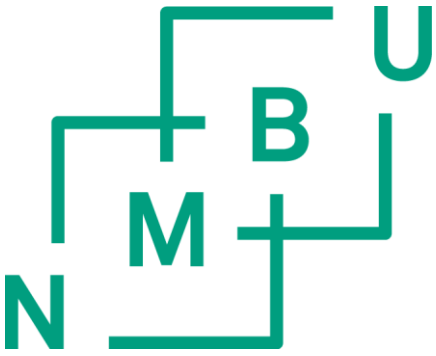

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Oslo, 20.12.2016

Abbreviations

ACF	Aberrant crypt foci
AOM	Azoxymethane
APC/ Apc	*Adenomatous polyposis coli;* lower case: rat or murine form of *adenomatous polyposis coli*
B6	C57BL/6J
CHF	Cytotoxic heme factor
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
CpG	Cytosine-phosphate-guanine
CRC	Colorectal cancer
CRP	C-reactive protein
DMH	1,2-dimethylhydrazine
DSS	Dextran sodium sulfate
FAP	Familial adenomatous polyposis
HCAs	Heterocyclic amines
H_2S	Hydrogen sulfide
IARC	International Agency for Research on Cancer
IBD	Inflammatory bowel disease
KRAS	Kirsten rat sarcoma viral oncogene homolog
LOH	Loss of heterozygosity
MDA	Malondialdehyde
Min	Multiple intestinal neoplasia
Mom	Modifier of min
MSI	Microsatellite instability
Neu5Gc	N-glyconylneuraminic acid
Nrf2	Nuclear factor (erythroid derived 2)-like 2
NOCs	N-nitroso compounds
OTUs	Operational taxonomic units
Pirc	Polyposis in the rat colon
PUFA	Polyunsaturated fatty acids
SCFA	Short-chain fatty acids
TBARS	Thiobarbituric acid reactive substances
Tp53	Tumor protein p53
WCRF	World Cancer Research Fund
Wnt	Wingless-related integration site
WT	Wild type

List of papers

Paper I:

Steppeler C, Haugen JE, Rødbotten R, Kirkhus B.

Formation of Malondialdehyde, 4-Hydroxynonenal, and 4-Hydroxyhexenal during *In Vitro* Digestion of Cooked Beef, Pork, Chicken and Salmon.

Journal of Agricultural and Food Chemistry. 2016. 64(2):487−496

Paper II:

Steppeler C, Sødring M, Paulsen JE.

Colorectal Carcinogenesis in the A/J Min/+ Mouse Model is Inhibited by Hemin, Independently of Dietary Fat Content and Lipid Peroxidation Rate.

BMC Cancer. 2016. 16:832

Paper III:

Steppeler C, Sødring M, Egelandsdal B, Kirkhus B, Oostindjer M, Alvseike O, Gangsei LE, Hovland EM, Pierre F and Paulsen JE.

Effects of Beef, Pork, Chicken and Salmon Meat on Intestinal Carcinogenesis in A/J Min/+ mice.

Submitted manuscript (PLOS ONE)

Paper IV:

Rud I, Steppeler C, Boysen P, Paulsen JE, Måge I.

Inflammation-induced colonic carcinogenesis in A/J Min/+ mice is dependent on the intestinal microbiota.

Manuscript

Summary

Colorectal cancer (CRC) represents a major public health burden worldwide, and particularly in the developed countries. Sporadic CRC has been associated with a number of lifestyle factors, including smoking, alcohol consumption, physical inactivity, and dietary habits. Despite of being a source for high biological value proteins and essential micronutrients, in 2015, the International Agency for Research on Cancer (IARC) classified red and processed meat as "probably carcinogenic to humans" (Group 2A) and "carcinogenic to humans" (Group 1), respectively. The conclusion was based on evidence from epidemiological investigations that support the association, as well as mechanistic evidence from animal studies. One potential mechanism that may explain the link between red or processed meat and CRC, involves heme iron from red meat. This hypothesis suggests that the ingestion of heme iron may affect the intestinal, epithelial homeostasis by enhancing unfavorable chemical processes, e.g. lipid peroxidation.

Heated beef, pork, chicken, or salmon was digested in an *in vitro* digestion model, comprised of a simulated oral, gastric and small intestinal phase. During *in vitro* digestion, lipid peroxidation was monitored by measuring the formation of malondialdehyde (as thiobarbituric reactive substances, TBARS), 4-hydroxynonenal and 4-hydroxyhexenal. Salmon and chicken, both containing high levels of unsaturated fat, were shown to be more prone to peroxidation than beef and pork. Nevertheless, lipid peroxidation rates were found to be enhanced by both heme iron and fat level, and the combination of beef and fish oil resulted in the highest rates of lipid peroxidation under *in vitro* conditions.

The association between red meat and CRC was then tested directly in the A/J Min/+ mouse, a model for *Apc*-driven CRC. A/J Min/+ mice develop numerous tumors in the small intestine and in the colon, and malignant tumors (carcinomas) are observed at high age. In contrast to what was expected, dietary heme iron, provided by hemin, inhibited colonic carcinogenesis in young adult A/J Min/+ mice. When the effects of heated beef and pork (red meat) were compared to chicken (white meat) and salmon (fish), dietary salmon was found to result in the lowest tumor load, whereas the effects of dietary red meat on intestinal carcinogenesis did not differ from the effects of dietary white meat. As in the *in vitro* digestion study, gastrointestinal formation of TBARS was enhanced by dietary heme iron and fat. However, no association could be established between intestinal carcinogenesis and luminal lipid peroxidation. In addition, fecal water

cytotoxicity tested on cultivated *Apc*$^{-/+}$ cells, was not related to intestinal carcinogenesis. Indications were given, however, that the amount of heme iron that reaches the colon in A/J Min/+ mice, may be lower than the amount of heme iron that reaches the colon in other rodent models.

As no carcinogenic effects of heme iron or red meat were observed in the intestines of A/J Min/+ mice, the susceptibility of the A/J Min/+ mouse model to intestinal carcinogenesis was confirmed in an additional study. Dextran sodium sulfate (DSS), a non-genotoxic carcinogen, which induces inflammatory processes in the colon, was tested in the A/J Min/+ mouse, and was shown to enhance colonic carcinogenesis substantially. Moreover, the study revealed a role of microbiota in the colonic carcinogenesis in A/J Min/+ mice.

Sammendrag (Summary in Norwegian)

På verdensbasis, og spesielt i de rike industrilandene har kolorektalkreft (CRC) blitt et folkehelseproblem. Sporadisk CRC har blitt knyttet til livsstilsfaktorer som røyking, alkohol, fysisk inaktivitet og ernæring. Kjøtt er en biologisk høyverdig proteinkilde og kilde til essensielle mikronæringsstoffer. Allikevel ble rødt kjøtt og bearbeidet kjøtt klassifisert som henholdsvis «sannsynlig carcinogen for mennesker» (Gruppe 2A) og «carcinogen for mennesker» (Gruppe 1) av International Agency for Research on Cancer (IARC) i 2015.

Konklusjonen var i hovedsak basert på holdepunkter fra epidemiologiske studier som støtter assosiasjonen, og dyrestudier som undersøkte de mekaniske sammenhengene. En av de foreslåtte mekanismer som forklarer assosiasjonen mellom rødt og bearbeidet kjøtt og CRC er knyttet til hemjern i rødt kjøtt. Hypotesen er at hemjernet fremmer kjemiske prosesser som har en ugunstig effekt på tarmslimhinnen, som for eksempel økt lipid peroksidering.

Varmebehandlet storfekjøtt, svinekjøtt, kyllingkjøtt og laks ble fordøyd i en *in vitro* fordøyelsesmodell for simulert munn-, mage-, og tynntarmsfase. Under fordøyelsen ble lipid peroksidering undersøkt ved å måle dannelsen av malondialdehyde (som thiobarbituric reactive substances, TBARS), 4-hydroxynonenal og 4-hydroxyhexenal. Laks og kylling, som inneholder et høyt nivå av umettet fett, viste seg å være mer utsatt for peroksidering enn storfe og svin. Allikevel ble det observert en sammenheng mellom både mengden hemjern og fett i dietten og peroksidering. Den største dannelsen av peroksideringsprodukter ble funnet under *in vitro* fordøyelsen av storfe innblandet fiskeolje.

Sammenhengen mellom rødt kjøtt og CRC ble undersøkt direkte i A/J Min/+ mus, som er en modell for arvelig *Apc*-relatert CRC. Disse musene utvikler et stort antall svulster både i tykktarm og tynntarm, og ondartet tarmkreft dannes ved høy alder. I et innledende forsøk ble hemjern i form av isolert hemin innblandet i fôret testet. I motsetningen til hva som var forventet, reduserte hemjern kreftprosessen i tykktarmen til A/J Min/+ musene. I neste fôringsforsøk hvor effektene av varmebehandlet storfekjøtt og svinekjøtt (rødt kjøtt) ble sammenlignet med kyllingkjøtt (hvit kjøtt) og laks (fisk), var det inntaket av laks som resulterte i det laveste nivået av svulstutviklingen. Det ble ikke observert noe forskjell mellom effektene av rødt og hvitt kjøtt på kreftutviklingen. I likhet med resultatene ved *in vitro*-fordøyelse, økte dannelsen av TBARS i mage-tarm-trakten i takt med mengde hemjern og fett i diettene, men det ble ikke funnet noen

sammenheng mellom kreftutviklingen i tarmen og lipid peroxidering. Cytotoksisiten av fekalvann, som ble testet på en *Apc*$^{-/+}$ cellelinje, var heller ikke relatert til kreftutviklingen i tarmen. Imidlertid ble det funnet indikasjoner på at mengden hemjern, som nådde tykktarmen i A/J Min/+ mus, var mindre enn mengden av hemjern målt i lignende studier utført med gnagere.

På grunn av manglende effekter av hemjern og rødt kjøtt på kreftutviklingen i tarmen til A/J Min/+ mus, ble det satt i gang en tilleggsstudie for å bekrefte følsomheten til modellen. Dekstran sodium sulfat (DSS), et ikke-genotoksisk karsinogen som induserer inflammasjon i tykktarmen, ble testet i A/J Min/+ mus. Resultatene viste at DSS i stor grad økte karsinogenesen i tykktarmen. I tillegg viste studien at sammensetningen av tarmbakteriene i A/J Min/+ mus var med på å påvirke kreftprosessen.

1. GENERAL INTRODUCTION

1.1 The intestine

The intestine can be considered a site of complex interaction between the inner and outer environment. As the intestinal surface is continuously exposed to ingested food with potentially harmful substances as well as microorganisms and their metabolites, an intact intestinal barrier is crucial for the maintenance of the intestinal homeostasis [1]. Major elements of the intestinal barrier are the intestinal epithelium, which is a rapidly renewing monolayer of columnar cells, and the protective mucus layer, containing antimicrobial molecules and oxygen [2, 3]. Besides, almost 70% of the total human immune system is located in the gut [4], where it is continuously exposed to a wide range of antigens and immune stimuli [5]. Irrespective of being a defensive system, the barrier ensures the selective passage of compounds through the intestinal mucosa and controls symbiotic interactions between microorganisms and the digestive tract [1]. Imbalance of the barrier function is related to dysbiosis and inflammation, and may result in several gastrointestinal diseases [1, 2].

1.1.1 Functional morphology of the intestine

The morphology, physiology and biochemistry of the digestive tract is related to the nature of the food sources [6], and the digestive tract of humans and mice, both of which are omnivorous mammals, share many similarities **(Figure 1)** [7]. In respect to its functionality, every region of the gastrointestinal tract has evolved a specialized structure.

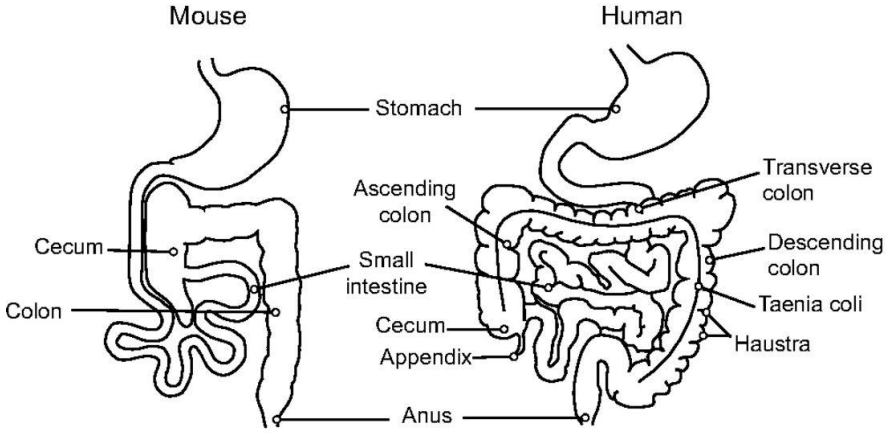


Figure 1: Anatomy of the mouse and human gastrointestinal tract. Reprinted from [7] with permission under the Creative Commons Attribution Non-Commercial License.

The primary function of the small intestine is the enzymatic digestion and the absorption of nutrients. In both humans and mice, the small intestine is comprised of the duodenum, the jejunum and ileum and runs from the pyloric sphincter to the ileocecal valve. Digestive juice from the pancreas and bile and is released into the digestive tract in the duodenum [8], and finger-like evaginations (villi) reach into the lumen to increase the absorptive surface area of the small intestine. These intestinal villi are taller in mice than humans. In the human small intestine, however, perpendicular folds (plicae) compensate for the relatively shorter villi [7].

The lower gastrointestinal tract is made up of the cecum, colon and rectum, and mainly serves to dehydrate fecal material [9]. Moreover, fermentation products, such as beneficial short-chain fatty acids (SCFA), are produced by microbiota predominantly in the proximal region of the colon [10]. Additionally, mice have a relatively large cecum, where bacterial breakdown of plant material leads to the production of vitamin K and B [7]. The human cecum is small, with the appendix, a blind-ended tube, attached to the inferior end. Unlike the relatively smooth appearance of the mouse colon, the human colon is characterized by longitudinal sacculations (haustra), caused by three longitudinal smooth muscle ribbons (taenia coli), which are slightly shorter than the colon [9]. The intestinal wall with its multilayer structure is comprised of the outermost serosa; the muscularis propria, mediating the peristaltic activity of the intestine; the submucosa with blood vessels, lymphatic vessels and nerve plexi; and the mucosa, the innermost layer surrounding the lumen. The mucosa is further comprised of the muscularis propria, the lamina propria and the epithelium [8]. Besides the villous structure, which is restricted to the small intestinal mucosa, invaginations of the epithelium, the crypts of Lieberkühn, are found in both small intestine and colon **(Figure 2)**.

1.1.2 The intestinal crypt

The intestinal epithelium is characterized by rapid turnover: a renewal of cells is accomplished every 3-5 days in the small intestine and every 5-7 days in the colon of mice [11]. Intestinal turnover in humans is less well explored, but is also thought to be accomplished every 3-4 days [12]. To secure epithelial homeostasis, cell proliferation is restricted to the crypt niche **(Figure 2)**. Moreover, there is a continuous migration of differentiating cells from the base to the top of the colonic crypts or small intestinal villi, where the fully differentiated cells turn apoptotic and eventually shed [13]. Only Paneth cells, the main function of which is the secretion of bactericide

compounds, reside at the bottom of the crypts and are renewed only every 3-6 weeks [11]. Paneth cells are found in the small intestine, appendix and cecum in humans, but are restricted to the small intestine in mice [9]. Intermingled with Paneth cells in the small intestine, or at the very bottom of the colonic crypts, there are base columnar stem cells which divide to generate rapidly proliferating transit-amplifying cells. These progenitor cells give rise to Paneth cells, as well as various other fully differentiated cells types, as they migrate towards the lumen. [11]. Cell populations in the epithelium include a vast number of absorptive enterocytes, mucus producing goblet cells, hormone-secreting endocrine cells, and less characterized cell types like tuft cells, cup cells and M cells [14, 15].

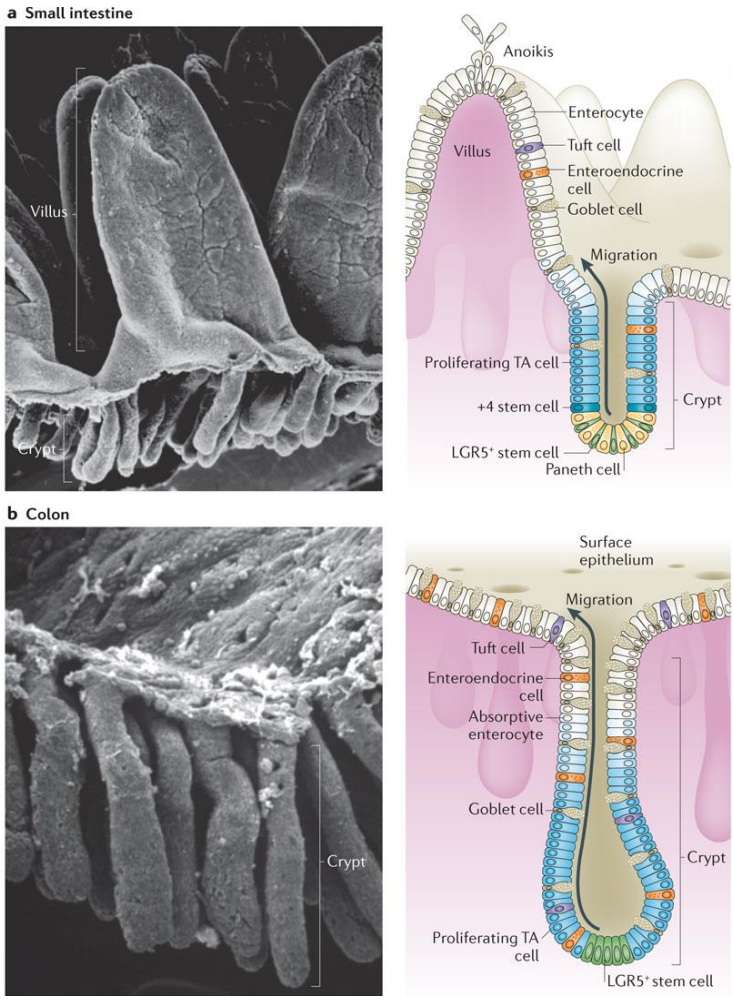


Figure 2: Scanning electron micrograph and schematic illustration of the (a) small intestinal and (b) colonic epithelium. Stem cells at the base of the crypts of Lieberkühn give rise to rapid proliferating transit-amplifying (TA) cells. These migrate towards the lumen and differentiate into cells of various functions located on the small intestinal villi or the colonic surface epithelium. Adapted by permission from Macmillan Publisher Ltd: Nature Reviews Molecular Cell Biology [11], copyright 2013.

1.2 Colorectal cancer

Colorectal cancer (CRC) is the second most common form of cancer in women, and the third most common form of cancer in men [16]. In 2014, the age-standardized incidence rates for colon and rectal cancer in Norway were respectively 51.4 and 20.7 per 100 000 person-years in women, and 59.2 and 33.1 in men. That year, a total number of 1138 and 408 cancer deaths were registered for colon and rectal cancer, respectively [17]. With the incidence of CRC increasing over the last decades, incidence and mortality rates in Norwegian men and women in particular are among the highest in Europe and the world [16] **(Figure 3)**.

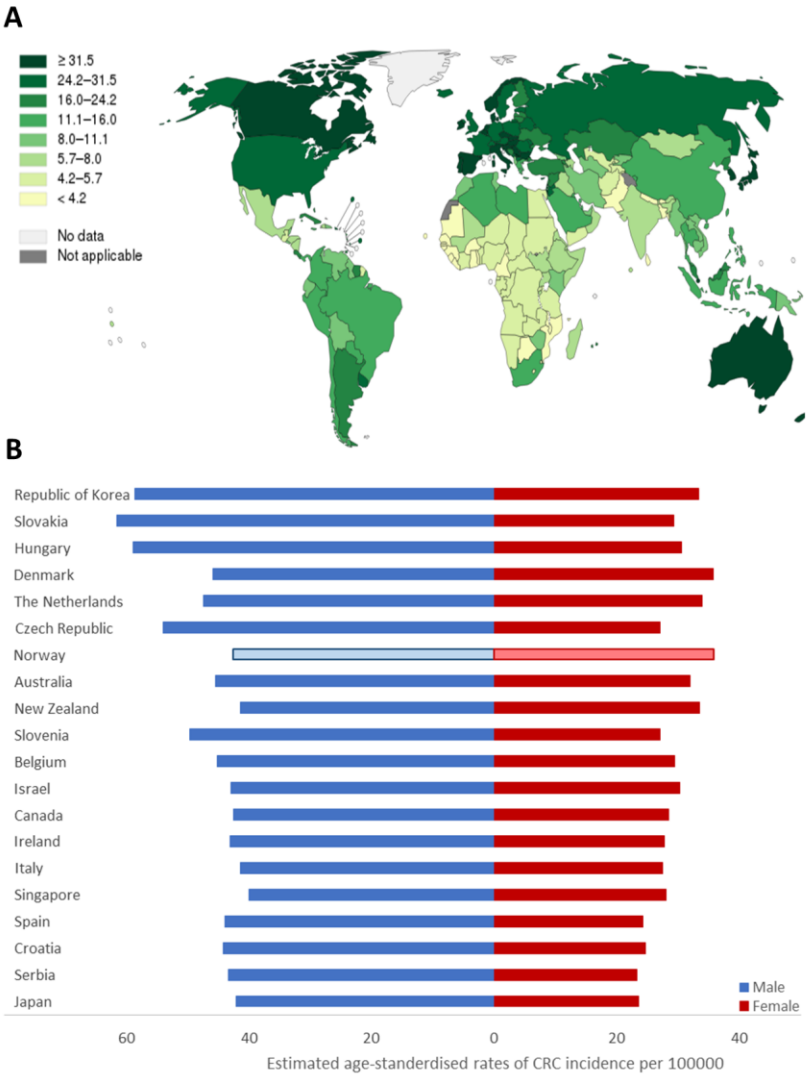
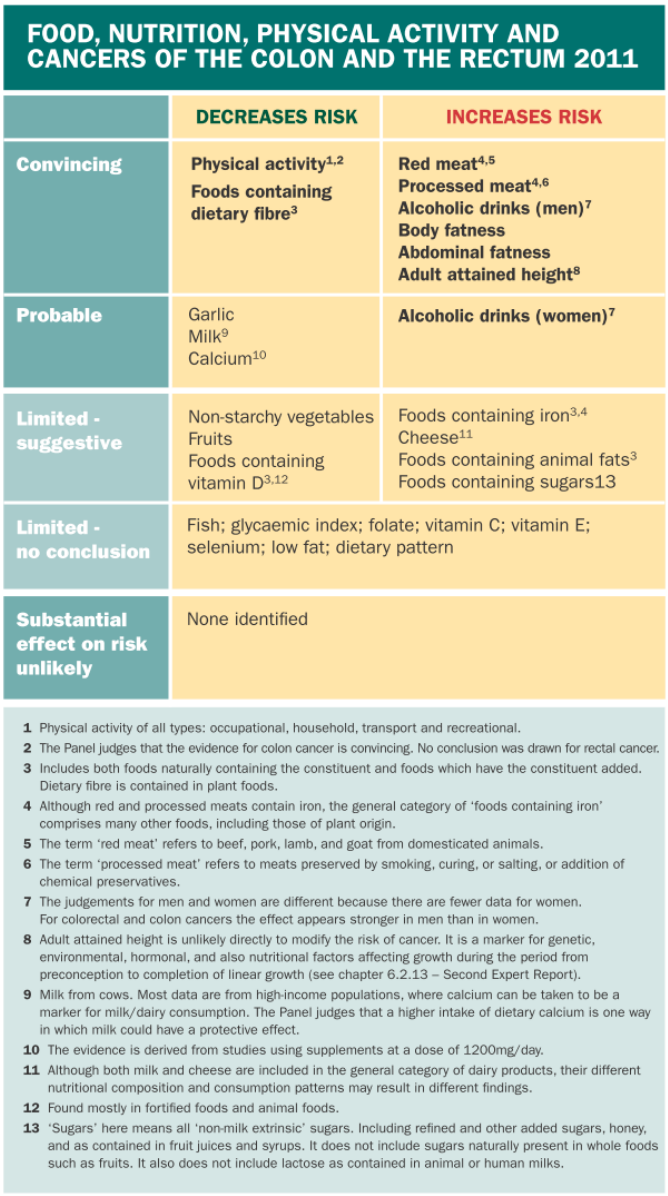


Figure 3: Estimated age-standardized rates of CRC incidence cases in 2012. (A) Worldwide, both sexes, and (B) Top 20 countries in the world with the highest CRC incidence rates. Data source: GLOBOCAN 2012, Graph production panel A: Cancer Today.



FOOD, NUTRITION, PHYSICAL ACTIVITY AND CANCERS OF THE COLON AND THE RECTUM 2011

	DECREASES RISK	INCREASES RISK
Convincing	**Physical activity[1,2]** **Foods containing dietary fibre[3]**	**Red meat[4,5]** **Processed meat[4,6]** **Alcoholic drinks (men)[7]** **Body fatness** **Abdominal fatness** **Adult attained height[8]**
Probable	Garlic Milk[9] Calcium[10]	**Alcoholic drinks (women)[7]**
Limited - suggestive	Non-starchy vegetables Fruits Foods containing vitamin D[3,12]	Foods containing iron[3,4] Cheese[11] Foods containing animal fats[3] Foods containing sugars13
Limited - no conclusion	Fish; glycaemic index; folate; vitamin C; vitamin E; selenium; low fat; dietary pattern	
Substantial effect on risk unlikely	None identified	

1 Physical activity of all types: occupational, household, transport and recreational.
2 The Panel judges that the evidence for colon cancer is convincing. No conclusion was drawn for rectal cancer.
3 Includes both foods naturally containing the constituent and foods which have the constituent added. Dietary fibre is contained in plant foods.
4 Although red and processed meats contain iron, the general category of 'foods containing iron' comprises many other foods, including those of plant origin.
5 The term 'red meat' refers to beef, pork, lamb, and goat from domesticated animals.
6 The term 'processed meat' refers to meats preserved by smoking, curing, or salting, or addition of chemical preservatives.
7 The judgements for men and women are different because there are fewer data for women. For colorectal and colon cancers the effect appears stronger in men than in women.
8 Adult attained height is unlikely directly to modify the risk of cancer. It is a marker for genetic, environmental, hormonal, and also nutritional factors affecting growth during the period from preconception to completion of linear growth (see chapter 6.2.13 – Second Expert Report).
9 Milk from cows. Most data are from high-income populations, where calcium can be taken to be a marker for milk/dairy consumption. The Panel judges that a higher intake of dietary calcium is one way in which milk could have a protective effect.
10 The evidence is derived from studies using supplements at a dose of 1200mg/day.
11 Although both milk and cheese are included in the general category of dairy products, their different nutritional composition and consumption patterns may result in different findings.
12 Found mostly in fortified foods and animal foods.
13 'Sugars' here means all 'non-milk extrinsic' sugars. Including refined and other added sugars, honey, and as contained in fruit juices and syrups. It does not include sugars naturally present in whole foods such as fruits. It also does not include lactose as contained in animal or human milks.

Figure 4: Food, Nutrition, Physical activity and Cancers of the Colon and the Rectum 2011. This material has been reproduced from the World Cancer Research Fund (WCRF) International Continuous Update Project (CUP) (www.wcrf.org) [18].

CRC is a slowly progressing and multifactorial disease, and the risk to develop CRC increases with age. A genetic contribution is estimated to be present in 30% of CRC cases, of which 2-5% are thought to be caused by inherited syndromes, e.g. familial adenomatous polyposis (FAP) or lynch syndrome. Other recognized genetic predisposition include less frequent gene variants that, due to single nucleotide polymorphisms (SNPs), modulate CRC susceptibility directly or through interaction with other genetic or environmental factors [19]. Along the updated report of the

World Cancer Research Fund (WCRF), life-style and dietary factors, such as smoking, alcohol consumption, obesity and the consumption of red and processed meat are considered risk factors for CRC, whereas physical activity and a high intake of fiber, fish, fruits and vegetables, calcium and vitamin D may favorably affect intestinal health, and decrease the risk of CRC **(Figure 4)** [18]. Over the last years, the role of microbiota in CRC has been extensively investigated, and dysbiosis of microbiota or the presence of so-called "driver bacteria" is believed to contribute to colorectal carcinogenesis. Besides the formation of microbial metabolites, gut microbiota interacts with the immune system. Sporadic CRC is often accompanied by an increase in inflammatory markers, and also chronic inflammation, as in cases of inflammatory bowel disease (IBD), is associated with an increased risk of developing CRC [20, 21].

1.2.1 Colorectal carcinogenesis

Human CRC is a stepwise, progressive disease. The molecular mechanisms involved in the early onset of sporadic cancers are still unknown, whereas the genetic and epigenetic events associated with the development of various molecular subtypes of CRC have been extensively investigated. During carcinogenesis, premalignant adenomas develop from normal epithelium, and progress into invasive and potentially metastatic adenocarcinomas [13, 22]. The sequential histological changes during CRC development are driven by an accumulation of genomic alterations on the genetic and epigenetic level [23, 24]. These affect physiologic cell functions and jointly contribute to carcinogenesis by interfering with processes like proliferation, differentiation, survival and apoptosis [25].

In connection with the acquisition of genomic instability, several molecular pathways have been identified (**Figure 5**). Microsatellite instability (MSI) is present in about 15-20% of sporadic CRC cases. Here, genetic alterations occur on the nucleotide level in the form of numerical changes of short repetitive gene sequences (microsatellites), which are prone to replication errors. The MSI pathway is linked to an impaired function of the DNA mismatch repair (MMR), and microsatellite unstable tumors are also characteristic for patients with Lynch syndrome, a hereditary form of CRC, caused by autosomal dominant germline mutations in MMR genes (mostly *MSH2*, *MSH6*, *MLH*1 and *PMS2*) [26]. The majority of sporadic microsatellite unstable cancers are associated with epigenetic silencing of *MLH1*, as a result of somatic hypermethylation [27].

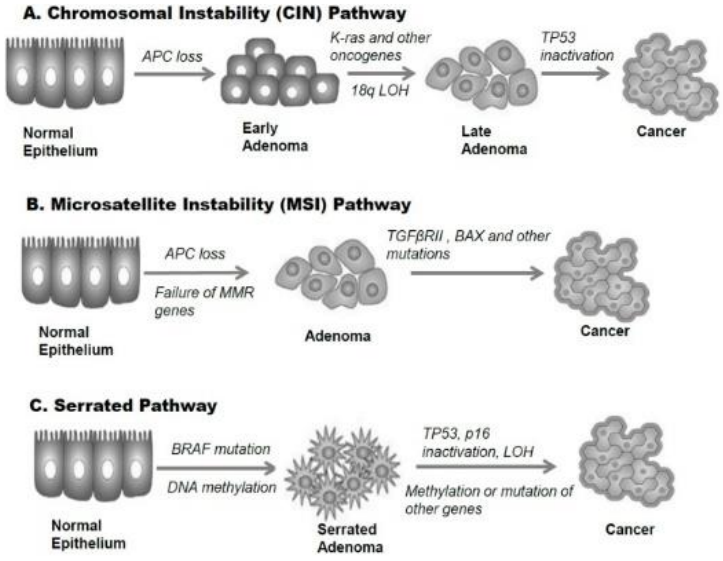


Figure 5: Genetic pathways implicated in the adenoma-carcinoma sequence in colorectal cancer. (A) The chromosomal instability (CIN) pathway is initiated by mutations in the tumor suppressor gene adenomatous polyposis coli (*APC*) and results in aneuploidy or loss of heterozygosity (LOH). CIN is accompanied by activation of numerous oncogenes and inactivation of tumor suppressor genes. (B) Failure of DNA repair genes leads to microsatellite instability (MSI), and thus genetic alterations on the nucleotide level. (C) The CpG island methylator phenotype (CIMP, here: serrated pathway) is characterized by epigenetic changes (hypermethylation) in CpG islands in promoter regions, with gene silencing of mostly tumor suppressor genes as a consequence. Reprinted by permission under the Creative Commons Attribution Non-Commercial License [28].

The different molecular pathways are not mutually exclusive and have been shown to partly overlap [29, 30]. As such, silencing of *MLH1* and a wide range of other genes (mostly tumor suppressor genes) by hypermethylation is the characteristic feature of the CpG Island Methylator Phenotype (CIMP) pathway. CpG (cytosine-phosphate-guanine) islands are often found in promoter regions, and are involved in the epigenetic regulation of the gene expression, mediated by DNA methyltransferases. Depending on the degree of aberrant hypermethylation and the nature of the affected genes, CIMP tumors present a high degree of heterogeneity [24].

The most common pathway in CRC development is the chromosomal instability pathway (CIN), which accounts for 80-85% of sporadic CRC [26]. CIN is considered a hallmark of cancer [31], and is characterized by partial or complete losses or gains of chromosomes due to errors in mitosis [32]. Although CIN leads to aneuploidy through mitotic missegregation, the most frequent mutations coupled with CIN are not typically found in genes coding for proteins that are directly involved in mitosis. Instead, the most frequent mutations are found in tumor suppressor genes

and oncogenes, that drive oncogenic signaling [31]. Often, the loss or the emergence of mutated transcription products of particular genes is related to specific phases during colorectal carcinogenesis **(Figure 5)** [22]. Mutations in the *APC* (adenomatous polyposis coli) gatekeeper gene, which are found in more than 80% of sporadic CRC cases, are an early event during carcinogenesis [33]. Besides its role in sporadic CRC, heterozygous germline mutations in *APC* are also recognized as the genetic predisposition causing FAP, an autosomal syndrome characterized by the early development of hundreds of adenomas in the intestine. By age 50, some of the adenomas will have progressed to carcinomas in 95% of the patients [19]. Examples of frequently occurring late events in regard to the CIN pathway are mutations in *KRAS* (Kirsten rat sarcoma viral oncogene homolog), which is involved in the adenoma-carcinoma-transition, and is mostly mutated in late adenomas. Moreover, the loss of 18q, where *DCC* (Deleted in colorectal cancer) and the tumor suppressor genes *SMAD2* (Mothers against decapentaplegic homolog 2) and *SMAD4* resides, interferes with the TGF-β signaling pathway. Another late event is the allelic loss of 17p, locus of *TP53,* coding for the tumor suppressor and transcription factor p53 [22, 31, 34]. Despite the high frequency of CIN in sporadic CRC, mechanisms behind CIN and new roles of CIN during tumor development are still in the process of being defined. While most previous research has focused on the promoting effect of CIN in colorectal carcinogenesis, Zasadil *et al.* [35] has recently proposed the induction of CIN as a therapeutic strategy, as they found a high CIN to inhibit tumor progression in late tumors.

Beside the aforementioned diseases Lynch syndrome and FAP, there are other, less frequent hereditary forms of CRC. Likewise, these forms of CRC are caused by germline mutations rather than somatic mutations and include MUTYH-associated polyposis (MAP), Peutz-Jeghers syndrome (PJS), juvenile polyposis syndrome (JPS) or Cowden syndrome [19].

1.2.2 *APC* in colorectal cancer

APC is located on the chromosomal band 5q21, and is comprised of 8535 base pairs. Its protein product of 312kDA consists of 2843 amino acids [33, 36]. *APC* is attributed a central role in colorectal carcinogenesis, as the stem cell specific inactivation of *APC* results in CRC initiation [37]. *APC*s role in CIN is largely explained through its role in chromosome segregation, as APC directly interacts with kinetochores, spindles and centrosomes during mitosis. Additionally, in consequence of the loss of *APC*, CIN may arise in connection with disturbances in the

Wingless-related integration site (Wnt) signaling pathway [31, 36]. The Wnt signaling pathway is crucial during embryonic development and adult cell homeostasis, and the importance of Wnt signaling in crypt renewal and intestinal carcinogenesis has been recognized for decades [38]. β–Catenin is a central protein in the Wnt-pathway **(Figure 6)**. In presence of extracellular Wnt signals, β-catenin translocates into the nucleus, where it associates with the T cell factor/lymphoid enhancer factor (TCF/LEF) family of proteins to enable the transcription of Wnt target genes. Wnt target genes are mainly involved in cellular proliferation, survival and motility, and include c-myc and cyclin D1 [39]. In absence of Wnt-signals, translocation of β–catenin is prevented through continuous degradation of β–catenin by a destruction complex.

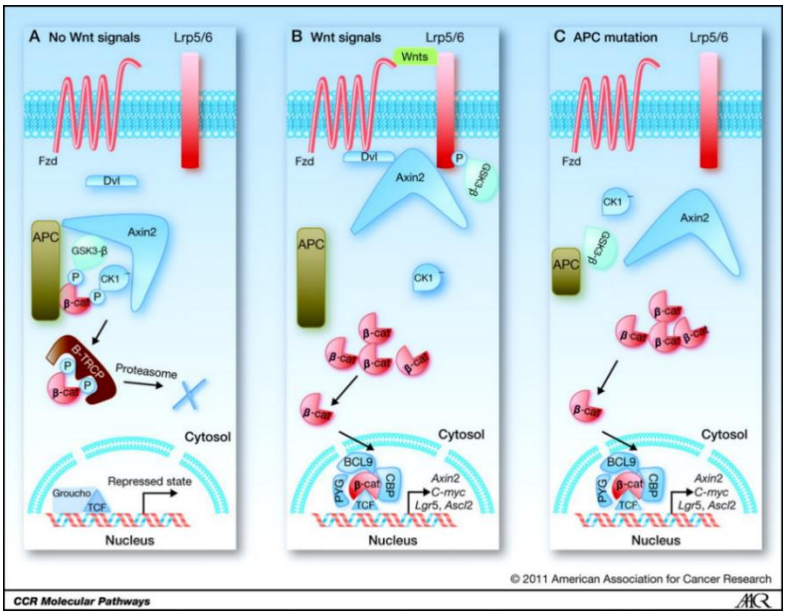


Figure 6: The Wnt canonical pathway. (A) In absence of Wnt signals, β-catenin is phosphorylated and degraded by a destruction complex. (B) In presence of Wnt signals, cytosolic β-catenin translocates to the nucleus, where it activates the transcription of target genes. (C) Mutations in *APC* prevent the formation of the destruction complex and β-catenin accumulates and translocates to the nucleus even in absence of Wnt signals. Minimally modified and reprinted from [40] with permission from AACR.

Indispensable for its assemblage, APC functions as an essential scaffolding protein in the β-catenin destruction complex, and under physiological conditions, the level of APC expression in epithelial cells increases from the bottom to the top of the crypts **(Figure 7)**. The constant activation of the Wnt pathway in absence of APC leads to dysfunctional cell proliferation and differentiation within the intestinal crypts, mediated by nuclear β–catenin [38]. Additionally, APC functions as an inhibitor on canonical Wnt signaling via several other mechanisms.

Full-length APC was shown to inhibit β–catenin/TCF–dependent transcription, and the β–catenin binding site in APC enables APC to actively block the interaction with TCF, and export β-catenin from the nucleus [36].

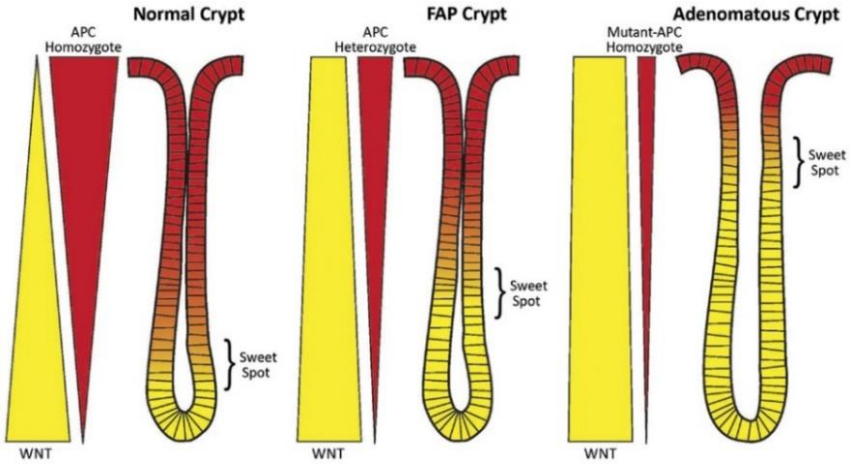


Figure 7: Physiological and pathophysiological APC and Wnt signaling gradients in the intestinal crypt. APC and WNT signaling is required in mitotic processes, and the balanced level between APC and Wnt signaling defines the "sweet spot" location, the optimal region of cell proliferation. In the normal crypt ($APC^{+/+}$), the "sweet spot" is located near the bottom of the crypt (right panel). Heterozygous mutations of *APC* ($APC^{-/+}$) decrease cellular APC concentrations and subsequent Wnt suppression. The "sweet spot" is located in the middle of the crypt (middle panel). Homozygous mutations of *APC* ($APC^{-/-}$) lead to a shift of the "sweet spot" to the top of the crypts and result in an accumulation of immature cells in the crypts (stem cell overpopulation) (right panel). Reprinted from [38] by permission under the Creative Commons Attribution Non-Commercial License.

APC also contributes to the maintenance of intestinal homeostasis by controlling the cellular distribution of β–catenin and E-cadherin, thereby mediating cell adhesion. APC impacts the formation of the cytoskeleton through binding to actin filaments, and regulating cell polarity and migration. Moreover, APC is able to associate with microtubule ends and is involved in the spindle formation during mitosis [33, 36]. Yet, the wide range of consequences following the loss of APC are still expanding and an direct role in apoptosis [41] and gene hypermethylation [42] has been described.

FAP patients are born with an inherited heterozygous germline mutation in *APC*, which in most cases results in a truncated APC protein with an abnormal, but partially retained, functionality. In contrast, in sporadic CRC the first mutation in *APC* occurs in somatic cells. However, in line with Knudsen two-hit hypothesis [43], a loss of function of the remaining wild type (WT) allele

in form of a second somatic mutation, loss of heterozygosity (LOH) or gene silencing is required for the onset of carcinogenesis of both CRC forms [44]. In both inherited and sporadic CRC, the position and type of the first and second hit were shown to be interdependent, and a depletion of both *APC* is seldom seen. This suggests that the nature of the first and second hit combined, establish a state of "just right" between APC function and Wnt signaling, which creates an optimal environment for cancer development, characterized by an increased cell proliferation and decreased cell differentiation along the crypts of Lieberkühn [38, 44, 45] **(Figure 7)**.

1.3 The A/J Min/+ mouse

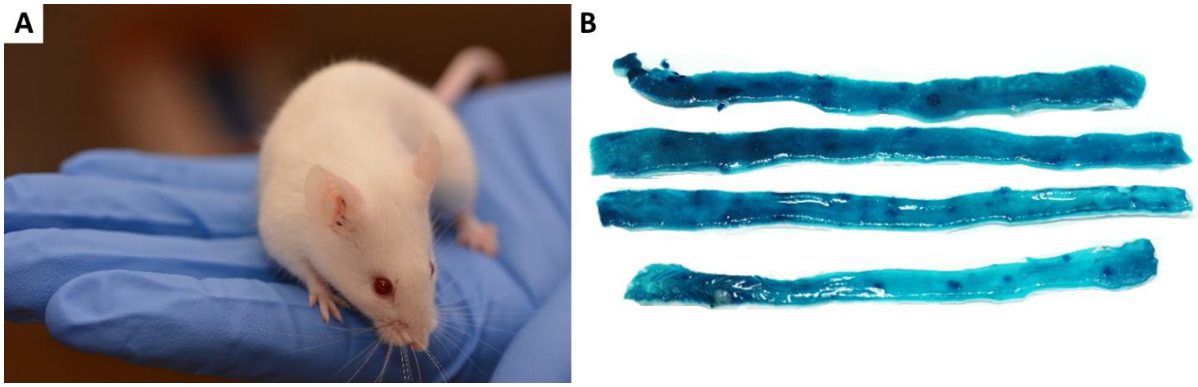


Figure 8: (A) The A/J Min/+ mouse (Photo H. Hjelmseth), (**B) Representative example of a methylene blue stained intestine of a 13 weeks old A/J Min/+ mouse.** The upper three sections show the small intestine, the forth section the colon. Dark stained marks are tumors, or in some cases gut associated lymphoid aggregates.

The high prevalence of CRC emphasizes the necessity for suitable animal models in cancer research. Mouse models represent a valuable tool in research, as regions of conserved synteny between mouse and human extends over more than 90% of the genome. Human homologs can be found for 99% of the mouse genes [46]. Among the genes that are highly conserved between humans and mice is the *APC/Apc* gene, which presents a homology of 86% at the nucleotide level, and is identical in 90% of the amino acids after being transcribed into the APC/Apc protein [47]. Due to the central role of APC in colorectal carcinogenesis, and the similar genetic etiology of FAP and sporadic CRC, multiple intestinal neoplasia (Min/+) mice, harboring heterozygous mutations at different regions in *Apc*, have been widely applied in both FAP and sporadic CRC related research [44, 48]. More recently, also two *Apc* mutant rat models, the polyposis in the rat colon (Pirc) [49] and Kyoto Apc Delta (KAD) rat [50] have been developed. As an alternative to models harboring a germline mutations in *Apc*, models have been generated that incorporate the

Cre/*lox* system, and allow for the conditional depletion of *Apc* [51, 52]. The various *Apc* rodent models exhibit wide phenotypic disparities, and the multiplicity of polyps in the models is largely defined by the site of the first hit in *Apc* and the mode of inactivation of the remaining *Apc* WT allele. Moreover, the inbred strain background, the intestinal flora and dietary factors modulate the models' susceptibility towards colonic carcinogenesis and the course of the disease [44, 53, 54].

The most widely applied model is the conventional C57BL/6J Min/+ (B6 Min/+) mouse. The first mutation in *Apc* in these mice was randomly induced by exposure of C57BL/6 (B6) mice to ethylnitrosurea (ENU) [55]. Later, the Min/+ mouse was found to carry a heterozygous truncation mutation in *Apc* at codon 850, which mainly leads to subsequent LOH of the WT allele [56, 57]. Murine embryos carrying bi-allelic mutations in *Apc* are not viable [58], and analogous to FAP patients, the Min/+ mouse develops intestinal neoplastic lesions spontaneously. However, as opposed to humans, where carcinogenesis mainly occurs in the colon and rectum, the main site of tumor formation in the conventional B6 Min/+ mouse is the small intestine. Another shortcoming of B6 Min/+ mice is that benign adenomas only rarely develop into invasive adenocarcinomas [48].

Compared to B6 WT mice, the A/J mouse strain is known to be highly susceptible towards colon-specific carcinogens like azoxymethane (AOM) and colitis-associated CRC induced by coadministration of AOM and dextran sodium sulfate (DSS) [59, 60]. Therefore, the Min/+ trait was introduced onto the A/J mouse strain to generate an A/J Min/+ mouse **(Figure 8)**. According to the expectations, the novel A/J Min/+ mouse exhibits a larger number of colonic lesions and a greater susceptibility towards AOM-induced colonic carcinogenesis than B6 Min/+ mice [61]. In 2016, Sødring *et al.* [62] described the continuous transition from preneoplastic lesions (flat ACF, flat aberrant crypt foci) to adenoma and eventually carcinoma in A/J Min/+ mice for the first time. In A/J Min/+ mice older than 30 weeks, the incidence of intestinal carcinomas reached 100%.

1.3.1 Aberrant crypt foci

The formation of preneoplastic lesions is considered the initial step in colonic carcinogenesis, and the early detection of precancerous lesions presents a valuable biomarker for CRC [63]. The first neoplastic crypts were identified by Bird *et al.* [64, 65] after exposing CF1 and B6 mice to

AOM. These aberrant crypt foci (ACF) were described as crypts of larger size and wider pericryptal zones, elevated from the mucosa. However, despite being observed in FAP and sporadic CRC patients [66], ACF were not found in untreated Min/+ mice [67]. Instead, Paulsen *et al.* [67] identified ACF_{Min} in Min/+ mice, which were renamed flat ACF after also being observed in AOM-treated rats [68] and A/J WT mice [60] and humans [66] **(Figure 9)**. The detection of colonic flat ACF is dependent on methylene blue staining, and lesions can only be observed by transillumination. In an inverted light microscope, flat ACF can be identified as blue-green crypt foci, with compressed pit patterns of luminal crypt openings. Flat ACF, as opposed to the classical ACF, originally described by Bird [64], are not elevated but usually lie flat against the surrounding epithelium [60, 67, 68]. Most importantly, unlike classic ACF, which mainly show signs of hyperplasia or mild dysplasia, dysplastic features were histologically described in flat ACF already at the monocryptal stage, and a direct relationship between flat ACF and tumorigenesis could be established in F344 rats, A/J WT and A/J Min/+ mice. The overexpression of β-catenin and cyclin D1 that was evident for flat ACF and tumors, but not classical ACF, provides evidence of active Wnt signaling and loss of APC functionality [60, 62, 69].

In parallel to these developments, lesions like β-catenin accumulated crypt (BCAC), mucin depleted foci (MDF) or dark ACF have been characterized and described as precancerous by other research groups [70–73]. However, despite the different nomenclature and identification methods, the described foci seem likely to be corresponding lesions [73–75].

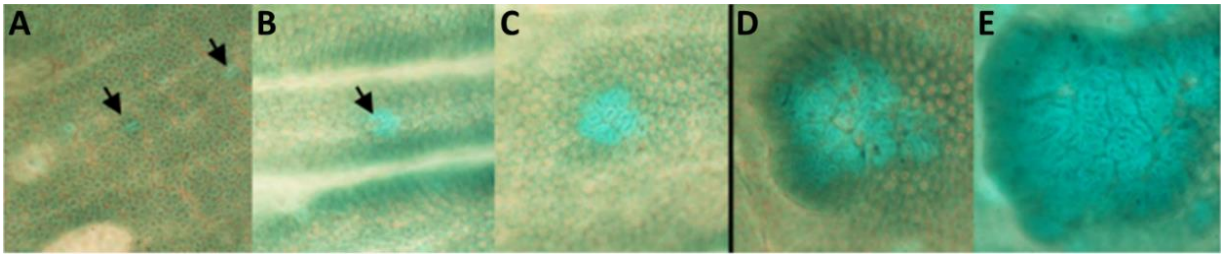


Figure 9: Colonic lesions in A/J Min/+ mice. Continuous development from flat ACF (A-C) into tumors (D-E). Arrows mark small flat ACF. Reprinted from [62] with permission under the Creative Commons Attribution Non-Commercial License.

1.4 Muscle food and CRC

Muscle food, i.e. animal protein, is a firm component of the human diet, and associations between muscle food consumption and pathological conditions have long been the subject of investigations.

1.4.1 Muscle food

Muscle food refers to commonly consumed seafood and meat. Seafood, which includes finfish as well as shellfish, presents a valuable source of high quality proteins, iodine, selenium, vitamin B12 and D and, in case of saltwater fish, essential long-chain polyunsaturated fatty acids (PUFA) [76]. On the other hand, seafood may contain variable concentrations of heavy metals, e.g. mercury, cadmium, lead or arsenic, and other toxic contaminants or environmental pollutants [77]. Based on the content of the heme iron **(Figure 10)** the oxygen-binding pigment in myoglobin the in sarcoplasm, meat is differentiated into white meat (consisting of mainly rapidly contracting white muscle fibers) and red meat (myoglobin-rich, slow-twitch red muscle fibers). Meat of any type that has been processed can be considered an additional category [78].

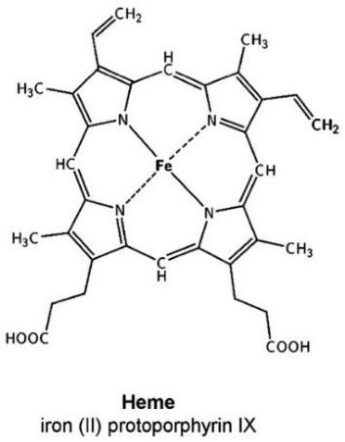


Figure 10: Structure of heme iron. Reprinted from [79] with permission from AACR.

Yet, the classification of red and processed meat has not been coherent over the last decades, complicating the interpretation of epidemiological studies regarding meat consumption and pathological conditions. Discrepancies exist e.g. in the categorization of pork. Pork meat contains concentrations of myoglobin, and thereby heme iron, that lie in between beef and chicken [80]. As it is the main muscle type used in processed meat, however, it is defined as red meat in most epidemiological studies [78]. For the evaluation of the carcinogenicity of the consumption of red

and processed meat in 2015, red meat was defined as unprocessed muscle meat from e.g. beef, pork, lamb, mutton, horse, goat, and veal, and processed meat included meat that was subjected to salting, curing, fermentation, smoking or other flavor enhancing or shelf-life prolonging processes [81]. Meat is attributed a high nutritional value as it contains high quality protein and high levels of vitamins and minerals e.g. group B vitamins (B12 in particular), vitamin D, zinc, selenium, and various amounts of heme iron [82]. Compared to red meat, white meat like chicken, guinea fowl and turkey [83], presents a more favorable n-6:n-3 fatty acid ratio and may, in addition to fish, present a source of long chain n-3 PUFA, if fed with a chow fortified by rapeseed- or linseed oil [84]. Moreover, white meat is generally considered lean, as fat is mainly located in the skin and can be easily removed [85]. More so than white meat, red meat, and beef in particular, is rich in the iron porphyrin pigment heme iron **(Figure 10)**, which is highly bioavailable for humans, and more readily absorbed than non-heme iron from plant food [86]. On the other hand, red meat is often criticized for its high amount of saturated fat and cholesterol [82].

With the consumption of meat steadily increasing worldwide, the consumption of meat has also been raising ethical and environmental concerns. One such concern is the carbon footprint of 32.0 kg CO_2-eq/kg beef, which is considerable higher than the carbon footprint of pork (4.5 kg CO_2-eq/kg pork) or chicken (2.9 kg CO_2-eq/kg chicken) [87]. The carbon footprint of most seafood products in Norway range between 1.0-4.0 CO_2-eq/kg [88], while the carbon emission from potatoes, in comparison, is only 0.43 kg CO_2-eq/kg [87].

1.4.2 The link between muscle food and CRC

Red and processed meat consumption has been associated with various pathological conditions, including cardiovascular disease, obesity, type 2 diabetes mellitus, all-cause mortality and cancer [82, 89]. Besides the limited or inconsistent evidence for the role of red and processed meat in cancer of the esophagus, lung, pancreas, prostate, endometrium and stomach, the association between red and processed meat and colorectal cancer is extensively described and considered convincing [83]. The coherence of the association from epidemiological studies is more pronounced for processed meat than red meat, and subsequently, in 2015, the International Agency for Research on Cancer (IARC) classified processed meat as “carcinogenic to humans” (Group 1) and red meat as “probably carcinogenic to humans” (Group 2A) [81, 83]. In the report, the evidence from mechanistic studies was considered strong for red meat and moderate for

processed meat. While AICR classified red and processed meat based on a hazard analysis, conclusions drawn from risk assessments suggest a dose-response relationship. Baseline risk for developing CRC is estimated to increase by 18% for every 50 g of processed meat and 17% for every 100 g of red meat consumed **(Figure 11)** [90]. Lowering the intake of red meat to 70 gram per week is proposed to lower CRC risk by 7-24% [91].

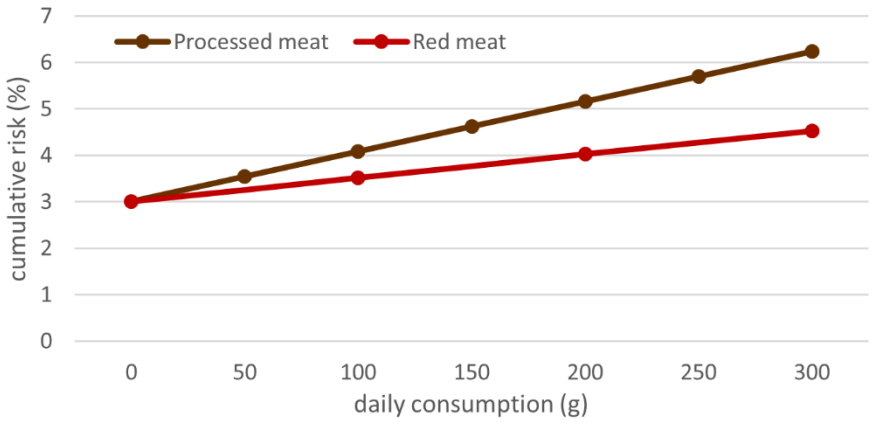


Figure 11: Processed meat- and red meat-related increase of the cumulative risk to develop CRC by age 75 in Norway. Basic cumulative risk to develop CRC by age 75 in Norway is about 3% [17]. Processed meat intake increases basic risk by 18% for every 50 g consumed, while red meat intake increases basic risk by 17% for every 100 g consumed [90].

In contrast to the effects of red meat consumption, there is some but limited evidence that fish prevents CRC, and the protective effect of fish is thought to be attributed to its high content of n-3 long-chain PUFA and vitamin D [83]. The effects of white meat (such as chicken) on CRC is less conclusive. Nevertheless, poultry consumption is unlikely to increase CRC risk, and may rather have protective properties [92, 93]. Additionally, the selective effect of unprocessed pork, generally classified as red meat, needs to be further established [94].

It also has to be kept in mind, that diet is only one of the factors that modulate CRC risk and that many questions remain yet unclarified. In Japan and Korea, CRC incidence rates have been increasing over the last decades, as has the intake of meat [95]. In other countries, in contrast, there are discrepancies between processed and red meat consumption and CRC incidence rates. Mongolia and Bolivia, for instance, present very low CRC incidences despite of a considerable intake of red meat [95], and the same applies for the Sami population in Northern Europe, which consume large amounts of reindeer meet [96]. The consumption of red meat in the United States has been slightly decreasing since the 1970s and is now comparable to the European red meat

intake [97]. Despite of comparable CRC incidence rates in the United States and the European countries, over the last decades, CRC incidence in the United States has been slightly increasing in the population below 50, while it has been decreasing by 30% in the population older than 50 years [98].

1.4.3 Consumption of muscle food in Norway

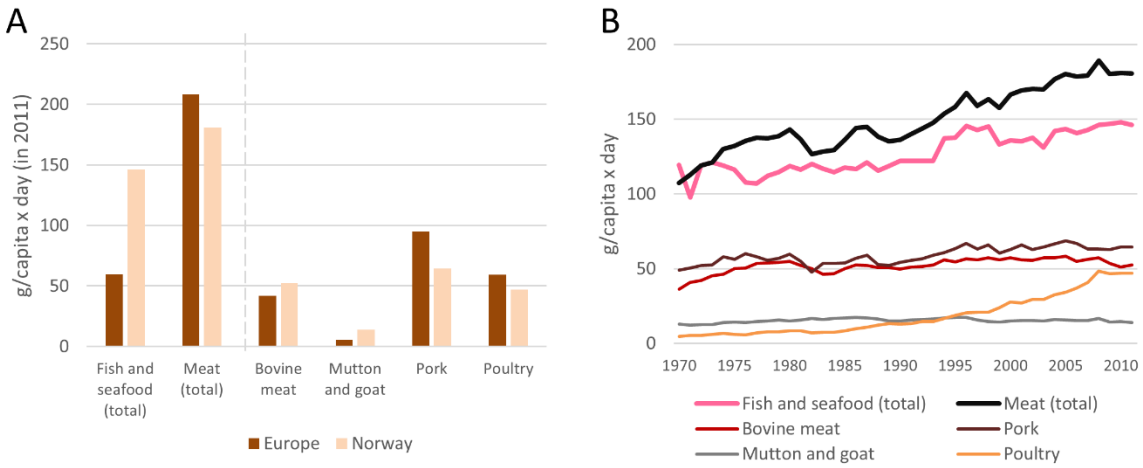


Figure 12: Food supply of muscle food in (A) Europe and Norway in 2011 and (B) in Norway from 1970-2011 ©FAO 2016, data accessed 09-07-2016 from http://fenix.fao.org/faostat/beta/en/#compare. This is an adaptation of an original work by FAO. Views and opinions expressed in the adaptation are the sole responsibility of the author or authors of the adaptation and are not endorsed by FAO.

In Norway, incidence rates for colon cancer in men and women have been doubled since the 1970, while incidence rates for rectal cancer have been increasing by approximately 50% [17]. With the aim to reduce the risk for developing CRC and other diseases, the WCRF recommends to avoid the consumption of processed meat and limit the intake of red meat to 500 g raw meat per week [83]. This corresponds roughly to 700-750 g of prepared meat. Distributed evenly over the week, it is thereby encouraged to consume no more than 107 g prepared red meat per day. According to Norkost 3, the total consumption of red and processed meat was 146 g/day for men and 89 g/day for women in 2010-2011 [99]. More differentiated data on female meat consumption from the European Investigation into Cancer and Nutrition (EPIC) study from 1999-2000 indicates that the total meat consumption in Norway at the turn of the century (ca. 95 g/day*capita (women)) generally reflected the average consumption in Europe. However, in comparison with other European countries included in the study, the intake of processed meat was highest in Norway (ca. 45-50 g/day), and the intake of sausages (20-25 g/day) was particularly high [100].

The total meat supply has been increasing in Norway over the last decades, but since the 1980s, poultry has been the main contributor to the increase. Consumption of seafood in Norway is among the highest in Europe and the world (data for 2011: 146 g/day, 60 g/day and 52 g/day for Norway, Europe and the world respectively) [101] **(Figure 12)**.

1.4.4 Suggested mechanisms linking CRC to red and processed meat consumption

There are multiple proposed mechanisms that aim to explain the link between meat intake and CRC. Of these mechanisms, some are more specific for red and processed meat than others.

Heme iron

A mechanism extensively investigated in rodents concerns heme iron **(Figure 10)** which is found in higher concentrations in red meat than in white meat [80]. In carcinogen-induced CRC in rats, an increased colonic tumor load in response to dietary beef and black pudding coincided with an increased formation of the luminal secondary lipid peroxidation products malondialdehyde (MDA, often measured as thiobarbituric acid reactive substances (TBARS)) and fecal water cytotoxicity, and a similar outcome was observed when beef was replaced by an equivalent amount of dietary heme iron, provided as hemoglobin [102]. Short-term exposure to heme iron (two weeks) induced hyperproliferation and decreased apoptosis in the intestinal mucosa of mice. In a time-course study, the increase of the cytotoxic potential of luminal content was proposed to be the crucial event in heme-mediated hyperproliferation, as there was a lag time between the rise in lipid peroxidation products and fecal water cytotoxicity, the latter coinciding with cell proliferation. Potentially, heme iron catalyzes lipid peroxidation and subsequently forms a cytotoxic heme factor (CHF) through covalent binding of reactive lipid peroxides to its porphyrin ring [103, 104]. Pierre *et al.* [105] demonstrated that cultured $Apc^{-/+}$ cells were more resistant to heme-related fecal water cytotoxicity than $Apc^{+/+}$ cells, thereby proposing an advantage for survival of cells with aberrant Apc functionality. Alternatively, heme iron may catalyze lipid peroxidation within cell membranes, and the oxidized fatty acids may impair the fluidity of cell membranes and increase permeability, eventually leading to inflammation. Peroxidation products can also function as signaling transducers, induce oxidative stress or form protein- and DNA-adducts, with the latter increasing the risk of gene mutations [106].

Another putative pathway which links heme iron to CRC is connected to the endogenous formation of NOCs, which are formed in response to the nitrosylation of heme iron in meat

products or in the gut. [79, 107, 108]. Thus, meat consumption was linked to an increase in fecal NOCs and NOC-specific DNA adducts (O^6-carboxymethyl guanine (O^6CMG)) in the intestinal mucosa in humans [109]. Also in rodents, dietary nitrite resulted in an increased level of fecal Apparent Total Nitroso Compounds (ATNC), but the intake of nitrite was not related to either fecal water cytotoxicity [110] or colonic carcinogenesis [111, 112]. Advanced detection methods are required, sensitive enough to differentiate between individual NOCs [108].

More recent investigations targeted the role of microbiota in heme-induced CRC. Ingestion of heme iron was shown to induce changes in microbiota in mice, which affected the mucin layer and subsequently impaired the integrity of the intestinal barrier function [113]. Hydrogen sulfide producing and mucin-degrading bacteria were proposed to play a central role in this process that may eventually result in compensatory hyperproliferation. Use of antibiotics partially inhibited the formation of heme-induced lipid peroxidation products, decreased fecal water cytotoxicity and prevented hyperproliferation [113, 114].

Although mechanisms are not conclusively understood, a role of heme iron in CRC is supported by epidemiological data [115, 116]. Notably, in rodents, the carcinogenic effect of heme iron, red and processed meat is prevented by an adequate calcium supply from the diet [117–121], and no adverse effect of dietary meat was found, before basal diets were adjusted to mimic a "Western style diet", meaning low calcium and fiber, high fat etc. [103, 122–125]. It is likely, that the protective effect of calcium is based on chelation of heme iron by calcium [121, 126], which is supported by the finding that dietary calcium inhibits the absorption of heme iron also in humans [127].

Dietary Fat

The high content of fat in red meat, and particularly processed red meat, may lead to obesity and other conditions associated with CRC [128]. It may give rise to harmful lipid peroxidation products [129], or enhance intestinal secretion of bile acids, which in turn are made accessible for bacteria, and may be converted into potentially toxic or carcinogenic secondary bile acids [130–133]. Moreover, diets rich in saturated fat, but not unsaturated fat, enhance taurine conjugation of hepatic bile acids, which provides a growth advantage to the sulfide-reducing pathobiont *Bilophila wadsworthia*. Metabolites of *B. wadsworthia* and other pathogens that flourish in presence of bile acids may partially degrade the mucus layer, facilitating inflammatory processes [134]. A recently published rodent study also provides evidence that high dietary fat

enhances stemness and self-renewal of intestinal crypt stem cells via peroxisome proliferator-activated receptor delta (PPARδ)-signaling [135]. A high intake of fat, however, is not necessarily coupled with a high consumption of red and processed meat, and despite the evidence from animal studies [71, 136–139], epidemiological data does not support a role of dietary total or animal fat in CRC [140–142].

Dietary Protein

Meat represents a valuable source of protein. An excessive consumption of meat, however, may increase the amount of protein that reaches the colon, where it is accessible for the intestinal microbiota. Microbiota-derived metabolites from protein fermentation include protective compounds like SCFA, but also potentially harmful substances like hydrogen sulfide (H_2S), amines, ammonia, phenol, *p*-cresol and phenyl acetate, which may affect the colonic epithelium, impair the mucin layer and lead to inflammation [143]. Although protein fermentation occurs independently of the protein source, fermentation products like H_2S may augment the adverse effects of other carcinogenic pathways connected to red and processed meat [113].

Meat-related mutagens and carcinogens

Persistent organic pollutants (POPs) like dioxin-like polychlorobiphenyls (DL-PCBs) are one class of carcinogens which accumulate in animal fat and may affect CRC risk [144]. Other carcinogens are formed during meat processing (e.g. smoking) or preparation (e.g. cooking at high temperatures, grilling), and these include heterocyclic amines (HCAs), polycyclic aromatic hydrocarbons (PAHs) and N-nitroso compounds (NOCs), e.g. N-nitrosamines and N-nitrosamides. Many of these compounds are alkylating agents or able to directly react with DNA to form adducts, and have documented mutagenic or carcinogenic effects in animals [145, 146]. Mutagens produced during processing and cooking, however, are formed to various extents in red meat, white meat and fish [145, 146], and evidence from human studies regarding their association with CRC remains inconclusive [147, 148]. However, it remains to be established, whether meat-derived carcinogens from various muscle food may differentially affect intestinal carcinogenesis. Recently, indications were given that red meat- but not white meat-derived HCAs, namely 2-amino-1-methyl-6-phenylimidazo pyridine (PhIP), 2-amino-3,8-dimethylimidazo quinoxaline (MeIQx) and 2-amino-3,4,8-trimethylimidazo quinoxaline (DiMeIQx) were positively associated with CRC [149, 150]. Also, susceptibility towards cooking mutagens varies within populations due to differences in the enzyme efficiency of genetic

variants of carcinogen-metabolizing genes (e.g. N-acetyltransferase 2 (NAT2), Cytochrom P450 1A2 (CYP1A2)) [151].

Bovine virus infection

Low CRC incidences despite of high red meat consumption in countries like Mongolia and Bolivia, and the altering CRC risk for migrants moving from low to high incidence countries or *vice versa* has led to the hypothesis of a role of infectious factors from specific cattle breeds in CRC. Circular single-stranded DNA, assumingly from viral origin, has been detected in cattle sera and milk, and it requires further clarification if certain species of cattle may carry and transmit nonpathogenic persistent viral infections, which may contribute to the onset of CRC in humans [95].

N-glyconylneuraminic acid (Neu5Gc)

Another newly proposed hypothesis linking red meat to an increased CRC risk builds upon the incorporation of Neu5Gc from mammalian meat into human epithelial cells. Subsequent recognition of Neu5Gc by auto-reactive antibodies in humans may promote an inflammatory response and increase the risk of CRC development. Neu5Gc is endogenously produced in most mammals and concentrations are higher in red than white meat. Humans, in contrast, are lacking the ability to anabolize Neu5Gc, due to the evolutionary loss of the cytidine monophospho-N-acetylneuraminic acid hydroxylase (CMAH). The hypothesis regarding Neu5Gc is highly specific for red-meat consumption and deserves further attention [152, 153].

1.5 *In vitro* digestion models

In scientific research, the development of relevant *in vitro* models that simulate *in vivo* conditions is desirable for many reasons. Apart from ethical concerns, *in vivo* studies are generally time- and cost intensive. Accessibility of gastric and intestinal content and tissue is limited under *in vivo* conditions, and the feasibility of continuous sampling is restricted. Therefore the main focus of *in vivo* gastrointestinal studies often remains limited to endpoint data [154]. To study the human digestion *in vitro*, sophisticated models are required that are able to mimic the structural and functional complexity of the gastrointestinal tract **(Figure 13)**. Typically, the *in vitro* digestion process is divided into an oral, gastric and small intestinal phase, where factors like pH, transit time, digestive enzymes, temperature, and electrolyte concentrations are adapted to match *in vivo* conditions. Optionally, undigested material obtained after dialysis can be added to an *in vitro* fermentation model based on human feces, in order to study the effect on microbiota

composition and metabolites. Despite their versatile applications in studies on digestibility, bioavailability and bioaccessibility of food components and pharmaceuticals [155], the relevance of *in vitro* models is arguable in comparison to *in vivo* digestion studies in humans or animals. A major drawback is the missing food-body interaction, as *in vitro* digestion models lack immunological and regulating feedback responses, as well as a neuroendocrine system [154, 156]. Much effort is being made to provide and validate refined *in vitro* digestion models. Most progress has been made in regard to the development of dynamic models, which reproduce the gradual addition of gastrointestinal fluids and transit of digesta through the gastrointestinal tract with the help of dynamic multicompartment simulators [157–160].

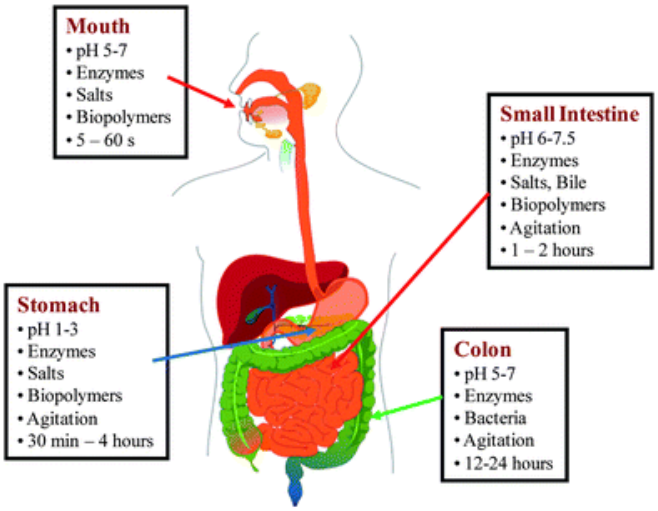


Figure 13: Schematic diagram of the physiologic conditions in the different regions of the human gastrointestinal tract, commonly used in *in vitro* digestion models. Reproduced from [161] with permission of the Royal Society of Chemistry.

1.5.1 *In vitro* digestion models in CRC research

Also in the field of CRC research, the number of *in vitro* digestion studies has been increasing. So far, the focus of these investigations has been on the formation of potentially harmful substances in the digesta (e.g. peroxidation products) [160, 162], the DNA-adduct generating potential of the digesta [162, 163], or the effect of digesta on cultured colon cells, dendritic cells or yeast (e.g. cytotoxicity, gene expression, inflammatory response) [164–166]. Results from *in vitro* studies can contribute to generating new approaches and hypotheses in the field, and comparative studies are needed to validate *in vitro* digestion models.

2 KNOWLEDGE GAPS

In 2007, the WCRF reported that there was convincing evidence for a link between the risk of CRC and intake of red and processed meat [83]. Yet, at the beginning of the present research work (January 2013), much effort was put into clarifying the molecular mechanisms behind this link, as well as evaluating the risks and benefits of red meat consumption. With the aim to publish a consensus about the role of red meat consumption in public health, leading experts in the field were invited to join a workshop in Oslo in November 2012 [78]. Despite a broad agreement within the main aspects, the strength of the epidemiological evidence for the association between red and processed meat and CRC risk became a topic of debates in the period after publication [167, 168].

Already as early as the 1990s, feeding experiments were conducted in rodents with the aim of identifying molecular mechanisms that could explain the epidemiological evidence linking red and processed meat to CRC [122–124]. However, results from these studies were not unequivocal, and it was only after the experimental diets were adjusted to reflect characteristics of a 'western style diet', that the first indications of a possible adverse effect of red meat or its components emerged. The most crucial alteration in this connection was the reduction of the dietary calcium level [117–119]. Mechanistic hypotheses were generated, focusing on heme iron and its role in luminal cytotoxicity, and formation of lipid peroxidation products and N-nitrosamines. Nonetheless, mechanistic evidence, as well as knowledge about possible interactions between various food components is sparse, and yet, no animal study has been able to show a promoting effect of red meat by comparing the effects of gently heated red meat with white meat or fish. Hitherto, the majority of animal studies that investigated the effect of red meat or meat components on CRC have been performed on 1) rodents, after chemical induction of CRC, 2) rodents, without including tumorigenesis as and endpoint, or 3) Min/+ mice, which mainly develop tumors in the small intestine.

Much effort has been made to characterize the novel A/J Min/+ mouse model, which spontaneously develops intestinal lesions, and presents a tumor distribution more similar to that of humans than the conventional B6 Min/+ mouse. The use of the model opened the possibility to examine the relationship between red meat and CRC under conditions that resemble the pathology of human sporadic CRC more closely than most other models.

3 AIMS OF THE STUDY

The overall aim of the study was to gain new knowledge about the relationship between the intake of red meat and the carcinogenesis of CRC, focusing on the effects of heme iron and differential effects of red meat, white meat and fish (Objective 1). A sub-goal was to better understand the role of dietary fat and lipid peroxidation in the carcinogenesis of CRC (Objective 2), and another sub-goal was to further characterize the potential of the A/J Min/+ mouse as a model for CRC (Objective 3).

Objective 1: Enhancing knowledge about the role of red meat in CRC was achieved by:

- Using the A/J Min/+ mouse model to test the hypothesis of a role of heme iron in CRC **(Paper II)**
- Comparing the effects of gently heated dietary cooked beef, pork, chicken, and salmon on CRC in the A/J Min/+ mouse model **(Paper III)**

Objective 2: Gaining further insight into the role of dietary fat and lipid peroxidation in the carcinogenesis of CRC was accomplished by:

- Evaluating the susceptibility of heated beef, pork, chicken and salmon towards lipid peroxidation in the gastrointestinal tract in an *in-vitro* digestion model **(Paper I)**, as well as under *in vivo* conditions **(Paper III)**
- Testing the effects of beef tallow **(Paper II)**, and the effects of the combination of dietary beef and n-6 PUFA **(Paper III)** on intestinal carcinogenesis and lipid peroxidation in the A/J Min/+ mouse model
- Relating the rate of luminal lipid peroxidation to the intestinal carcinogenesis in A/J Min/+ mice **(Paper II+III)**

Objective 3: The potential of the A/J Min/+ mouse as a model for CRC was further characterized by:

- Testing effects of DSS-induced inflammation on intestinal carcinogenesis and composition of microbiota in A/J Min/+ mice **(Paper IV)**

4 SUMMARY OF PAPERS

Paper I

Formation of Malondialdehyde, 4-Hydroxynonenal, and 4-Hydroxyhexenal during *In Vitro* Digestion of Cooked Beef, Pork, Chicken and Salmon. *Christina Steppeler*, John-Erik Haugen, Rune Rødbotten, Bente Kirkhus. **Journal of Agricultural and Food Chemistry (2016)**

Red meat high in heme iron may promote the formation of potentially genotoxic aldehydes during lipid peroxidation in the gastrointestinal tract. In this study, the formation of malondialdehyde (MDA) equivalents measured by the thiobarbituric acid reactive substances (TBARS) method was determined during *in vitro* digestion of cooked red meat (beef and pork), as well as white meat (chicken) and fish (salmon), whereas analysis of 4-hydroxyhexenal (HHE) and 4-hydroxynonenal (HNE) was performed during *in vitro* digestion of cooked beef and salmon. Comparing products with similar fat contents indicated that the amount of unsaturated fat and not total iron content was the dominating factor influencing the formation of aldehydes. It was also shown that increasing fat content in beef products caused increasing concentrations of MDA equivalents. The highest levels, however, were found in minced beef with added fish oil high in unsaturated fat. This study indicates that when ingested alone, red meat products low in unsaturated fat and low in total fat content contribute to relatively low levels of potentially genotoxic aldehydes in the gastrointestinal tract.

Paper II

Colorectal Carcinogenesis in the A/J Min/+ Mouse Model is Inhibited by Hemin, Independently of Dietary Fat Content and Fecal Lipid Peroxidation Rate. *Christina Steppeler*, Marianne Sødring, Jan Erik Paulsen. **BMC Cancer (2016)**

Intake of red meat is considered a risk factor for colorectal cancer (CRC) development, and heme, the prosthetic group of myoglobin, has been suggested as a potential cause. One of the proposed molecular mechanisms of heme-induced CRC is based on an increase in the rate of lipid peroxidation catalyzed by heme. In the study, the novel A/J Min/+ mouse model for *Apc*-driven colorectal cancer was used to investigate the effect of dietary heme (0.5 μmol/g), combined with high (40 energy %) or low (10 energy %) dietary fat levels, on intestinal carcinogenesis. At the end of the dietary intervention period (week 3–11), spontaneously developed lesions in the colon

(flat aberrant crypt foci (flat ACF) and tumors) and small intestine (tumors) were scored and thiobarbituric reactive substances (TBARS), a biomarker for lipid peroxidation, was analyzed in feces. Results showed that dietary hemin significantly reduced colonic carcinogenesis. The inhibitory effect of hemin was not dependent on the dietary fat level, and no association could be established between colonic carcinogenesis and the lipid oxidation rate measured as fecal TBARS. Small intestinal carcinogenesis was not affected by hemin. Fat tended to stimulate intestinal carcinogenesis. In conclusion, contradicting the hypothesis, dietary hemin inhibited colonic carcinogenesis in the presented study, and indications are given, that fecal TBARS concentrations are not directly related to intestinal lesions.

Paper III

Pork, Chicken and Salmon Meat on Intestinal Carcinogenesis in A/J Min/+ mice. *Christina Steppeler*, Marianne Sødring, Bjørg Egelandsdal, Bente Kirkhus, Marije Oostindjer, Ole Alvseike, Lars Erik Gangsei, Ellen-Margrethe Hovland, Fabrice Pierre and Jan Erik Paulsen. **Submitted manuscript (PLOS ONE).**

The International Agency for Research on Cancer classified processed meat as "carcinogenic to humans" (Group 1) and red meat as "probably carcinogenic to humans" (Group 2A). In mechanistic studies exploring the link between intake of red meat and CRC, heme iron, the pigment of red meat, is proposed to play a central role as a catalyzer of luminal lipid peroxidation and cytotoxicity. In the presented work, the novel A/J Min/+ mouse was used to investigate the effects of dietary beef, pork, chicken, or salmon (40% muscle food (dry weight) and 60% powder diet) on *Apc*-driven intestinal carcinogenesis, from week 3-13 of age. Muscle food diets did not differentially affect carcinogenesis in the colon (flat ACF and tumors). In the small intestine, salmon intake resulted in a lower tumor size and load than did meat from terrestrial animals (beef, pork or chicken), while no differences were observed between the effects of white meat (chicken) and red meat (pork and beef). Additional results showed, that intestinal carcinogenesis was not influenced by fat, and not related to the intestinal formation of lipid peroxidation products (TBARS), or cytotoxic effects of fecal water on $Apc^{-/+}$ cells. Notably, the amount of heme reaching the colon appeared to be relatively low in this study. The greatest tumor load was induced by the reference diet RM1, underlining the importance of the basic diets in experimental

CRC. The presented study in A/J Min/+ mice does not support the hypothesis of a role of red meat in intestinal carcinogenesis.

Paper IV

Inflammation-induced colonic carcinogenesis in A/J Min/+ mice is dependent on the intestinal microbiota. Ida Rud, *Christina Steppeler*, Jan Erik Paulsen, Preben Boysen, Ingrid Måge. **Manuscript.**

Dysbiosis of the intestinal microbiota or the presence of so-called "bacteria drivers" is believed to contribute to colorectal carcinogenesis through the formation of potentially harmful metabolites or interaction with the immune system. Also, inflammatory bowel disease is associated with an increased risk for colorectal cancer (CRC). The aim of this study was to further elucidate the role of inflammation, microbiota and short chain fatty acids (SCFAs) in the pathogenesis of CRC. The A/J Min/+ mouse model was used, which is characterized by spontaneous formation of numerous intestinal tumors, and a high susceptibility to colon-specific carcinogenesis. Dextran sodium sulfate (DSS) was administrated to female mice (10 and 12 weeks of age) for 4 days via drinking water to promote inflammation. Changes in fecal microbiota and SCFAs were monitored throughout the study, and effects on inflammation and intestinal carcinogenesis were determined at the end of the study (day 24). DSS-treatment was shown to induce an immunological response and initiate colonic carcinogenesis, measured as flat aberrant crypt foci (ACF) and tumors, whereas small intestinal carcinogenesis remained unaffected. However, different susceptibilities to colonic carcinogenesis were observed within the DSS-treated mice, which differentiated the mice into two subgroups. The subgroups were found to differ in the initial microbiota, age and parents. Furthermore, DSS-treatment resulted in temporal changes of the fecal microbiota and SCFAs profile, where dysbiosis was most apparent on day 5, with decreased alpha diversity, higher relative levels of propionic acid, and lower levels of butyric acid. Bacteria related to the colonic carcinogenesis were identified (e.g. *Bacteroides*, *B. acidifaciens* and *[Prevotella]*), as well as bacteria related to the SCFAs. To conclude, the presented study shows that the A/J Min/+ mouse model is susceptible to inflammation-induced carcinogenesis, where the intestinal microbiota is involved in mediating the development, and the initial microbiota seems to modulate the degree of susceptibility.

5 GENERAL DISCUSSION

5.1 *In vitro* digestion models in meat-related CRC research

Due to ethical constrains, toxicological studies cannot be conducted in humans. Instead, robust and efficient *in vitro* and *in vivo* models are necessary to be able to test the toxicological potential of food, xenobiotics and other chemical substances and compounds. In regard to reliability and relevance of toxicological studies in risk assessment, i*n vivo* studies performed in rodents and other mammals are superior to *in vitro* studies. *In vitro* studies, however, represent a valuable screening tool and contribute to the generation of new mechanistic hypotheses and approaches. Based on knowledge gained from *in vitro* models, i*n vivo* studies can be conducted to evaluate to which extend it may be possible to extrapolate the observed effects to animals and eventually humans. Indeed, findings from the *in vitro* digestion of meat and fish **(Paper I)** laid the basis for the design of a large feeding study on A/J Min/+ mice **(Paper III**). During the *in vitro* digestion of heated beef, pork, chicken and salmon, described in **Paper I**, lipid peroxidation rates, i.e. the formation of secondary lipid oxidation products MDA and hydroxyalkenals, in salmon and chicken were found to be higher than in beef and pork. Also, heme iron did not substantially contribute to the formation of aldehydes, unless combined with an increased amount of unsaturated fat (fish oil). These findings were somewhat controversial in respect to the purported protective effect of fish on CRC [18], and questioned the importance of intestinal lipid peroxidation as a proposed mechanisms in meat-related CRC.

Analysis of TBARS in fecal water of mice fed meat or fish **(Paper III)** confirmed the high susceptibility of salmon to lipid peroxidation in the gastrointestinal tract. However, with similar levels of fecal TBARS in mice fed beef and salmon, the effect of heme iron on lipid peroxidation appeared to be more pronounced under *in vivo* conditions. Notably, no colonic phase was included in the *in vitro* digestion model, which could increase the consistency between the *in vitro* and *in vivo* analysis results. A colonic phase should be included in the *in vitro* model, as Martin *et al.* [114] and Ijssennagger *et al.* [113] reported a role of microbiota in the heme iron-induced formation of TBARS in the colon, and found fecal TBARS concentrations in rats and B6 mice to be decreased by antibiotic treatment. Moreover, heme iron intake was found to induce alterations in the microbiota composition [169], and hence, lipid peroxidation rates in the colon may vary in respect to the type of meat consumed.

Besides, the static *in vitro* digestion model used in **Paper I** may be improved. Larsson *et al.* [160] mimicked intestinal absorption by means of a semi-permeable membrane in a dynamic digestion model. Lipid peroxidation is a self-propagating chain reaction, which is accelerated in presence of peroxidation products in the digesta [170]. The continuous removal of lipids and peroxidation products in a dynamic *in vitro* digestion model may prevent an overestimation of lipid peroxidation products. Moreover, decreasing oxygen exposure in samples by nitrogen blanketing may be another helpful tool to limit overestimation of peroxidation products [171].

5.2 The A/J Min/+ mouse as a model in dietary intervention studies

In vivo animal studies allow to take into account the entirety of physiological and pathophysiological processes of complex organisms. Rodents, like mice and rats, feature a high rates of genetic homogeneity when compared to humans [46, 172], and are therefore widely used as model organisms in scientific research. However, just like in the human population, the spontaneous development intestinal neoplasms in WT rodents is generally low [173–175]. In order to reduce the number of laboratory animals and decrease study durations, models with an accelerated rate of tumor formation were developed. Meeting these requirement, genetically engineered rodent models for CRC, e.g. the Min/+ mouse, the Pirc rat or the $Msh2^{-/-}$ mouse [49, 55, 176], can be distinguished from models, in which intestinal carcinogenesis is chemically induced [59, 102].

The adequacy of the use of rodents as models of human diseases is determined by the degree of phenotypic and mechanistic similarities in regard to the human pathogenesis. The main focus of the presented work was to elucidate the role of red meat in sporadic CRC, using the A/J Min/+ mouse, a model for spontaneous, *Apc*-driven intestinal carcinogenesis. Results in **Paper II and III** did not support the largely accepted hypothesis of a role of heme iron in CRC, and hence qualities and characteristics of the A/J Min/+ mouse model are discussed below.

5.2.1 Genetic events in CRC development

Sporadic CRC

APC is a negative regulator of the Wnt signaling pathway, and in sporadic CRC, mutations in the *APC* gene are considered the rate-limiting step in the adenoma-carcinoma sequence [23]. As mutations in *APC* are found in more than 80% of all sporadic CRC cases [33], the use of Min/+ mouse models in sporadic CRC research is highly relevant **(Paper II and Paper III)**. Also the

exposure of WT rodents to carcinogens, e.g. 1,2-dimethylhydrazine (DMH) or AOM, may result in aberrant Wnt signaling, but here, mutations are more frequently found in the gene coding for β-catenin (77% of tumors) than in *Apc* (33% of tumors) [177]. In A/J Min/+ mice, genetic and epigenetic events during the carcinogenesis have not yet been studied. However, studies that focused on the acquisition of mutations in the adenoma-carcinoma sequence in other Min/+ mouse models, found only low frequencies of mutations in both *Kras* and *Tp53* [178]. Also DMH/AOM-treated rodents rarely exhibit mutations in *Tp53*, whereas mutations in *Kras* are found at similar rates as in humans [177]. The potential of AOM-treatment of Min/+ mice in intervention studies should be further assessed, as the exposure of conventional Min/+ mice with AOM resulted in an increased transition of adenomas to invasive carcinomas, without enhancing the rate of mutations in the gene coding for β-catenin [179, 180]. Chemical induction of A/J Min/+ mice may be relevant in case of prevention trials [181], or when only small effects of the intervention can be expected, e.g. in case of dietary red meat.

Meat-related CRC

A number of studies have been carried out with the attempt to relate genetic and epigenetic alterations in human colorectal tumors to dietary and lifestyle habits. Hitherto, no conclusions can be drawn, in which way red meat or heme iron intake is related to specific mutations in key regulatory genes. In some studies, positive associations or trends were found between tumors, harboring a truncating mutation in *APC*, and the intake of red meat or heme iron from red meat and meat products [182, 183]. In contrast, other studies report positive associations between red meat or heme iron intake, and tumors that do not harbor a truncating mutation in *APC* [184–186]. Similarly, data on the influence of meat on *KRAS* mutations is inconsistent [183, 186, 187]. In a more consistent manner, intake of meat products and heme iron seems to be related to a higher frequency of G>A transitions [183, 186], which are commonly induced by alkylating compounds, e.g. NOCs [183, 186, 187], as well as AOM [48]. More studies are required to assess the relevance of *APC* and Min/+ mouse models in meat-related CRC.

Inflammation and CRC

In **Paper II and III,** heme iron and the various muscle foods did only have a minor impact on colonic carcinogenesis in A/J Min/+ mice. To confirm the models susceptibility towards colonic carcinogenesis, in **Paper IV**, A/J Min/+ mice were exposed to DSS, a non-genotoxic carcinogen. DSS-administration of rodents stimulates inflammatory processes in the colon and is a popular

model for human IBD or, when preceding AOM-treatment, a model for colitis-associated CRC [188]. Human colitis-associated CRC does not follow the typical histological and genetic changes of the adenoma-carcinoma sequence seen in sporadic CRC, and mutations in *APC* are only found to occur in later stages of the disease. Instead, in 85% of colitis-associated CRC cases, mutations or loss of function of *Tp53* are detected early, or even prior to the development of dysplasia [189]. Nevertheless, DSS-administration of conventional Min/+ mice was shown to enhance intestinal carcinogenesis, suggesting an interplay between *Apc* inactivation and inflammation. Moreover, higher rates of adenoma-carcinoma transitions in Min/+ mice can be induced by DSS-administration alone [190, 191]. Inflammation is attributed a significant role also in sporadic CRC [192] and hence, rather than being considered a model for colitis-associated CRC, DSS-administration of A/J Min/+ mice may represent a promising tool for investigations of the role of inflammation in sporadic CRC. The role of inflammation can be examined by DSS alone, or in combination with dietary interventions.

5.2.2 Intestinal carcinogenesis in the A/J Min/+ mouse

The main disadvantage of most models carrying mutations in *Apc* is that neoplasms are preferably located in the small intestine, and that tumor distribution poorly reflects the human phenotype of CRC [44]. Moreover, mice do not live until tumors turn metastatic, and even adenoma-to-carcinoma transitions are rare in these animals [55]. The A/J Min/+ mouse model, on the other hand, mirrors the pathology of human CRC more closely. In comparison with the conventional B6 Min/+ mouse, the proportion of colonic lesions in A/J Min/+ mice is increased **(Paper II, III and IV)**, and even without chemical induction, the incidence of invasive carcinoma development is a 100% in old animals [62].

Colonic carcinogenesis in the A/J Min/+ mouse

Most sporadic mutations are acquired during DNA-replication, and based on the assumption that the mutation rate of cells does not dependent on tissue or cell type, Tomasetti and Vogelstein [193] proposed that the risk to develop cancer in a specific organ is largely defined by the lifetime number of divisions of the respective stem cells. Taking into account a multitude of tissues and organs, they found a remarkable correlation between the human lifetime risk to develop a specific type of cancer and the number of stem cell divisions in the respective organ. They concluded that the human lifetime risk to develop colon cancer (0.048%), which is higher than the risk to develop cancer in the small intestine (0.0007%), goes along with a number of colonic stem cell divisions

($1.2x10^{12}$), which is four times higher than the number of divisions in the total small intestine ($2.9x10^{11}$), and more than 150 times higher than the number of divisions in the duodenum ($7.8x10^{9}$). They further argued that this ratio is the opposite of what has been observed in mice [193]. Compared to B6 Min/+ mice, Min/+ mice on the A/J genetic background exhibit an improved colonic-to-small-intestinal-lesion-ratio, and accordingly, WT mice of the A/J strain were shown to have a higher colonic proliferative index than WT mice of the B6 strain [194]. In the latter study, the high mitotic activity of the colonic tissue in A/J mice was also reflected by a high susceptibility to DMH-induced colonic carcinogenesis [194].

Wu *et al.* [195] put the adequacy of Tomasetti's and Vogelstein's estimations on division rates of several tissues into question, but, nonetheless, supports the theory that stem cell division rate represents an intrinsic key parameter in the acquisition of mutations. They conclude, however, that such intrinsic processes may influence cancer risks to a far lesser extent than extrinsic factors, e.g. inherited mutations in key regulatory genes. B6 and A/J Min/+ mice, however, share the same mutation in *Apc*, and it cannot be ruled out that the differences in the susceptibility to develop colonic neoplasms are partly due to varying rates of stem cell divisions.

Besides the mitotic rate, several cancer modifier loci in the murine genome have been recognized that modulate the susceptibility to develop cancer. In respect to colonic carcinogenesis, genome analyses of recombinant congenic strains have contributed to identify gene loci that either directly modulate susceptibility (Susceptibility to Colon Cancer loci (*Scc*) or Colon Cancer Susceptibility loci (*Ccs*)) [54, 59, 196], or have an indirect, modulating effect on susceptibility by modifying cancer-inducing gene mutations, e.g. mutations in *Apc* (Modifiers of Min (Mom)) [197, 198]. Indeed, the susceptibility of B6 and A/J WT mice towards AOM (with or without DSS-coadministration) was shown to be largely determined by the haplotypes of the susceptibility loci *Ccs3*, *Ccs4* and *Ccs5*, which are considered 'resistant' for B6 and 'susceptible' for A/J mice [54, 59, 199]. Likewise, the haplotype of *Mom*7, located on chromosome 18, has been found to be repressive in Min/+ mice on a B6 genetic background, while the homologous haplotype of A/J mice seems to be enhancing.

In summary, genetic variation in rodent models may affect the outcomes of experimental studies. Enhancing the expertise within the field of murine modifier of cancer susceptibility, in combination with the attempt to characterize the role of individual genes within the loci, has great

translational relevance, as orthologous regions of murine *Scc*s and *Mom*s are also found in the human genome [54, 197]. Regardless of the cause of the improved colonic carcinogenesis in A/J Min/+ mice, the opportunity to test effects of dietary compounds in the colonic environment of the mice represents a major advantage of the A/J Min/+ mouse model.

Advanced CRC in the A/J Min/+ mouse

Intervention studies with heme iron and meat, presented in **Paper II and III,** have been performed in young animals, covering the early phase of spontaneous carcinogenesis. Due to the unexpected results of these studies, it should be considered to study effects of dietary heme iron or meat also during later phases of CRC. The high frequency of adenoma-carcinoma transitions, as well as the invasive growth of cancerous tissue into a local lymph vessel that was documented in an old untreated A/J Min/+ mouse [62], suggests that the A/J Min/+ mouse model is well suited for this purpose. The risk of developing CRC, both sporadic and hereditary, increases with age [19, 200, 201], and one likely explanation for the frequent development of invasive carcinomas in A/J Min/+ mice is the lifespan of the animals. While Min/+ mice bred on an A/J genetic background can survive beyond the age of 50 weeks, B6 Min/+ mice require termination at about 17 weeks of age [55].

Hitherto, the availability of models that allow to study advanced CRC is limited, and the most common technique to mimic metastatic processes, is the transplantation of cancerous cells or tissues into animals [52]. Invasive adenocarcinomas have also been documented in the Pirc rat, one of the available rat models for *Apc*-driven tumorigenesis, but so far, no indications of metastases were given in these animals [49]. A model that covers the entire adenoma-carcinoma sequence, and even develops liver metastases 24 weeks after tumor induction, is the Apc CKO/LSL-Kras mouse. The mouse is a conditional *Apc* knockout mouse, which carries an additional mutation in *Kras*. However, despite of presenting many features that characterize a good model for human CRC, the main disadvantage of the model is that a surgical procedure is required to induce the local adenoviral infection that initiates the loss of *Apc* [52]. Hence, the A/J Min/+ mouse model seems to be unique in its potential to study all stages of CRC development.

5.2.3 Framework for designing of studies in A/J Min/+ mice

Despite the evidence of a link between processed and red meat and CRC [18, 81], the estimated risk of CRC associated with red and processed meat intake in humans is moderate **(Figure 11)**

[90]. This underlines the need for relevant models and study designs that allow to detect small differences between study groups within reasonable timeframes.

Determination of the study period

A general limitation of rodent models for CRC is the lack of feasible methods to assess tumor burden in living animals. Despite of the possibility to perform colonoscopies in mice and rats [49, 202, 203], reliable and less invasive methods are desired to detect early intestinal neoplasia, as well as to monitor intestinal carcinogenesis during animal studies. Advances have been made in Magnetic Resonance Colonography and other *in vivo* imaging techniques that represent promising tools of colonic tumor burden assessment in living animals [204, 205]. Alternatively, tumor burden can only be evaluated after termination of the animals, and reliable predictions of study outcomes are crucial for the choice of the duration of the experimental period. Although it is important to choose an experimental period adequate to avoid type II errors during statistical evaluation, a prolonged experimental period may give rise to ethical concerns and lead to challenges during the scoring of the intestines. Large tumors, for instance, may prevent the flattening of the intestines for microscopic examination and partially obscure the intestinal surface. The most important factor for the determination of the study duration is, however, the physiological relevance in regard to the hypothesis. In **Paper II and III,** the dietary interventions with heme iron or meat began at three weeks of age, directly after weaning. Various compounds may differentially affect the various stages of cancer development [206], and as susceptibility to AOM-induced intestinal carcinogenesis was shown to decrease with age in B6 Min/+ mice [207], an early exposure to heme iron and meat was chosen. The terminal age of mice in the studies included in this work **(Paper II, III and IV)** was 11-15 weeks, which falls into the period, in which the influx rate of flat ACF is at its highest [62]. The age of 11-15 weeks is equivalent to the age of approximately 20 years in humans [208], an age at which more than half of all FAP patients will already have developed intestinal adenomas. According to Half *et al.* (2009) [200], this percentage increases to 95% by age 35, and, if untreated, the onset of CRC is likely to occur one decade later. In **Paper IV** it was shown that inflammatory processes substantially accelerate carcinogenesis in young adult A/J Min/+ mice (10 to 12 weeks, until 13 to 15 weeks of age). In contrast, the effects of heme iron and muscle foods in **Paper II and III**, respectively, were less pronounced. However, the exposure time of 8 and 10 weeks in **Paper II and III**, respectively, seems appropriate. In similar intervention studies with intestinal neoplasia as an endpoint, a study

duration of approximately 14 weeks was chosen for AOM- or DMH-treated rats, which have a relatively longer life span than mice [112, 118, 209]. In B6 Min/+ mice, exposure to heme irons lasted over 7 weeks [112], and other dietary intervention studies with heme iron or red meat were conducted on B6 WT and $Msh^{-/-}$ mice, and lasted for 18 month and 26 weeks, respectively [174, 176].

Interindividual variation and determination of group sizes in experimental studies

The world population is characterized by a high level of genetic variation. Varying CRC incidence rates of ethnic groups, the existence of hereditary forms of CRC, and the role of specific gene polymorphisms in CRC demonstrate, how genetic factors influence the susceptibility of individuals to CRC development [19, 210]. As opposed to humans, in laboratory inbred mice (>20 brotherXsister matings), heterozygosity of gene loci reaches more than 98.6% [211], and the basic rational of using inbred strains in scientific research is to increase reproducibility. Although inbred, the range of the multiplicity of intestinal lesions in A/J Min/+ mice was found to vary substantially between individual animals in **Paper II and III**. Hence, in case of minor effects of the dietary interventions, large group sizes are required to detect differences between experimental groups. It should be noted, however that the biological relevance of the respective effects must be weighed against the inclusion of a large number of individuals.

Identifying determinants that influence phenotypic variation is a key objective within both epidemiological and animal studies. Factors that modulate susceptibility to carcinogenesis include the individual epigenetic signature [212] and microbiota composition. Despite of being housed in the same room and receiving the same kind of diet, microbiota varies between individuals and influences intestinal carcinogenesis. With the majority of tumors assumingly being initiated before the start of the study, tumor load in the DSS study **(Paper IV)** was explained by more than 50% by the microbiota composition prior to DSS-exposure. Additionally, even in inbred strains a certain degree of genetic drift cannot be avoided [213].

In humans, female sex hormones may prevent CRC development, and hormone replacement therapy may have protective effects in postmenopausal women [214]. Also in the Pirc rat, another model for *Apc*-driven CRC, male animals generally develop a greater tumor load than female animals [49]. A common tool to increase the statistical power of a study is therefore to restrict study groups to only one gender. Compared to the Pirc rat, however, gender differences are not as explicit in the A/J Min/+ mouse, and in **Paper III**, gender did not significantly affect intestinal

carcinogenesis. Hence, the inclusion of only one gender of A/J Min/+ mice in an experimental study **(Paper IV)** may not increase the reproducibility as much as it may in other rodent models.

5.3 Meat and intestinal carcinogenesis in A/J Min/+ mice

Ever since the inhibitory effect of calcium on heme iron-induced carcinogenesis was revealed, the number of rodent intervention studies focusing on the clarification of the association between red and processed meat and CRC, has been constantly increasing. Rodents have been provided heme iron in form of hemin (ferriprotoporphyrin IX chloride) [103, 111, 113, 118, 174, 215, 216] or hemoglobin (heme iron coupled with globular proteins) [102, 112, 114, 118, 216], as well as unprocessed [102, 119, 176, 217, 218] and processed meat [121, 209, 216, 219]. In most, but not all studies the hypothesis of a role of heme iron in meat-related CRC was supported. In the studies included in the present work, adverse effects of heme iron or red meat could not be reproduced in A/J Min/+ mice. While dietary heme was found to inhibit colonic carcinogenesis **(Paper II)**, the effects of red meat on intestinal carcinogenesis did not differ from the effects of white meat **(Paper III)**.

5.3.1 Dietary heme iron as model for meat consumption, and effects of preparation methods on muscle food

Both hemin (free heme iron) and hemoglobin (globin-bound heme iron) have been used to mimic the consumption of red meat in rodent studies, and hemin was shown to stimulate intestinal carcinogenesis more effectively than hemoglobin [118]. The association between CRC and meat consumption is stronger for processed meat, than red meat [81], and accordingly, the effects of hemin were compared to the effects of processed meat [216], while hemoglobin was proposed a model for fresh meat [102]. The rationale behind this hypothesis was based on the assumption that heme iron is released from myoglobin during meat processing, which may result in larger amounts of heme iron reaching the colon after ingestion. Free heme iron from processed meat may form polymers in the acid environment of the stomach, thereby decreasing the solubility and small intestinal absorption rate of heme [118, 220]. In accordance with this theory, fecal heme concentrations in rats were higher in response to hemin than in response to equivalent amounts of heme iron provided as hemoglobin [118]. In the digestive tract, however, heme iron is also released from the globin by enzymatic processes [221]. As the pH in the human stomach is lower than the pH in mice, protein denaturation and digestion in rodents may be less efficient than in

humans, and therefore, Ijssennagger [222] argues against the use of globin-bound heme iron as a model for red meat. To avoid a missing effect of heme iron in consequence of an insufficient release of heme iron from myoglobin in mice, A/J Min/+ mice were exposed to hemin in **Paper II.**

The more pronounced effect of processed meat on CRC is discussed to be related to the addition of substrates, e.g. nitrite, to meat products, as well as the formation of potentially carcinogenic compounds during processing [223]. In AOM-treated rats, intestinal tumor load was increased by dietary interventions with cooked ham, cured meat and hot dogs [121, 216, 219], but also unprocessed red meat [102, 119]. In these studies, however, the latter was blended into the diets after being freeze-dried, a process known to facilitate peroxidation processes [209]. In **Paper III,** moist muscle sources were added to the powder diet after being cooked sous vide, which is considered one of the most gentle methods to prepare meat [224]. The results indicate that gentle sous vide processing does not generate carcinogenic compounds in red meat that stimulate intestinal carcinogenesis in A/J Min/+ mice **(Paper III).** Also red meat, which was cooked and oven-dried, did not affect proliferation and in B6 WT and $Msh^{-/-}$ mice, and even increased the survival of $Msh^{-/-}$ mice [176, 218].

5.3.2 Meat and intestinal carcinogenesis in Min/+ mice

Hitherto, there are only three published studies that have tested the effect of meat or heme iron on *Apc*-driven carcinogenesis in Min mice [111, 112, 225]. In the study by Mutanen *et al.* [225] conducted on the conventional B6 Min/+ mouse, beef increased the number of distal small intestinal tumors, but not the total number of tumors in the small intestine or colon. However, in this study, calcium levels of the diet were not adjusted to match a 'Western style diet'. Bastide *et al.* [112] found the small intestinal tumor load in B6 Min/+ mice to be positively affected by dietary hemoglobin, and the effect of hemoglobin was accompanied by an increase in biomarkers for lipid peroxidation and fecal water cytotoxicity. Yet, despite the increase in lipid peroxidation and cytotoxicity, colonic carcinogenesis remained unaffected in this experimental setting. However, as mentioned earlier, the rate of colonic tumor formation in B6 Min/+ mice is generally low. Sødring *et al.* [111] investigated the effect of hemin in the A/J Min/+ mouse for the first time, and observed, in accordance with the study described in **Paper II**, an inhibitory effect of hemin on colonic carcinogenesis. Interestingly, despite of the protective effect of hemin in the

colon, Sødring *et al.* [111] found an increase in small intestinal tumor size. This was, however, not observed in **Paper II**. The lack of a promoting effect of hemin on colonic carcinogenesis in the study of Sødring *et al.* [111] may be explained by a rather low dietary fat content (4%). In **Paper II,** the fat content was higher, but consisted of mainly saturated fat, which is less susceptible to oxidation. In the study presented in **Paper III**, the colonic carcinogenesis remained unaffected by the different types of muscle foods, and also in the small intestine, the only significant effects of dietary muscle foods were found for dietary salmon, which resulted in a lower tumor burden than meat from terrestrial animals.

To summarize, evidence from studying the effects of red meat or heme iron on intestinal carcinogenesis in Min/+ mice is inconclusive, and the transferability of findings from the small intestine in B6 Min/+ mice to the colon remains questionable. Besides anatomical differences between small intestine and colon, the composition of the intestinal content changes along the intestine. Nature and quantity of compounds that colonic epithelium is exposed to, is largely determined by digestive and absorptive processes in the upper intestinal tract, and intestinal microbiota. The density of bacteria increases along the small intestine towards the colon (10^4 to 10^8 microorganisms per gram), where the highest densities are found (10^{10} to 10^{11} microorganisms per gram) [226]. A role of microbiota in CRC is likely [21], and a reduction of the bacterial colonization by antibiotic treatment decreased heme-induced proliferation in the colon of rodents [113, 114].

5.3.3 Other rodent models in meat-related CRC

In studies conducted with rodents other than Min/+ mice, animals were exposed to carcinogens (AOM/DMH) to induce the formation of intestinal lesions [102, 112, 118, 219]. Alternatively, findings were related to histological changes in the integrity of the intestinal epithelium or changes in carcinogenesis-related biomarkers, like proteins involved in proliferation or apoptosis [104, 114, 169].

In chemically initiated animals, dietary red meat and heme iron were shown to promote carcinogenic processes [118, 216]. AOM and DMH are alkylating compounds, resulting in DNA-adduct formation and an increased rate of DNA replication errors [48]. Hence, treatment with carcinogens accelerates the accumulation of mutations in key regulatory genes [177], readily inducing intestinal carcinogenesis [60, 207]. As a consequence, susceptibility towards additional

stimuli may be potentiated in carcinogen-treated animals. The novel A/J Min/+ mouse, however, provides the possibility to test the effects of dietary interventions on the spontaneous, *Apc*-driven intestinal carcinogenesis without the initial use of carcinogens. In this model, aging of the animals can be considered the driving factor in the acquisition genomic instability [62]. In **Paper II and III**, heme iron levels in the colon did not substantially enhance the acquisition of genetic alterations, but it cannot be excluded, however, that dietary heme iron could create an environment, in which the intestinal barrier function and tissue homeostasis is impaired or challenged. Potentially, these processes may contribute to CRC development provided that certain conditions, e.g. an advanced genomic instability, are met. Long time studies, or exposure of A/J Min/+ mice to AOM or DMH may help to elucidate in which way later stages of colonic carcinogenesis are influenced by dietary red meat or heme iron in A/J Min/+ mice.

The hypothesis that heme iron affects epithelial homeostasis, is in line with conclusions drawn from previous studies. Heme iron is suggested to damage the intestinal epithelium by enhancing the cytotoxicity of the intestinal content [102, 104, 105, 227], or by inducing unfavorable changes in microbiota composition, which may impair the integrity of the mucus barrier [113]. In either case, experimental results suggest that heme-induced epithelial damage may trigger compensatory hyperproliferation in epithelial cells [113, 169]. In these studies, however, the effects of heme were tested in untreated WT mice after short-term exposure (2 weeks), and hence, no connection between compensatory hyperproliferation and intestinal carcinogenesis could be established. Notably, only chronic proliferation is considered a hallmark of cancer [25], and whether compensatory hyperproliferation may eventually lead to malignant growth and cancer development is likely to be dependent on the degree of genomic integrity, and the functionality of repair and regeneration mechanisms within the epithelium [26]. Winter *et al.* [174] demonstrated that that an enhanced rate of cell proliferation in healthy animals does not inevitably result in uncontrolled cell division, as enhanced epithelial proliferation in B6 WT mice in response to the short term exposure to heme iron (4 weeks) did not persist in a long term study (18 month) [174].

Other mechanisms that have been associated with sporadic CRC and may impact tissue homeostasis in the intestine include inflammatory processes [20]. Red meat intake was positively related to the inflammatory marker C-reactive protein (CRP) in cross-sectional studies [228, 229], and was shown to impair DDS-induced colitis in mice [230]. Nevertheless, the role of diet in

inflammatory processes needs to be seen from a wider perspective, as increasing the intake of red meat at the expense of the energy intake from carbohydrates, may have rather positive effects on inflammation status and oxidative stress [231]. In rats, plasma CRP levels were not affected by heme iron [232], and in comparison with dietary chicken, plasma CRP was increased by dietary lean red meat, but not fat red meat [217].

5.3.4 Iron metabolism

Discrepancies between findings from **Paper II and III,** and most other experimental studies on meat-related intestinal carcinogenesis, may alternatively be explained by biologic and metabolic characteristics of A/J Min/+ mice, related to their genetic background. Identification of such factors has great relevance in regard to the assessment of the translational potential of studies, and therefore, common fecal biomarkers were analyzed in **Paper II and III**. In **Paper III**, fecal water content of heme correlated with levels of heme iron in muscle foods, but concentrations were up to ten times lower than in comparable studies conducted by Pierre and colleagues on rats [102, 110, 112, 121, 209, 223]. Hence, the observed lack of tumor induction by heme iron could be due to low concentrations of heme iron reaching the colon of A/J Min/+ mice. Iron metabolism varies largely between various species. While the absorption rate of heme iron in rats was found to be 60% lower than the absorption of non-heme iron, in humans heme iron is absorbed at 2-5 times higher rates than non-heme iron [233]. In case of iron deficiency in rats, only the absorption rates of non-heme is enhanced, while iron deficiency in humans and dogs is compensated by an increase in the absorption of both non-heme and heme iron [234]. Organic iron is also the superior source of iron in mice [235], but little is known about the effects of genetic backgrounds on iron absorption. A/J mice were shown to absorb free iron approximately twice as efficient as B6 mice [236], and active Wnt signaling may additionally increase the expression of organic iron transporter [237]. It should be noted, however, that a more effective absorption of non-heme does not imply that differences in iron absorption also exist for heme-iron. Reason for this is that the uptake of non-heme iron into enterocytes is mainly mediated by the divalent metal transporter 1 (DMT1), while heme iron is absorbed via receptor-mediated endocytosis or alternative transporters like Slc46a1 [238]. In case of a more efficient heme iron absorption in A/J Min/+ mice, the model may reflect the human iron metabolism more closely than the above mentioned rat and B6 mouse models.

Alternatively, the high fecal content of heme in the studies conducted by Pierre and colleagues may be related to the use of carcinogens. AOM does not only induce tumor formation in the colon, but may also affect epithelial homoeostasis in the small intestine. Kikuchihara *et al.* [239] found AOM to induce small intestinal tumors in rats, which were located posterior to the pyloric ring. Tumor development proceeds histological changes within the epithelium, e.g. decreased differentiation rates of cells within the intestinal crypts. Gene expression of the folate and heme transporter SLc46a1 [240] was shown to be increased in differentiated cells [240, 241], and it may therefore be speculated that AOM negatively influences the differentiation of small intestinal crypt cells, thereby decreasing the rate of heme iron absorption. As no non-AOM control (saline-treated) was included in these studies, there is no insight given into possible additional effects of AOM on metabolism in rats. However, also in separate studies, hemoglobin substantially increased the amount of fecal heme in untreated rats [110, 126]. Knowledge about the physiology of heme absorption is essential to understand the effects of heme iron in the intestine, and to evaluate whether findings from animal models may be applied to humans.

Irrespective of the iron metabolism, the low levels of fecal heme in A/J Min/+ mice may be related to the fact that fecal heme concentrations were not analyzed in total feces, but only in the soluble fraction, which is suspected to interact more strongly with the intestinal epithelium [121]. The solubility of heme depends on various factors, e.g. the precipitation of heme iron by dietary compounds like calcium [126], digestive processes [221], or chemical modifications of heme iron during meat processing and preparation. Incorporated in polypeptide chains (globins), heme iron from myoglobin is highly soluble and bioavailable in humans. Protein denaturation, however, taking place at cooking temperatures above 60°C, will increase the fraction of insoluble heme iron. Moreover, long cooking durations at high temperature will also increase the amount of iron set free from the porphyrin moiety, and raise the amount of luminal non-heme iron [242–244]. It must therefore be assumed, that the heating of muscle foods at 70°C for 50 minutes led to an increased fraction of non-heme and insoluble iron in **Paper III**.

Iron requirement is elevated during growth [245], and iron absorption in rats was shown to strongly depend on the age of the animals [246]. Thus, the rate of iron absorption, iron metabolism and possibly intestinal carcinogenesis may be determined by the initial age of animals receiving dietary heme iron in feeding experiments. In **Paper II** and in the previously published study on A/J Min/+ mice by Sødring *et al.* [111], dietary heme protected against the

development of colonic lesions. In these studies, as well as in **Paper III**, animals were assigned to experimental diets on day 19 to 21. In comparison, the initial age of B6 mice used in studies by the research group of van der Meer, was approximately 8 weeks of age [113, 169], and the initial age of rats in studies, performed by Pierre and colleagues, was approximately 5 weeks or older [118, 119]. Growth rate, i.e. the increase in body weight, in **Paper II** was greatest from week 3 to 4 (app. 60%) and decreased significantly in the following weeks (data not shown). Hence, the beneficial health effects of heme iron in regard to intestinal carcinogenesis in **Paper II** may be a result of the increased supply of heme iron during the growing phase of A/J Min/+ mice. While the total amount of dietary iron differed between the control and heme iron group in **Paper II**, in the study conducted by Sødring at al. [111], diets were balanced for total iron content by the addition of ferric chloride to the control diet. The inhibitory effect of heme iron, however, was the same in both studies, indicating distinct effects of heme iron and free iron on intestinal carcinogenesis in A/J Min/ mice.

Last but not least it should be mentioned that injection of rodents with heme iron (subcutaneous or intraperitoneal) was shown to induce HO-1, an enzyme regulating oxidative stress responses, and to prevent inflammation and mucosal damage [247, 248].

In conclusion, it needs to be established, whether the missing promoting effect of heme iron and red meat on intestinal carcinogenesis in A/J Min/+ mice may be related to a high absorption of heme iron in the small intestine, which lowers the concentration of heme iron in the colon.

5.4 Role of fat, lipid peroxidation and fecal water cytotoxicity in the intestinal carcinogenesis of A/J Min/+ mice

There is increasing evidence of a role of lipid peroxidation in CRC and other pathological conditions (e.g. metabolic syndrome, obesity, cardiovascular disease and diabetes mellitus) [106, 249–251], and in regard to the pathogenesis of red meat-related CRC, lipid peroxidation products, e.g. genotoxic aldehydes, have been related to heme-mediated cytotoxicity: In rodents, there was a lag time between the rise in luminal peroxidation products and fecal water cytotoxicity [104], and fecal water cytotoxicity on colonic epithelial cells was reduced when aldehydes were removed from fecal water prior to cellular exposure [112].

In accordance with other studies, dietary heme iron and fat increased TBARS formation in the studies presented in **Paper I, II and III**. However, while small intestinal and colonic carcinogenesis in A/J Min/+ mice was enhanced by dietary fat in **Paper II**, dietary heme iron was found to inhibit colonic carcinogenesis. Moreover, in **Paper III**, the addition of n-6 PUFA to beef meat enhanced TBARS formation, without affecting intestinal carcinogenesis. In comparison with diets containing salmon or pork, the cytotoxic potential of fecal water from mice fed beef was limited in **Paper III**, and hence, the results from **Paper II and III** do not give any indications of a direct relationship between luminal TBARS concentration, fecal water cytotoxicity and intestinal carcinogenesis in A/J Min/+ mice.

Fecal water cytotoxicity was proposed to be mediated though the formation of a CHF in the intestine [104, 215, 252]. Therefore, it cannot be excluded, that the low cytotoxicity of fecal water in response to the intake of beef in **Paper III** was related to the small amounts of heme reaching the colon, preventing the formation of a CHF. Also concentrations of fecal TBARS in **Paper II and III** appeared to be slightly lower than previously found concentrations in feces of rats and B6 Min/+ mice [79, 232]. Nonetheless, the variations in fecal water cytotoxicity in response to the experimental diets in **Paper III** cannot be easily interpreted, and as the cytotoxicity assay (MTT assay) does not allow to distinguish between apoptotic and necrotic processes, no clear conclusions can be drawn, whether an increased rate of cell death of *Apc*$^{-/+}$ cells represents an advantage or disadvantage in respect to intestinal carcinogenesis.

The lack of an association between lipid peroxidation and intestinal carcinogenesis indicates that the levels of peroxidation products in fecal water in **Paper II and III** did not harm the integrity of the intestinal crypts in a manner that resulted in malignant proliferation. Furthermore, the results indicate that the carcinogenic effect of fat, observed in **Paper II**, is likely to be related to other mechanisms than lipid peroxidation alone. MDA, 4-hydroxyhexenal (4-HHE), 4-hydroxynonenal (4-HNE) and 1,4-dihydroxynonane mercapturic acid (DHN-MA), the major urinary metabolite of 4-HNE, are commonly used markers for lipid peroxidation in meat-related CRC research [118, 165, 253]. These α,β-unsaturated aldehydes are generated from unsaturated fatty acids via several radical intermediates and have a wide range of biological effects. At low tissue concentrations, lipid peroxidation products may function as biological stimuli and affect cellular gene expression and signal transduction pathways [254]. In contrast, a dramatic increase of lipid peroxidation products may lead to the generation of DNA-adducts and unbalance in the

cellular redox potential, thereby inducing oxidative stress [249]. However, cellular oxidative stress does not arise before concentrations of reactive compounds exceed the capacity of the cellular defense mechanisms [106, 249, 250]. One oxidative stress response, which has been associated with resistance against heme-induced fecal water cytotoxicity, is mediated by the transcription factor nuclear factor (erythroid derived 2)-like 2 (Nrf2), which is involved in the expression of various antioxidant enzymes [255]. Cell culture studies performed on *Apc*$^{-/+}$ and *Apc*$^{+/+}$ cells, derived from B6 Min/+ mice and WT mice respectively, propose a more efficient antioxidative defense in *Apc*$^{-/+}$ cells, which may partly be explained by a stronger activation of Nrf2 in these cells. Aging is accompanied by a decline in the activity of Nrf2 in rats [256], and hence, it cannot be ruled out that the levels of lipid peroxidation products in **Paper II and III** may have a different effect on colonic tissue homeostasis in older mice.

One major point of criticism related to the proposed link between lipid peroxidation and meat-induced intestinal carcinogenesis is the fact that lipid peroxidation products are, in most rodent studies, measured in the intestinal content and not the colonic tissue [104, 112, 118]. The absorption of both endogenously formed and dietary peroxidation products, however, is documented in both humans and rodents [232, 257], and in a recent study on beef- and chicken fed rats, the MDA contents of colonic tissue (measured as TBARS) increased in parallel to higher concentrations of MDA in the gastrointestinal content in stomach and intestine of beef fed rats [217].

5.5 Role of meat-independent factors and microbiota in the A/J Min/+ mouse model

According to the American Institute for Cancer Research, 50% of all CRC cases can be prevented by following the recommendations for cancer prevention. This means that the number of all CRC could be halved, if people started to be more active, limit alcohol consumption, stop smoking and eat more healthy [83]. The main reason for the role of diet in CRC development is the fact that dietary components are in direct contact with the intestinal epithelium, and contribute to shaping the composition of microbiota by providing nutrients to the bacteria. In this connection, the avoidance of potentially harmful compounds is considered at least just as important as the ingestion of protective bioactive compounds [181, 237, 258].

In **Paper II,** dietary heme iron did not affect intestinal carcinogenesis as anticipated, and in order to confirm the responsiveness of the A/J Min/+ mouse model, a group of animals fed RM1 was included as a reference in the meat study **(Paper III)**, and the effects of intestinal inflammation on CRC were tested in **Paper IV**, by the administration of DSS. Taken together, the natural ingredient diet RM1 presented a substantially stronger tumor-inducing potential than the purified powder diet with added muscle foods **(Paper III)**, and A/J Min/+ mice were highly responsive to DSS-exposure **(Paper IV)**.

The effects of RM1 on the spontaneous intestinal carcinogenesis during the lifetime of A/J Min/+ mice have been described in detail [62], and also the capability of RM1 to induce intestinal carcinogenesis more strongly than various powder diets has been reported previously [62, 259]. Various plausible factor may explain the CRC-promoting effect of RM1 observed in **Paper III**. On the one hand, RM1 contains high levels of non-heme iron. Non-heme iron has been related to an increased carcinogenesis in Min/+ mice, and activation of Wnt signaling was shown to enhance protein expression of iron transporters in cultivated colon cells [237]. On the other hand, a substantial amount of energy from RM1 is provided by carbohydrates, and a high glycemic load has been found to be associated with CRC development [260]. Indications are also given that replacing carbohydrate-rich foods by red meat favorably affects markers of inflammation and oxidative stress [231]. Another ingredient of RM1 is de-hulled, extracted, toasted soya, and it cannot be excluded that soya may contain low concentrations of carcinogenic compounds, formed in response to structural changes during heat treatment (Maillard reaction) [261]. Taking together the results form **Paper II and III**, it appears that unknown components or the different proportion of macronutrients in the RM1 diet determine the rate of intestinal carcinogenesis in maturing A/J Min/+ mice to a much larger extent than the presence of dietary heme iron.

Besides the effects of inflammation on colonic carcinogenesis in A/J Min/+ mice, in **Paper IV**, temporal changes in microbiota composition and SCFA profile were investigated, and related to carcinogenesis. The role of microbiota in inflammation and intestinal carcinogenesis was not within the main scope of the present work, but as could be expected, DSS-administration was accompanied by alterations in microbiota composition, which could be further linked to intestinal carcinogenesis. The most pronounced effect of DSS-administration was an increase in the relative abundance of *Bacteroides, B. acidifaciens* and *[Prevotella]*. Interestingly, while the load of colonic flat ACF was mainly related to the bacteria composition after DSS-administration, the

load of colonic tumors, which have most likely been initiated prior to DSS-exposure, was mainly associated with microbiota composition prior to DSS-administration. Also Zackular *et al.* [262] found the susceptibility to intestinal carcinogenesis to vary in mice, when microbiota composition was altered in response to antibiotic therapy. Operational taxonomic units (OTUs), negatively related to tumor load were e.g. OTUs of *Lactobacillus*. Exposure to DSS temporarily decreased microbiota diversity, and modulated intestinal concentrations of SCFA. The study emphasizes the overall importance of microbiota composition in the intestinal carcinogenesis of A/J Min/+ mice.

6 FUTURE PERSPECTIVES

- ✓ Lipid peroxidation during *in vitro* digestion of meat only reflected *in vivo* conditions to a certain extent. As only the oral, gastric and small intestinal phase was simulated in the *in vitro* model, the inclusion of a colonic phase, combined with further optimizations would enhance the applicability of the model in regard to peroxidative processes along the gastrointestinal tract.
- ✓ Heme iron and cooked red meat did not enhance intestinal carcinogenesis in maturing/young adult A/J Min/+ mice. Long-term studies, or studies on carcinogen-treated A/J Min/+ mice (AOM, DMH or DSS) are warranted to examine the effect of heme iron or red meat during advanced stages of CRC.
- ✓ A carcinogenic effect of heme iron has been observed in other rodent models, and comparative studies with A/J Min/+ mice and B6 Min/+ mice or carcinogen-induced CRC models will help to identify crucial factors and mechanisms that account for the observed discrepancies between the studies. Isotope-labeled, dietary heme can be used to elucidate the fate of heme iron in the gastrointestinal tract of rodents.
- ✓ The association between processed meat and CRC in epidemiological studies is stronger than the association between CRC and unprocessed red meat. Testing the effects of dietary processed meat, e.g. bacon or sausage, on intestinal carcinogenesis presents a new approach to study the effects of meat in the A/J Min/+ mouse model.
- ✓ The rate of tumor formation varies substantially between individual A/J Min/+ mice and carcinogenesis has been associated with the initial microbiota composition in A/J Min/+ mice. Analysis of microbiota and their metabolites may contribute to a better understanding of the role of microbiota in CRC, and to explain variations in susceptibility to intestinal carcinogenesis in individual mice.
- ✓ To better understand the interindividual variation in experimental settings, it is also of interest to assess the epigenetic variation of animals, and to investigate genomic alterations that go along with intestinal carcinogenesis in A/J Min/+ mice. Quantification of epigenetic changes and mutations in key regulator genes like *KRAS* or *Tp53* in the adenoma-carcinoma sequence, will also contribute to further evaluation of the relevance and translational potential of the A/J Min/+ mouse model.
- ✓ Colonic cancer tissue from A/J Min/+ mice could be used to derive cells lines of the $Apc^{-/-}$ genotype as a tool in *in vitro* studies.

7 CONCLUSIONS

The main conclusions that can be drawn from this work are as follows:

- ✓ The link between red meat and intestinal carcinogenesis is not supported in A/J Min/+ mice, as heme iron and red meat intake did not accelerate tumor development in maturing/young adult A/J Min/+ mice under the given conditions. On the contrary, indications were given that heme iron inhibits colonic carcinogenesis in these mice.
- ✓ In regard to intestinal carcinogenesis in A/J Min/+ mice, salmon intake resulted in a more beneficial outcome than the intake of meat from terrestrial animals (white meat and red meat). Intake of white meat did not result in a more favorable effect on intestinal carcinogenesis than intake of red meat.
- ✓ Intestinal carcinogenesis in the small intestine and colon was enhanced when energy from fat in the diet was raised from 10% to 40%. Increasing the level of n-6 PUFA in beef did not modulate the tumor-promoting potential of beef in A/J Min/+ mice.
- ✓ Intestinal carcinogenesis in maturing/young adult A/J Min/+ mice was not related to luminal lipid peroxidation or fecal water cytotoxicity. In addition, there was no evident relationship between lipid peroxidation and fecal water cytotoxicity.
- ✓ Intestinal carcinogenesis in A/J Min/+ mice is enhanced by DSS-administration, and the mouse model is highly susceptible to inflammation-induced colonic carcinogenesis.
- ✓ Intestinal carcinogenesis in A/J Min/+ mice is related to microbiota composition.
- ✓ When investigating lipid peroxidation in *in vitro* digestion models, a colonic phase (fermentation phase) should be included to mirror *in vivo* conditions more closely.

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Errata

Table of content	**Conclutions** replaced by **Conclusions**
II	**MDA Malondialdehyde** *(added)*
IV, l. 15	**hydroxyhexanal** replaced by **hydroxyhexenal**
VI, l. 9	**til** *(added)*
VI, l. 15	**hydroxyhexanal** replaced by **hydroxyhexenal**
VI, l. 24	**Hemjernkreftprosessen** replaced by **hemjern kreftprosessen**
VI, l. 25	**ble** (added)
VII, l. 7	**carsinogensom** replaced by **karsinogen som**
p. 15, l. 16	**32 kg** *(in one line)*
p. 17, l. 18	**20-25 g/d** *(spacing added)*
p. 28, l. 14	**Malondialdehyde** replaced by **MDA**
p. 30, l. 11	**b-catenin** replaced by **β-catenin**
p. 37, l. 12	**results indicates** replaced by **results indicate**
p. 42, l. 14	**mentioned that** *(comma removed)*
p. 43, l. 24	**Malondialdehyde** *(removed)*
p. 43, l. 24	**hydroxyhexanal** replaced by **hydroxyhexenal**
p. 44, l. 5	**, which** *(comma added)*
p. 44, l. 8	**defence** replaced by **defense**
p. 44, l. 24	**can** replaced by **could**
p. 47, l. 32	***Apc*$^{-/-}$** *(in one line)*
p. 48, l. 1	**Conclutions** replaced by **Conclusions**
p. 49, reference 21	*List of authors corrected*
p. 50, reference 26	*List of authors corrected*
p. 59, reference 181	*List of authors corrected*
p. 61, reference 205	*List of authors corrected*
p. 61, reference 213	*List of authors corrected*
p. 61, reference 217	*List of authors corrected*

Appendix: Scientific Papers I-IV

I

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

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Formation of Malondialdehyde, 4-Hydroxynonenal, and 4-Hydroxyhexenal during *in Vitro* Digestion of Cooked Beef, Pork, Chicken, and Salmon

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ABSTRACT: Red meat high in heme iron may promote the formation of potentially genotoxic aldehydes during lipid peroxidation in the gastrointestinal tract. In this study, the formation of malondialdehyde (MDA) equivalents measured by the thiobarbituric acid reactive substances (TBARS) method was determined during *in vitro* digestion of cooked red meat (beef and pork), as well as white meat (chicken) and fish (salmon), whereas analysis of 4-hydroxyhexenal (HHE) and 4-hydroxynonenal (HNE) was performed during *in vitro* digestion of cooked beef and salmon. Comparing products with similar fat contents indicated that the amount of unsaturated fat and not total iron content was the dominating factor influencing the formation of aldehydes. It was also shown that increasing fat content in beef products caused increasing concentrations of MDA equivalents. The highest levels, however, were found in minced beef with added fish oil high in unsaturated fat. This study indicates that when ingested alone, red meat products low in unsaturated fat and low in total fat content contribute to relatively low levels of potentially genotoxic aldehydes in the gastrointestinal tract.

KEYWORDS: *red meat, salmon, malondialdehyde, TBARS, 4-hydroxynonenal, 4-hydroxyhexenal, in vitro digestion, lipid peroxidation*

INTRODUCTION

Intake of red meat (beef, pork, lamb, and goat) and processed meat is associated with increased risk of colorectal cancer (CRC).[1,2] Both epidemiologic[3,4] and experimental evidence[5,6] indicate that the high level of heme iron in red meat may contribute to the promotion of CRC. Besides acting as a catalyst that facilitates the endogenous formation of potentially carcinogenic N-nitroso compounds (NOCs),[7] heme iron may provoke carcinogenesis through the formation of cytotoxic and genotoxic aldehydes by lipid peroxidation.[8]

Several studies have monitored the oxidation occurring during digestion of meat in the gastric phase[9−11] and identified the stomach as a bioreactor where an acidic and oxygen-rich environment promotes peroxidation.[12] One of the most abundant secondary oxidation products and commonly used biomarker for oxidative stress is malondialdehyde (MDA), which is formed during the decomposition of lipid hydroperoxides produced during peroxidation of unsaturated fatty acids, preferentially long-chain polyunsaturated fatty acids.[9,13,14] The mutagenic and genotoxic properties of MDA have been extensively described.[15] Other potentially genotoxic secondary oxidation products are 4-hydroxynonenal (4-HNE) and 4-hydroxyhexenal (4-HHE), which are products of the oxidative breakdown of hydroperoxides derived from n-6 and n-3 polyunsaturated fatty acids, respectively. Next to MDA, they represent the major aldehydes formed during peroxidation.[16−18] In contrast to other biomarkers of oxidative stress *in vivo,* such as the isoprostanes, the levels of MDA and 4-hydroxyalkenals in blood and urine are highly affected by dietary factors, for example, peroxidation during digestion.[8] In particular, MDA and 4-HNE has been shown to be involved in a number of pathologies such as metabolic diseases, neurodegenerative diseases, and cancers, probably due to their chemical reactivity and ability to form covalent adducts with macromolecules.[19] High levels of MDA and 4-HNE in serum, urine and feces have been associated with various types of cancers, including cancers in the digestive tract.[8,20−22]

The present study investigates the formation of 4-HNE and 4-HHE and MDA equivalents measured as TBARS (thiobarbituric acid reactive substances) during *in vitro* digestion of fish and meat products commonly used for domestic cooking. Since the formation of these aldehydes during digestion may contribute to an increased risk of CRC, it is hypothesized that red meat (beef and pork) due to a high content of heme iron may induce higher levels of aldehydes than white meat (chicken) and fish (salmon), which are not associated with CRC. Since differences in total fat content and fatty acid composition may influence the rate of peroxidation in the gastrointestinal tract, beef products with increasing fat contents and a beef product with added fish oil were included in the study. To our knowledge, a comparative study of gastrointestinal peroxidation of marine, mammalian, and avian protein sources has not been reported before.

MATERIALS AND METHODS

Chemicals. Pepsin (porcine, P7000, 683 U/mg solid), pancreatin (porcine, P1750), bile extract (bovine/ovine, B8381), thiobarbituric acid (TBA), 1,1,3,3-tetraethoxypropane (TEP), propyl gallate, and ethylenediaminetetraacetic acid (EDTA) were obtained from Sigma-Aldrich Co (St. Louis, MO). Trichloroacetic acid (TCA) was

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Table 1. Contents of Fat, Nitrite, Iron, and Peroxide Value (PV) in Raw Materials

	study 1				study 2			
sample	minced beef	minced pork	minced chicken	salmon loin	beef sirloin	minced beef	minced beef	minced beef + fish oil
fat, declared (%)	10.0	9.0	9.5	14.0	2.3	10.0	14.0	14.0[a]
fat, analyzed (%)	8.9	6.4	8.5	11.4	2.3	12.2	13.6	16.2[a]
iron (mg/kg)	20.0	9.7	7.2	3.2	24.3	21.7	15.7	21.7[a]
pv (mequiv/kg fat)	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<1.5[a]
nitrite (mg/kg)	<0.16	<0.16	<0.16	<0.16				

[a]Calculated value.

Table 2. Content of Fatty Acids (%) and Relative Number of Double Bonds (DB, Arbitrary Units) in Raw Materials

	study 1				study 2			
	minced beef (10.0%)	minced pork (9.0%)	minced chicken (9.5%)	salmon loin (14.0%)	beef sirloin (2.3%)	minced beef (10.0%)	minced beef (14.0%)	minced beef + fish oil (14.0%)
fatty acids (%)								
C14:0	2.6	1.3	0.6	1.9	2.8	2.8	2.7	2.0[a]
C16:0	24.3	22.7	18.5	8.9	27.3	25.9	24.7	18.6[a]
$C16:1_{n-7}$	3.6	2.7	3.1	2.1	3.5	3.3	3.3	2.2[a]
C18:0	16.6	12.1	5.6	2.8	15.8	18.0	19.0	13.1[a]
$C18:1_{n-9}$	42.0	44.8	35.1	43.1	43.5	42.5	42.6	30.9[a]
$C18:2_{n-6}$	1.6	10.6	30.9	4.3	1.8	1.5	1.7	1.1[a]
$C18:3_{n-3}$	0.4	0.9	2.9	4.8	0.5	0.5	0.5	0.3[a]
$C18:4_{n-3}$	0.0	0.0	0.1	0.5	0.0	0.0	0.0	0.1[a]
$C20:1_{n-9}$	0.2	1.0	0.6	3.8	0.1	0.2	0.2	2.2[a]
$C20:4_{n-6}$	0.1	0.1	0.5	1.5	0.2	0.1	0.1	0.9[a]
$C20:5_{n-3}$	0.0	0.0	0.1	2.9	0.1	0.0	0.0	16.0[a]
$C22:5_{n-3}$	0.0	0.0	0.1	1.5	0.1	0.1	0.1	0.6[a]
$C22:6_{n-3}$	0.0	0.0	0.2	4.1	0.0	0.0	0.0	6.3[a]
DB[b]	50.60	72.80	113.90	126.60	54.00	51.40	51.90	162.63[a]

[a]Calculated value. [b]DB is the relative number of double bonds per g fat in the product.

purchased from Merck KGaA (Darmstadt, Germany). Chemical standards of undeuterated and deuterated (D_3) 4-hydroxy-2-hexenal (4-HHE) and 4-hydroxy-2-nonenal (4-HNE) were purchased from Cayman Chemical (Ann Arbor, MI). Derivatization reagent *O*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine (PFBHA) hydrochloride was obtained from Fluka Analytical (Buchs, Germany), and *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane was obtained from Sigma-Aldrich Co (St. Louis, MO). All other chemicals used were of analytical grade.

Raw Materials. Fresh beef sirloin, minced beef, minced pork, minced chicken leg, and salmon loin (Atlantic salmon, *Salmo salar* L.) were purchased in local retail stores. The minced beef product is traditionally made from several low-value beef cuts. The products did not contain additional salt, water, antioxidants, or other additives. Samples of all products were immediately stored at −80 °C and sent to Eurofins Food & Agro Testing AS (Moss, Norway) within 1 week for analysis of fat content (NMKL 131, 1989), fatty acid composition,[23] contents of nitrite (NMKL 100, 2006), and total iron (NMKL 161, 1998) and peroxide value (PV) (AOAC Official Method 965.33, 1969) (Tables 1 and 2). Eurofins laboratories are accredited according to the ISO 17025 standard. The degree of unsaturation, that is, the relative number of double bonds per gram of fat (DB) (Table 2), was calculated by the formula

$$\sum_{n=1}^{6} n(\%\ \text{fatty acids with } n \text{ double bonds})$$

Fish oil (PronovaPure 500:200 TG (triglycerides) with high levels of eicosapentaenoic acid (EPA, 55.6%) and docosahexaenoic acid (DHA, 21.7%)) was provided by Pronova BioPharma Norge AS (BASF, Germany). The oil was stabilized with antioxidants, for example, tocopherols, and peroxide value (PV) was 5.0 mequiv/kg and anisidine value (AV) was 8.7.

Sample Preparation. All samples were vacuum-packed without exceeding a thickness of 1.5 cm and cooked for 50 min at 70 °C in a water bath. Beef sirloin and salmon loin were ground (3 mm grid) before vacuum packing to match the texture of minced meat products. Hence, there was no need for further processing (chewing) in the oral phase. After cooking, the samples were cooled on ice and stored at −40 °C until use (less than a month). In order to study the effect of additional homogenization before digestion, samples of cooked minced beef were homogenized for 10 s at 7000 rpm with a Polytron PT 1300 D homogenizer (Kinematica, Lucerne, Switzerland). In order to study the effect of increased amount of unsaturated fat in minced beef, cooked minced beef (10% fat) was manually blended with 4% fish oil to obtain a final fat content of 14%.

***In Vitro* Digestion.** A static *in vitro* digestion model was used. The model is in principle based on the EU Cost Action 1005 INFOGEST harmonized digestion method[24] and the procedure for lipid digestion described by Aarak et al.[25] For each time point investigated, that is, undigested (UD), end of gastric phase (GP), and end of intestinal phase (IP), individual sample tubes were prepared in triplicate. Digestive fluids mirroring the physiologic electrolyte composition, pH, and enzyme concentrations in humans were prepared for the oral, gastric, and intestinal phases (Table 3). $CaCl_2{\cdot}(H_2O)_2$ was added to the digestive fluids separately before the addition of digestive enzymes.

One gram of product was blended with 1 mL of simulated saliva fluid (SSF) (Table 3) simulating the oral phase. No saliva amylase was added because the content of carbohydrates in meat and salmon is negligible. Thereafter, 2 mL of simulated gastric fluid (SGF) (Table 3) was added resulting in a final pepsin concentration of 2000 U/mL. The pH was adjusted to 3.0 with 10 M HCl before incubation in a shaking incubator (37 °C, 215 rpm) for 120 min (Innova 40/40R, New Brunswick Scientific, Edison, NJ). After completing the gastric step, 4 mL of simulated intestinal fluid (SIF) was added to the tubes (Table 3) resulting in a final bile salt concentration of 10 mM and

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Table 3. Composition of Digestive Fluids: Simulated Saliva Fluid (SSF), Simulated Gastric Fluid (SGF), and Simulated Intestinal Fluid (SIF)

	SSF, pH 7	SGF, pH 3	SIF, pH 7
KCl	0.936 g/L	2.621 g/L	
KH_2PO_4	2.720 g/L	0.122 g/L	
$NaHCO_3$	0.672 g/L	1.092 g/L	5.929 g/L
NaCl	0.240 g/L	2.400 g/L	
$MgCl_2·(H_2O)_6$	0.061 g/L	0.122 g/L	
$CaCl_2·(H_2O)_2$	0.588 μg/L	0.176 μg/L	
pepsin		8.439 g/L (4000 U/mL)	
bile extract			20 mM
pancreatin			2.4 g/L

pancreatin concentration of 1.2 g/L. The pH was adjusted to 7.0 with 10 M NaOH before a further incubation period of 80 min. Sample tubes removed at the end of gastric phase (GP) were adjusted to pH 7.0 by addition of NaOH and placed on ice in order to stop the enzymatic activity. When digestion was completed, all samples (UD, GP, and IP) were immediately prepared for TBARS measurement. Blank samples, that is, samples where distilled water replaced meat, were prepared following the same procedure as the other samples.

Determination of Malondialdehyde (MDA) Equivalents. The analysis of TBARS (thiobarbituric acid reactive substances) was adapted from the method developed by Lemon.[26] The TBARS assay is nonspecific and also monitors other compounds than MDA such as aldehydic products, alkanals, protein, and urea.[27] Still it is the most common method used for estimation of MDA. The assay is based on the formation of a stable chromophore through the binding of aldehydes like malondialdehyde (MDA), that is, MDA equivalents, to thiobarbituric acid (TBA) under acid conditions and heat. The chromophore, TBA_2−MDA, can be quantified spectrophotometrically by means of the absorbance at 532 nm and a standard curve generated from 1,1,3,3-tetraethoxypropane (TEP). For TBARS analysis, undigested samples and samples from the gastric phase and intestinal phase, as well as blank samples, were replenished with distilled water to 8.5 mL. Samples were kept on ice during the entire preparation. In order to precipitate proteins and stop the enzymatic reactions, 1 mL of a solution containing trichloroacetic acid (TCA), propyl gallate, and EDTA was added to the samples, resulting in final concentrations of 7% TCA, 0.1% propyl gallate, and 0.1% EDTA. All samples were then homogenized for 10 s using a Polytron PT 1300 D homogenizer (Kinematica, Lucerne, Switzerland) at 7000 rpm and incubated on ice for another 10 min. Finally, the samples were filtered through an ashless quantitative filter paper (Grade 589/2 white ribbon, Whatman, Little Chalfont, UK). Hence, only the free fraction of TBARS was measured.

At this stage, aliquots of the filtrate were frozen at −20 °C for further analysis of 4-hydroxyalkenals (see below); the rest was used for TBARS analysis. For preparation of the standard stock solution, TEP was dissolved in distilled water. On the day of use, the stock solution was further diluted with a 7% TCA-solution containing 0.1% propyl gallate and 0.1% EDTA, to cover the range from 0 to 10 μM MDA. After the addition of freshly prepared 0.94% TBA-solution (0.25 mL was added to 4.7 mL sample), standards and samples were heated at 100 °C for 35 min and subsequently cooled on ice. Their spectral images were recorded with a full UV/vis spectrum absorbance microplate reader (SPECTROstar nano, BMG Labtech, Ortenberg, Germany) in the visible range of 400−700 nm. A baseline correction was applied by subtracting the absorbance at 573.5 nm from the absorbance at 532 nm. Results are presented as mean values after subtraction of blank values. Accuracy and recovery was determined by spiking the samples with TEP before the addition of TBA.

Determination of 4-Hydroxyalkenals (4-HNE, 4-HHE). A modified method based on Luo et al.[28] was validated in-house. Only the free fraction of 4-hydroxyalkenals was measured. Frozen (−20 °C) digested samples prepared with TCA and filtered (see above) were thawed, and internal standards, deuterated 4-HHE-D3 and 4-HNE-D3, were added to 0.5 mL of sample. Prior to GC/MS analysis, *O*-pentafluorobenzyl-oxime-trimethylsilyl ether (PFB-oxime-TMS ether) derivatives of the syn and anti stereoisomers of the respective 4-hydroxyalkenals were generated during a two-step derivatization; after the initial formation of the oxime derivative using PFBHA in methanol, samples were evaporated to dryness. The second step was the silylation of the hydroxyl group with BSTFA. An Agilent 7890A gas chromatograph interfaced with a 5975C mass selective detector (Agilent Technologies, Little Falls, DE) was used with 1 μL splitless injection. The PFB-TMS derivatives were separated on a HP-5MS fused silica capillary column (30m × 0.25 mm × 0.25 μm) using helium as carrier gas at a flow rate of 1 mL/min. The oven temperature was programmed from 50 °C (1 min) at 10 °C/min to 240 °C, followed by 20 °C/min to 300 °C (5 min.). Transfer line temperature was maintained at 280 °C. Derivatized aldehydes were measured in negative ion chemical ionization mode. Methane was used as reagent gas with source pressure 2.3×10^{-4} Torr. Ion source temperature was 230 °C, with electron ionization energy of 100 eV. Mass spectra of derivatized standard compounds of 4-HHE and 4-HNE were first recorded in full scan for identification of target ions for quantitation in selected ion monitoring (SIM) mode. The two syn and anti isomers of their respective PFB-oxime-TMS ether derivatives were monitored at m/z 291 (4-HHE), and m/z 283 (4-HNE), and quantification was done by measuring m/z 294 and m/z 286, respectively, of the deuterated 4-HHE-D_3 and 4-HNE-D_3 internal standards. Repeatability of the analysis of the two 4-hydroxyalkenals measured in replicate digest samples was within 10%. Limit of quantification ($S/N = 10$) was 0.7 ng/mL digest and limit of detection ($S/N = 3$) was 0.2 ng/mL digest.

Experimental Design and Statistical Analyses. Two separate studies were performed. Study 1 compared products of meat and salmon with similar fat contents (about 10−14% fat), and study 2 compared beef products with various fat contents (about 2−14% fat) and fatty acid composition, that is, minced beef with added fish oil (14% fat) (Tables 1 and 2). All experiments were performed in triplicate. In study 1, only one to two products were investigated per day, always including minced beef as a control, and the formation of MDA equivalents was measured in both gastric phase and intestinal phase. In study 2, the formation of MDA equivalents was only measured at the end of intestinal phase. 4-Hydroxyalkenals were measured in both gastric phase and intestinal phase. Statistical analyses of designed experiments were performed with SigmaPlot, version 13. Variations in MDA equivalents during *in vitro* digestion (UD, GP, and IP) due to day-to-day variation, product type, or fat content were determined by two-way ANOVA followed by Holm−Sidak post hoc test. Comparison of blank samples and effects of homogenization and addition of fish oil to minced beef were examined using Student's *t* test (two-tailed). Nominal level for significance was 5%. Associations between two variables (cross correlations) were analyzed by calculating Pearson correlation coefficient (r).

RESULTS

Evaluation of TBARS Measurements. Repeated experiments with minced beef (10% fat, study 1) during a period of 1 month showed no significant day-to-day variation in TBARS measurements, but clearly demonstrated significantly higher levels of MDA equivalents in intestinal phase compared with gastric phase and undigested material (Figure 1). Blank samples also showed a significant increase in MDA equivalents from gastric phase (1.4 ± 1.8 μmol/kg) to intestinal phase (4.3 ± 0.7 μmol/kg); however, levels were generally low compared with minced beef samples, indicating only minor contribution from the digestive fluids to the formation of MDA equivalents.

Effect of Homogenization of Minced Beef. In order to examine the effect of food texture on gastrointestinal peroxidation, minced beef samples (10% fat, study 1) were homogenized or left in the minced state prior to *in vitro*

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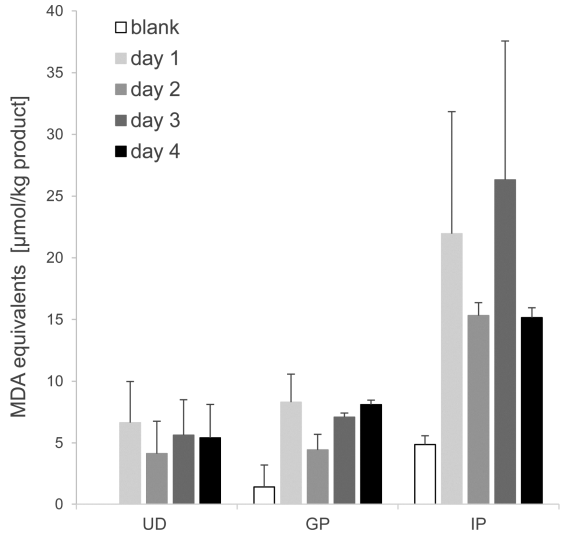

Figure 1. Average concentration of MDA equivalents in blank samples (white bars) and minced beef (10%) in repeated *in vitro* digestion experiments (day-to-day variation, gray and black bars). In each experiment, MDA equivalents (μmol/kg product) were measured before digestion (UD) and at the end of gastric phase (GP) and intestinal phase (IP). Results are presented as mean values. Vertical bars represent the standard deviations.

digestion. Results showed no significant effect of homogenization on the formation of MDA equivalents during *in vitro* digestion (Figure 2).

Determination of MDA Equivalents during *in Vitro* Digestion of Minced Meat and Salmon. The formation of MDA equivalents during *in vitro* digestion was compared between minced red meat (beef and pork), minced chicken, and salmon loin, all commercial products with comparable fat contents (study 1, Table 1). Since there was no significant day-to-day variation in TBARS measurements (Figure 1), MDA equivalents in minced beef was presented as the average of all measurements, and all products could be compared with each other. The results (Figure 3) showed that the levels of MDA equivalents (μmol/kg product) in undigested samples were low, ranging from 2.9 μmol/kg in salmon to 6.7 μmol/kg in minced pork, and that the levels increased significantly from gastric to intestinal phase in all samples. In minced chicken and salmon, a significant increase was also observed from undigested to gastric phase.

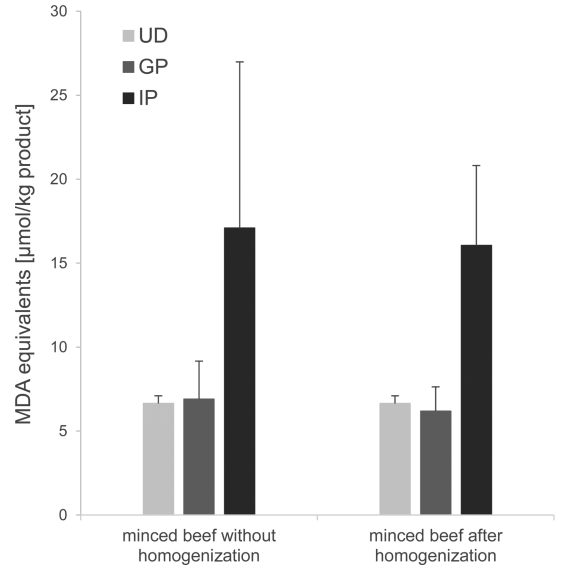

Figure 2. Effect of homogenization on the formation of MDA equivalents in cooked minced beef (10% fat) during *in vitro* digestion. MDA equivalents (μmol/kg product) were measured before digestion (UD, light gray) and at the end of gastric phase (GP, dark gray) and intestinal phase (IP, black). Results are presented as mean values after subtraction of blank values. Vertical bars represent the standard deviations.

Compared with minced beef, both salmon and chicken showed significantly higher levels of MDA equivalents (μmol/kg product) in the gastric phase and intestinal phase, whereas minced pork only showed slightly higher levels in gastric phase. Further comparison showed that salmon had significantly higher levels of MDA equivalents in gastric and intestinal phases compared with minced chicken and pork, and minced chicken had significantly higher levels in gastric and intestinal phases than minced pork.

Cross correlations showed that the relative number of double bonds per gram fat (DB) in the raw materials was clearly the factor that was best correlated to both the level of MDA equivalents (μmol) per kilogram of fat and per kilogram of meat in the intestinal phase (r = 0.97 and r = 0.95, respectively).

Determination of MDA Equivalents during *in Vitro* Digestion of Beef Products with Different Fat Contents. Two-way ANOVA was used to compare minced beef sirloin (2.3% fat) to regular minced beef products with higher fat contents of 10% and 14% (analyzed values 12.2% and 13.6%) (study 2, Table 1). The two minced beef samples varied in iron content (21.7 nd 15.7 mg/kg) and contained somewhat lower amounts of iron than beef sirloin (24.3 mg/kg). MDA equivalents (μmol/kg product) increased significantly in all samples during *in vitro* digestion but were generally lower in beef sirloin than in the minced beef products, although the level of MDA equivalents per kilogram fat was higher (Figure 4). No difference was found between the two minced meat samples. Although not significantly different, the percentage increase during digestion was highest for 10% fat minced beef (69.3% ± 13.4%), followed by 14% fat minced beef (57.0% ± 22.6%). The lowest increase was found for beef sirloin (47.2% ± 16.5%).

Determination of MDA Equivalents during *in Vitro* Digestion of Minced Beef with Added Fish Oil. In order to investigate the effect of combining beef high in heme iron with fish oil high in unsaturated fat, 4% fish oil was added to cooked minced beef (10% fat, study 2) prior to *in vitro* digestion. The intention was to obtain the same fat content as in 14% fat minced beef (study 2, Table 1), but when the fat content was calculated on the basis of analyzed values, it came out slightly higher (16.2% fat). Addition of fish oil caused a substantial increase in the formation of MDA equivalents during *in vitro* digestion (Figure 4). Even though the level of MDA equivalents per kilogram of fat was similar in undigested samples (56.9 ± 7.6 and 48.9 ± 3.3 μmol/kg fat), a much higher rise in MDA equivalents was observed in intestinal phase for minced beef with fish oil (3853.3 ± 83.0 versus 76.8 ± 11.0 μmol/kg fat), which was even higher than that observed in salmon loin (2432.4 ± 289.6 μmol/kg fat). In comparison, *in vitro* digestion of fish oil alone showed very low levels of MDA equivalents (59.8 ± 5.4 μmol/kg fat in gastric phase and 210.2 ± 10.0 μmol/kg fat in intestinal phase).

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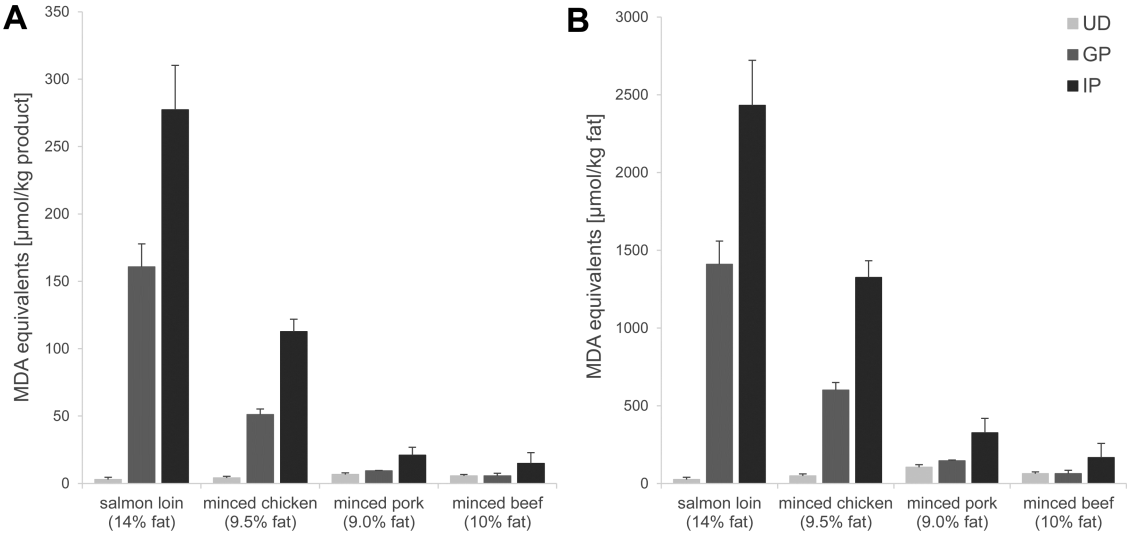

Figure 3. MDA equivalents per kilogram of product (A) and per kilogram of fat in the product (B) in cooked minced meat products and salmon loin during *in vitro* digestion. Measurements were performed before digestion (UD, light gray) and at the end of gastric phase (GP, dark gray) and intestinal phase (IP, black). Results are presented as mean values after subtraction of blank values. Vertical bars represent the standard deviations.

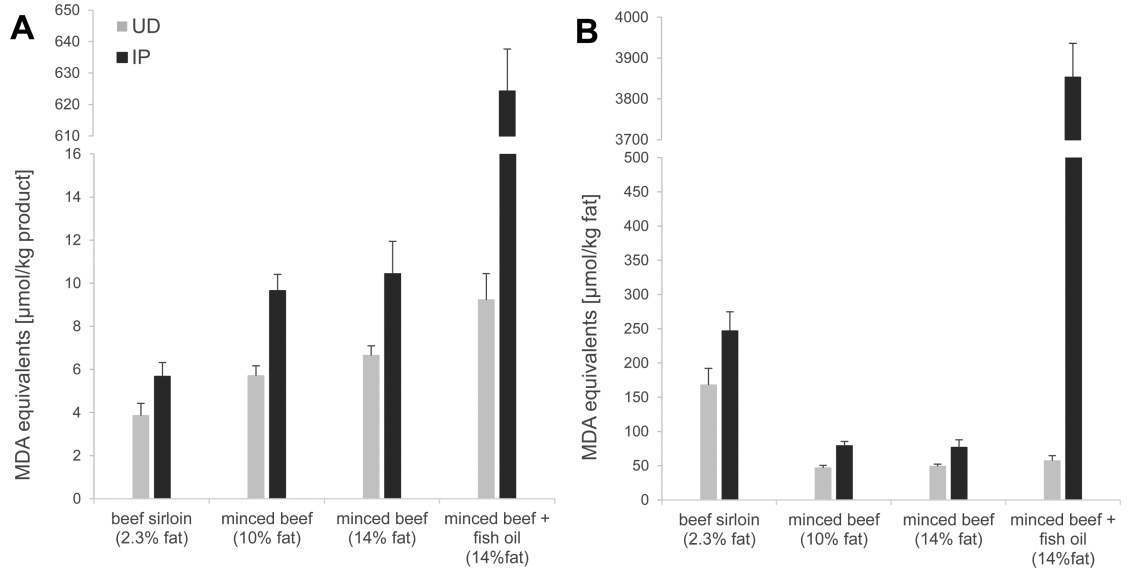

Figure 4. MDA equivalents per kilogram of product (A) and per kilogram of fat in the product (B) in beef sirloin, minced beef (10% and 14% fat), and minced beef (10% fat) enriched with 4% fish oil during *in vitro* digestion. Measurements were performed before digestion (UD, gray) and at the end of intestinal phase (IP, black). Values are presented as mean values after subtraction of blank values. Vertical bars represent the standard deviations.

Determination of 4-Hydroxyalkenals during *in Vitro* Digestion of Beef and Salmon. The formation of 4-hydroxyalkenals (4-HHE and 4-HNE) during *in vitro* digestion of minced beef (14% fat, study 2), salmon loin (14% fat, study 1) and blank samples (digestive fluids) is shown in Figure 5. The levels of 4-HHE increased significantly from gastric to intestinal phase, and intestinal levels were significantly higher in salmon (3.6 ± 0.1 μmol/kg) than in minced beef (2.3 ± 0.1 μmol/kg) and blank samples (2.1 ± 0.1 μmol/kg), whereas there were no differences between minced beef and blank samples.

The levels of 4-HNE in digests from minced beef and salmon were generally lower than the levels of 4-HHE (Figure 5), and no significant differences were observed between minced beef and salmon, except for a significantly lower level of 4-HNE in undigested salmon compared with undigested beef. In contrast to 4-HHE, the levels of 4-HNE in minced beef and salmon were significantly lower in the intestinal phase than in the gastric phase. In blank samples, however, the levels of 4-HNE was highest in the intestinal phase. Hence, 4-HNE levels in minced beef and salmon were significantly higher than blank samples in the gastric phase only.

DISCUSSION

The formation of potentially genotoxic aldehydes, that is, malondialdehyde (MDA) equivalents and 4-hydroxyalkenals (4-HNE, 4-HHE), was determined during *in vitro* digestion of red meat (minced beef and minced pork), which has been associated with CRC, as well as white meat (minced chicken) and fish (salmon loin). Results showed that in products with

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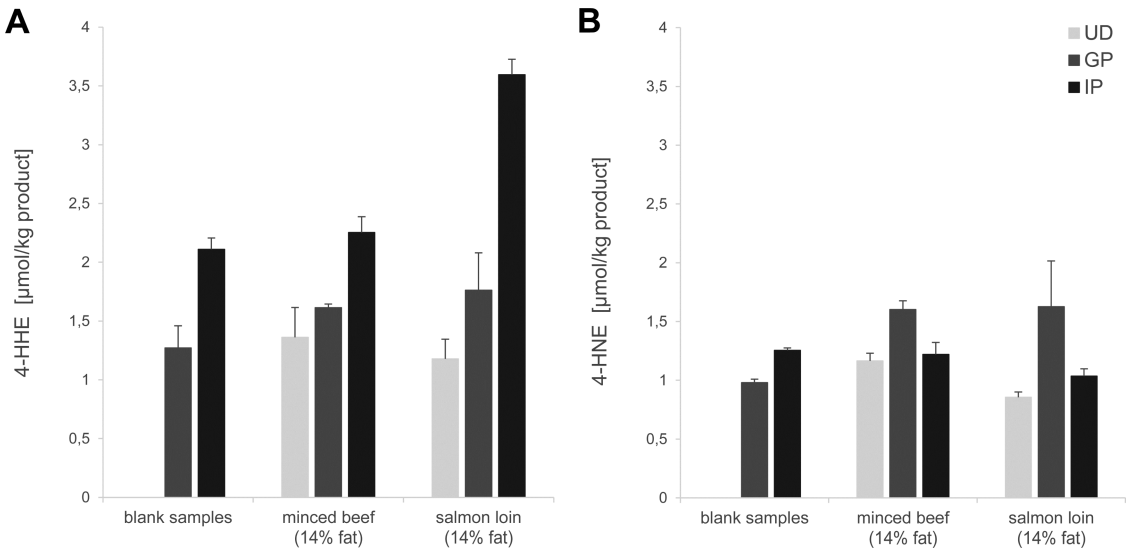

Figure 5. 4-HHE (A) and 4-HNE (B) (μmol/kg product) in minced beef (14% fat) and salmon (14% fat) during *in vitro* digestion. Measurements were performed before digestion (UD, light gray) and at the end of gastric phase (GP, dark gray) and intestinal phase (IP, black). Results are presented as mean values. Vertical bars represent the standard deviations.

similar fat content, MDA equivalents were formed to a higher extent in salmon and chicken than in red meat samples, indicating that the amount of unsaturated fat was the dominating factor determining the degree of gastrointestinal peroxidation. The relative number of double bonds per gram of fat (DB) in the raw materials was clearly the factor that was best correlated to the level of MDA equivalents in the intestinal phase. Also 4-HHE, which was determined during *in vitro* digestion of minced beef and salmon loin, showed significantly higher levels in salmon compared with beef. The results further indicated a positive association between the fat content and formation of aldehydes in beef, demonstrating significantly lower levels of MDA equivalents (μmol/kg product) in beef sirloin (low fat, 2,3%) than in minced beef products (high fat, 10−14%). However, the concentration of MDA equivalents per kilogram of fat was higher in beef sirloin, but whether this can be explained by a slightly higher number of double bonds and higher total iron content is uncertain. The highest formation of MDA equivalents occurred during *in vitro* digestion of minced beef enriched with fish oil, suggesting that not the ingestion of red meat alone but the combination of red meat high in iron and unsaturated fat in the diet may lead to a higher formation of aldehydes in the gastrointestinal tract.

Other factors that may affect lipid oxidation in various muscle sources are, for example, contents of pro- and antioxidants (both enzymatic and nonenzymatic), salt, muscle type, cooking, and oxygen availability.[29] Also lipid oxidation products present in the food before digestion may cause increased peroxidation during digestion.[30,31] Measurement of MDA equivalents performed after cooking and storage at −40 °C revealed low levels of MDA equivalents in all products (undigested material), indicating that the cooking process had minor effect on peroxidation, although it cannot be excluded that some aldehydes may bind to proteins during cooking and therefore be left out in the TBARS measurement. In the present study, no salt (sodium chloride), antioxidants, or other additives were added to the salmon and meat products, and they were all very low in PV (<0.1 mequiv/kg fat). The fish oil was stabilized with antioxidants, for example, tocopherols, and had a PV well below acceptable limit (5 mequiv/kg).[32,33] It is not known, however, in which way antioxidants present in the fish oil affected lipid peroxidation during digestion of fish oil enriched minced beef, since antioxidants may function as either anti- or pro-oxidants depending on concentration and surrounding conditions.[10,34,35]

The total iron content, as well as the content of unsaturated fatty acids, varied considerably among the products investigated. Although minced beef had twice the iron content of minced pork, gastric levels of MDA equivalents were significantly lower, probably due to the lower content of unsaturated fat. Similarly, salmon showed the highest levels of MDA equivalents and 4-HHE in digested samples although the iron content was lowest. Also the results from fish oil enriched minced beef suggest that although iron is a primary catalyst of lipid oxidation, the presence of unsaturated fatty acids is crucial for the formation of MDA equivalents during digestion. Iron may be represented in muscle tissue by, for example, hemoglobin/myoglobin, transferrin, or ferritin or as ionic iron, and the concentrations of the various iron compounds vary with species and muscle type.[35−37] It has also been suggested that fish hemoglobin is more active as pro-oxidant than mammalian hemoglobin;[38] thus a lower level of heme iron in salmon can still be much more powerful than an equal amount in beef. Additionally, the release of iron from heme during cooking processes may vary between muscle sources.[36] Whether such differences influenced the present results is unknown.

The involvement of different iron compounds in lipid peroxidation in meat products is not known in detail,[10,29,39] but some studies have shown that hemoglobin and myoglobin increases MDA equivalent levels during gastrointestinal digestion of marine and vegetable oils.[30,31,40] Although heme iron accounts for 80−90% of the total iron in beef[35,36] and seems to be a more potent initiator of lipid peroxidation than nonheme iron, acidic conditions in the stomach may lead to a degradation of the heme iron complex.[41] More investigation is needed to determine the release and impact of different iron compounds on the formation of peroxidation products in the gastrointestinal tract.

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Our results are in agreement with Van Hecke et al.[42] who showed that increasing fat contents in pork caused increased TBARS during *in vitro* digestion.[42] In another study by Van Hecke et al.[43] pork fat was added to various types of lean meat (chicken, pork, and beef) to obtain similar fatty acid compositions. The results showed that the amount of iron compounds significantly affected the level of MDA equivalents. A study by Vanden Bussche et al.[44] showed increased lipid peroxidation in beef samples compared with chicken after *in vitro* intestinal digestion. However, neither meat type, fat content, nor fatty acid composition were specified. Also a feeding study with rats exhibited increased plasma concentration of MDA equivalents in rats that were fed beef compared with rats that were fed chicken.[45] However, in that study less than 50% of the animal fat originated from chicken, reducing the overall content of unsaturated fat in the chicken diet and therefore probably the rate of lipid peroxidation. Nevertheless, the most plausible explanation for the deviation between these results and the present results may be that the meat products investigated in the present study had higher fat contents. The minced chicken had a fat percentage of 10%, which is much higher than for other typical chicken products, like chicken breast (about 1% fat).[46]

The highest levels of MDA equivalents formed during *in vitro* digestion were found in salmon, showing a dramatic increase in gastric phase followed by a further increase in intestinal phase. This is in accordance with published data on emulsified cod liver oil during *in vitro* digestion.[30] Also chicken demonstrated a smaller, but significant increase in gastric phase, whereas the increase in MDA equivalents in pork and beef mainly took place in the intestinal phase. Protein digestibility is related to the protein source and can vary between species.[47] Whether differences in protein digestibility and disintegration of the food matrix in the gastric phase may influence the amount of lipids accessible for peroxidation needs more investigation. It is well know that mechanical processing, like chewing, is important for the digestive process. A study by Bax et al.[48] showed that protein denaturation in cooked pork influenced the speed of pepsin digestion, whereas mincing had no effect. The present study also showed that extensive homogenization of minced beef before digestion did not have any effect. Still, it cannot be excluded that differences in texture and poorer digestibility of beef compared with chicken and salmon may have contributed to the lower formation of MDA equivalents during gastric phase.

The increase in MDA equivalents and 4-HHE during intestinal phase may partly be due to the emulsifying nature of bile that leads to an increased lipid droplet surface area susceptible to lipid peroxidation. Lipid compounds present in the bile, for example, phospholipids and cholesterol, may also be readily oxidized and contribute to the increase in aldehydes. Furthermore, bile contains lipophilic bilirubin, which is known for its antioxidant capacity,[49] whereas some bile acids may act as pro-oxidants. Sreejayan and von Ritter[50] associate the presence of taurodeoxycholic acid and some other hydrophobic bile acids with a rise in iron-induced lipid peroxidation, and a study by Larsson et al.[30] using simulated human or porcine bile during *in vitro* digestion of cod liver oil showed that the absence of bile in the intestinal phase led to reduced levels of TBARS. Although there are some differences between porcine, bovine, and human bile composition,[51] it is likely that the increase in MDA equivalents and 4-HHE observed in the present study is partly attributed to the presence of ovine/bovine bile.

As opposed to MDA equivalents and 4-HHE, the level of 4-HNE was lower in the intestinal phase than in gastric phase. This was also recently observed by Van Hecke et al.[42] who suggested that the decrease may result from the reaction of 4-HNE with proteins. The binding of 4-HNE to protein amino groups at neutral pH is well-known,[16] and such reactions might have taken place in the intestinal phase and influenced the measurement of free 4-HNE. This may also explain why the blank samples, which contain much less protein, showed higher levels of 4-HNE in intestinal phase. Since 4-HNE is derived from n-6 polyunsaturated fatty acids, the formation of 4-HNE during digestion was expected to be higher in minced beef than in salmon, but this could not be confirmed. However, as expected, the levels of 4-HHE (derived from n-3 fatty acids) were higher in salmon than in minced beef.

In vitro digestion models are widely utilized for food applications in order to study structural changes, digestibility, and release of food components under gastrointestinal conditions.[52] The models have so far been less common in CRC research but have been used to determine the impact of the food matrix on bioaccessibility of contaminants[53,54] and heterocyclic aromatic amines,[55,56] as well as the formation of endogenic mutagens, including NOCs.[57] The models have also been shown to be well suited for the investigation of peroxidation in the gastrointestinal tract.[30,31,41,43]

The *in vitro* digestion model used in the present study was a static model based on the harmonized model of EU Cost Action 1005.[24] The model utilizes commercial enzymes, for example, porcine pepsin, bovine/ovine bile, and porcine pancreatin, to simulate the gastric and duodenal phase. Whether human gastric and intestinal juices contain components that give better protection against oxidation *in vivo* is not known, but a recent study by Kristinova et al.[40] indicated no difference between oxidation of marine lipids in gastric juice and hydrochloric acid solution. The model used in the present study also deviates from the *in vivo* situation in that the exposure to oxygen was probably higher, particularly in the intestinal phase. However, the highest increase in the level of MDA equivalents (salmon) was observed in the gastric phase where oxygen is available also *in vivo*. The occurrence of *in vivo* peroxidation in gastric phase was recently demonstrated in minipigs receiving a standard Western diet containing minced beef and sunflower oil high in unsaturated fat.[58]

In a static *in vitro* model, digested compounds that are normally absorbed in the body are not removed and may therefore influence the results. This applies also for peroxidation products that can be absorbed or scavenged by the antioxidant system,[59] as well as antioxidants, for example, peptides derived from protein digestion.[60] However, in the present study all products were exposed to the same conditions during digestion, and results are therefore comparable, reflecting differences in susceptibility to lipid peroxidation and formation of potentially genotoxic aldehydes in the gastrointestinal tract.

Based on our findings, red meat does not contribute to a high level of potentially genotoxic aldehydes in the gastrointestinal tract when ingested alone. Minced chicken and salmon showed significantly higher levels of aldehydes, and fish oil enriched minced beef showed by far the highest levels. This indicates that products and diets combining high contents of iron and unsaturated fat may induce increased peroxidation in the digestive tract. Although the vast majority of existing literature indicates that intake of red meat, in contrast to white meat and

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fish, is associated with CRC, there are a few studies indicating that intake of marine n-3 fatty acids may cause increased levels of toxic aldehydes in feces from rats[8] and increase the risk of distant colon cancer in humans.[61] However, whether the increased levels of aldehydes observed in the present study represent a health hazard with regard to development of CRC remains to be established. So far, the bioavailability and safe dose for these aldehydes is not known. Since meat and fish are usually ingested together with other foods, future research should investigate potential interaction effects of relevant meal components. It would be useful to include the colon phase since peroxidation may also take place here, as well as in the cell membranes of the intestinal epithelium.

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ABBREVIATIONS

CRC, colorectal cancer; DB, relative amount of double bonds; EDTA, ethylenediaminetetraacetic acid; MDA, malondialdehyde; NOCs, *N*-nitroso compounds; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; TCA, trichloroacetic acid; TEP, 1,1,3,3-tetraethoxypropane; 4-HHE, 4-hydroxyhexenal; 4-HNE, 4-hydroxynonenal; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; UD, undigested samples; GP, gastric phase; IP, intestinal phase; SSF, simulated saliva fluid; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; PFB-oxime-TMS ether, *O*-pentafluorobenzyl-oxime-trimethylsilyl ether; PFBHA, *O*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine; BSTFA, *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide; SIM, selected ion monitoring

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Colorectal Carcinogenesis in the A/J Min/+ Mouse Model is Inhibited by Hemin, Independently of Dietary Fat Content and Fecal Lipid Peroxidation Rate

Christina Steppeler*, Marianne Sødring and Jan Erik Paulsen

Abstract

Background: Intake of red meat is considered a risk factor for colorectal cancer (CRC) development, and heme, the prosthetic group of myoglobin, has been suggested as a potential cause. One of the proposed molecular mechanisms of heme-induced CRC is based on an increase in the rate of lipid peroxidation catalysed by heme.

Methods: In the present work, the novel A/J Min/+ mouse model for Apc-driven colorectal cancer was used to investigate the effect of dietary heme (0.5 μmol/g), combined with high (40 energy %) or low (10 energy %) dietary fat levels, on intestinal carcinogenesis. At the end of the dietary intervention period (week 3–11), spontaneously developed lesions in the colon (flat aberrant crypt foci (flat ACF) and tumors) and small intestine (tumors) were scored and thiobarbituric reactive substances (TBARS), a biomarker for lipid peroxidation was analysed in feces.

Results: Dietary hemin significantly reduced colonic carcinogenesis. The inhibitory effect of hemin was not dependent on the dietary fat level, and no association could be established between colonic carcinogenesis and the lipid oxidation rate measured as fecal TBARS. Small intestinal carcinogenesis was not affected by hemin. Fat tended to stimulate intestinal carcinogenesis.

Conclusions: Contradicting the hypothesis, dietary hemin did inhibit colonic carcinogenesis in the present study. The results indicate that fecal TBARS concentration is not directly related to intestinal lesions and is therefore not a suitable biomarker for CRC.

Keywords: Colorectal cancer, Intestinal carcinogenesis, Red meat, Heme iron, Min mouse model, Lipid peroxidation, TBARS

Background

Globally, colorectal cancer (CRC) is the third most frequent form of cancer in men and the second most frequent in women. More than half of all CRC cases recorded in 2012 occurred in developed countries [1]. Therefore, an association between western lifestyle factors and incidence of CRC has been suggested. In 2007, the World Cancer Research Fund considered intake of red and processed meat to be a convincing risk factor for CRC [2], and in 2015 the International Agency for Research on Cancer (IARC) classified processed meat carcinogenic to humans (Group 1) and red meat as probably carcinogenic to humans (Group 2A) [3]. Even though several experimental studies in rodents have suggested a relationship between red meat intake and CRC [4–6], the role of red meat in initiation, promotion and progression of CRC is not clarified. Interestingly, animal studies were not able to reproduce epidemiological findings until basal diets were modified to reflect a "Western style diet" characterized by high fat, low calcium, and low antioxidants [7, 8], indicating complex mechanisms of action. Potential mechanisms involving heme iron, the red pigment in meat, seem promising, as these may explain why red meat, but not white meat (low in heme iron) is associated with CRC

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[9, 10]. Dietary heme iron (hemin) was found to cause similar colonic changes as meat-based diets in azoxymethane-treated rats [11], and changes in gene expression linked to cancer and proliferation were detected in colon scrapings of mice after only 4 days of heme iron (hemin) administration [12]. Two main hypotheses connect heme iron to CRC: its catalytic effect on peroxidation of lipids and its catalytic effect on the formation of N-nitrosamines (NOCs). Many lipid peroxidation products, including thiobarbituric reactive substances (TBARS) like malondialdehyde, as well as NOCs are potentially cytotoxic and mutagenic [4, 9, 10, 13].

Fat is susceptible to lipid peroxidation, and TBARS, a biomarker for lipid peroxidation, have repeatedly been linked to heme-induced tumor promotion [14, 15]. It has previously been suggested that reactive lipid peroxides may be covalently added to the protoporphyrin ring of heme, which may result in the formation of a cytotoxic heme factor (CHF) [12, 16]. As lipid peroxidation was found to occur before cytotoxicity, it was hypothesized that peroxidation products need to accumulate before the CHF forms [12].

Germline mutations in the tumor-suppressor gene adenomatous polyposis coli (*APC*) causes familial adenomatous polyposis (FAP), an inherited colorectal cancer syndrome. Similarly, the multiple intestinal neoplasia (Min/+) mouse, which is heterozygous for a truncation mutation at codon 850 of *Apc*, develops multiple spontaneous intestinal lesions. *Apc* controls the proliferation [17], apoptosis [18] migration and differentiation [19] of enterocytes by interfering with the Wnt signaling pathway. Complete somatic inactivation of *APC/Apc* in discrete crypts of the intestinal epithelium appears to be the initial carcinogenic event in Min/+ mice, human FAP and the majority of sporadic colorectal cancer in humans [20]. The Min/+ mouse model is frequently used to study factors that may influence critical events in *Apc*-driven intestinal carcinogenesis. However, in contrast to human FAP, conventional C57BL/6 J Min/+ mice develop tumors predominantly in the small intestine [21–24]. Recently, a novel Min/+ mouse on an A/J genetic background was suggested to provide a better model for colon cancer, as these mice also develop numerous adenomas in the colon that eventually progress to carcinomas in old individuals [25]. Furthermore, this novel A/J Min/+ mouse model demonstrated a continuous developmental growth of colonic lesions highlighted by the transition of early lesions, flat aberrant crypt foci (flat ACF), to tumors over time.

Recently, the A/J Min/+ mouse model was used to test the effect of dietary hemin, either alone or in combination with nitrite on intestinal carcinogenesis [26]. Surprisingly, dietary hemin was found to suppress the development of colonic lesions, independently of the presence of nitrite, and it was speculated whether the lack of the expected stimulation could be related to the low level of fat (4 %) in the AIN-93 M diet. Sesink et al. [27] observed enhanced the heme-induced cytolytic activity of colonic content as well as a greater rate of epithelial proliferation in rat colons with increasing dietary fat level. Therefore, the present study aimed to investigate the effects of heme in the A/J Min/+ mouse model when fat levels were taken into account. Beef tallow was chosen as the fat source to reflect the fatty acid composition of red meat.

The aim of the present study was to: i) examine the effect of dietary heme on intestinal carcinogenesis in A/J Min/+ mice fed a low or high fat diet; ii) examine whether intestinal carcinogenesis is related to the production of fecal TBARS.

Methods

Animals

The experiment was approved by the Norwegian Animal Research Authority (application ID: 6704) and conducted in compliance with local and national regulations on animal experimentation. The animals were maintained in open top plastic cages on a 12-h light/dark cycle at 20–22 °C and 55–56 % humidity. Weight gain was monitored once every 2 weeks during the experiment. Animals were sacrificed by cervical dislocation.

The A/J Min/+ mouse model was developed at the Norwegian Institute of Public Health [28], and later transferred, and subsequently maintained, at the experimental animal facility at the Norwegian University of Life Science, Campus Adamstuen. For breeding, two female A/J wild-type mice were placed together with one male A/J Min/+ mouse. On day 19–21 after birth, offspring were weaned and randomly assigned to the experimental diets, being allowed free access to diet and water. As only A/J Min/+ mice were included in the experiment, DNA was extracted from ear punch samples and subjected to allele-specific PCR for determination of the genotype. The following primer set was used for DNA amplification: MAPC MT (5'-TGAGAAAGACAG AAGTTA -3'), MAPC 15 (5'-TTCCACTTTGGCATAA GGC-3'), and MAPC 9 (5'-GCCATCCCTT- CACGTT AG-3'). The PCR product of a wild-type allele consists of 618 bp and is visible as a band for both wild type (+/+) and Min/+ mice. In the presence of the Min allele, an additional PCR product of 327 bp is generated [29].

Diets and study design

From weaning at 3 weeks until termination at 11 weeks, the A/J Min/+ mice were fed four different experimental diets (Table 1): $Hemin^{-}$, Low fat (low fat control with no hemin); $Hemin^{+}$, Low fat (low fat with hemin); $Hemin^{-}$, High fat (high fat control with no hemin); $Hemin^{+}$, High

Table 1 Study groups and composition of the experimental diets

	Hemin$^-$ Low fat	Hemin$^+$ Low fat	Hemin$^-$ High fat	Hemin$^+$ High fat
N (female/male)	11/14	12/13	10/10	10/10
Metabolisable energy (MJ/kg)	13.79	13.78	16.61	16.61
% as fat	10 %	10 %	40 %	40 %
% as protein	20 %	20 %	20 %	20 %
% as carbo	70 %	70 %	40 %	40 %
Moisture (g/100 g)	4.35	4.35	4.45	4.45
Rice starch (g/100 g)	29.88	29.88	19.9	19.9
Sucrose (g/100 g)	36.11	36.11	25.59	25.59
Crude protein (g/100 g)	18.7	18.7	22.49	22.49
Crude fat (g/100 g)	4.22	4.22	20.39	20.39
Crude fiber (g/100 g)	2	2	2.23	2.23
AIN-93G-MX (adjusted for Ca and P) (g/100 g)	3.5	3.5	4.18	4.18
AIN-93-VX (w/o Vit D3) (g/100 g)	1	1	1.20	1.20
l-Cystine (g/100 g)	0.323	0.323	0.38	0.38
Choline Bitartrate (g/100 g)	0.24	0.24	0.29	0.29
Hemin (μmol/g)	–	0.5	–	0.6
adjusted minerals/vitamins level				
Total Ca (%)	0.08 %	0.08 %	0.10 %	0.10 %
Total P (%)	0.15 %	0.15 %	0.18 %	0.18 %
Total Vit D3 (ui/kg)	<21.5	<21.5	<25.9	<25.9

fat (high fat with hemin). Beef tallow was used as a fat source, providing 10 % (low fat diet) and 40 % (high fat diet) of the energy. The number of animals per study group is indicated in Table 1. Based on the assumption that the total daily caloric intake would be equivalent between the low fat and high fat groups, high fat diets were formulated on an isocaloric exchange basis to compensate for the increase in energy density in the high fat diets. After balancing, all diets contained corresponding amounts of nutrients per megajoule. Heme was added in the form of hemin, a protoporhyrin IX with a chloride ligand associated with the central, ferric iron ion. All diets were customized to be deficient in calcium (0.08/0.10 % in low and high fat diet, respectively) and vitamin D3, as these are natural protectants against CRC development [30]. Vitamin D3 was removed from the vitamin mix, and vitamin D3 level in casein was confirmed to be <100 iu/kg. Hence, the low fat and high fat diet contained no more than 21.5 and 25.9 iu/kg vitamin D3, respectively. Additionally, diets were deficient in linoleic acid (0.18/0.92 %) as beef tallow was used as the only source of fat to mimic red meat consumption. The level of phosphorus was 0.15 and 0.18 % in the low fat and high fat diets, respectively. All other nutrients were met by the NRC requirements for rodents. Diet consumption was registered cagewise during the last week of the experiment.

Fecal water content

Fresh fecal pellets were collected, weighed and freeze-dried. Fecal water content was calculated as the weight difference before and after freeze-drying.

Scoring of lesions

After termination by cervical dislocation, the intestines were excised and extensively flushed with phosphate-buffered saline (PBS). Small intestine and colon were cut open longitudinally, and the small intestine was divided into three sections (proximal, middle, distal part). All parts of the intestine were then flattened between to filter papers. The intestinal preparations were fixed in 10 % neutral buffered formalin overnight and subsequently stained (5–10 s) in 0.2 % methylene blue dissolved in the formalin solution. After another 24 h in 10 % formalin, the intestines were scored for intestinal lesions by surface microscopy. The number of lesions was recorded, and the size of each lesion was calculated based on the diameter, measured with an eyepiece graticule. The total surface area covered by lesions was defined as load. The scoring was performed blindly, by one observer. Stained lesions appeared bright blue in contrast to the brownish-green surrounding epithelium (Additional file 1: Figure S1). Colonic lesions were classified into two categories: flat aberrant crypt foci (flat

ACF) and tumors. Flat ACF are suggested to be the early stages of tumors, as both flat ACF and tumors share morphologic features such as enlarged, compressed crypt openings, which form gyrus-like pit patterns as they increase in size. Tumors are defined by a crypt multiplicity of more than 30 crypts, and commonly show, in contrast to flat ACF, structures that appear elevated compared to the surrounding epithelium. In the A/J Min/+ mouse model, colonic lesions demonstrate continuous development from flat ACF to tumors [25], therefor merged data for colonic lesions was used to generate a size distribution graph. For presentation of the size distribution, lesions were allocated into the following size classes: 0–0.008 mm^2, 0.009–0.064 mm^2, 0.065–0.512 mm^2, 0.512–4.096 mm^2, and >4.096 mm^2. The size classes are based on a logarithmic scale to improve the readability of the graph. The categories build upon a base-eight logarithm, which allows the smallest lesions (approximately 1–4 crypts) to be grouped within the first size class.

TBARS

To assess the rate of lipid peroxidation in the lumen, TBARS were analysed in fecal water. The procedure of TBARS analysis was adapted from previously described protocols [31, 32]. Fecal water was prepared from freeze-dried 24-h feces collected from 1–3 mice. 150 mg grounded feces was incubated with 1000 μl distilled water for 60 min at 37 °C. After centrifugation at 20,000×*g* for 15 min, supernatants were frozen at −20 °C until use. For the assay, 40 μl of sample was replenished with 60 μl distilled water and mixed with 100 μl sodium dodecyl sulphate (8.1 %). After the addition of 1 ml 2-thiobarbituric acid solution (0.05 % in 10 % acetic acid), samples were incubated for 75 min at 82 °C. Absorption spectra (450 to 700 nm) were read using an Epoch Microplate Spectrophotometer (Biotek, Winooski, United States) with Gen5TM Microplate Data Analysis Software. Peak absorption at 532 nm was corrected for baseline absorbance by subtracting the absorbance at 700 nm. 1,1,3,3,-tetramethoxypropane was used as a standard (covered range: 0, 25, 50, 100, 200 μM) and underwent the same procedure as samples. Results are expressed as μM malondialdehyde equivalents per millilitre fecal water.

Statistics and data presentation

The distribution of the intestinal lesion parameters was heavily skewed and could not be transformed to meet the assumptions of parametric tests. Hence, relationships between outcome variables and dietary factors (high fat and hemin) were analyzed using quantile regression. Due to the low incidence of colonic tumors, a cut-off point of 75 % was used for tumor number, average size and load in the colon, and odds ratios were calculated for tumor incidence. Median regression was used for all other variables. The relationship between lesions and fecal parameters was evaluated in the entire data set and within groups (within $Hemin^-$ and $Hemin^+$; within Low fat and High fat) by determination of the Spearman's correlation coefficient. A p-value of $p < 0.05$ was considered significant. Figures present results as median [interquartile range percentile (IQR): percentile 25-percentile 75] and mean. Raw data are provided in Additional file 2.

Results

Animals and food consumption

After 8 weeks on the experimental diets, body weight and food consumption were not related to dietary hemin or fat level (Additional file 2: Table S2).

Effects of hemin and fat on intestinal carcinogenesis

The tumorigenic potential of dietary hemin and fat, as well as the interaction of the two factors, was tested on the following variables: number of colonic lesions (flat ACF and tumors), number of small intestinal tumors, average lesion size (mm^2) and load (total lesion area per animal). No significant interactions of the dietary interventions were observed for any of the outcome parameters, thus, the hemin x fat interaction was removed from all subsequent analyses.

Colon

Independent of the fat level, dietary intervention with hemin caused a significant decrease in the number of flat ACF ($p = 0.036$) (Table 2), as well as the total area covered by flat ACF (load, $p = 0.040$) in the colon. As presented in Fig. 1, the inhibitory effect of hemin was also apparent for the average size of flat ACF and colonic tumor parameters, albeit statistical significance was not reached (Table 2). The proportion of mice developing colonic tumors was significantly decreased by dietary hemin (odd ratio = 0.40, 95 % CI: [0.16–0.99], $p = 0.046$).

No relationship could be established between dietary fat level and formation of flat ACF. Likewise, tumor incidence (odd ratio 1.0, 95 % CI: [0.44–2.51], $p = 0.92$), tumor number and tumor load were not significantly affected by dietary fat level. The growth of colonic tumors, however, was enhanced by high fat diets and led to a significantly increased average tumor size ($p = 0.002$). Fig. 1b indicates that also the average size of flat ACF may be equally affected.

The size distribution of colonic lesions (Fig. 2a) builds upon the merged data from flat ACF and tumors, as a transition of flat ACF to tumors can be assumed [25]. The graph further illustrates the presented results: while only minor differences can be observed between the low and high fat diets, mice fed diets devoid of hemin exhibited a

Table 2 Relationship between dietary interventions (hemin and fat) and outcome variables in A/J Min/+ mice

		Hemin$^+$ vs. Hemin$^-$		High fat vs. low fat	
		Coefficient	*p*-value	Coefficient	*p*-value
Colon					
Flat ACF	**Number per animal**	**−13.0 [−25.1– −0.9]**	**0.036**	5.0 [−7.2–17.2]	0.421
	Average Size	−0.000 [−0.003–0.002]	0.862	0.002 [−0.001–0.004]	0.142
	Load	**−0.18 [−0.35– −0.01]**	**0.040**	0.14 [−0.03–0.31]	0.105
Tumor	**Number per animal**	−1.0 [−4.0–2.0]	0.513	1.0 [−2.0—4.0]	0.515
	Average Size	−0.28 [−0.67–0.13]	0.181	**0.66 [0.23–1.07]**	**0.002**
	Load	−0.55 [−3.60–2.50]	0.723	1.39 [−1.69–4.46]	0.377
Small intestine					
Tumor	**Number per animal**	2.0 [−5.2–9.2]	0.588	6.0 [−1.3–13.3]	0.107
	Average Size	0.00 [−0.06–0.58]	1.000	**0.12 [0.07–0.18]**	**<0.001**
	Load	1.13 [−2.52–4.78]	0.544	**5.54 [1.86–9.21]**	**0.031**
Fecal Parameters					
	TBARS	**7.1 [3.8–10.4]**	**<0.001**	**5.3 [1.9–8.6]**	**0.002**
	Fecal water content	**4.5 [1.9–7.0]**	**0.001**	**−2.7 [−5.2– −0.1]**	**0.045**

Regression coefficients [95 % confidence interval] from quantile regression are presented. Significant results ($p < 0.05$) are shown in bold text

greater amount of lesions across all size categories than mice fed hemin-enriched diets.

Small intestine

The number of tumors, average tumor size and tumor load in the small intestine was found to be independent of dietary hemin (Fig. 3). High dietary fat content significantly enhanced carcinogenesis (Table 2), reflected by a significant increase in average tumor size ($p < 0.001$) and tumor load ($p < 0.031$). Tumor number tended to be increased by dietary fat, although not significant (Fig. 1a). The size distribution of the small intestinal tumors

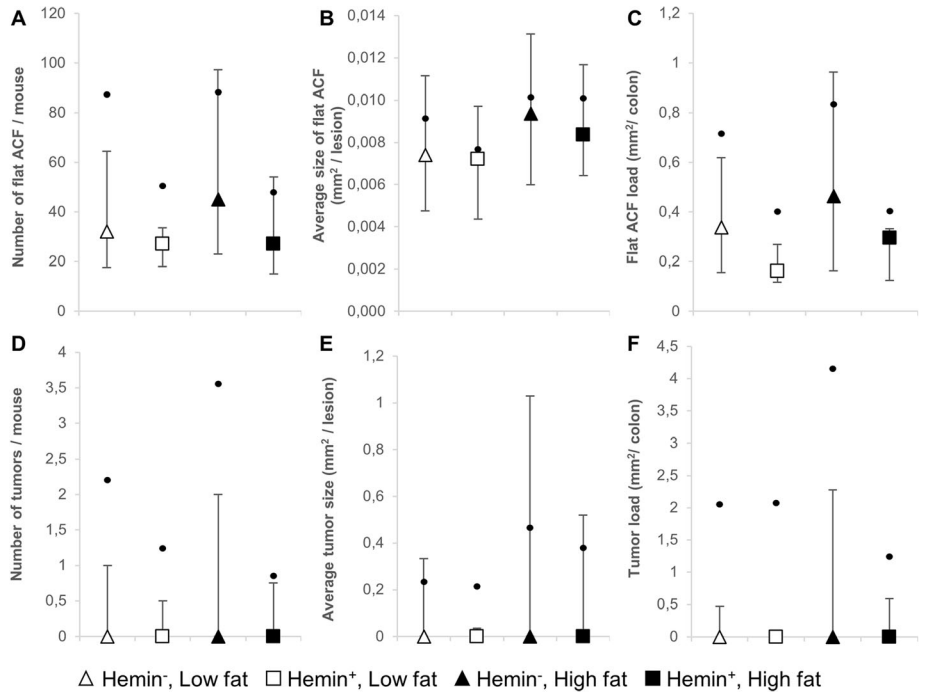

Fig. 1 Development of intestinal lesions in the colon of A/J Min/+ mice. **a**–**c** shows data for flat ACF, while **d**–**f** represents data for colonic tumors. **a** and **d** number of lesions, **b** and **e** average size of lesions, **c** and **f** load of lesions. Values are presented as median [IQR] and mean. Dots indicate means

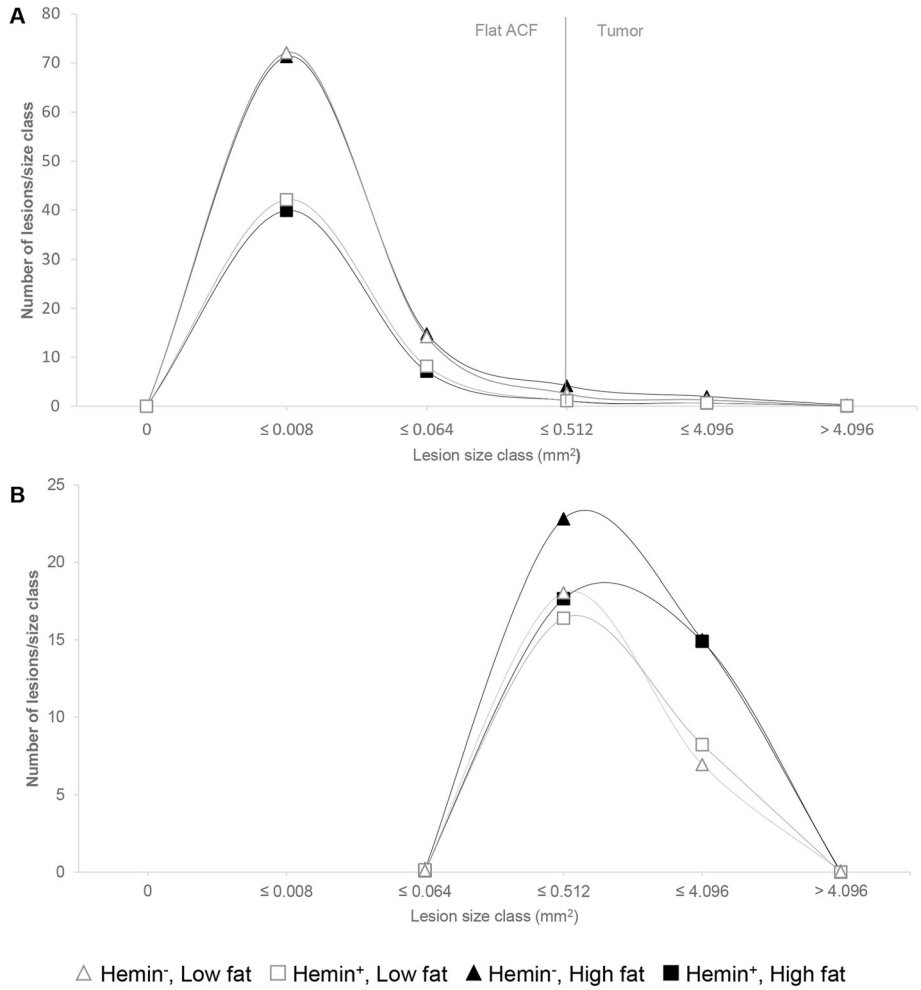

Fig. 2 Size distribution of intestinal lesions in A/J Min/+ mice. **a** colon: flat ACF and tumors, **b** small intestine: tumors

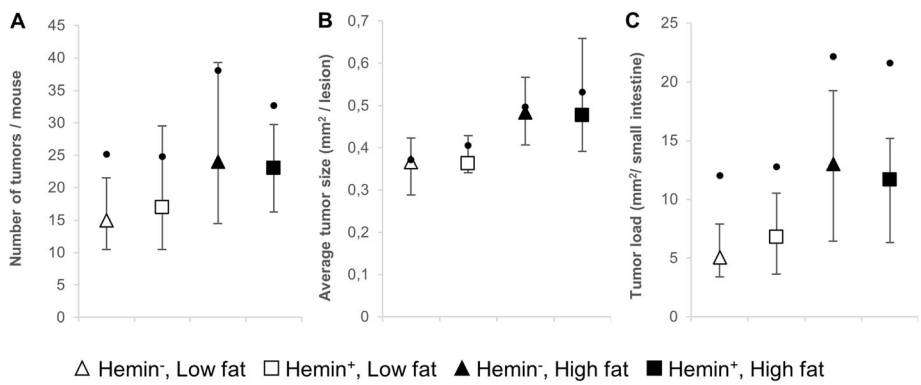

Fig. 3 Development of intestinal lesions in the small intestine of A/J Min/+ mice. **a** Number of tumors, **b** average tumor size, **c** tumor load. Values are presented as median [IQR] and mean. Dots indicate means

(Fig. 2b) clearly illustrates how elevated dietary fat caused a shift towards larger tumor classes (low fat vs. high fat, 1.3 fold increase in average tumor size).

Effects of hemin and fat on fecal parameters

TBARS

Analysis of fecal water showed that dietary hemin caused an increase in fecal TBARS concentration ($p < 0.001$) (Table 2, Fig. 4a). Furthermore, a significantly higher TBARS yield was observed in response to high fat diets than to low fat diets ($p = 0.002$).

To identify possible relationships between intestinal carcinogenesis and fecal parameters, Spearman's rank correlation coefficients were determined (Table 3). No association was found between fecal TBARS concentration and colonic carcinogenesis. In the small intestine, in contrast, fecal TBARS concentration was positively linked to the number, average size, and load of the tumors (Table 3). These correlation data were then grouped by hemin level to explore the possible influence of variations of dietary fat, and subsequently by fat level to explore the possible influence of variations of hemin level. Significant correlation persisted only in the groups with varying levels of dietary fat. Figure 5 illustrates how a significant relationship between small intestinal average tumor size and TBARS concentration was seen in animals grouped by hemin level (fat level varied) and not in animals grouped by fat level (hemin level varied). This is consistent with the observation that dietary hemin increased TBARS concentration but did not affect small intestinal carcinogenesis.

Fecal water content

Fecal water content has previously been related to colonic reabsorption capacity [27]. At the end of the intervention, water content of feces was decreased by high fat diets ($p = 0.045$) (Fig. 4b). In contrary, dietary hemin increased water content in feces ($p = 0.001$). Fecal water content was not associated with intestinal tumorigenesis (Additional file 3: Table S3).

Discussion

In the present study we examined the effect of dietary heme iron on intestinal carcinogenesis and fecal water concentration of TBARS, a biomarker of lipid peroxidation, in A/J Min/+ mice fed a low or high fat diet. Although contradicting the current prevailing opinion regarding hemin and CRC, this work did confirm the results of a recent study by our group [26]. Instead of the expected promoting effect [12], heme iron was found to inhibit carcinogenesis in the colon of A/J Min/+ mice. While the growth of colonic lesions remained unaffected, dietary hemin apparently reduced tumor initiation by decreasing the number of flat ACF, which represent newly formed colonic lesions.

In our recent study [26], we speculated whether the lack of a stimulatory response of dietary heme iron was related to the low level of fat in the diet (4 %) and that the conditions were insufficient for lipid peroxidation and cytotoxic heme factor (CHF) formation. Therefore, the dietary fat level was included as a variable in the present study. Although high dietary fat content increased colonic tumor growth, the results clearly showed that changes in dietary fat level were not capable of reversing or changing the inhibitory effect of dietary heme iron on colonic carcinogenesis.

In contrast to what was observed in the colon, dietary hemin exposure did not influence carcinogenesis in the small intestine. In hemoglobin-fed C57BL/6 J Min/+ mice, Bastide et al. [33] observed a significant increase in the number of jejunal tumors and a greater number of tumors with increased diameter (>1 mm^2) along the entire small intestine. In A/J Min/+ mice, we recently found an increase in small intestinal tumor size in response to dietary heme [26]. It is not clear why no effect of heme on small intestinal carcinogenesis was observed in the present study. As in the colon, high dietary fat

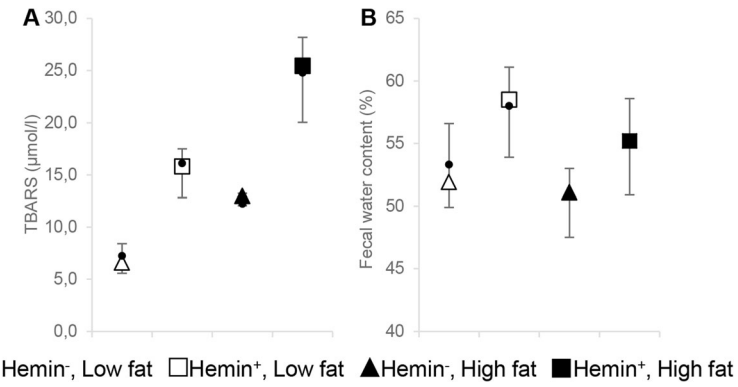

Fig. 4 Analysis of feces: **a** TBARS (µmol/l) in fecal water and **b** fecal water content. Results are shown as median [IQR] and mean. Dots indicate means

Table 3 Correlation between fecal TBARS and small intestinal lesions

	Number of lesions		Average lesion size		Lesion load	
	ρ	*p*-value	ρ	*p*-value	ρ	*p*-value
Colon, flat ACF						
Total	−0.079	0.477	0.039	0.724	−0.084	0.446
Colon, tumor						
Total	−0.132	0.233	−0.098	0.377	−0.109	0.324
Small intestine, tumor						
Total	**0.227**	**0.038**	**0.286**	**0.008**	**0.265**	**0.015**
Within Hemin$^-$	**0.354**	**0.025**	**0.422**	**0.007**	**0.374**	**0.018**
Within Hemin$^+$	0.213	0.165	**0.329**	**0.029**	0.286	0.060
Within Low fat	0.178	0.242	0.232	0.126	0.243	0.108
Within High fat	0.002	0.991	0.031	0.853	−0.049	0.768

ρ, Spearman's rank correlation coefficient. Significant results from Spearman's ρ ($p < 0.05$) are shown in bold text

induced a significant stimulation of carcinogenesis in the small intestine.

The hypothesis of a contribution of lipid peroxides to the carcinogenesis of colorectal cancer is widely supported in the literature [5, 10]. In the present study, however, correlation analysis revealed no indication that fecal TBARS are related to colonic carcinogenesis. Although a correlation was found between TBARS and small intestinal tumors, the observed association was dependent on varying dietary fat level and was not verifiable when investigated within the high and low fat groups separately. Despite the enhanced concentration of fecal TBARS following the ingestion of dietary heme iron, hemin did not affect small intestinal carcinogenesis, and even inhibited carcinogenesis in the colon. An increased TBARS concentration in fecal water has previously been linked to heme-induced cell proliferation [12], and when calcium phosphate was added to a beef-based diet, a decrease in the promotion of colonic lesions was accompanied by a reduced level of TBARS and cytotoxicity of fecal water [15]. In contrast, however, Santarelli et al. [34] did not find an association between the level of peroxidation and the promotion of colonic lesions, and despite an elevated concentration of TBARS, Martin et al. [35] also did not observe a change in cell proliferation in response to dietary hemoglobin. Levels of malondialdehyde (as TBARS) and 4-hydroxynonenal, two conventional biomarkers for lipid peroxidation, are tightly related to the fat source used in experimental diets [36, 37]. Therefore it may be difficult to make predictions about the carcinogenic potential of experimental diets based on these particular peroxidation products. Further studies are needed to define the role of individual peroxidation products in the carcinogenesis of colorectal cancer, but based on the present results, the heme-induced formation of TBARS appears to occur as an independent event within the carcinogenesis in the colon. The relevance of fecal TBARS as a biomarker for colorectal cancer development is further questioned, as Bastide et al. [33] did not find any cytotoxic or genotoxic effects of malondialdehyde, the most prevalent TBARS, on cultured $Apc^{+/+}$ and $Apc^{+/-}$ cells in vitro.

In the present study, carcinogenesis in both the colon as well as the small intestine was enhanced when the

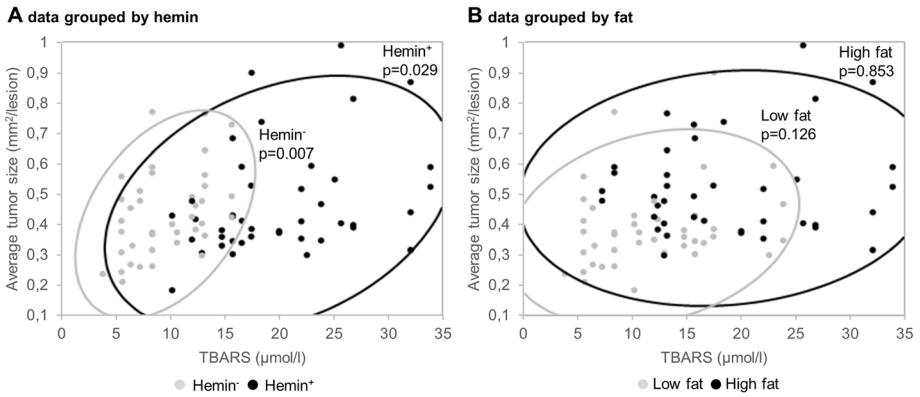

Fig. 5 Relationship between average tumor size in the small intestine and fecal TBARS. A 95 % bivariate normal density ellipse and *p*-values from Spearman's ρ are shown to reflect the degree of correlation within the **a** Hemin$^-$ and Hemin$^+$ group, and **b** Low fat and High fat group

level of fat in the diet was increased. The fatty acid composition of the experimental diets was designed to reflect consumption of red meat, and beef tallow was used as the only fat source. Animal fat from red meat mainly consists of saturated fat, omega-6 polyunsaturated fatty acids (n-6 PUFAs) and cholesterol. Beside its susceptibly to oxidative processes, it is still under debate how fat level and fatty acid composition of the diet may affect CRC. High levels of fat have been shown to stimulate the secretion of bile acids, which can be harmful to the intestine after being metabolized by microbiota in the gut [38, 39]. Additionally, n-6 PUFAs can modulate the immune response after being subjected to enzymatic conversion and being further metabolized into eicosanoids with mainly pro-inflammatory properties [40]. Although a high dietary fat content is associated with increased tumor formation in various animal studies [41–45], the link is generally not supported by epidemiological evidence [46, 47].

The percentage of dietary linoleic acid (C18:2, n-6) in the current study, as well as the estimated percentage of linoleic acid provided by soybean oil in our recent study [26] was below the concentration of the safflower oil based diets used by Pierre and colleagues [32], or the mixture of corn and palm oil commonly used by van der Meer and colleagues [48]. Hence, it cannot be excluded, that the formation of a CHF, as proposed by Ijssennagger et al. [12] is dependent on a critical level of n-6 fatty acids or specific PUFAs. However, in a long term study by Winter et al. [49], dietary heme tended to decrease the incidence of colonic neoplasms in mice, despite a high level of linoleic acid, provided by sunflower oil (16.8 g/100 g diet).

Fecal water content and content of cations have previously been used as parameters for the colonic reabsorption capacity [27]. Fecal cation content in rat feces was shown to increase in response to heme, and was linked to the degree of colonic epithelial damage [27, 50]. In the present study, however, increased fecal moisture in response to hemin was not associated with carcinogenesis, which may indicate that the colonic epithelium was not severely damaged. These contradicting findings may be the result of other underlying factors that have the ability to modulate fecal water content, such as the richness and composition of microbiota. For instance, the Bacteroidetes: Firmicutes ratio which was previously found to be increased by dietary heme [51], is positively correlated with stool consistency in humans [52].

We have tested the effects of dietary heme by exposing A/J Min/+ mice from 3 to week 11 of age, a period where the majority of flat ACF are formed spontaneously [25]. This window of exposure was also chosen based on the idea that young mice, in particular, may be highly susceptible to stimuli that may enhance colon carcinogenesis. This has previously been demonstrated in young Min/+ mice treated with the colon carcinogen azoxymethane (AOM) [28, 53]. Although dietary hemin appeared to be protective in mice at this early stage of life, we cannot rule out potential stimulatory effects of long time exposure. Long-term studies are required to investigate the effect of exposure during periods of tumor progression in old mice [25].

Conclusions

When testing the dietary heme hypothesis in the A/J Min/+ mouse model, we found that dietary hemin inhibited colonic carcinogenesis and enhanced fecal TBARS concentration independent of dietary fat level. Small intestinal carcinogenesis was not affected by hemin. High dietary fat stimulated intestinal tumor growth as well as increased TBARS concentration. Further research is needed to clarify the role of lipid peroxidation during intestinal carcinogenesis, and whether interactions between heme iron and other dietary compounds may be responsible for the link between red meat and CRC observed in epidemiological studies.

Additional files

Additional file 1: Figure S1. Representative examples of methylene blue-stained intestinal lesions. (PDF 156 kb)

Additional file 2: Table S2. Dataset. Number, average size and load of intestinal lesions, fecal TBARS and fecal water content, body weight and daily food intake. (XLS 86 kb)

Additional file 3: Table S3. Final body weight and daily food intake. (PDF 7 kb)

Additional file 4: Table S4. Correlation between fecal water content and intestinal lesions. (PDF 330 kb)

Abbreviations

AOM: Azoxymethane; APC: Adenomatous polyposis coli; CHF: Cytotoxic heme factor; CRC: Colorectal cancer; flat ACF: Flat aberrant crypt foci; IRQ: Interquartile range percentile; Min: Multiple intestinal neoplasia; n-6 PUFA: Omega-6 poly unsaturated fatty acids; NOCs: N-nitrosamines; TBARS: Thiobarbituric acid reactive substance

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Availability of data and materials

Images of representative examples of intestinal lesions are provided in Additional file 1: Figure S1. Raw data is provided in the Additional file 2: Figure S2. Data on final body weight and food intake is provided in Additional file 3: Table S3, and results from the correlation analysis between fecal water and intestinal lesions is provided in Additional file 4: Table S4.

Authors' contributions

Conceived and designed the experiments: CS MS JEP. Performed the experiments: CS MS. Analyzed the data: CS JEP. Wrote the article: CS. Critically reviewed the manuscript: MS JEP. All authors read and approved the final manuscript.

Authors' information
Not applicable.

Competing interests
The authors have declared that no competing interests exist.

Ethics approval
The experiment was approved by the Norwegian Animal Research Authority (application ID: 6704) and conducted in compliance with local and national regulations on animal experimentation.

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III

Effects of Beef, Pork, Chicken and Salmon Meat on Intestinal Carcinogenesis in A/J Min/+ mice

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Abbreviations: APC: Adenomatous polyposis coli; CMAH: monophospho-N-acetylneuraminic acid hydroxylase; CRC: Colorectal cancer; flat ACF: Flat aberrant crypt foci; Neu5Gc: N-glycolylneuraminic acid; Min: Multiple intestinal neoplasia; n-6 and n-3 PUFA: Omega-6 and omega-3 poly unsaturated fatty acids; PCA: Principal component analysis; TBARS: Thiobarbituric acid reactive substance

Abstract

The International Agency for Research on Cancer has classified red meat as "probably carcinogenic to humans" (Group 2A). In mechanistic studies exploring the link between intake of red meat and CRC, heme iron, the pigment of red meat, is proposed to play a central role as a catalyzer of luminal lipid peroxidation and cytotoxicity. In the present work, the novel A/J Min/+ mouse was used to investigate the effects of dietary beef, pork, chicken, or salmon (40% muscle food (dry weight) and 60% powder diet) on *Apc*-driven intestinal carcinogenesis, from week 3-13 of age. Muscle food diets did not differentially affect carcinogenesis in the colon (flat ACF and tumors). In the small intestine, salmon intake resulted in a lower tumor size and load than did meat from terrestrial animals (beef, pork or chicken), while no differences were observed between the effects of white meat (chicken) and red meat (pork and beef). Additional results indicated that intestinal carcinogenesis was not related to dietary n-6 polyunsaturated fatty acids, intestinal formation of lipid peroxidation products (thiobarbituric acid reactive substances, TBARS), or cytotoxic effects of fecal water on $Apc^{-/+}$ cells. Notably, the amount of heme reaching the colon appeared to be relatively low in this study. The greatest tumor load was induced by the reference diet RM1, underlining the importance of the basic diets in experimental CRC. The present study in A/J Min/+ mice does not support the hypothesis of a role of red meat in intestinal carcinogenesis.

Introduction

Colorectal cancer (CRC) represents a major global health concern, particularly in developed countries. Besides genetic predispositions, CRC development is influenced by various life style factors, and the consumption of red and processed meat has been associated with an increased risk for CRC [1–3]. In 2015, the International Agency for Research on Cancer (IARC) concluded that the evidence on the carcinogenicity of processed and red meat was sufficient to classify processed meat as "carcinogenic to humans" (Group 1) and red meat as "probably carcinogenic to humans" (Group 2A) [4]. In their report, red meat is defined as meat from beef, veal, pork, lamb, mutton, horse, or goat, while processed meat includes all types of meat that have been subjected to flavor enhancement or preservation, e.g. salting, curing, fermentation or smoking. Genotoxicity, lipid peroxidation and the formation of mutagenic N-nitrosamines are discussed as possible molecular mechanisms behind the link between CRC and red and processed meat intake. In this context, heme, the red pigment in red meat, is suggested to play a central role, and function as a catalyzer [5,6]. The Word Cancer Research Fund guidelines advise to limit the intake of red meat to 500 g (corresponding to 700-750 g raw meat) weekly, with very little if any to be processed [7]. Fish consumption, in contrast to red meat consumption, may be inversely related to CRC [7–10], and its protective effect has been attributed to the high contents of n-3 fatty acids and vitamin D [11]. However, the underlying mechanisms are not known in detail, and a few studies have also indicated no connection, or an increased risk of CRC, after intake of marine n-3 fatty acids [1,12]. The effect of poultry consumption on CRC has not been extensively investigated [7], but, if any, protective implications have been suggested [1]. The Word Cancer Research Fund recommendations are to choose poultry and fish instead of red meat [7].

Types and amounts of dietary fat may influence CRC risk, and it has been suggested that n-6 polyunsaturated fatty acids (PUFA) and saturated fat, in contrast to n-3 PUFA, may have unfavorable effects [13]. High dietary fat, in particular saturated fat, has been shown to increase the synthesis of taurine conjugated bile acids that promote the growth of gut bacteria that generate genotoxic H_2S gas, and increased levels of potentially carcinogenic secondary bile acids [14,15]. Dietary arachidonic acid (C20:4 n-6), or arachidonic acid derived from other n-6 PUFA, is a precursor of mainly pro-inflammatory eicosanoids [13,16], and n-6 PUFA are, just as n-3 PUFA, generally susceptible towards lipid peroxidation [17,18].

The APC multiple intestinal neoplasia (Min/+) mouse has been widely used as a model to study mechanisms of human CRC pathology. On a molecular level, the development of CRC follows a successive multi-step sequence, and is accompanied by an accumulation of genetic and epigenetic alterations [19]. The majority of human sporadic CRC cases (ca. 85 %) follow the chromosomal instability pathway, where mutations in one allele of the tumor suppressor gene *APC* (adenomatous polyposis coli) are typically followed by mutations in, or by loss of heterozygosity (LOH) of the remaining allele. This second event defines the rate-limiting step for tumor initiation [20]. Familial adenomatous polyposis (FAP) patients, in contrast, carry a heterozygous germline mutation in the *APC* gene, and only one hit is required for the complete loss of APC. Similarly, Min/+ mice spontaneously develop CRC as a result of an inherited germline truncating mutation in the *Apc* gene (Min allele), followed by the subsequent loss of the wild-type allele [21,22]. Compared to the conventional Min/+ mouse, which is bred on a C57BL/6 genetic background, and mainly develops lesions in the small intestine, the A/J Min/+ mouse, used in the present work, demonstrates increased colonic

carcinogenesis and thus, a tumor development more similar to that seen in humans [23].

The main objective of the present study was to compare the effects of mildly heated beef-, pork-, chicken- and salmon-based diets on the development of intestinal lesions in A/J Min/+ mice. A second objective was to explore the influence of dietary fatty acid composition, amount of fat, and heme level, as well as fecal levels of lipid peroxidation (TBARS), fecal heme and cytotoxicity of fecal water on intestinal carcinogenesis. To our knowledge, the effects of beef, pork, chicken and salmon on spontaneous intestinal carcinogenesis have never been directly compared before.

Material and Methods

Animals

The experiment was approved by the Norwegian Animal Research Authority (application ID: 7528) and conducted in compliance with local and national regulations on animal experimentation. A/J Min/+ mice were maintained in open-top plastic cages under standard laboratory conditions with free access to food and water. Animals were bred in breeding trios (two female A/J wild-type mice and one male A/J Min/+ mouse), and the resulting offspring (male and female) were genotyped and randomly assigned to the experimental diets at the age of 19-21 days. For genotype determination, DNA was extracted from ear punch samples and the following primer set was used for DNA amplification via PCR: MAPC MT (5'-TGAGAAAGACAGAAGTTA -3'), MAPC 15 (5'-TTCCACTTTGGCATAAGGC-3'), and MAPC 9 (5'-GCCATCCCTT- CACGTTAG-3'). Min/+ mice were identified by the 327 bp Min fragment of *Apc*, which is generated in addition to the 618 bp wild-type PCR product [24]. Four to five mice were housed in one cage and animals were sacrificed by cervical dislocation at the end of the experiment.

Study Design

To compare the effects of dietary beef, pork, chicken and salmon on intestinal carcinogenesis, A/J Min/+ mice of both sexes (n=18-19 per group) were fed four main experimental diets (Table 1 and S1 Table). Beef, Pork, Chicken and Salmon contained approximately 40% (dry weight) muscle source and 60% purified powder diet (SDS special diet services, Witham, UK); the latter representing some features of a 'western style diet', e.g. low levels of calcium (0.08%), vitamin D (<12 IU/kg) and fiber (1.96%), but being fat-free (S2 and S3 Tables). The fat content of these four diets (15-17% of dry weight) was adjusted to equal the physiological high fat level of Salmon. Accordingly, the effects of the four muscle sources could be compared directly. Since the high fat level of the Chicken diet poorly mimics a chicken-breast based meal, a low-fat chicken breast diet (Chicken Low Fat) was also included in the study.

In previous studies, where heme iron was found to induce intestinal carcinogenesis, ca. 5% or more of the dietary fat was provided in form of n-6 PUFA [6,25], and to gain additional information about the potential role of n-6 unsaturated fat, a Beef n-6 diet was designed. This diet resembled the Beef diet, i.e. it had the same total fat content, but contained 5% safflower seed oil, which is high in n-6 PUFA. An additional group of mice, fed the standard rodent maintenance diet RM1 (Table 1, and S1 and S2 Tables), was included as a reference for the mouse model. RM1 was also used, when the A/J Min/+ mouse was described as a relevant model for initiation, promotion and progression of CRC [26]. The experimental period lasted for 10 weeks, from weaning of the A/J Min/+ mice at the age of 19-21 days, until termination at 13 weeks of age. Every day, diet leftovers from the previous day were removed from the cage before fresh food, thawed in the fridge overnight, was provided. Both the initial, halfway and terminal body weights were recorded. During the last week of the experiment, fresh feces was collected. Energy intake during the final week was calculated as average food intake, registered on 5 different days.

Table 1: Composition of study diets (dry weights).

	Muscle food diets						Reference diet
	Salmon	**Chicken Low Fat**	**Chicken**	**Pork**	**Beef**	**Beef n-6**	**RM1[e]**
Muscle source including fat (% of dry weight)	40 (salmon, fat not adjusted)	40 (chicken breast, fat not adjusted)	40 (chicken leg, adjusted with chicken fat)	40 (pork, adjusted with pork fat)	40 (beef, adjusted with beef fat)	35 (beef, adjusted with beef fat)	-
Safflower seed oil	-	-	-	-	-	5	-
Powder diet (% of dry weight)	60	60	60	60	60	60	-
Energy (MJ/kg diet)[a]	22.4	20.8	22.9	22.9	21.9	22.7	14.7
Fat (g/100g)[a]	15.6	4.5	16.8	16.6	15.6	13.6	2.7
SFA[b]	2.2	1.4	4.8	5.8	7.8	5.3	0.5
MUFA[b]	8.1	2.0	7.5	7.8	6.2	4.6	0.9
PUFA	4.8	0.9	4.1	2.7	0.5	2.8	0.8
n-6/n-3	1.1	8.6	9.2	7.5	2.9	28.6	11.5
Protein (g/100g)[a]	36.2	47.5	37.2	39.4	38.8	39.2	14.4
sugar as glucose after hydrolysis (g/100g)[a]	28.7	25.8	24.6	24.2	25.0	25.2	49.0
Fiber (g/100g)[a]	2.2	1.5	1.8	1.4	1.7	1.9	17.1
Calcium (g/100g)[c]	0.06	0.06	0.06	0.07	0.07	0.06	0.73
Vit D3 (µg/100g)[a]	6.0	0.6	0.8	0.5	0.6	0.5	15.5
Iron (mg/kg)[a]	32.3	32.8	33.7	35.6	62.4	53.8	177.0
Heme iron[d]	3.41	3.86	4.53	6.11	27.51	20.68	0.0
Non-heme iron[d]	28.9	29.0	29.1	29.5	34.9	33.2	177.0

[a]Analyses performed by Eurofins Food & Agro Testing AS (Moss, Norway): calorific value (EN14918/15400/ISO1928, EN 15400:2011, EN 14918:2010, EN14918:2010), fat (NMKL 131, 1989), carbohydrate (total carbohydrates as glucose, Eurofins in-house method based on Luff Schoorl titration), fiber (ISO 5498), protein (NMKL 6), vitamin D3 (EN 12821: 2009-08), total iron (NMKL No 161)

[b]SFA – saturated fatty acids, MUFA – monounsaturated fatty acids

[c]calculated values; based on reference values from www.matvaretabellen.no

[d]calculated values; based on the estimation that heme iron accounts for 80% of the total iron in muscle sources

[e]Diet composition declared by producer

Diet Production

The experimental diets were produced at the pilot facility of Animalia (Oslo, Norway). Meat and fish were provided by at least two different Norwegian producers. Raw materials were first processed in a bowl cutter machine, and fat and water content of salmon was analyzed using LF-NMR (low field Nuclear Magnetic Resonance) as previously described [27], whereas meat was analyzed with a FoodScan™ Meat Analyzer (Foss, Denmark). Chicken-, pork- and beef fat were then added to the respective meat type to match the fat content of salmon. Safflower seed oil (S2821, Sigma Aldrich) was blended to one batch of beef meat for the preparation of the Beef n-6 diet. All samples were heated Sous Vide in air-tight bags at 70°C (1.5 cm thickness). After 50 minutes, bags were cooled in cold water and their contents mixed with the powder diet. The finished diets were portioned and vacuumed in small plastic bags,

providing sufficient food for one cage of 4 to 5 mice for 24h. Diets were stored at -80°C to prevent lipid peroxidation. For diet characterization, samples of all diets were sent to Eurofins Food & Agro Testing AS (Moss, Norway) (Table 1 and S1 Table).

Intestinal preparations and scoring of intestinal lesions

A/J Min/+ mice were sacrificed at 13 weeks, by cervical dislocation, before colonic and small intestinal preparations were made as previously described [26]. The formalin-fixed, methylene blue stained preparations were examined by transillumination in an inverted microscope. Intestinal lesions were measured with an eyepiece reticle and the location of each lesions was registered in intervals of 1 cm along the length of the intestine. Based on this data, the number of lesions, average size of lesions and load of lesions (total areal covered by lesions) were assessed for each individual animal. In contrast to the brownish-green coloration of healthy epithelium, stained lesions appear bright blue-green and are characterized by having enlarged crypts and compressed luminal openings, which give each lesion a gyrus-like appearance. In the early stages, colonic lesions are defined as flat aberrant crypt foci (flat ACF). Flat ACF usually lay flat against their surrounding epithelium, however, a small number of lesions may appear somewhat polypoid. As these early lesions continuously develop into tumors, the topology of the lesions typically becomes elevated [23,28]. A cut-off point was set, and lesions of ≥0.196 mm^2 were defined as tumors. Small intestinal tumors have the same physical features as colonic tumors, but are located enclosed within adjacent villi.

Fecal water preparation and fecal parameters

Fresh feces from mice of each group were pooled, and 1.0 ml distilled water were added to 400 mg feces. Fecal water was prepared as previously described [25]. TBARS were quantified in fecal water as previously described by Ohkawah et al. [29] and Pierre and colleagues [30], and results are expressed as MDA (malondialdehyde) equivalents. The content of heme was analyzed in fecal water by fluorescence as described by Van Den Berg et al. [31] and Pierre and colleagues [25]. For the analysis of fecal water cytotoxicity (MTT assay), fecal water was diluted 1:20 with culture medium and added to cultivated *Apc*$^{-/+}$ cells derived from C57BL/6J Min/+ mice [32]. The MTT assay, as well as the validation and authentication of Apc$^{-/+}$ cells was performed as previously described [33,34].

Statistics and data presentation

All tests were conducted using 5% confidence levels. Colonic tumor incidence, meaning the rate of colonic tumor formation, was low in the muscle food groups, and dependency between diet and the colonic tumor incidence was compared by chi-square independency test. One-way ANOVA testing on log-transformed responses for the total number, average size and load (total area covered) of colonic flat ACF and small intestinal tumors was used to identify differences between experimental groups, applying the model:

$$y_{ji} = \mu + \tau_j + \epsilon_{ji}, \qquad \sum \tau_j = 0, \qquad \epsilon_i \sim N(0, \sigma^2),$$
$$i = 1, \dots, n_j \qquad j = B, \dots, RM1$$

where y_{ji} denotes the response variable, μ denotes the overall mean for all groups and τ_j the effects of treatment j (7 in total, abbreviations used as subscripts). Model assumptions were controlled by visual inspection of the Q-Q plot of residuals against theoretical normal quantiles, and residuals vs. predicted values. For responses with significant overall effect, post hoc multiple comparison analyses were conducted by testing contrasts, defined prior to the analysis, using Sheffé's method, which accounts for the multiple testing within each response variable. Body weight and food intake were analyzed by two-way ANOVA, using diet and gender as independent factors. Box plots indicate the median, and the 10th, 25th, 75th

and 90th percentiles as vertical boxes with error bars, and dots indicate data points. For the presentation of the tumor load distribution along the intestine, intestinal lesions were allocated into 5 location categories, encompassing the area of 0-19%, 20-39%, 40-59%, 60-79% and 80-100% of the small intestine and colon (proximal to distal). Principal component analysis (PCA) on experimental groups was performed using parameters of intestinal carcinogenesis and fecal parameters, and total amounts of individual constituents of the experimental diets as variables. To adjust for the large differences in magnitudes of variables, weighing by 1/Sdev was used.

Results

Animal body weight, diet consumption and stability of experimental diets

There were no significant differences in initial body weight between genders or study groups at weaning at three weeks (p=0.79), whereas final weight was differentially affected by gender and diet (p<0.01). Male animals gained more weight than female animals (p<0.01) (Table 2), and animals on meat and fish diets gained significantly more weight than animals in the RM1 group (p<0.01). Yet, average energy intake during the final week of the study did not differ between groups or gender (p=0.59) (Table 2). TBARS levels were analyzed in the fresh diets and diet leftovers after 24 h in the cage (S1 Fig). In fresh diets, TBARS levels were generally low, with the highest concentration analyzed in Salmon. The highest rates of TBARS formation after 24h were found for Salmon and Beef n-6, while the remaining diets proved to be relatively unaffected by peroxidation.

Effects of muscle foods on intestinal carcinogenesis

Results from one-way ANOVA showed that the experimental diets had significantly different effects on the number and load of colonic flat ACF,

Table 2: Size of study groups, body weight and daily energy intake.

	Muscle food diets						Reference diet
	Salmon	Chicken low fat	Chicken	Pork	Beef	Beef n-6	RM1
N (male/female)	9/9	9/9	9/9	9/10	10/9	9/9	9/9
Final body weight (g)	22.8 [21.1-24.5]	22.8 [21.4-24.2]	22.2 [20.5-24.0]	23.0 [21.6-24.3]	22.6 [21.5-23.8]	23.0 [21.5-24.5]	19.9 [19.2-20.5]
Energy intake (KJ/animal*day)	45 [43-47]	49 [44-53]	45 [40-50]	45 [42-49]	45 [41-48]	46 [43-50]	48 [45-52]

Results are presented as mean [95% confidence interval].

Table 3: Results from one-way ANOVA of the effects of the experimental diets on intestinal carcinogenesis.

	Colonic flat ACF			Small intestinal tumors		
	number	average size	load	number	average size	load
(Intercept)	2.82	-5.05	-2.23	2.95	-0.72	2.23
Beef	-0.09	-0.07	-0.17	0.13	0.18	0.32
Beef n-6	-0.21	-0.06	-0.27	-0.15	-0.06	-0.21
Pork	0.14	0.02	0.17	-0.01	-0.03	-0.04
Chicken	-0.26	-0.03	-0.23	-0.04	0.00	-0.03
Chicken Low Fat	-0.72	-0.06	-0.79	-0.05	0.07	0.02
Salmon	-0.63	-0.06	-0.69	-0.70	-0.32	-1.02
RM1	1.78	0.20	1.98	0.81	0.16	0.97
sigma_sq (σ^2)	1.45	0.32	1.91	0.65	0.14	1.13
F-value	8.78	0.55	8.17	5.47	3.85	5.66
p-value	<0.01	0.77	<0.01	<0.01	<0.01	<0.01

Columns indicate the 6 different responses. Rows 1-9 show estimates for the parameters and row 10 and 11 gives the F-statistic (at df 6 and 121) and associated p-value.

and on the number, average size and load of small intestinal tumors (Table 3). Contrast analyses revealed that Beef, Pork, Chicken and Salmon with similar fat content did not differentially affect the number, average size or load of flat ACF in the colon. In the small intestine, the number of tumors did not significantly differ between the four groups (Table 4, S2 Fig); however, when compared to Salmon, Beef resulted in a significantly increased average tumor size (p=0.01) and tumor load (p=0.03) in the small intestine (Fig 1A).

Results from contrast analyses, investigating the effects of fat on colonic and small intestinal carcinogenesis, showed that there was no significant difference between the effects of the two chicken diets, where fat levels, but not fatty

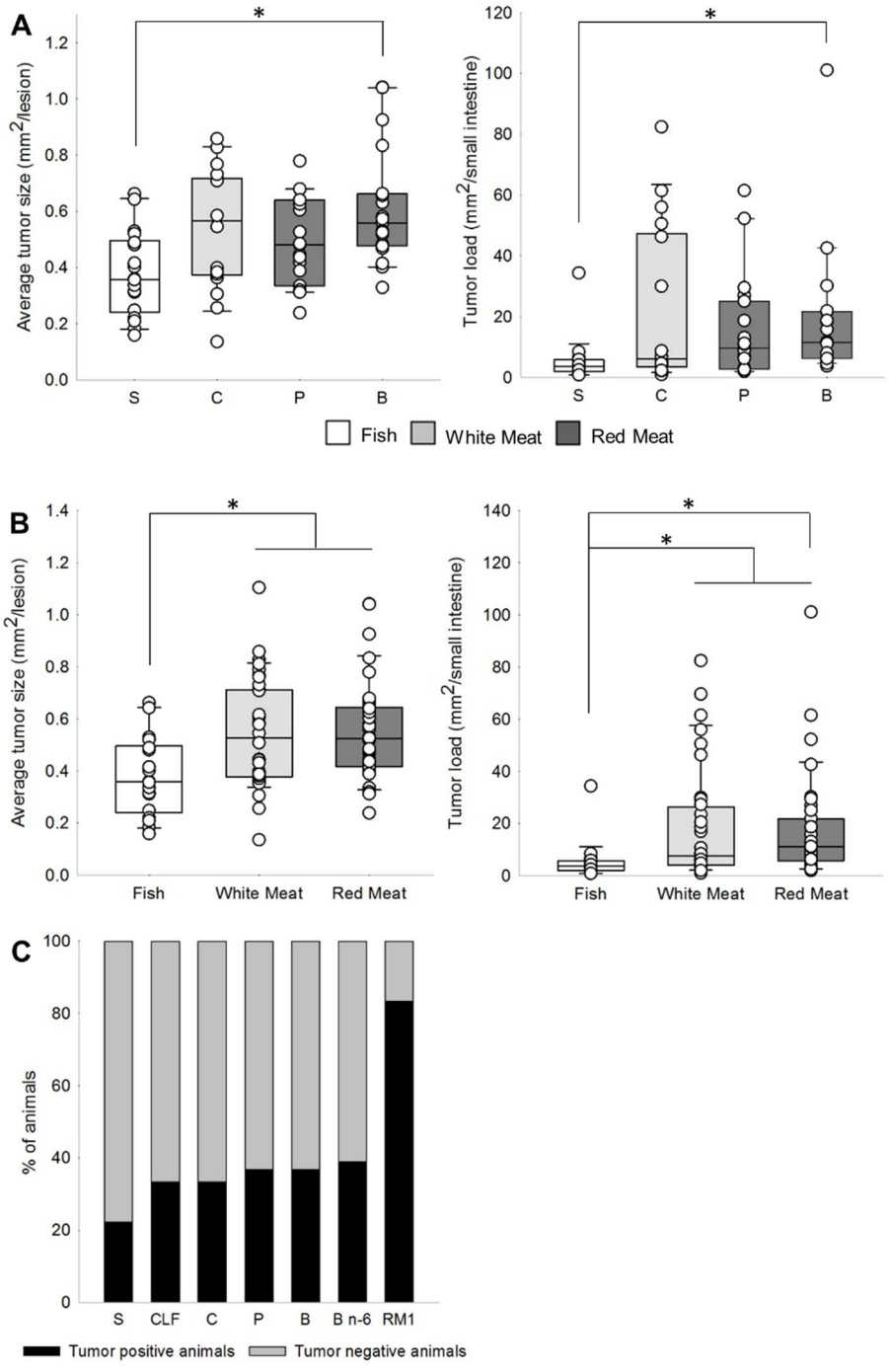

Fig 1: Effects of experimental diets on intestinal carcinogenesis. (A) Effects of Salmon [S], Chicken [C], Pork [P] and Beef [B] on average small intestinal tumor size and tumor load. (B) Effects of Fish [Salmon], white meat [Chicken, Chicken Low Fat] and red meat [Pork, Beef, Beef n-6] on average small intestinal tumor size and tumor load. (C) Effects of Salmon [S], Chicken Low Fat [CLF], Chicken [C], Pork [P], Beef [B], Beef n-6 [B n-6] and RM1 on colonic tumor incidences (proportion of tumor positive and negative animals). Significant differences are indicated by asterisks.

Table 4: Results from Scheffe's method for multiple testing of the number, average size and load of colonic flat ACF and small intestinal tumors (post hoc).

Test	Contrast		Colonic flat ACF			Small intestinal tumors		
			number	average size	load	number	average size	load
Effects of diets with similar fat content	Beef - Pork	*Estimate*	-0.23	-0.10	-0.33	0.14	0.22	0.36
	$\tau_B - \tau_P$	p-value	1.00	1.00	1.00	1.00	0.77	0.98
	Beef - Chicken	*Estimate*	0.17	-0.11	0.07	0.17	0.18	0.35
	$\tau_B - \tau_C$	p-value	1.00	1.00	1.00	1.00	0.9	0.98
	Beef - Salmon	*Estimate*	0.54	-0.01	0.53	0.83	**0.51**	**1.34**
	$\tau_B - \tau_S$	p-value	0.93	1.00	0.97	0.14	**0.01**	**0.03**
	Pork - Chicken	*Estimate*	0.41	-0.01	0.40	0.03	-0.04	0.00
	$\tau_P - \tau_C$	p-value	0.98	1.00	0.99	1.00	1.00	1.00
	Pork - Salmon	*Estimate*	0.78	0.08	0.86	0.69	0.29	0.98
	$\tau_P - \tau_S$	p-value	0.70	1.00	0.73	0.35	0.46	0.25
	Chicken - Salmon	*Estimate*	0.37	0.09	0.46	0.66	0.33	0.99
	$\tau_C - \tau_S$	p-value	0.99	1.00	0.99	0.42	0.33	0.26
Effects of fat	Chicken - Chicken Low Fat	*Estimate*	0.46	0.09	0.55	0.01	-0.06	-0.06
	$\tau_C - \tau_{CLF}$	p-value	0.97	1.00	0.96	1.00	1.00	1.00
	Beef - Beef n-6	*Estimate*	0.12	-0.02	0.10	0.29	0.24	0.53
	$\tau_B - \tau_{B\,n-6}$	p-value	1.00	1.00	1.00	0.98	0.69	0.89
Effects of food groups	Red Meat - White Meat	*Estimate*	0.49	-0.01	0.48	0.25	0.12	0.37
	$\frac{1}{3}(\tau_B + \tau_{B\,n-6} + \tau_P) - \frac{1}{2}(\tau_C + \tau_{CLF})$	p-value	0.61	1.00	0.77	0.85	0.84	0.77
	Red Meat - Fish	*Estimate*	0.58	0.02	0.60	0.69	**0.36**	**1.04**
	$\frac{1}{3}(\tau_B + \tau_{B\,n-6} + \tau_P) - \tau_S$	p-value	0.79	1.00	0.86	0.14	**0.06**	**0.05**
	White Meat - Fish	*Estimate*	0.14	0.04	0.18	0.66	0.36	1.02
	$\frac{1}{2}(\tau_C + \tau_{CLF}) - \tau_S$	p-value	1.00	1.00	1.00	0.25	0.09	0.10
	Meat - Fish	*Estimate*	0.40	0.03	0.43	0.68	**0.36**	**1.03**
	$\frac{1}{5}(\tau_B + \tau_{B\,n-6} + \tau_P + \tau_C + \tau_{CLF}) - \tau_S$	p-value	0.94	1.00	0.96	0.11	**0.04**	**0.03**
Effects of basic diets	RM1 vs. Powder-based diets	*Estimate*	**2.08**	0.24	**2.31**	**0.94**	0.19	**1.13**
	$\tau_{RM1} - \frac{1}{6}(\tau_B + \tau_{B\,n-6} + \tau_P + \tau_C + \tau_{CLF} + \tau_S)$	p-value	**0.00**	0.85	**0.00**	**0.00**	0.69	**0.01**

Each part consist of two rows showing the contrast estimate as defined in column 2, and the p-value. τ_i is the effect of a group/ treatment i, with Salmon [S], Chicken Low Fat [CLF], Chicken [C], Pork [P], Beef [B], Beef n-6 [B n-6] and RM1 [RM1]. Significant results ($p < 0.05$) are shown in bold text.

acid composition differed.

Likewise, a higher concentration of n-6 unsaturated fat in beef meat did not significantly affect the development of colonic flat ACF or small intestinal tumors, as there was no difference between mice fed Beef and Beef n-6 (Table 4 and S2 Fig).

For further analyses, the muscle food groups were grouped into Red Meat (Beef, Beef n-6, Pork), White Meat (Chicken, Chicken Low Fat) and Fish

(Salmon). Chicken Low Fat was included into the White Meat group and Beef n-6 was included into the Red Meat group, based on the findings that the formation of intestinal lesions was not affected by dietary fat.

Again, no differences were found for colonic carcinogenesis. Also, no significant differences were found when the effects of Red Meat were compared to White Meat in the small intestine. However, Red Meat induced a greater small intestinal tumor load (p=0.05) than Fish, and the difference was reflected by a borderline significant increase in average tumor size (p=0.06) (Table 4 and Fig 1B). Indications are also given that White Meat resulted in an increased average small intestinal tumor size (p=0.09) and tumor load (p=0.10), when compared to Fish. Accordingly, Red Meat and White Meat combined induced a significantly greater average tumor size (p=0.04) and tumor load (p=0.03) than Fish.

When comparing the reference diet RM1 to the muscle food diets based on the powder diet, mice fed RM1 showed a significantly larger number and load of colonic flat ACF (both p<0.01) and small intestinal tumors (p<0.01 and p=0.01, respectively) (Table 4 and Fig 2). The average size of lesions was not affected (S2 Fig).

In regard to tumor formation in the colon, tumor incidence varied significantly between the experimental groups (p<0.01, χ^2 = 17.2 at df 6). Fig 1C indicates a larger probability of tumor development in mice fed RM1 and a slight protective effect of salmon on tumor incidence.

Fig 3 presents the average area covered by lesions (load) along the intestine. All study groups present a similar distribution, where the majority of intestinal lesions are found in the distal sections of both the small intestine and the colon. The graph shows that mice fed the reference diet RM1 had a greater tumor load than mice fed any of the other diets, in both small intestine and colon, and that differences between the powder-based experimental groups were comparatively modest. Only the tumor load profile of mice fed Salmon appeared to be lower than the profiles of mice fed meat diets, and the difference was more pronounced in the small intestine than in the colon. Size distributions of intestinal lesions (S3 Fig) further illustrate the presented results.

To test the robustness of our results, gender and parents were separately added as additional predictors. Importantly, the number of animals versus variables reduces the degrees of freedom considerably, especially for “parents”. The effects of gender were in general small, whereas the effects of parents (26 combinations of dam and

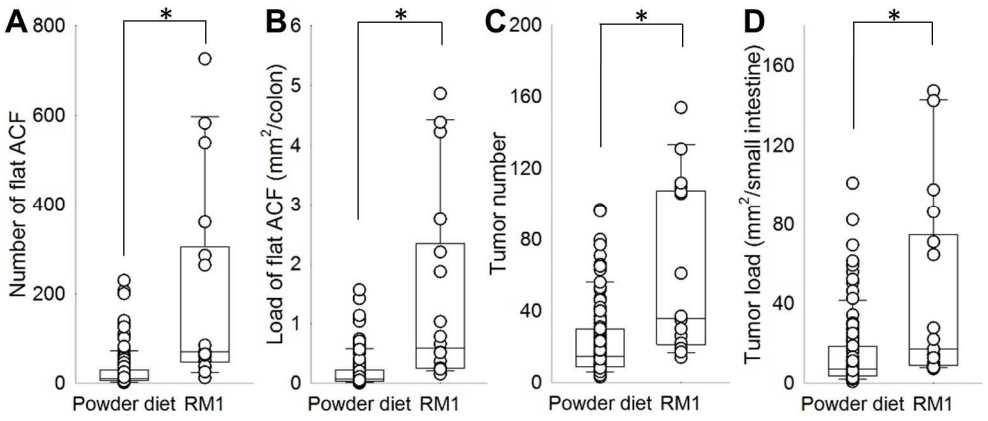

Fig 2: Effects of powder-based diets and RM1 on number and load of intestinal lesions. (A) Number of colonic flat ACF, (B) Load of colonic flat ACF, (C) Number of small intestinal tumors, (D) Load of small intestinal tumors. Significant differences are indicated by asterisks.

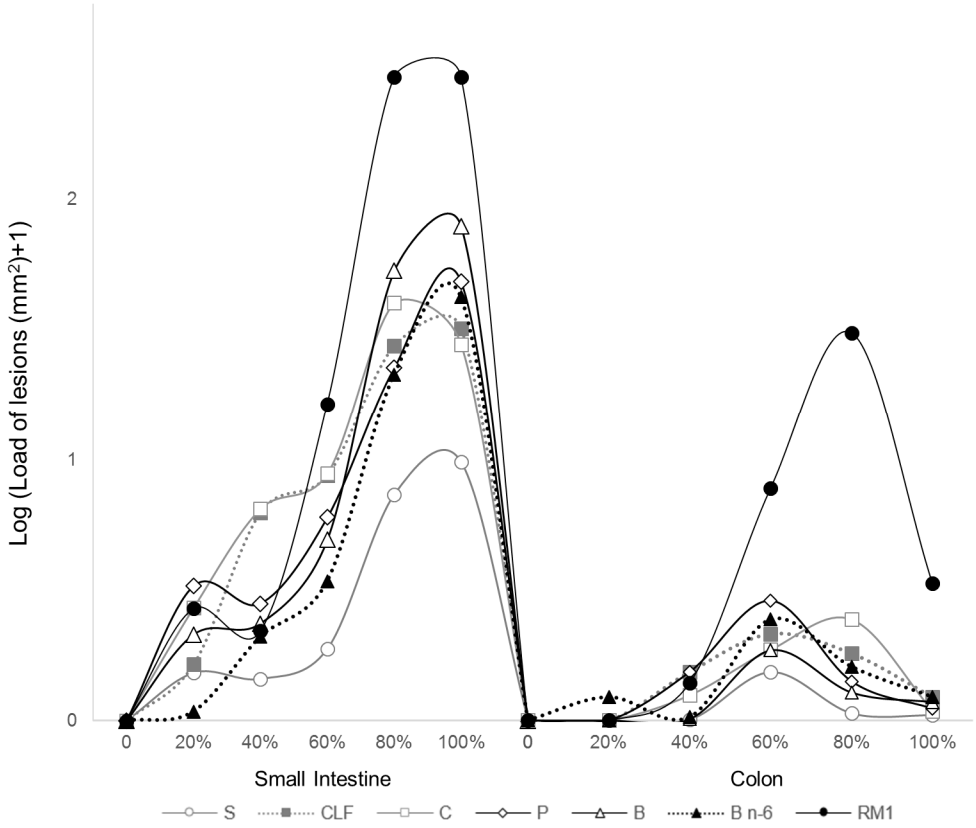

Fig 3: Effects of experimental diets on the load of lesions along the intestine. Salmon [S], Chicken Low Fat [CLF], Chicken [C], Pork [P], Beef [B], Beef n-6 [B n-6]. Values represent means from log-transformed data.

sire) were clear for most responses, suggesting epigenetic or environmental factors for the interindividual variation. The effects of parents on the estimates for the significant one-way contrasts, however, were small, but led to smaller differences, i.e. marginally larger p-values.

Fecal water analyses

Fecal heme concentrations (Fig 4A) were highest in the sample from animals fed Beef n-6, followed by Beef, Pork, Chicken, Chicken Low Fat and Salmon. The sample from mice fed RM1 presented the lowest concentrations of fecal heme. The highest concentration of fecal TBARS (Fig 4B) were found in the sample from animals fed Salmon and Beef n-6, followed by samples from animals fed Beef, Pork and the Chicken diets. Fecal TBARS concentration were lowest in the sample from the RM1 group.

Apc$^{-/+}$ cells were incubated with fecal water to assess fecal water cytotoxicity (Fig 4C). Fecal water samples from mice fed Salmon, Pork and RM1 induced the greatest reduction of cell viability, while the highest cell viability of all experimental groups was observed in response to fecal water of mice fed Chicken.

Principal component analysis (PCA)

PCA (Fig 4D) shows the relationship between characteristics of fecal water, constituents of the experimental diets and intestinal carcinogenesis. PC 1 and 2 explained more than 75% of variance. Experimental groups fed meat from terrestrial animals clustered around the intersection of PC 1 and 2, while the group fed Salmon was better defined by PC 2, i.e. high levels of unsaturated fatty acids in the diet and low heme content. The RM1-group was associated with parameters of intestinal carcinogenesis, dietary non-heme iron, calcium, vitamin D, carbohydrates and fiber.

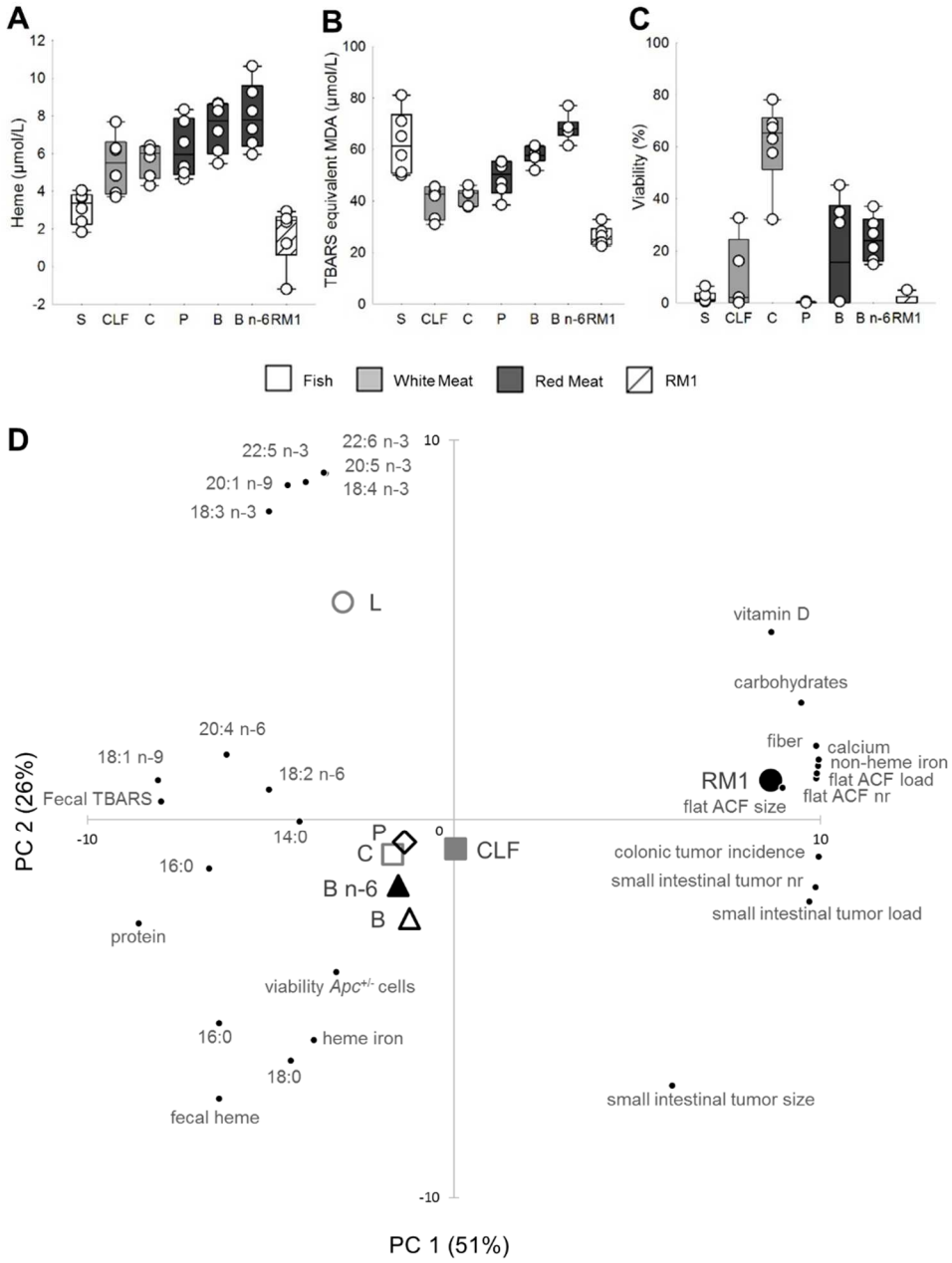

Fig 4: Fecal water analyses and scatter plot of PCA on experimental groups. Fecal water content of (A) Heme and (B) TBARS, and (C) Cytotoxic effect of fecal water on *Apc*$^{-/+}$ cells (n=1, fecal water from pooled fresh feces of 18 to 19 animals per group). Data points and box plots indicate measurement uncertainties of the methods. (D) PCA biplot showing scores from PC1 and PC2: associations between experimental groups, and parameters of intestinal carcinogenesis and fecal water, and constituents of the experimental diets. Salmon [S], Chicken Low Fat [CLF], Chicken [C], Pork [P], Beef [B], Beef n-6 [B n-6].

Colonic tumor development was negatively correlated to fecal heme and TBARS.

Discussion

In epidemiological and experimental studies, high intake of red meat has been associated with an increased risk for CRC. Fish consumption may have a protective effect on CRC, while meat from poultry is considered neutral [7]. To our knowledge, this is the first experimental study to directly compare the effects of intake of red meat (beef and pork), white meat (chicken) and fish (salmon) on intestinal carcinogenesis. Results suggest that replacing beef with salmon with similar fat content may reduce small intestinal tumor burden in A/J Min/+ mice, and more generally, fish intake, represented by salmon, was found to result in lower cancer burden than meat from terrestrial animals (approx. factor 3). Similarly, a protective effect of dietary long chain n-3 PUFA from fish oil on intestinal carcinogenesis has previously been documented in the

conventional C57BL/6 Min/+ mouse [35,36]. In the present study, white meat did not prompt a more beneficial outcome than red meat, but instead affected intestinal carcinogenesis in the same manner as red meat. Also, tumor burden induced by the chicken diet (16.8% fat) did not differ from the tumor burden in mice fed the low-fat chicken diet, a diet characterized by a similar composition of fatty acids but lower levels of fat (4.5%). This gives reason to assume that the lack of differences between the effects of red and white meat was not attributed to the relatively high level of fat in the chicken diet. One of the main hypothesis linking red meat to an increased risk of CRC, concerns the idea that heme iron from red meat enhances oxidative stress through lipid peroxidation [5,6,25]. Thus, the combination of heme iron and a high level of PUFA may adversely affect intestinal health [17,18]. In the present study, however, the formation of colonic or small intestinal lesions was not affected by the quality of fat, and additional n-6 PUFA in beef meat did not affect the outcome. The results of the present study do not give any indications of a role of heme iron in CRC.

Remarkably, the largest tumor burden, in both the small intestine and colon was induced by the reference diet RM1. Albeit similar tumor initiating effects of RM1 have been reported before [26,37], the result is surprising due to various reasons: In contrast to the semi-synthetic powder diet, the natural ingredient diet RM1 was not adjusted for nutrients with documented protective properties, e.g. calcium, vitamin D or fiber. Moreover, despite the similar calorie intake between study groups, consumption of RM1 resulted in a lower body weight, which is considered beneficial in regard to CRC risk [8]. Diet compositions of the powder-based diets differ from RM1 in many aspects, which makes it impossible to assess whether the observed differences between the basic diets (RM1 and powder diet) may be attributed to the level of particular macronutrients, micronutrients or unknown, bioactive compounds. Nevertheless, based on the PCA biplot, it may be speculated that the tumor inducing potential of the RM1 feed is, to some extent, connected to the high level of organic iron, which was previously shown to enhance intestinal carcinogenesis in Min/+ mice [38]. Besides, the outcome may be associated with the higher proportion of energy provided by carbohydrates [8]. Most importantly, the results underline that differences provoked by the different muscle foods were small in comparison to differences provoked by unknown factors in the basic diets. Nutritional factors that are typically included as confounding variables in epidemiological CRC risk assessments are total energy intake, fiber, calcium, folate and use of multivitamin supplements [1,2,39]. As meat and fish meals may be typically consumed along with certain accompaniments (e.g. vegetables, potatoes or bread) [40], the results emphasis the importance of the inclusion of certain food groups and macronutrients as putative confounding factors in epidemiological studies.

Several experimental studies link lipid peroxidation and fecal water cytotoxicity to intestinal carcinogenesis [25,41]. Fecal TBARS formation appeared to be enhanced by unsaturated fat and heme iron, but in line with the findings from a previous study on A/J Min/+ mice [42], the present study does not suggest a direct link between luminal peroxidation and intestinal carcinogenesis. Moreover, fecal water cytotoxicity on *Apc*$^{-/+}$ cells of the various experimental groups did not follow an evident pattern, and there was no indication for a relationship between fecal water cytotoxicity on *Apc*$^{-/+}$ cells, fecal TBARS, and intestinal carcinogenesis. Despite the favorable effects of dietary salmon on small intestinal carcinogenesis, levels of TBARS and fecal water cytotoxicity were amongst the highest in response to salmon. In turn, a strong fecal water cytotoxicity in the RM1 groups coincided with high rates of tumor formation, but was not connected to lipid peroxidation. Hence, the use of TBARS in

predicting carcinogenic effects, and the relevance of the cytotoxicity assay in the present study seem controversial. The MTT assay that was used to assess fecal water cytotoxicity, does only indicate decreases in cell viability, and does not allow to differentiate between apoptotic and necrotic processes. Hence, it needs to be established whether the induction of cell death of epithelial cells presents an advantage or disadvantage in regard to intestinal carcinogenesis.

Ingested heme iron is presumed to increase fecal water cytotoxicity, and heme in the soluble fraction of the feces is thought to interact more strongly with the intestinal epithelium than the non-soluble fraction [33]. Despite of higher fecal concentrations of heme in response to dietary red meat than white meat and fish, concentrations were low in comparison to fecal concentrations detected in rats fed equivalent amounts of beef or heme iron [30,33,34,43]. The amount of heme iron reaching the colon may be influenced by possible precipitation of heme by compounds like calcium [30,44], but calcium levels in the muscle meat diets were low (0.06-0.07%). Besides, fecal heme concentration is determined by the absorption rate of heme in the small intestine. As opposed to humans, in rats and mice, heme iron is absorbed at lower rates than non-heme iron [45,46], and the adaption of the absorption of heme is limited even in case of iron deficiency [47]. Nevertheless, absorption rates of heme and non-heme iron were shown to correlate in rats [45], and in comparison with C57BL/6J wild type mice, mice on an A/J genetic background were shown to absorb free iron twice as efficient [48]. Hence, the discrepancies in the effects of red meat between the present study and previously performed studies by Pierre and colleagues [30,33] may be related to a more efficient removal of intestinal heme in A/J Min/+ mice. More work is necessary to evaluate the translation potential of rodent studies in regard to heme metabolism.

Carcinogenesis is divided into initiation, promotion and progression, and, depending on their mode of actions, carcinogens have the ability to interfere with molecular processes at either stage of tumor development [49]. The period where the majority of new intestinal lesions are spontaneously initiated in A/J Min/+ mice covers the time span from birth to approximately 30 weeks of age, and peaks at the age of 7 to 12 weeks. While small intestinal lesions are characterized by a relatively uniform growth throughout the lifespan of the mouse, an extensive growth acceleration in the colon is not seen before the age of 30 weeks [23]. In the present work, similar tendencies of tumor induction were observed in the small intestine and colon after 13 weeks, but differences between study groups were more pronounced in the small intestine, where the average size and load of tumors was significantly increased in response to meat from terrestrial animals. Thus, it needs to be established how intestinal lesions, and, in particular, colonic lesions, are influenced by different muscle foods during later stages of tumor development. The possibility that CRC may not be influenced by red meat at the stage of tumor initiation is supported by a long-term study conducted by Winter et al. [50], where no increased rate of colonic neoplasms was observed in heme iron-fed C57BL/6 WT mice, and two previous studies on A/J Min/+ mice that documented an inhibitory effect of heme iron on colonic carcinogenesis [26,42]. In rodent studies that reported promoting effects of red meat, intestinal carcinogenesis was accelerated by azoxymethane (AOM) or 1,2-demethylhydrazine (DMH) [30,33,43]; two colon-specific carcinogens, which stimulate the acquisition of mutations in key regulatory genes.

Due to an evolutionary loss of the cytidine monophospho-N-acetylneuraminic acid hydroxyl-lase (CMAH), humans, as opposed to other mammals, are not able to endogenously produce N-glycolylneuraminic acid (Neu5Gc) through enzymatic conversion of N-actetylneuraminic acid

(Neu5Ac) [51]. Nevertheless, Neu5Gc from dietary sources, e.g. red meat, seems to be incorporated into human tissue and recently, inflammatory processes induced through recognition of Neu5Gc by auto-reactive antigens have been proposed as a mechanism in red-meat related CRC [52]. If this mechanism is proven to be valid, mice with a functional *Cmah* gene, like the A/J Min/+ mouse, will most likely not represent a suitable model for red meat-related CRC. However, crossing A/J Min/+ with *Cmah*$^{-/-}$ mice (mice that do not express Neu5Gc due to deletion of exon 6 of the *Cmah* gene [53]) could provide a helpful tool in the investigation of the role of Neu5Gc in red meat related CRC development.

In summary, the results of the present work do not indicate that the intake of cooked red meat is less favorable for CRC development than the intake of white meat. However, it was shown that consumption of salmon may inhibit intestinal carcinogenesis. The present study could not confirm a link between TBARS, fecal water cytotoxicity and intestinal carcinogenesis, but underlines the importance of the basic diet during carcinogenesis. Long-term studies are needed to increase knowledge on the effects of red meat on initiation, promotion and progression of CRC in A/J Min/+ mice.

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Conflict of interest

Ole Alvseike, Lars Erik Gangsei and Ellen-Margrethe Hovland are employed in Animalia. Animalia is a Norwegian non-profit organization mainly financed by the national purchase tax, supervised by the Agricultural Marketing Board, and organized by the Norwegian Meat cooperative. The Agricultural Marketing Board is a committee appointed by the Ministry of Agriculture and Food to administer the regulations of markets for agricultural products in Norway. No potential conflicts of interest were disclosed by the other authors.

Author’s contribution

Conceived and designed the experiments: CS MS BEG BK MO OA LEG EMH FP JEP. Performed the experiments: CS MS FP. Analyzed the data: LEG CS JEP. Wrote the article: CS. Critically reviewed the manuscript: MS BEG BK MO OA LEG EMH FP JEP. All authors read and approved the final manuscript.

Supporting Information

S1 Table: Fatty acid composition of the experimental diets.

S2 Table: Ingredients of the powder diet and RM1.

S3 Table: Composition of the powder diet.

S1 Fig: TBARS concentrations in fresh muscle food diets (T0) and after 24h in the cage (T24).

S2 Fig: Effect of Salmon (S), Chicken Low Fat (CLF), Chicken (C), Pork (P), Beef (B), Beef n-6 (B n-6) and RM1 on the number, average size and load of intestinal lesions in A/J Min/+ mice.

S3 Fig: Size distribution of (A) flat ACF and tumors in the colon, and (B) small intestinal tumors of A/J Min/+ mice fed Salmon [S], Chicken Low Fat [CLF],

Chicken [C], Pork [P], Beef [B], Beef n-6 [B n-6] and RM1.

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Supporting Information:

The Effect of Beef, Pork, Chicken and Salmon meat on the intestinal carcinogenesis in A/J Min/+ mice

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Experimental diets

S1 Table: Fatty acid composition of the experimental diets.

	Muscle food diets						Reference diet
	Salmon	**Chicken Low Fat**	**Chicken**	**Pork**	**Beef**	**Beef n-6**	**RM1**
% of total fatty acids[a]							
C14:0	2.2	1.2	0.8	1.5	3.1	2.2	5.2
C16:0	8.8	23.2	21.2	22.0	23.6	19.1	11.4
C16:1 $_{n-7}$	2.5	4.2	4.2	2.3	2.9	2.1	3.3
C18:0	2.4	6.6	6.0	10.5	21.0	16.0	1.5
C18:1$_{n-9}$	42.9	39.6	40.1	43.6	36.1	31.4	28.4
C18:2 $_{n-6}$	13.4	17.6	21.2	12.8	2.5	19.9	25.5
C18:3 $_{n-3}$	4.8	1.7	2.1	1.4	0.8	0.5	2.2
C18:4 $_{n-3}$	0.6	-	-	-	-	-	-
C20:1 $_{n-9}$	3.7	0.4	0.4	0.9	0.2	0.2	-
C20:4 $_{n-6}$	0.3	0.6	0.4	0.4	0.1	0.1	-
C20:5 $_{n-3}$	2.6	-	-	-	-	-	-
C22:5 $_{n-3}$	1.6	0.2	-	0.2	0.1	0.1	-
C22:6 $_{n-3}$	3.9	0.2	-	0.1	-	-	-
DB[b]	**138.3**	**89.1**	**95.0**	**79.8**	**47.5**	**75.9**	**89.3**

[a]Analyses performed by Eurofins Food & Agro Testing AS (Moss, Norway): fatty acid composition (1)

[b]DB is the relative number of double bonds per g fat, and was calculated by the formula:
DB= $\sum_{n=1}^{6}$ n(% fatty acids with n double bonds)

S2 Table: Ingredients of the powder diet and RM1.

Powdered diet	RM1
Rice Starch	Wheat
Sucrose	Barley
Casein	Wheat feed
AIN-93G-MX, adjusted for Ca and P	De-hulled extracted Toasted Soya
Cellulose	Soya Protein Concentrate
AIN-93-VX, without supplementary vitamin D3	Macro Minerals
L-Cystine	Soya Oil
Choline Bitatrate	Whey powder
	Amino Acids
	Vitamins
	Micro Minerals

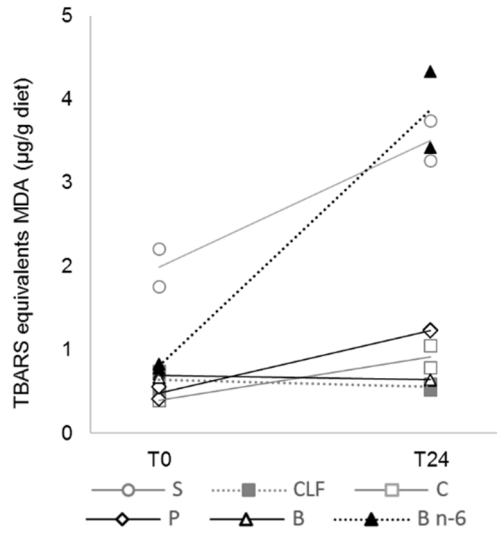
S3 Table: Composition of the powder diet.

	Powder diet
Energy (MJ/kg)	14.7
Fat (g/100 g)	0.0
Protein (g/100 g)	18.3
Sugar as glucose after hydrolysis (g/100 g)	64.6
Fiber (g/100 g)	2.0
adjusted mineral/vitamin level	
total Ca (%)	0.08
total P (%)	0.14
total Vit D3 (iu/100 g)[a]	<18.3

[a]vitamin D from casein (<100 iu/kg casein)

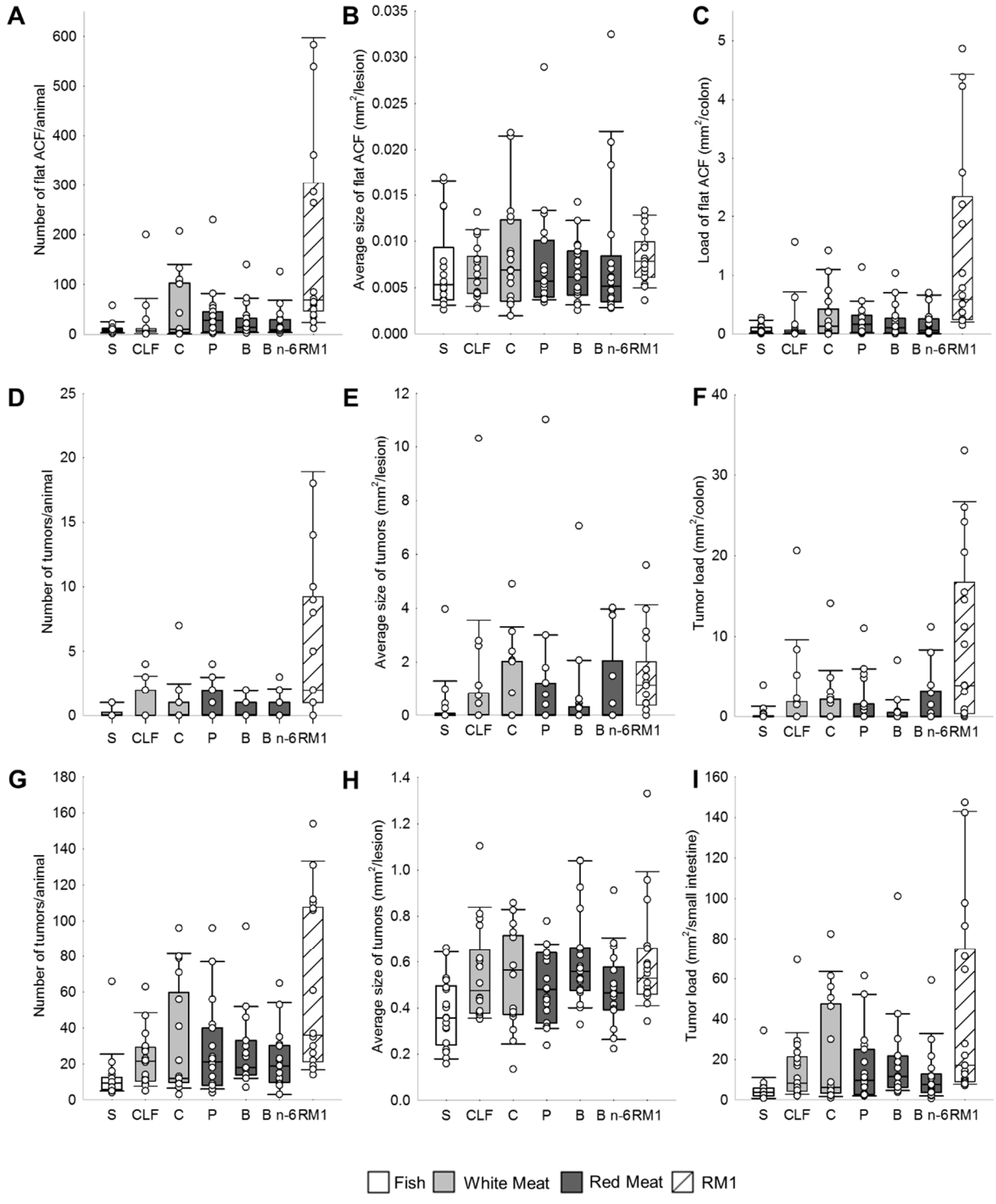
Stability of the experimental diets

TBARS were analysed in fresh diet and diet leftovers after 24h in the cage. To 2 g of grounded diet, 10 ml of 5% trichloroacetic acid, 40 µl of butylated hydroxytoluene (1 mg/ml) and 40 µl ethylenediaminetetraacetic acid (0.1 M) were added and homogenized for 2 x 15 s with a mechanical grinder. Homogenized mixtures were centrifuged at 3000 x g for 3 minutes at 4°C. The supernatant was collected and filtered through Whatman® qualitative filter paper (grade 1). TBARS were analyzed as previously described by Ohkawah et al. (2).



S1 Fig: TBARS concentrations in fresh muscle food diets (T0) and after 24h in the cage (T24). Salmon [S], Chicken Low Fat [CLF], Chicken [C], Pork [P], Beef [B] and Beef n-6 [B n-6].

Effect of Salmon, Chicken, Pork, Beef and RM1 on intestinal carcinogenesis in A/J Min/+ mice

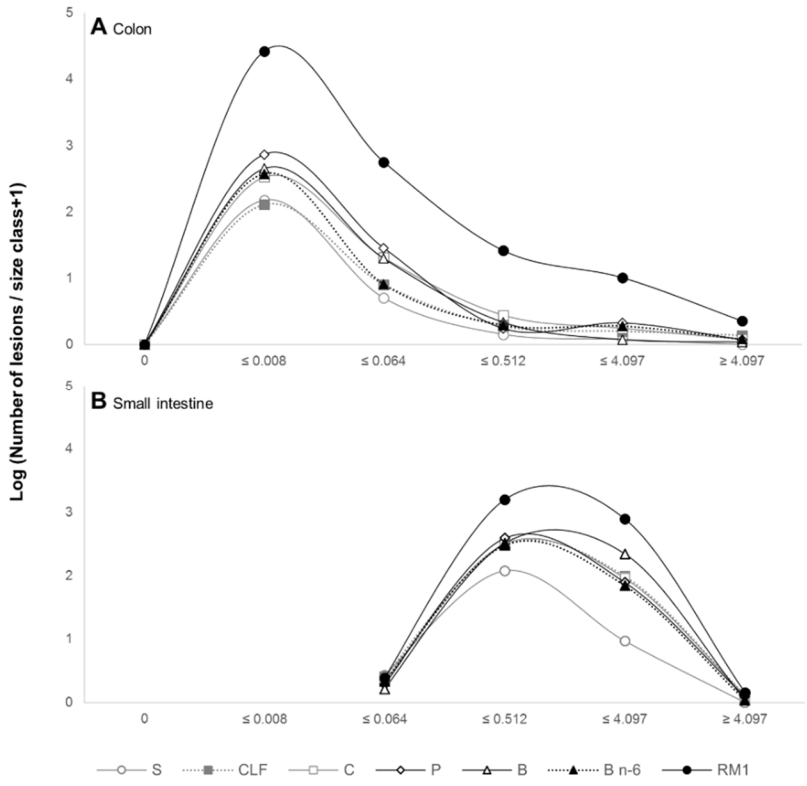


S2 Fig: Effect of Salmon [S], Chicken Low Fat [CLF], Chicken [C], Pork [P], Beef [B], Beef n-6 [B n-6] and RM1 on number, average size and load of intestinal lesions in A/J Min/+ mice. (A-C) flat ACF, (D-F) colonic tumors and (G-I) small intestinal tumors.

Size distribution of intestinal lesions in A/J Min/+ mice

For the size distribution, intestinal lesions were allocated into five size classes, based on a logarithmic scale: 0-0.008 mm^2; 0.009-0.064 mm^2; 0.065-0.512 mm^2; 0.513-4.096 mm^2 and >4.096 mm^2. Thereby, the smallest lesions (approx. 1-4 lesions) are grouped within the first size class. The presented average numbers of lesions within size classes were calculated from log-transformed data to correct for the large variation between individual mice within the study groups.

Colonic and small intestinal size class distributions (Supplementary Fig 3) illustrate the number of lesions within each size category. It becomes apparent that RM1 had the strongest ability to induce intestinal carcinogenesis in the colon and small intestine of the A/J Min/+ mouse. In the colon, the comparable profiles of the distribution curves illustrated that differences between groups were marginal. In the small intestine, Salmon resulted in a size distribution curve below the other meat diets. In addition, a shift towards larger lesions was only observed for the meat diets from terrestrial animals, but not for Salmon.



S3 Fig: Size distribution of (A) flat ACF and tumors in the colon, and (B) small intestinal tumors of A/J Min/+ mice fed Salmon [S], Chicken Low Fat [CLF], Chicken [C], Pork [P], Beef [B], Beef n-6 [B n-6] and RM1.

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IV

Inflammation-induced colonic carcinogenesis in A/J Min/+ mice is dependent on the intestinal microbiota

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Abbreviations: APC: Adenomatous polyposis coli; CRC: Colorectal cancer; DSS: Dextran sodium sulfate; flat ACF: Flat aberrant crypt foci; IgA: Immunoglobulin A; Min: multiple intestinal neoplasia; PCA: Principal component analysis; SCFA: Short chain fatty acids

Abstract

Dysbiosis of the intestinal microbiota or the presence of so-called "bacteria drivers" is believed to contribute to colorectal carcinogenesis through the formation of potentially harmful metabolites or interaction with the immune system. Also, inflammatory bowel disease is associated with an increased risk for colorectal cancer (CRC). The aim of this study was to further elucidate the role of inflammation, microbiota and short chain fatty acids (SCFAs) in the pathogenesis of CRC. The A/J Min/+ mouse model was used, which is characterized by spontaneous formation of numerous intestinal tumors, and a high susceptibility to colon-specific carcinogenesis. Dextran sodium sulfate (DSS) was administrated to female mice (10 and 12 weeks of age) for 4 days via drinking water to promote inflammation. Changes in fecal microbiota and SCFAs were monitored throughout the study, and effects on inflammation and intestinal carcinogenesis were determined at the end of the study (day 24). DSS-treatment was shown to induce an immunological response and initiate colonic carcinogenesis, measured as flat aberrant crypt foci (ACF) and tumors, whereas small intestinal carcinogenesis remained unaffected. However, different susceptibilities to colonic carcinogenesis were observed within the DSS-treated mice, which differentiated the mice into two subgroups. The subgroups were found to differ in the initial microbiota, age and parents. Furthermore, DSS-treatment resulted in temporal changes of the fecal microbiota and SCFAs profile, where dysbiosis was most apparent on day 5, with decreased alpha diversity, higher relative levels of propionic acid, and lower levels of butyric acid. Bacteria related to the colonic carcinogenesis were identified (e.g. *Bacteroides*, *B. acidifaciens* and *[Prevotella]*), as well as bacteria related to the SCFAs. To conclude, the presented study shows that the A/J Min/+ mouse model is susceptible to inflammation-induced carcinogenesis, where the intestinal microbiota is involved in mediating the development, and the initial microbiota seems to modulate the degree of susceptibility.

Introduction

In 2012, colorectal cancer (CRC) was the second common form of cancer in Europe, causing nearly 215.000 of the total 1.75 million cancer deaths estimated that year [1]. Besides of CRC as a consequence of hereditary conditions like lynch syndrome or familial adenomatous polyposis, the majority of all colorectal cancer cases (>85 %) occur sporadically, and are related to environmental or lifestyle factors [2]. Moreover, inflammatory conditions like inflammatory bowel disease (e.g. Chron's disease and ulcerative colitis) are associated with an increased risk for CRC

development, and the degree of the association is affected by the duration and severity of the disease [3-5]. Inflammatory pathways and mediators are attributed a central role also in the carcinogenesis of non-colitis associated CRC [6], and long-term use of non-steroidal anti-inflammatory drugs (NSAIDs) like sulindac or aspirin may have chemopreventive effects on the disease [7]. Microbiota composition has been related to both inflammation and CRC, and has been acknowledged as one of the factors that mediates the link between inflammation and CRC. Sporadic CRC commonly goes along with alterations in the microbiota compositions [8], and treatment with antibiotics was found to ameliorate colitis, and inhibit colonic carcinogenesis in a rodent model for colitis-associated cancer [9].

The administration of dextran sodium sulfate (DSS) via drinking water induces intestinal inflammation, and is a well-established model for colitis in rodents. Although mechanisms are not conclusively understood, the condition induced by DSS mirrors the course of the disease of ulcerative colitis [10], and goes along with impairments of the intestinal barrier function [11]. DSS-treatment has also been shown to enhance tumor formation initiated by a loss of heterozygosity in the *adenomatous polyposis coli (Apc)* gene in Min/+ mice [10, 12-14]. Mutations in the tumor suppressor gene *APC* are common events in the onset of sporadic CRC in humans (>80 %), and also in colitis-induced CRC, later stages of tumor formation are accompanied by mutations in *APC* in 4-27 % of the cases [15]. Min/+ mice carry a heterozygous mutation in the *Apc* gene, and are characterized by spontaneous intestinal carcinogenesis. Compared to the conventional C57BL/6 Min/+ mouse, Min/+ mice bred on an A/J genetic background present an increased susceptibility to colonic carcinogenesis, and reflect the pathology of CRC in humans more closely. In A/J Min/+ mice, intestinal crypts become dysplastic and develop into flat aberrant crypt foci (flat ACF), which increase in size and continuously progress into adenomas and eventually carcinomas [16].

In the present study, A/J Min/+ mice were exposed to DSS, with the aim to further elucidate the role of inflammation on microbiota composition and pathogenesis of CRC.

Materials and methods

Animals and intervention

The experiment was approved by the Norwegian Animal Research Authority and executed in compliance with the local and national regulations associated with animal experiments (application ID: 6906). A/J Min/+ mice were bred and housed at the experimental animal facility at the Norwegian University of Life Science, Campus Adamstuen, and were allowed free access to a standard maintenance or breeding diet (RM1/RM3) and drinking water. For mating, two female A/J +/+ were housed together with one male A/J Min/+ in breeding trios, and offspring was weaned between day 19 and 21. The genotype of mice was determined by allele-specific polymerase chain reaction (PCR) after extracting DNA from ear punch samples as previously described [17]. For the intervention study (Fig. 1A), 16 young adult A/J Min/+ mice (10 and 12 weeks old) were randomly assigned to the control- and DSS-group and housed in pairs for 24 days. Mice of 12 weeks of age descended from only one breeding cage, while 10 weeks old mice descended from 4 different breeding cages. The DSS-group was exposed to 1.8% of DSS (40 kDa, Alfa Aesar, J63606) via drinking water for 4 days, and the moderate exposure to DSS was chosen based on previous published studies on the conventional C57BL/6J Min/+ mouse model [10, 12-14], and the comparably higher susceptibility of A/J mice towards colon-specific stimuli [18]. A score sheet was used to monitor health status, weight development and food intake. At the end of the experimental period, the animals were euthanized by cervical dislocation.

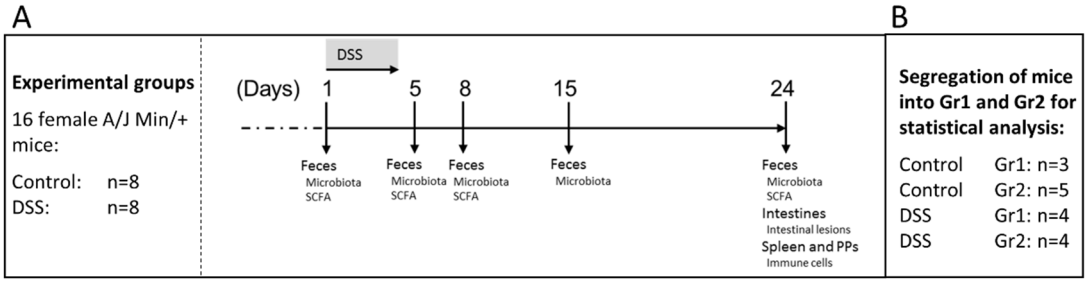

Figure 1: Experimental design (A), and segregation of mice into subgroups (B).

Sampling

For the analysis of microbiota and short chain fatty acids (SCFA), fresh feces was collected prior to DSS-administration (day 1), at the final day of DSS-administration (day 5), day 8 and 15, and on the final day of the experimental period (day 24), and frozen at -80°C. Spleen and Peyer's patches (PPs) were dissected for immunobiological analysis and weights of spleens and ceca were registered. The intestine was dissected and subsequently prepared for visual examination.

Scoring of intestinal lesions

Preparation and staining of intestines was carried out as previously described [17] . Shortly, intestines were rinsed with ice-cold phosphate buffered saline solution (PBS) and cut open longitudinally. The small intestine was cut into three sections, before small intestine and colon were flattened between filter paper and fixed in 10% neutral buffered formalin solution. After 24 hours, the formalin-fixed preparations were stained for 10-15 seconds in 0.2% methylene blue dissolved in neutral buffered formalin, and rinsed with 10% formalin to remove the exceeding methylene blue. Until examination by transillumination in an inverse light microscope, and at least for another 24 hours, the preparations were stored in 10% formalin. Surface examination of methylene blue-stained intestines was performed blindly by one observer. In the colon, flat ACF were differentiated from tumors. Flat ACF are characterized by a bright blue-green appearance, a moderately increased size of crypts and compressed crypt openings, which often create branched or gyrus-like pits seen in the microscope. Colonic tumors exhibit a similar appearance as flat ACF, but consist of more than 30 crypts, and exhibit elevated structures compared to the surrounding mucosa. In the small intestine, tumors are encircled by small intestinal villi. The size of every intestinal lesion was measured with an eyepiece graticule, and the total number, average size and the total areal (load) of lesions was determined for every animal. For the size distribution, lesions were categorized into size classes, with the smallest lesions (approx. 1-4 crypts) falling into the first category (0–0.008 mm^2). The remaining categories were based on a logarithmic scale (0.009– 0.064 mm^2, 0.065–0.512 mm^2, 0.512–4.096 mm^2, and >4.096 mm^2).

Flow cytometry

Leukocytes were extracted from tissues using a GentleMACS dissociator and mouse Spleen Dissociation Kit (Miltenyi Biotech), according to the manufacturer's instructions. Splenic suspensions were briefly treated with NH_4Cl solution to lyse erythrocytes. Single-cell suspensions were ensured by running the suspensions through a 70 µm cell strainer (BD Biosciences) and concentrations were standardized using a Countess automated cell counter (Thermo Fisher Scientific). Immuno-phenotyping was carried out on ice by incubating single-cell suspensions in PBS with 0.5% BSA and 10 mM NaN_3. After FcR-blocking with anti-CD32/16

antibody, cells were stained with Live/Dead Fixable Yellow Dead Cell Stain Kit (Thermo Fisher Scientific), followed by incubation with combinations of monoclonal antibodies listed in Supplementary Table S1. For intranuclear antigen staining, surface staining was followed by treatment with Foxp3/Transcription Factor Staining Buffer Set (eBioscience), according to the manufacturer's instructions. Cells were analyzed in a Gallios 3-laser flow cytometer, using Kaluza 1.3 software (both Beckman Coulter). Cell gates were designed to select for single and viable cells positive for the pan leukocyte marker CD45 (Supplementary Fig. S1).

SCFAs

Gas chromatography (GC) was used for the quantification of SCFA in all feces samples collected, except for day 15. The method followed was based on Anson *et al.* [19] and Jouany & Senaud [20]. Samples were homogenized in PBS, containing 2-ethyl butyric acid (2 mg/ml) as an internal standard. Samples were centrifuged (5000 x g, 10 min) and filtrated (0.22 µm). Filtered samples were diluted 50:50 with a mixture of formic acid (20%) and methanol. Acetic, propionic and butyric acids were used as external standards at various concentrations in methanol. 1 µl of the samples was injected into an Agilent GC HP-FFAP column (length 30m, diameter 0.32mm, film thickness 0.25µm). Agilent 7890A gas chromatography instrumentation was used, coupled with auto-sampler and flame ionization detector (240^{o}C). The column was heated at a rate of 8^{o}C/min from 100^{o}C to 180^{o}C and 20^{o}C/min from 180^{o}C to 200^{o}C.

DNA extraction and microbiota analysis

Bacterial DNA was extracted from fecal content by mechanical and chemical lysis using the PowerSoil®-htp 96 Well Soil DNA Isolation Kit (MoBio Laboratories), following the manufacture's protocol. The mechanical lysis step with bead beating was done twice using the FastPrep®-96 homogenizer (MP Biomedicals) for 60 seconds at 1600 rpm. Then samples were centrifuged for 6 minutes at 4500 x g as described in the protocol. The microbiota was analyzed by 16S rRNA amplicon sequencing (2 x 150 bp) of the variable region 4 following our in-house protocol [21], which is presented in detail in supplementary methods of Caporaso *et al.* [22]. The sequencing was done on a MiSeq (Illumina) at Nofima using pooled PCR samples, which were based on triplicate PCRs per DNA sample (in total 93) using sample-specific barcoded reverse primers. PhiX Control v3 was included and accounted for 10% of the reads. The MiSeq Control Software (MCS) version used was RTA 1.18.54.

Data processing of sequencing data

Data processing of the sequencing reads was performed using the pipelines in Quantitative Insight Into Microbial Ecology (QIIME) v.1.8 [23]. Briefly, the total number of reads was 14 236 703 followed by 10 899 685 reads after joining forward and reverse reads and removal of barcodes that failed to assemble. The sequences were demultiplexed into representative sample taqs and quality filtered, allowing zero barcode errors and a quality score of 30 (Q30), resulting in 7 879 508 sequences. Reads were assigned to their respective bacterial taxonomy (Operational Taxonomic Unit; OTU) by clustering them against the Greengenes reference sequence collection (gg_13_8) using a 97% similarity threshold. Reads that did not hit a sequence in the reference sequence collection were clustered *de novo*. Chimeric sequences were removed using ChimeraSlayer, and all OTUs that were observed fewer than 2 times were discarded. This resulted in an OTU table containing 13 626 different OTUs, which was based on a total of 7 396 079 reads. The OTU table was used for alpha diversity analysis using equal number of sequences across samples, where the OTU table was resampled to an even depth of 10 000 sequences per sample. Prior to the

statistical analysis on taxonomy, only those OTUs that satisfied at least one of two criteria were kept: 1) more than 0.005% in 50% or more of the individuals in at least one intervention group, or 2) more than 0.005% in 50% or more of all individuals. In total 549 OTUs passed this filter, and each of these represents a phylotype that may be a representative of a bacterial species. Square brackets around taxonomic names (e.g. [Prevotella]) are taxa proposed by Greengenes based on genomic trees, but not verified taxonomies.

Statistical analyses

In the statistical analysis, the two subgroups of the mice (Gr1 and Gr2) were included due to heterogeneity in susceptibility to intestinal carcinogenesis, microbiota composition, initial age of the animals and different parents (Fig. 1B). N-way ANOVA with fixed effects was used to analyze the experimental effects on single responses such as tumors, flat ACF, alpha diversity and SCFAs. Multivariate ANOVA (50–50 MANOVA [24]) was used to investigate the effect of the intervention on total microbiota (OTUs) and immune responses. The fixed factors Treatment (DSS/Control) and Subgroup (Gr1/Gr2) were included in both models, while the factor Time (day 1, 5, 8, 15, 24) was only present for the microbiota model. All main effects and two-factor interactions were included in the model. Rotation testing was used to compute adjusted single response p-values according to false discovery rates [25]. The experimental effects on microbiota was further investigated by Parallel factor analysis (PARAFAC), which can be seen as a multi-way extension of principal component analysis (PCA) [26]. Using PARAFAC, the three-dimensional data cube (dimensions represented by animals, OTUs and days) is decomposed into trilinear components which can be used to simultaneously interpret the experimental factors, time trajectories and OTU contributions. Unpaired t-test was also used for analyzing treatment effects on immune responses presented, as no significant effect of mice subgroups was identified using multivariate ANOVA.

The relationship between microbiota and carcinogenesis/SCFA was analyzed by Partial Least Squares (PLS) regression [27]. The microbiota before intervention (day 1) and after intervention (day 5, 8, 15 and 24) were used to predict load of flat ACF and tumor load in independent PLS regression models. The models were validated by leave-one-out cross-validation in order to get a conservative measure of the coefficient of determination (R^2). Important OTUs were identified by the Variable Importance in Projection (VIP) method [28, 29]. The statistical analyses were performed in MATLAB (R2014b, The MathWorks Inc.) with the 50–50 MANOVA toolbox (www.nofimamodeling.org) and the N-way toolbox (www.models.life.ku.dk/algorithms).

Results

Differentiation of mice into two subgroups

During the planning stage of the study, only two experimental groups (treatment and control, n=8) were intended. However, at the end of the study, susceptibility to colonic carcinogenesis within the treatment group was characterized by a large interindividual variation, whereas colonic carcinogenesis was found to be relatively uniform within the control group. Differences in susceptibility coincided with differences in the initial commensal microbiota composition (Supplementary Fig. S2) and subsequently mice were segregated into two subgroups (Gr1 and Gr2, Fig. 1B). At the beginning of the study, mice in Gr1 and Gr2 were respectively 12 and 10 weeks of age, and descended from one single breeding cage, and four different breeding cages, respectively.

Overview of key responses caused by DSS-intervention in A/J Min/+ mice

The effects of DSS-treatment (1.8%) on the immunological status, the formation of intestinal

lesions, and temporal chances in fecal microbiota and SCFA were tested in A/J Min/+ mice. Activity and appearance of the mice was not visibly affected by the DSS-treatment, and there were no signs of diarrhea. The food intake remained unchanged during the experimental period, and DSS-treatment did not have any significant effects on body weight, colon length or weight of cecum or spleen (Supplementary Table S2). ANOVA showed that the immunological status was significantly affected by DSS-treatment in the spleen, but not in the PPs (Table 1). DSS-treatment also affected carcinogenesis in the colon, where DSS was shown to significantly increase the load of flat ACF. Furthermore, an interaction effect between DSS-treatment and subgroups of mice (Gr1 and Gr2) was apparent for tumor load (Table 1). No significant effects of DSS-treatment were found for tumor development in the small intestine (Supplementary Table S2). All the SCFA's measured in the feces were significantly affected by DSS-treatment and time (Table 1). The fecal microbiota in terms of composition (operational taxonomic units; OTUs) and diversity (alpha diversity; number of observed OTUs) was also clearly affected by DSS-treatment, time, and interaction effects (Table 1). In addition, a subgroup effect was shown for microbiota composition (OTUs), and an interaction effect of DSS-treatment and subgroup was apparent for both microbiota composition and alpha diversity.

DSS-treatment induced effector-memory CD4+ T-cell response in PPs and spleen

The relative numbers of T-cells and its main subsets (CD4+ and CD8+) were investigated in DSS-treated mice and compared with the control group (Fig. 2). Differentiation into subgroup was not included in this analysis, since multivariate ANOVA did not shown any statistical differences in immunological status of subgroups, as well as no interaction effect (Table 1). In the spleen (Fig. 2A), significantly lower CD8+ T-cell numbers were found in mice following DSS-treatment, but no clear effect was observed on the numbers of CD4+

Table 1. Overview of the key responses caused by DSS-administration in A/J Min/+ mice (ANOVA). Responses are shown as explained variance (%).

	Immunology		**Colonic carcinogenesis**		**Fecal SCFA**			**Fecal microbiota**	
	PPs[a]	Spleen[a]	Flat ACF load[b]	Tumor load[b]	Acetic acid[b]	Propionic acid[b]	Butyric acid[b]	OTUs[a]	Alpha diversity[ac]
Factors	Explained variance (%)								
Trt	13.6	49.4*	55.0**	6.4	4.7*	41.0**	5.2*	10.0**	24.6**
Subgroup	12.7	1.4	4.9	8.6	1.0	0.2	4.4*	4.6**	2.0
Time	nd	nd	nd	nd	56.8**	10.8**	39.8**	10.6**	30.4**
Trt x Subgroup	2.8	1.7	9.9	24.1*	0.7	0.1	0.6	2.4**	2.6*
Trt x Time	nd	nd	nd	nd	1.3	18.8**	7.5*	7.4**	7.8*
Subgroup x Time	nd	nd	nd	nd	2.4	0.6	4.7	3.5**	0.6
Error	70.2	45.1	26.0	58.4	30.0	25.7	34.7	60.2	33.3

[a] Multivariate 50-50 MANOVA
[b] N-way ANOVA
[c] Observed OTUs
nd = not determined; Trt = DSS-treatment
Significance level *<5% **<1%

T-cells. However, when looking into subsets of the memory phenotype, a significant higher proportion of splenic CD4+ T-cells had an effector-memory phenotype (CD44+/CD62L-), whereas both naïve (CD44-/CD62L+) and central memory (CD44+/CD62L+) T-cells were reduced. A similar shift was present within the CD8+ subset. In small intestinal PPs (Fig. 2B), DSS-treatment caused no significant changes in CD4+ or CD8+ T-cell numbers, but, in parallel to the cell proportions in the spleen, a significant higher proportion of CD4+ effector-memory T-cells was observed. However, the proportions of the CD8+ subset did not differ between control and DSS-treated mice. The proportion of CD4+ cells with a regulatory T-cell (Treg) phenotype (CD4+/CD25+/FOXp3+) appeared slightly elevated in the spleen of DSS-treated mice, but not to a significant degree, and this effect was not observed in the PPs (Fig. 2C). B-cell numbers were measured in PPs only (Fig. 2D). Germinal center cells (IgD-/CD38-) among B-cells (B220+/CD3-) were not significantly different,

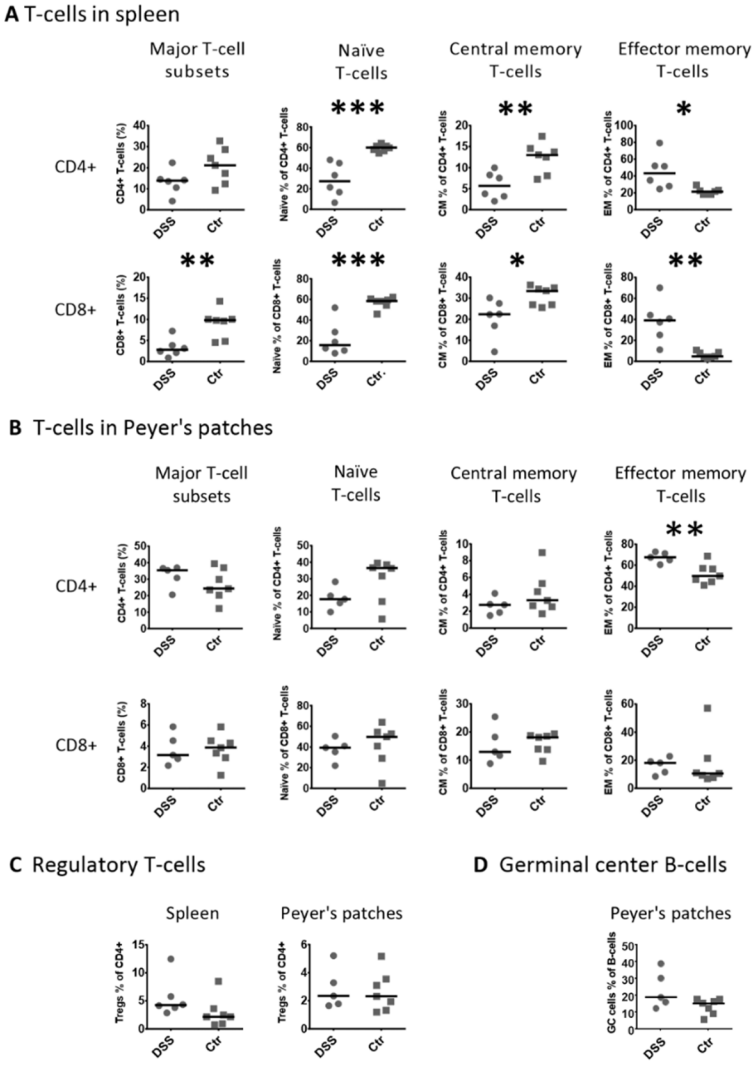

Fig. 2. Relative numbers of lymphocyte subsets derived from spleen and PPs of DSS-treated and control mice, measured by flow cytometry. Using markers and gating strategies shown in Supplementary Table S1 and Supplementary Fig. S1, relative numbers of CD4+ and CD8+ T-cells, as well as their corresponding naïve, central and effector memory subsets were measured in the spleen (A) and in PPs of the small intestine (B); regulatory T-cells were measured in the same two compartments (C) and germinal center B-cells were measured in PPs (D). CM: Central Memory; EM=Effector Memory; GC: Germinal Center; Treg: Regulatory T-cells. *n*=5-7 mice per group. *: p<0.05; **: p<0.01; ***: p<0.001 (unpaired t-test).

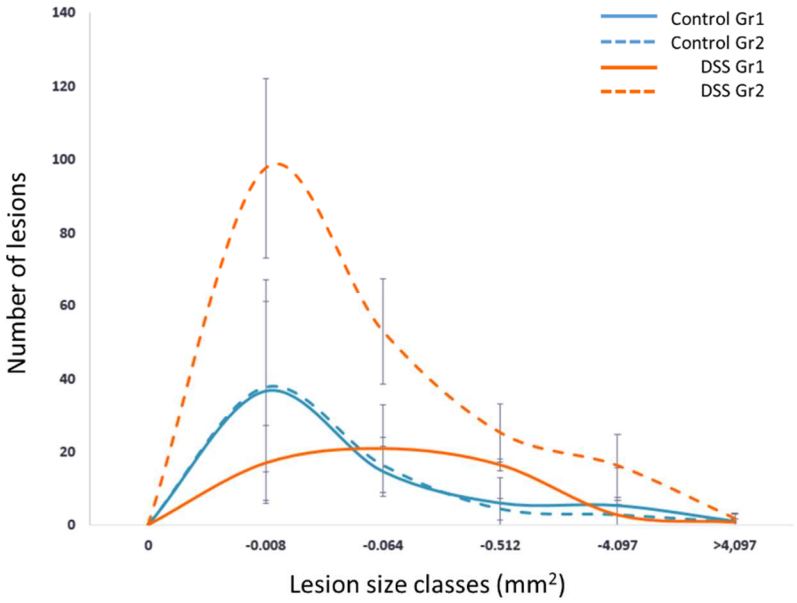

Figure 3: Number of colonic lesions per size classes in control and DSS-treated mice of Gr1 and Gr2. Results are presented as mean [95% confidence intervals].

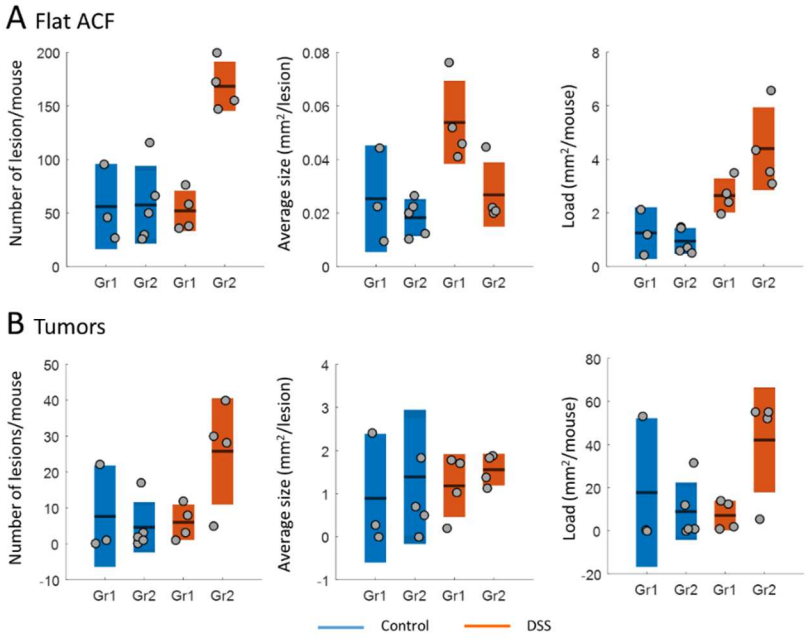

Figure 4: Colonic carcinogenesis presented as number, average size and load of flat ACF (A) and tumor (B) in control and DSS-treated mice of Gr1 and Gr2. Boxes indicate the mean [95% confidence intervals], and individual mice per box are indicated as grey circles.

although the results suggest a tendency of a small increase in DSS-treated mice.

Differential DSS-induced colonic carcinogenesis in mice subgroups

Fig. 3 presents the number of colonic lesions per size category for all experimental groups. Most lesions of the control group were flat ACF (early-stage lesions), and size distributions appeared to be similar between the two subgroups of mice. Within the treatment group, however, a substantial amount of newly formed lesions was induced by DSS in Gr2 animals, while DSS-

treatment in Gr1 animals led to an increase in lesion size. An overview over the effects of DSS on number, average size and load of lesions is presented in Fig. 4. DSS-treatment significantly affected the number, average size and load of flat ACF (p=0.004, p=0.015 and p<0.001, respectively) (Fig. 4A). An interaction between the effects of treatment and subgroups was observed for the number of flat ACF (p=0.002), with the highest numbers of flat ACF within Gr2 of DSS-treated mice. In contrast, the significant increase in the average size of flat ACF in DSS-treated mice was mainly attributed to an increased growth of flat ACF in the Gr1 mice. No significant effects of DSS-treatment were shown for tumor number, average size and tumor load (Fig. 4B). However, the formation of tumors followed the same trends as seen for flat ACF, with the largest increase in tumor number and load within Gr2 mice (interaction effect of p = 0.047 and p = 0.046, respectively).

Effects of DSS-treatment on SCFAs

The main SCFAs detected in the feces were acetic, propionic and butyric acid, and their time trajectories are presented in relative values in Fig. 5. ANOVA identified that all the three SCFAs were significantly affected by DSS-treatment and time, but did not vary between the subgroups of mice (Table 1, Fig. 5). A significant interaction between the effects of DSS-treatment and time was detected for both propionic and butyric acid, indicating that these SCFAs were differently changing over time within control and DSS-treated mice. A clear relative increase of propionic acid was observed from day 1 to 5 in DSS-treated mice, which was persistent until day 24. In contrast, butyric acid was relatively higher in the control groups on day 5, but the amounts seemed to coincide on day 8. A significant subgroup effect was detected for butyric acid, which from day 8 was observed at higher relative amounts within the Gr2 mice.

Initial microbiota in subgroups of A/J Min/+ mice

At the baseline of the intervention (day 1), Bacteroidetes was the major dominating bacterial phylum (85%), followed by Firmicutes (13%) and Proteobacteria (1.3%). The two dominating orders were Bacteroidales and Clostridiales. OTUs of S24-7 of Bacteroidales were highly dominating on day 1 (66%), followed by Bacteroideceae (10%) and unassigned OTUs within Clostridales (6%). Significant differences in the microbiota composition between Gr1 and Gr2 mice were identified by 50-50 MANOVA on day 1 (p=0.013) (Supplementary Fig. S2). The differences were mainly related to higher relative amounts of OTUs of S24-7 in Gr1 mice (except OTU 273208), and higher amounts of two *Lactobacillus* OTUs and one OTU of *Ruminococcus gnavus*, *Oscillospira*, Clostridales and *Prevotella*. No differences in initial microbiota composition were observed between mice of the control- and DSS-group (p=0.761).

DSS-treatment induced *Bacteroides*, *B. acidifaciens* and *[Prevotella]*

The microbiota composition and diversity were

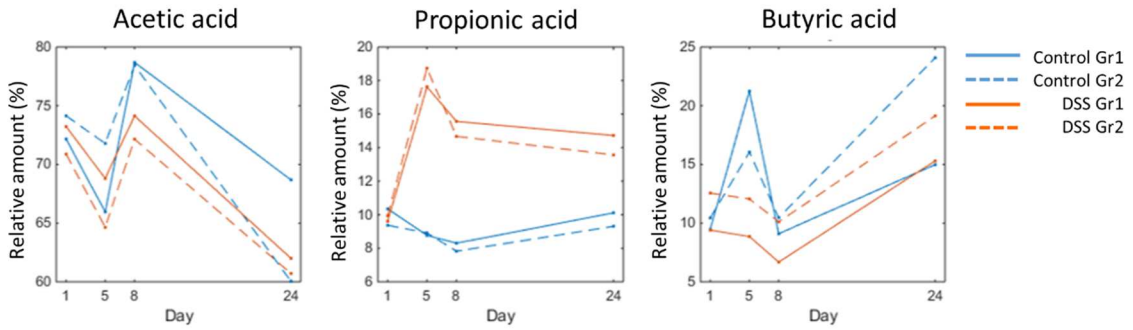

Figure 5. Temporal trajectories of fecal SCFAs (%) in control and DSS-treated mice of Gr1 and Gr2.

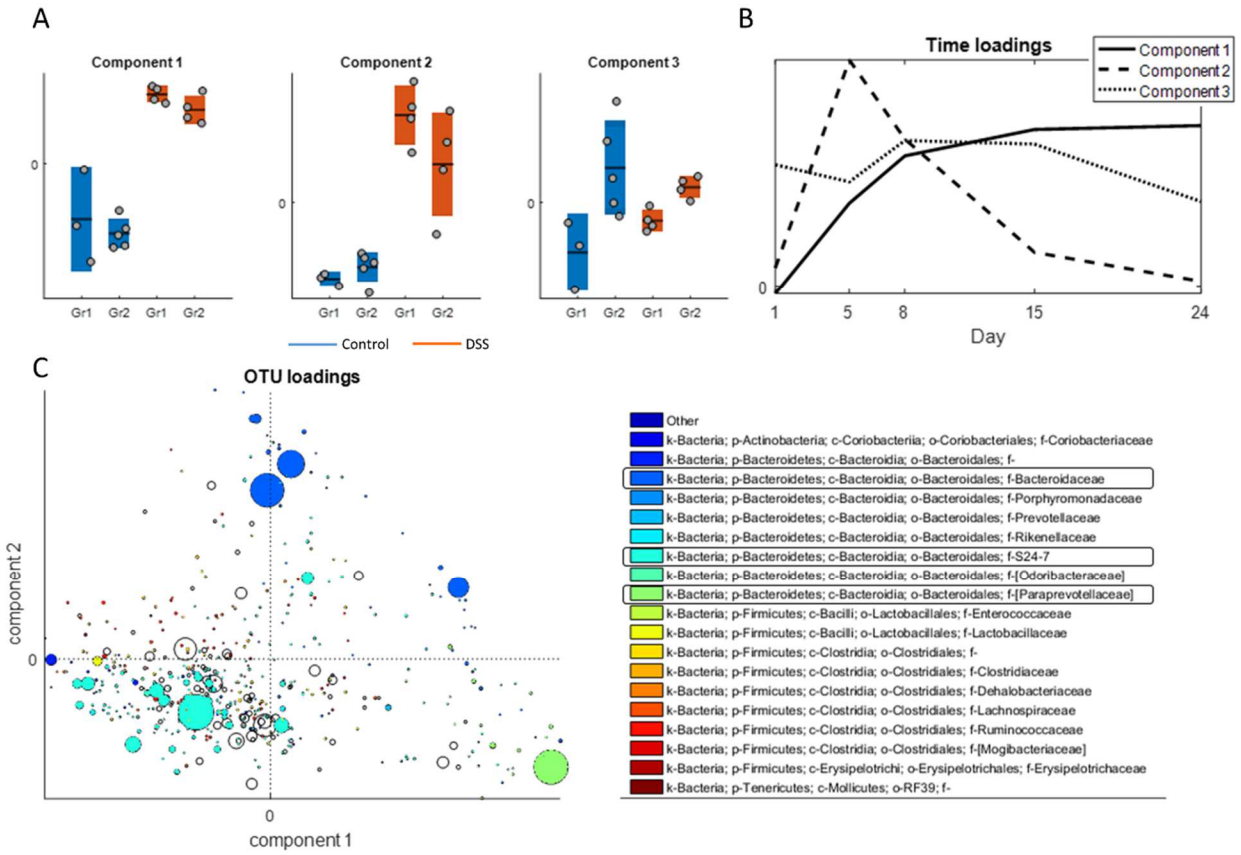

Figure 6. Differences in microbiota composition within DSS-treated mice, control mice and mice subgroups shown with three-component PARAFAC. Treatment effects are shown with component 1 and 2, and subgroup effect with component 3 (A). Temporal trajectories of the three components is indicated (B), and corresponding OTUs of component 1 and 2 are shown in a loading plot (C). Significant OTUs are colored according to their taxonomic family, and the dominating families are framed.

significantly affected by the DSS-intervention (Table 1). Alpha diversity was dramatically decreased on day 5 in DSS-treated mice, but was reestablished on day 8 (Supplementary Fig. S3). DSS-treatment was shown to explain 10% of the variance in the microbiota (Table 1), where 160 OTUs were significantly affected. A selection of these OTUs are presented in Supplementary Table S3, all with an average abundance >0.05%. A three-component PARAFAC model was fitted to further illustrate the differences in microbiota composition (Fig. 6). Component 1 and 2 represent a treatment effect ($p<0.001$ for both components), while component 3 represents the difference between subgroups ($p=0.003$) (Fig. 6A). Temporal trajectories of the three components are shown in Fig. 6B. Component 1 increases after the intervention and stays at a high level, while component 2 has a steep increase at day 5 followed by a gradual decrease. Component 3 is fairly stable over time, as expected since it is not related to treatment. The OTU loadings for the first two components are shown in Fig. 6C, where each OTU is sized according to the average abundance, and colored in case of significant differences between the experimental groups (treatment or subgroups). The dominating OTUs associated with DSS-treatment and component 1, were within the [Paraprevotellaceae] family, and assigned as *[Prevotella]*. The component 2 was dominated by OTUs within Bacteroidaceae, including *Bacteroides* and *B. acidifaciens*, and one OTU within the

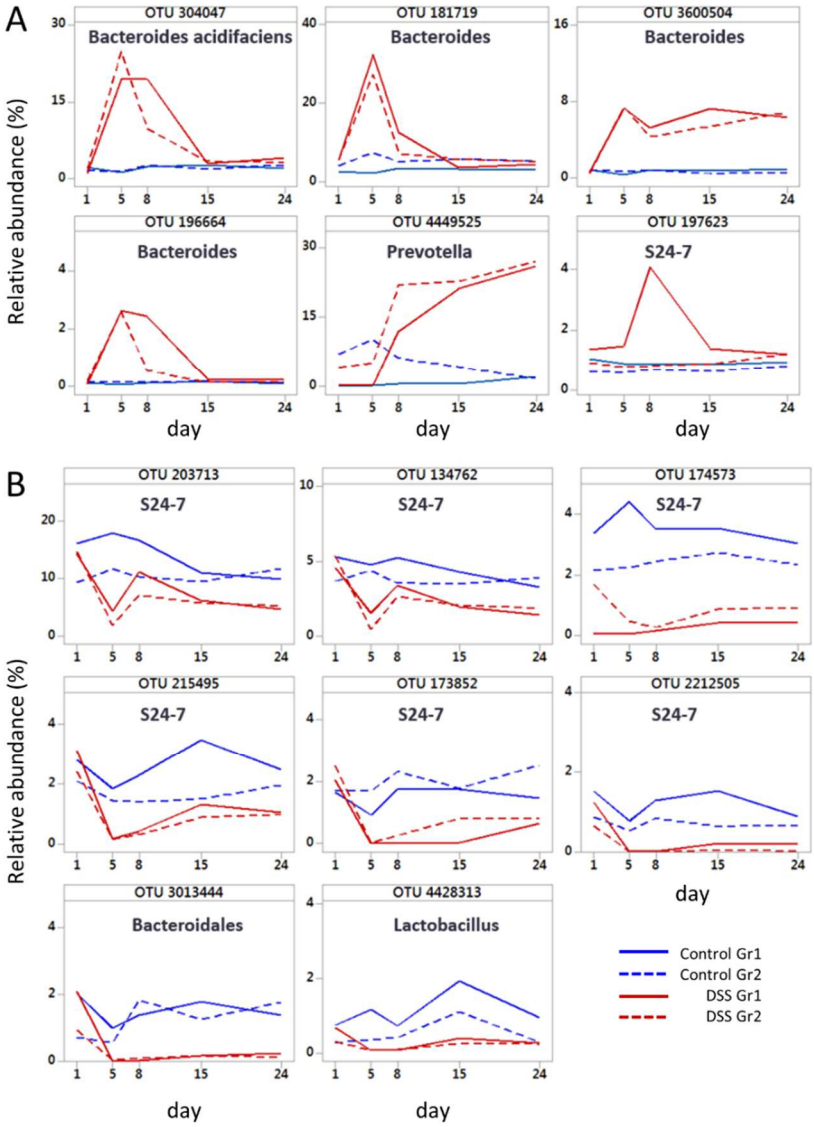

Figure 7. Temporal trajectories of dominating OTUs significant for DSS-treatment and correlated to flat ACF load. Positive correlations (A) and negative correlations to flat ACF (B). Only OTUs of >1% of the average abundance are presented.

family S24-7. Most of the other OTUs within S24-7 were negatively associated with DSS-treatment. Less dominating OTUs affected by DSS-treatment were also found within Bacteroidetes, and with a few exceptions within Firmicutes, i.e. OTUs within *Lactobacillus*, Ruminococcaceae and *Oscillospira* (Supplementary Table S3).

Relationship between microbiota and colonic carcinogenesis

Temporal changes of the microbiota during the intervention were shown to explain 10.6% of the variance in the microbiota (Table 1). In addition, an interaction effect between DSS-treatment and time was evident, which explained 7.4% of the variance in the microbiota (Table 1). The relationship between these temporal changes of the microbiota and carcinogenesis was analyzed with PLS analysis, using separate regression models against flat ACF load and tumor load.

The microbiota related to flat ACF load was only explained by the microbiota after the four days of DSS-treatment, with 30% explained variance on day 5 and above 50% on day 8, 15 and 24. Indeed, these OTUs related to flat ACF load coincided with

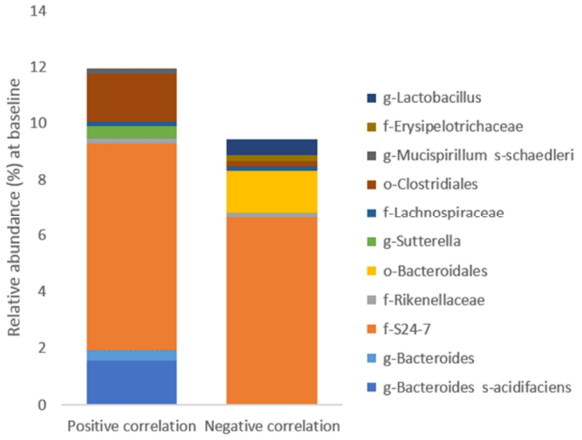

Figure 8. Overview (lowest taxonomic level) of the OTUs at baseline (day 1) with significant correlations to tumor load. Only OTUs of average abundance >0.1% at day 1 are included.

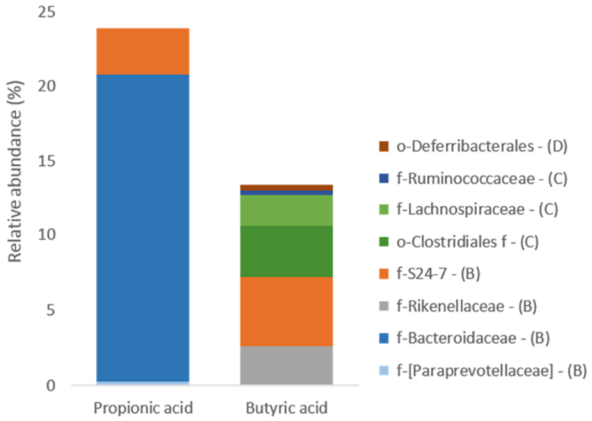

Figure. 9. Overview (family level) of OTUs with significant positive correlations to propionic acid and butyric acid. Sum families >0.1% of representative OTUs are included, which were based on average abundance per OTU during the intervention.

OTUs significantly affected by DSS-treatment, and time trajectories of the dominating OTUs are presented in Fig. 7. Dramatic relative increases of OTUs within *Bacteroides*, including *B. acidifaciens* were apparent on day 5 within DSS-treated mice (Fig. 7A). The increase in *Bacteroides* was declining for some of the OTUs after day 5, and partly differed between Gr1 and Gr2 mice. The increase in *Bacteroides* OTU 360054 lasted throughout the intervention. On day 8, a steep increase of *[Prevotella]* was apparent in the DSS-treated mice, which also lasted throughout the intervention. The OTUs negatively affected by DSS and negatively related to flat ACF were shown to have a clear decrease on day 5 in the intervention (Fig. 7B), mostly dominated by OTUs within S24-7, but also one OTU of Bacteroidales and *Lactobacillus*.

In contrast, tumor load was to a large extend explained by the microbiota before the intervention (>50%), and the positive and negative correlated OTUs are presented in Fig. 8. OTUs of *Bacteroides*, including *B. acidifaciens*, undetermined OTUs of Clostridales, *Sutterella* and *Mucispirillum schaedleri* were among the OTUs positively related to tumor load. Several OTUs within S24-7 were either positively or negatively related to tumor load. Other negatively related OTUs included undetermined OTUs within Bacteroidales and *Lactobacillus*.

Relationship between microbiota and SCFA

The relationship between microbiota and SCFAs during the intervention was investigated by PLS regression analysis. Correlations between OTUs and propionic and butyric acid were identified, but no valid regression model was identified between OTUs and acetic acid. Only OTUs within the order Bacteroidales, i.e. Bacteroidaceae (all *Bacteroides*) and S24-7 were positively correlated with propionic acid (Fig. 9). In contrast, butyric acid was correlated with OTUs within Clostridales (e.g. undetermined Clostridales and Lachnospiraceae), in addition to S24-7 and Rikenellaceae of Bacteroidales.

Discussion

In the present study, DSS was shown to enhance *Apc*-driven carcinogenesis in the A/J Min/+ mouse model, and a link between DSS-induced inflammation, intestinal microbiota and colonic carcinogenesis was identified. An immune response was demonstrated by higher amounts of effector memory T-cells in DSS-treated mice, and temporal changes in fecal microbiota and SCFAs

profiles were demonstrated in response to DSS-administration. Dysbiosis in DSS-treated mice was most apparent on day 5, characterized by a decrease in alpha diversity, and rearrangement of dominating bacteria and SCFAs. Bacteria related to the SCFAs and carcinogenesis were identified. This is the first study to characterize the fecal microbiota trajectory in the A/J Min/+ mouse model, as well as to demonstrate its susceptibility to inflammation-induced carcinogenesis.

DSS-administration was at lower doses (1.8%) than the standard proposed DSS protocol (3%), with the aim to promote mild inflammation, without influencing the general health status. This selection was based on previous investigations of the dose-response relationship between DSS and intestinal carcinogenesis in C57BL/6J Min/+ mice [12], as well as the high susceptibility of A/J mice towards DSS [18]. Indeed, the general health status of the animals was not affected, and none of the typical symptoms that often accompany DSS-administration, e.g. weight loss, diarrhea, occult blood in stool, piloerection, anemia or death in the acute phase, were observed. However, these symptoms do not necessarily reflect pathophysiological processes in the intestine [30], and cellular changes measured in immune cells of the PPs and spleens indicate that DSS-treated mice had gone through a significant degree of immunological experience. The immune response was particularly measurable in the effector memory T-cell subsets, and the changes were most prominent in the spleen. This might indicate that DSS-treatment affected the colon to a larger extent than the small intestine, from where PPs were derived. The involvement of PPs has nevertheless been indicated in DSS-induced colitis before [31-33], and immune responses in PPs may play a role in the protection against colon carcinogenesis [34]. In accordance with the observed results, persistent inflammation has been reported to decrease CD8+ T-cell numbers, and particularly the long-lived central memory subset [35]; however, details of the presented cellular snapshot should be interpreted with care, as lymphocyte differentiation and circulation is a complex and debated topic [36]. We conclude that in A/J Min/+ mice, the shift from naïve to memory T-cell phenotypes constitutes a useful marker for accumulated antigenic challenges in response to a major inflammatory episode, such as DSS-exposure.

As opposed to small intestinal carcinogenesis, colonic carcinogenesis was significantly affected by DSS-treatment, underscoring the evidence of a mediating role of the microbiota. A role of intestinal microbiota in the pathogenesis of DSS-induced colitis in mice has also been demonstrated in previous studies, and current evidence has been reviewed [30]. During statistical analysis, mice of the control- and DSS-group were segregated into two subgroups (Gr1 and Gr2). Segregation into subgroups was not intended, but appeared to be necessary when different susceptibilities to colonic carcinogenesis were observed in DSS-treated mice. In addition, the initial microbiota composition was found to vary between Gr1 and Gr2 mice, and an interaction effect of DSS-treatment and subgroup was found on microbiota composition and alpha diversity, which might explain the differences in colonic carcinogenesis. Tumor load was also shown be largely explained by the initial microbiota, which is in line with a study by Zackular *et al.* [37], who found that tumor burden could be predicted based on the initial microbiota. It should though be mentioned that the Gr1 and Gr2 mice in the present study differed in age at the initiation of the intervention (12 and 10 weeks old, respectively), and that all mice of Gr1 were from the same breeding cage, whereas mice of Gr2 were from four different breeding cages. However, unpublished data indicates that age of untreated A/J Min/+ mice has only minor effects on microbiota composition, and hence, differences in microbiota of the two subgroups may rather be related to environmental or epigenetic factors

than age. Hence, it appears to be likely, that microbiota composition at the initiation of the study affected susceptibility to carcinogenesis of DSS-treated mice to a lager extent than factors connected to age.

The lowest susceptibility to DSS-induced carcinogenesis was observed in Gr1 mice, which at baseline (day 1) had higher relative amounts of the OTUs that significantly differed between the two subgroups. For instance, Gr1 mice had higher amounts of *Lactobacillus* OTUs, and *Lactobacillus* OTU340960 was found to be negatively related to the load of tumors. Accumulating evidence shows that lactic acid bacteria, such as *Lactobacillus*, inhibit initiation or progression of carcinogenesis through various mechanisms [38], and the initial relative abundance has been shown to be predictive to the final tumor burden [37]. Accordingly, also one OTU of Clostridiales (OTU3176547) was higher in Gr1 and found to be negatively related to tumor load, whereas OTU273208 of S24-7 was higher in Gr2 mice (3%) and found to be positively related to tumor load. Recently, the latter OTU has been found to be positively related to tumor load also in another A/J Min/+ mouse feeding trial, though not related to the dietary intervention [21].

DSS-treatment mainly influenced the formation of flat ACF, and major DSS-induced changes in the fecal microbiota were observed within the OTUs that were related to flat ACF load. Disruption of the gut homeostasis was reflected in particular by a rapid decrease in alpha diversity on day 5 within DSS-treated mice, indicating dysbiosis, which is common in a colitis state. In accordance with other studies [39, 40], enrichment in members of *Bacteroides* with DSS-induced inflammation and carcinogenesis was observed. Recently, a relatively higher abundance of *Bacteroides* species has been found in feces of humans with advanced adenomas than healthy individuals, while other species of *Bacteroides* were increased in feces of carcinoma patients [41]. In the present study, a temporal enrichment of *Bacteroides* and *B. acidifaciens* at the end of DSS-treatment (day 5) was followed by enrichment of *[Prevotella]* from day 8. Similar temporal changes in the microbiota were also observed in another longitudinal study of murine microbiota during DSS-induced inflammation [42]. *B. acidifaciens* has been identified as an indicator for acute colitis, and acts as an important mucin-degrader [43, 44]. Schwab *et al.* [42] found *B. acidifaciens* to be positively correlated with mucin degrading enzyme transcripts. Interestingly, *B. acidifaciens* has been shown to promote Immunoglobulin A (IgA) production in the large intestine by inducing germinal center formation and increasing the number of IgA+ B cells [45]. IgA plays an important role in the maintenance of the intestinal homeostasis, and a role of *B. acidifaciens* in the immune regulation in response to DSS-treatment is likely. It should be mentioned that *B. acidifaciens* detected on day 1 also was positively related to tumor load on day 24, although the relative abundance was not found to significantly vary between Gr1 and Gr2 mice. The increase in *[Prevotella]* from day 8 lasted throughout the intervention, an increase at the expenses of *Bacteroides* that started to decline from day 8. A trade-off between *Prevotella* and *Bacteroides* is a highly common feature of these two genera [46]. Similar changes have also been observed in mice following subsequent rounds of DSS-treatment [40], in which *Bacteroides* were consistently enriched with DSS-treatment, while *Prevotella* declined. In general, *Prevotella* is associated with a plant-rich diet, and thought to be beneficial for humans [47-49]. However, *Prevotella* has also been linked with inflammatory conditions [50, 51]. Scher *et al.* [51] identified a potential role for *Prevotella copri* in the pathogenesis of rheumatoid arthritis in humans, and demonstrated that *P. copri* increased the severity of DSS-induced colitis in mice. It was hypothesized that *P. copri* thrives in a pro-inflammatory environment and might even increase inflammation for its own benefit.

Prevotella detected on day 1 was not found to be positive related to tumor load, and in the literature, evidence of a role of *Prevotella* in the development of CRC seems to be limited.

At baseline (day 1) of the study, the S24-7 family was highly dominating in the A/J Min/+ mice. However, a dramatic decrease in the S24-7 OTUs was observed on day 5 after DSS-treatment, and S24-7 bacteria were shown to be negatively related to flat ACF load. The S24-7 family is an uncultured family of Bacteroidales commonly inhabiting the gut of homeothermic animals [52]. The S24-7 family was also shown to be enriched in C57BL/6 Min/+ mice, when compared to their WT littermates [53]. Three trophic guilds have been described within the S24-7 family [52], indicating distinct roles for each in the gut. Recently, dietary inulin, which inhibits intestinal carcinogenesis, was shown to increase some of the S24-7 OTUs in the A/J Min/+ mice, while other OTUs of S24-7 were correlated with increased tumor load [21]. We also found various OTUs of S24-7 on day 1 that were either positive or negative related to tumor load, reflecting the heterogeneity of this family. Also *Lactobacillus* decreased with DSS-treatment in accordance with other studies [31, 37], and was negatively related to flat ACF load. The negative correlation between *Lactobacillus* and flat ACF load and tumor load in our study further supports the hypothesis of the suppressive role of this genus in the promotion of carcinogenesis. In parallel, several low abundant OTUs within Clostridiales were found to be decreased by DSS, and decreases in Clostridiales and transcripts related to butyrate formation have previously been observed during DSS-induced tumor formation [42]. Indeed, we found Clostridiales to be related to butyric acid, an order known to be comprised of many butyric acid producers [54], while Bacteroidales was related to intestinal propionic acid. SCFAs are known to have a major role in maintaining intestinal homeostasis, and act suppressive on inflammation and cancer [55]. Especially butyric acid is known to be anti-inflammatory and anti-tumorigenic, and the levels of butyrate and butyrate-producing species have been shown to be under-represented in patients with CRC compared to healthy individuals [56, 57]. We observed that the relative amounts of butyric acid were lower in DSS-treated mice on day 5. Independent of the treatment, from day 8 the relative amounts of butyric acid were higher in the Gr2 mice. In some studies, butyric acid has been shown to promote CRC [58, 59], and recently Belcheva *et al.* [60] demonstrated butyrate-fueled hyperproliferation in colonocytes of Min/+ mice with DNA mismatch repair (MMR)-deficiency. Whether the higher amounts of butyric acid in Gr2 mice could be involved in the increased tumor load and flat ACF load within the Gr2 mice, remains unknown.

In conclusion, the present study shows that the A/J Min/+ mouse model is highly responsive to intestinal stimuli, and that the *Apc*-driven colonic carcinogenesis is enhanced by DSS-induced intestinal inflammation. Moreover, DSS-administration in A/J Min/+ mice is accompanied by temporal changes in microbiota composition, and the initial microbiota composition of the animals appears to be an important factor that influences the susceptibility to colonic carcinogenesis.

Supplementary Material

Table S1. Antibodies used for immunophenotyping.

Fig. S1. Flow cytometric gating strategies and representative dot plots of results summarized in figure 2.

Fig. S2. PCA plot of the initial microbiota of mice in Gr1 and Gr2 (A); Relative abundance (%) of OTUs significantly different between Gr1 and Gr2 at baseline.

Table S2. Body and organ weight, colon length and carcinogenesis in the small intestine and in the colon (ANOVA) in control and DSS-treated mice of Gr1 and Gr2.

Fig. S3: Temporal trajectory of alpha diversity

(observed number of OTUs) of control and DSS-treated mice of Gr1 and Gr2.

Table S3: OTUs significantly affected by DSS-treatment with 50-50 MANOVA

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Conflict of interest

The authors declare that there is no conflict of interest.

Author Contributions

Conceived and designed the experiments: CS, JEP. Performed the experiments: CS, JEP, PB. Analyzed the data: IM, IR, PB. Contributed reagents/materials/analysis tools: JEP, PB, IM. Wrote the paper: IR, CS, IM, PB. Critically reviewed the manuscript: JEP.

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Supplementary Material

Inflammation-induced colonic carcinogenesis in A/J Min/+ mice is dependent on the intestinal microbiota

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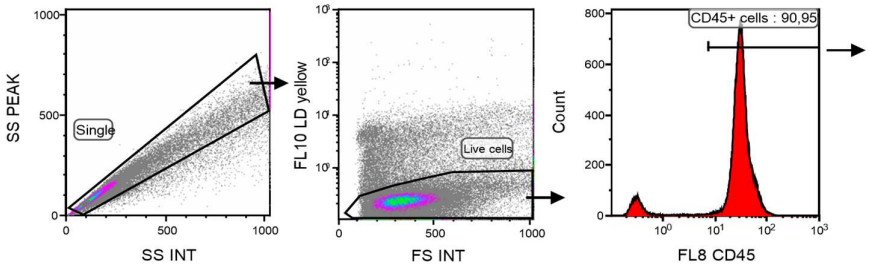
Immune responses

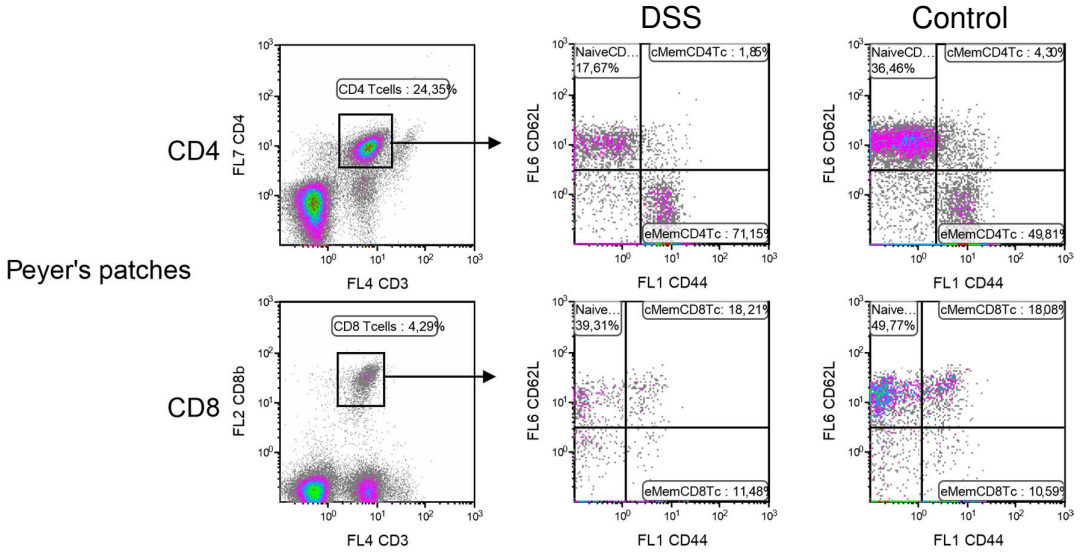
Table S1. Antibodies used for immunophenotyping.

Target molecule	Clone	Isotype	Conjugate used	Source
B220	RA3-6B2	Rat IgG2a	PE-Vio770	Miltenyi Biotech
CD3	145-2C11	Rat IgG2b	PerCp-eFluor710	eBioscience/Affymetrix
CD4	GK 1.5	Rat IgG2	Alexa 700	BioLegend
CD8b	H35-17.2	Rat IgG2b	PE	eBioscience/Affymetrix
CD16/32 (Fc-block)	93	Rat IgG2a	unconjugated	eBioscience/Affymetrix
CD25	PC61	Rat IgG1	Brilliant violet 421	eBioscience/Affymetrix
CD38	90.4	Rat IgG2a	VioBlue	Miltenyi Biotech
CD44	KM81	Rat IgG2a	FITC	Miltenyi Biotech
CD45	30-F11	Rat IgG2b	APC-eFluor780	eBioscience/Affymetrix
CD62L	MEL-14	Rat IgG2a	APC	eBioscience/Affymetrix
FOXp3	FJK-16s	Rat IgG2a	PE	eBioscience/Affymetrix
IgD	11-26c.2a	Rat IgG2a	PE	Miltenyi Biotech

A Overall gating strategy



B Memory T-cells



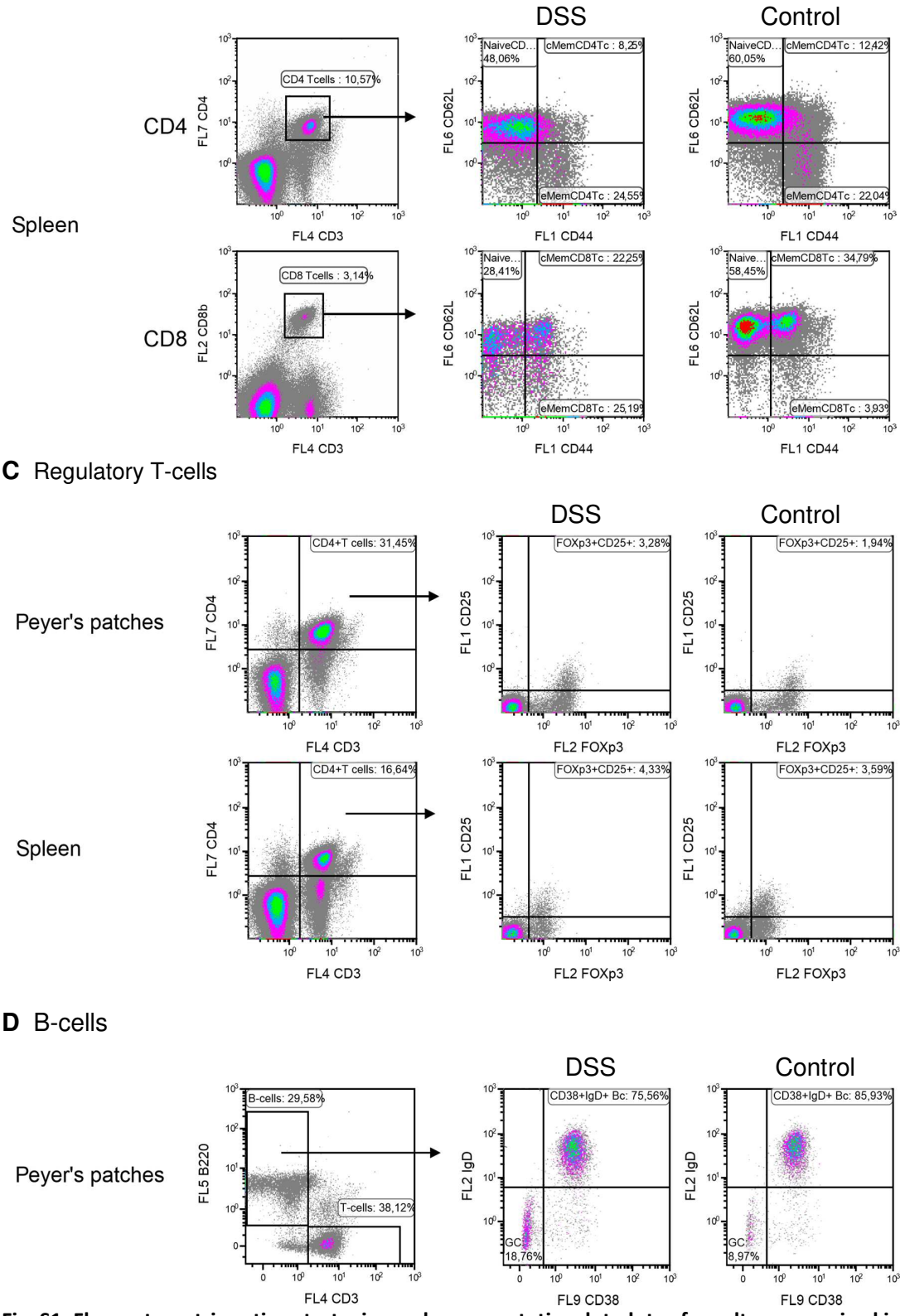


Fig. S1. Flow cytometric gating strategies and representative dot plots of results summarized in figure 2. Gating for single cells, live cells (staining negative for Live/Dead Fixable Yellow), and selected for pan leukocyte marker (CD45)+ (A). Memory T-cell phenotyping in PPs and Spleen (cMem= central memory; eMem=effector memory) of the indicated T-cell (Tc) subsets, using CD44 and CD62L (B). Regulatory T-cell phenotyping defined as CD3+/CD4+/FOXp3+/CD25+ (C). Germinal center (GC) B-cells defined as B220+/CD3-/CD38-/IgD-) (D).

Initial microbiota

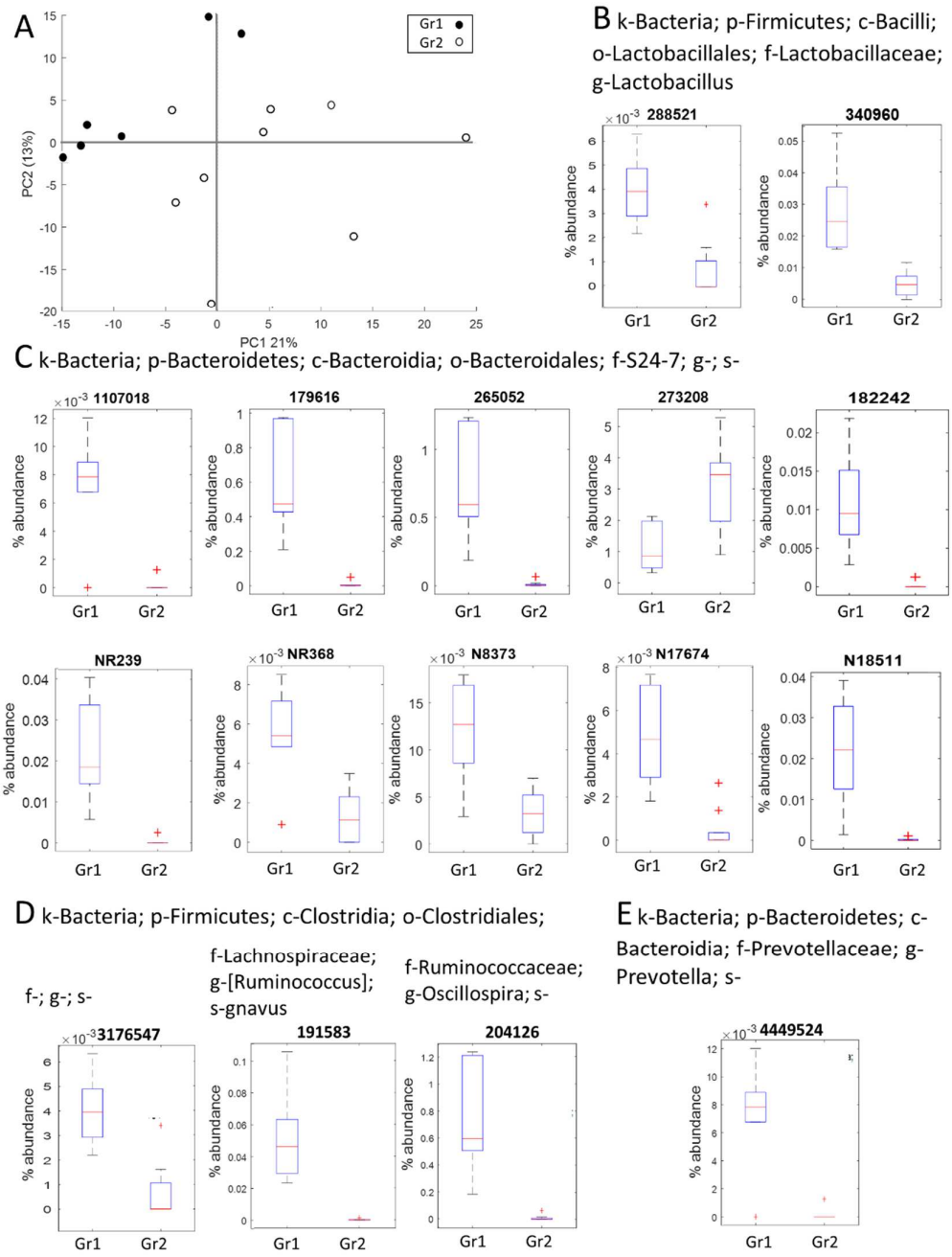


Fig. S2. PCA plot of the initial microbiota of mice in Gr1 and Gr2 (A); Relative abundance (%) of OTUs significantly different between Gr1 and Gr2 at baseline. Taxonomic information is indicated (B-E).

Key responses

Table S2. Body and organ weight, colon length and carcinogenesis in the small intestine and in the colon (ANOVA) in control and DSS-treated mice of Gr1 and Gr2.

	Rel. cecum weight	Rel. spleen weight	Body weight	Colon length
Factors	Explained variance (%)			
Trt	0.0	0.0	6.3	3.3
Subgroup	7.7	3.4	26.2	0.0
Trt x Subgroup	15.0	5.7	1.0	0.0
Error	76.6	90.6	68.3	96.5

Responses are shown as explained variance (%).
Trt = treatment
Significance level *<5% **<1%

	Small intestinal tumors			Colonic flat ACF			Colonic tumors		
	Number	Size	Load	Number	Size	Load	Number	Size	Load
Factors	Explained variance (%)								
Trt	0.9	0.2	0.0	22.6**	26.4*	55.0**	15.0	1.2	6.4
Subgroup	15.4	0.2	4.4	27.6**	22.3*	4.9	11.0	4.6	8.6
Trt x Subgroup	14.8	25.6	21.0	26.1**	7.7	9.9	20.6*	0.1	24.1*
Error	67.3	74.1	73.9	21.4	39.2	26.0	50.6	94.7	58.4

Responses are shown as explained variance (%).
Trt = treatment
Significance level *<5% **<1%

Alpha diversity

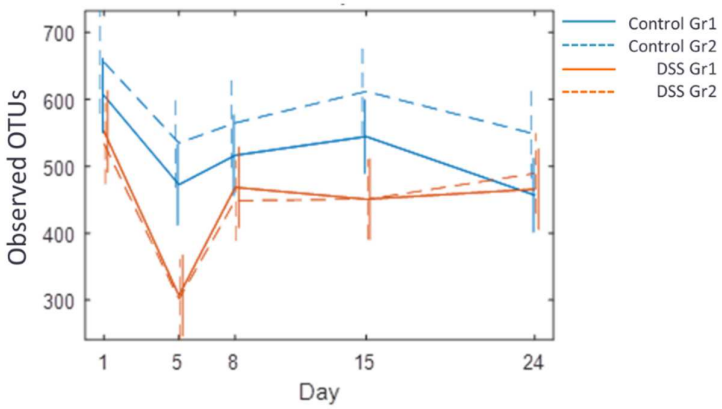


Fig. S3: Temporal trajectory of alpha diversity (observed number of OTUs) of control and DSS-treated mice of Gr1 and Gr2.

Correlation between OTUs and DSS-treatment

Table S3: OTUs significantly affected by DSS-treatment with 50-50 MANOVA[a]

OTU (average abundance >0.05%)	Correlation to trt	Average abundance (%)	P (trt)	p (subgroup)	p (time)	p (trt*subgroup)
4449525:k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-[Paraprevotellaceae]; g-[Prevotella]; s-	pos	8,774	0,001	1	0,001	0,001
181719 :k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-Bacteroidaceae; g-Bacteroides; s-	pos	8,508	0,001	1	0,001	0,001
304047 :k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-Bacteroidaceae; g-Bacteroides; s-acidifaciens	pos	5,514	0,001	1	0,001	0,001
3600504:k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-Bacteroidaceae; g-Bacteroides; s-	pos	3,178	0,001	1	0,552	0,712
197623 :k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-S24-7; g-; s-	pos	1,084	0,021	1	0,998	1
196664 :k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-Bacteroidaceae; g-Bacteroides; s-	pos	0,581	0,001	0,981	0,001	0,001
4401580:k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-Bacteroidaceae; g-Bacteroides; s-	pos	0,401	0,001	1	0,001	0,001
177539 :k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-[Paraprevotellaceae]; g-[Prevotella]; s-	pos	0,224	0,013	0,126	0,809	0,026
4449524:k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-Prevotellaceae; g-Prevotella; s-	pos	0,219	0,008	0,001	0,002	0,249
3588390:k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-Bacteroidaceae; g-Bacteroides; s-	pos	0,207	0,001	1	0,585	0,654
1992 :k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-Bacteroidaceae; g-Bacteroides; s-	pos	0,132	0,001	1	0,001	0,001
4468234:k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-Bacteroidaceae; g-Bacteroides; s-	pos	0,127	0,001	1	0,133	0,454
NR160 :k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-[Paraprevotellaceae]; g-[Prevotella]; s-	pos	0,116	0,005	1	0,996	0,982
3472078:k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-Bacteroidaceae; g-Bacteroides; s-acidifaciens	pos	0,110	0,001	1	0,002	0,002
NR198 :k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-[Paraprevotellaceae]; g-[Prevotella]; s-	pos	0,105	0,005	1	0,978	0,996
NR346 :k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-[Paraprevotellaceae]; g-[Prevotella]; s-	pos	0,093	0,001	1	0,13	0,146
NR242 :k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-Bacteroidaceae; g-Bacteroides; s-	pos	0,089	0,005	1	0,967	0,885
NR286 :k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-Bacteroidaceae; g-Bacteroides; s-	pos	0,078	0,002	1	0,881	0,889
NR135 :k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-Bacteroidaceae; g-Bacteroides; s-	pos	0,075	0,001	1	0,05	0,038
3563235:k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-Bacteroidaceae; g-Bacteroides; s-	pos	0,075	0,001	1	0,36	0,599
194429 :k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-[Paraprevotellaceae]; g-[Prevotella]; s-	pos	0,061	0,002	0,011	0,615	0,05
3426658:k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-Bacteroidaceae; g-Bacteroides; s-	pos	0,059	0,001	1	0,482	0,556

OTU (average abundance >0.05%)	Correlation to trt	Average abundance (%)	P (trt)	p (subgroup)	p (time)	p (trt*subgroup)
203713 :k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-S24-7; g-; s-	neg	9,509	0,002	1	0,153	0,219
134762 :k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-S24-7; g-; s-	neg	3,276	0,002	1	0,092	0,348
174573 :k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-S24-7; g-; s-	neg	1,793	0,001	0,525	0,988	0,67
215495 :k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-S24-7; g-; s-	neg	1,570	0,001	1	0,001	0,06
173852 :k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-S24-7; g-; s-	neg	1,286	0,001	0,908	0,035	0,038
3013444:k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-; g-; s-	neg	0,881	0,002	0,001	1	1
2212505:k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-S24-7; g-; s-	neg	0,610	0,001	1	0,504	1
4428313:k-Bacteria; p-Firmicutes; c-Bacilli; o-Lactobacillales; f-Lactobacillaceae; g-Lactobacillus; s-	neg	0,589	0,02	1	0,994	1
276509 :k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-S24-7; g-; s-	neg	0,553	0,012	1	0,044	1
166718 :k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-S24-7; g-; s-	neg	0,419	0,001	0,001	1	0,693
175646 :k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-S24-7; g-; s-	neg	0,408	0,002	1	0,173	1
312322 :k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-S24-7; g-; s-	neg	0,382	0,002	0,6	0,001	0,995
207284 :k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-S24-7; g-; s-	neg	0,350	0,001	1	0,001	0,983
206324 :k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-S24-7; g-; s-	neg	0,295	0,001	1	0,139	0,861
423455 :k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-S24-7; g-; s-	neg	0,288	0,001	0,02	0,005	0,948
216495 :k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-S24-7; g-; s-	neg	0,285	0,001	1	0,061	1
190673 :k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-S24-7; g-; s-	neg	0,238	0,002	0,002	0,025	1
276629 :k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-S24-7; g-; s-	neg	0,184	0,001	1	0,289	0,999
162539 :k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-Rikenellaceae; g-; s-	neg	0,176	0,024	0,314	1	1
NR193 :k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-; g-; s-	neg	0,169	0,004	0,02	0,95	1
264534 :k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-S24-7; g-; s-	neg	0,119	0,001	1	0,304	1
267689 :k-Bacteria; p-Firmicutes; c-Clostridia; o-Clostridiales; f-Ruminococcaceae; g-; s-	neg	0,117	0,027	0,435	1	1
162005 :k-Bacteria; p-Firmicutes; c-Clostridia; o-Clostridiales; f-Ruminococcaceae; g-Oscillospira; s-	neg	0,106	0,001	0,001	0,729	0,996

OTU (average abundance >0.05%)	Correlation to trt	Average abundance (%)	P (trt)	p (subgroup)	p (time)	p (trt*subgroup)
191994 :k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-S24-7; g-; s-	neg	0,089	0,005	1	0,003	1
263420 :k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-S24-7; g-; s-	neg	0,086	0,007	1	0,724	1
187959 :k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-S24-7; g-; s-	neg	0,077	0,001	1	1	1
195931 :k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-Rikenellaceae; g-; s-	neg	0,071	0,002	1	1	1
197890 :k-Bacteria; p-Firmicutes; c-Clostridia; o-Clostridiales; f-Ruminococcaceae; g-Oscillospira; s-	neg	0,056	0,03	1	0,069	0,478
356226 :k-Bacteria; p-Bacteroidetes; c-Bacteroidia; o-Bacteroidales; f-S24-7; g-; s-	neg	0,050	0,001	1	0,045	0,691

[a]Only OTUs with the average abundance >0.05% are included. p(trt*time) and p(subgroup*time) were not significant for any of the listed OTUs, and not included in the table.