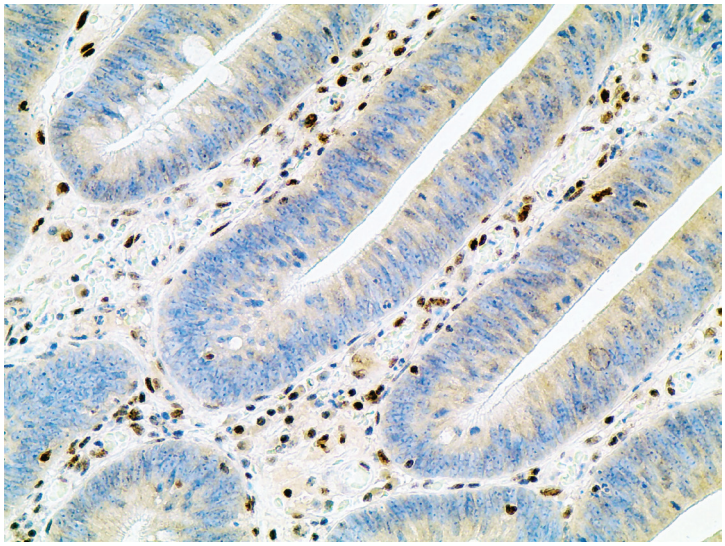
DISSERTATIONES SCHOLAE DOCTORALIS AD SANITATEM INVESTIGANDAM
UNIVERSITATIS HELSINKIENSIS

40/2016

SATU VALO

Western Diet and Genetic Predisposition as Risk Factors of Colon Cancer



DEPARTMENT OF BIOSCIENCES
FACULTY OF BIOLOGICAL AND ENVIRONMENTAL SCIENCES
DOCTORAL PROGRAMME IN INTEGRATIVE LIFE SCIENCE
UNIVERSITY OF HELSINKI

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WESTERN DIET AND GENETIC PREDISPOSITION AS RISK FACTORS OF COLON CANCER

DIVISION OF GENETICS, DEPARTMENT OF BIOSCIENCES
FACULTY OF BIOLOGICAL AND ENVIRONMENTAL SCIENCES

DEPARTMENT OF MEDICAL AND CLINICAL GENETICS
MEDICUM

INTEGRATIVE LIFE SCIENCE DOCTORAL PROGRAM
UNIVERSITY OF HELSINKI
HELSINKI, FINLAND

ACADEMIC DISSERTATION

To be presented for public examination with the permission of
the Faculty of Biological and Environmental Sciences of the University of Helsinki
in Biomedicum Helsinki Lecture hall 2, Haartmaninkatu 8, Helsinki
on the 1st of July 2016 at 12 o'clock noon.

Supervisors

Professor Minna Nyström
Division of Genetics, Department of Biosciences
Faculty of Biological and Environmental Sciences
University of Helsinki
Helsinki, Finland

Professor Päivi Peltomäki
Department of Medical and Clinical Genetics
Medicum, University of Helsinki
Helsinki, Finland

Thesis committee

Assistant Professor Ville Hietakangas
Division of Genetics, Department of Biosciences
Faculty of Biological and Environmental Sciences
University of Helsinki
Helsinki, Finland

Docent Nina Horelli-Kuitunen
Department of Genetics
United Medix Laboratories Ltd.
Helsinki, Finland

Reviewed by

Docent Anne-Maria Pajari
Department of Food and Environmental Sciences
University of Helsinki
Helsinki, Finland

Docent Markku Aarnio
Department of Surgery
Jyväskylä Central Hospital
Jyväskylä, Finland

Opponent

Professor Karl Heinimann
Medical Genetics
University Hospital Basel
Basel, Switzerland

Custos

Professor Minna Nyström

Cover layout by Anita Tienhaara
Layout by Tinde Päivärinta, PSWFolders Oy
Cover image: MLH1 deficient tubular adenoma with high-grade dysplasia. Image by Satu Valo.

Dissertationes Scholae Doctoralis Ad Sanitatem Investigandam Universitatis Helsinkiensis
ISBN 978-951-51-2235-3 (paperback)
ISBN 978-951-51-2236-0 (PDF)
ISSN 2342-3161 (paperback)
ISSN 2342-317X (PDF)

Hansaprint, Vantaa 2016

Quiet people have the loudest minds

- *Stephen Hawking*

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

I. Dermadi Bebek D, **Valo S**, Pussila M, Reyhani N, Sarantaus L, Lalowski M, Baumann M, Nyström M. 2014, *J Nutr Biochem*. Inherited cancer predisposition sensitizes colonic mucosa to address Western diet effects and putative cancer-predisposing changes on mouse proteome.

II. Dermadi D, **Valo S**, Ollila S, Solymani R, Sipari N, Pussila M, Sarantaus L, Linden J, Baumann M, Nyström M. Western diet causes deregulation of intracellular bile acid homeostasis, cell proliferation and tumorigenesis in colon, *manuscript submitted.*

III. **Valo S**, Kaur S, Ristimäki A, Renkonen-Sinisalo L, Järvinen HJ, Mecklin J-P, Nyström M, Peltomäki P. 2015, *Clin Epigenetics*. DNA hypermethylation appears early and shows increased frequency with dysplasia in Lynch syndrome-associated colorectal adenomas and carcinomas.

In studies I-II S.V. conducted/participated:
- design of the study
- preparation of tissue specimens
- extraction and quantification of DNA, RNA and proteins
- *Mlh1* genotyping
- 2D Difference gel electrophoresis (2D DIGE) and data analysis (DeCyder Software)
- Western blot validations
- extraction of metabolites
- Ki67 analysis
- writing of the manuscripts

In study III S.V. conducted
- design of the study
- preparation of tissue specimens
- design and optimization of MS-MLPA probes for methylation analysis
- methylation analysis by MS-MLPA
- microsatellite instability (MSI) analysis
- *KRAS* mutation analysis
- microarray analysis by GeneSpring
- all statistical analyses by SPSS
- writing of the manuscript

The publications are referred to in the text by Roman numerals I-III.

ABBREVIATIONS

2D DIGE	Two-dimensional difference gel electrophoresis
AC	Amsterdam criteria
ACF	Aberrant crypt foci
AFAP	Attenuated familial adenomatous polyposis
AIN	AIN-93, American Institute of Nutrition, purified diet for laboratory rodents
APC	Adenomatous polyposis coli
ASE	Allele-specific expression
BMI	Body mass index
bp	base pair
CA	Cholic acid
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
CRC	Colorectal cancer
DCA	Deoxycholic acid
Dm	Methylation dosage ration
DNA	Deoxyribonucleic acid
FA	Folic acid
FAO	Fatty acid oxidation
FABP	Fatty acid binding protein
FAP	Familial adenomatous polyposis
FCCX	Familial colorectal cancer type X
FFPE	Formalin fixed paraffin embedded
FXR	Farnesoid X receptor
GO	Gene Ontology
HNPCC	Hereditary non-polyposis colorectal cancer
IHC	Immunohistochemistry
IPA	Ingenuity Pathway Analysis
LCA	Lithocholic acid
LC-MS	Liquid chromatography coupled with mass spectrometry
LOH	Loss of heterozygosity
LS	Lynch syndrome
MALDI-MS	Matrix-assisted laser desorption ionization mass spectrometry
MCR	Mutation cluster region
MLH	MutL homologues
MMR	Mismatch repair
mRNA	messenger RNA
MSH	MutS homologues
MSI	Microsatellite instability
MSS	Microsatellite stable
MS-MLPA	Methylation-specific multiplex ligation-dependent probe amplification
NADPH	Nicotinamide adenine dinucleotide phosphate
PCA	Principal component analysis
PCR	Polymerase chain reaction

PMS	Post meiotic segregation increased
POI	Protein of interest
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
UPLC-MS	Ultra-performance liquid chromatography mass spectrometry
WD	Western-style diet

ABSTRACT

Lifestyle and diet have a major effect on the development of colorectal cancer (CRC). Dietary habits of Western populations in particular are recognized as a risk factor for CRC. However, the mechanisms that mediate the effects of Western-style diet (WD) on colorectal tumor development are largely unknown. CRC develops via multiple steps which involve genetic changes, such as mutations in growth-regulatory genes, and epigenetic alterations, such as CpG island hypermethylation. Lynch syndrome (LS) is one of the most common inherited cancer susceptibility syndromes. It is caused by inherited defects of the DNA mismatch repair genes (MMR), which together with other genetic and epigenetic changes are known to accelerate tumorigenesis. Genetic and epigenetic changes that promote the initiation and progression of cancer are called driver events whereas changes that accompany the transformation but have no effect on carcinogenesis are referred to as passengers. MMR defects are known to accelerate tumor progression in LS but the early events leading to polyp formation and the timing and order of the molecular “hits” remain unknown.

A long-term feeding experiment with mouse models for LS and sporadic CRC was conducted to characterize tumor-promoting changes in normal colonic mucosa caused by WD and/or genetic predisposition. Changes in the proteome of histologically normal colonic mucosa were monitored at different time points of feeding experiment (5 weeks, 12, 18 and 21 months) with two high-throughput proteomic methods followed by analysis of affected pathways. Data from proteomic analysis indicated that the proteome was more consistently changed by diet and aging than by genotype. Overall, 21 out of 26 colonic tumors were detected in mice fed with WD. Proteomic analysis indicated disrupted lipid metabolism and increased oxidative stress in the normal-appearing tissue in association with WD. Moreover, proteome analyses revealed increased cell proliferation and decreased apoptotic processes in the normal colon mucosa of mice fed with WD, which may promote colorectal tumorigenesis. Finally, proteomic data coupled with measurement of bile acids in tissue specimens indicated that WD induces downregulation of intracellular bile acid transport, resulting in disrupted bile acid homeostasis which may provide a possible mechanism underlying the tumor-promoting effects of the diet.

Studies on sporadic CRC have demonstrated that promoter hypermethylation leading to gene silencing can act as an alternative mechanism to mutations in early stages of tumor development but its importance in hereditary CRC remains unknown. We analyzed tissue specimens gathered during colonoscopy surveillances and colectomies performed on human LS mutation carriers to define changes in CpG island methylation that occur at different stages of the tumor progression sequence. Methylation changes at different stages of tumor progression were analyzed in relation to MMR gene expression, and normal tissue biopsies were studied for carcinogenic “fields”. In addition, we aimed to clarify the role of CpG island hypermethylator phenotype (CIMP) in the LS-associated tumorigenesis. Results indicate that the expression of the MMR protein corresponding to

the gene mutated in the germline decreases along with dysplasia but occurs as a relatively late event in the tumor progression sequence, suggesting the presence of other somatic events that drive neoplastic transformation. Indeed, significant increase in the average degree of methylation of two candidate genes (*SFRP1* and *SLC5A8*) was observed in normal colonic mucosa biopsies from patients with CRC (high-risk mucosa) when compared to those without (low-risk mucosa), indicating a possible carcinogenic field. Moreover, methylation was found to increase in LS adenomas and carcinomas along with dysplasia. These findings emphasize the importance and early appearance of epigenetic alterations in LS-associated tumorigenesis. In summary, the results offer new insights into the initiating molecular mechanisms through which Western-style diet and DNA methylation contribute to hereditary and sporadic colorectal carcinogenesis.

INTRODUCTION

The incidence of colorectal cancer (CRC) is increasing in all industrialized countries and dietary habits of Western populations in particular are recognized as a risk factor for CRC (1). Based on human and experimental evidence it has been concluded that a Western-type diet (WD) high in total energy, saturated fats and red meat, in addition with low levels of fiber, vitamin D, calcium and folic acid may predispose gut mucosa to CRC. The mechanisms that mediate the effects of diet on tumor development are largely unknown. CRC develops via progressive accumulation of genetic changes, such as mutations in growth-regulatory genes, and epigenetic alterations, such as CpG island hypermethylation of tumor suppressor genes. Unhealthy features in WD are likely to induce expression changes in key regulatory pathways affecting normal metabolic processes in colonic mucosa.

While diet may account for up to 70% of all CRCs (1), genetics and environmental influences intertwine. Lynch syndrome (LS) is one of the most common inherited cancer susceptibility syndromes and it is associated with inherited defects of the DNA mismatch repair genes (MMR) (2), which together with other genetic and epigenetic changes are known to accelerate tumorigenesis. Interestingly, the disease phenotype such as age of onset and tumor spectrum vary considerably between individuals that carry the same germline mutation, suggesting contribution of additional phenotype determinants on risk accumulation (3). Apart from genetic alterations, epigenetic effects play a major role in the initiation and progression of CRC (4). Epigenetic changes are however potentially reversible and can be modified by the environment. These changes can accumulate over time due to many environmental factors such as diet. Environmental influences have been implicated in LS and epigenetic regulation provides a possible link between genetics and variation observed in clinical phenotypes (5). In studies I-II mouse models for LS and sporadic CRC were used to study whether and how WD and genetic predisposition may separately or together affect the proteome of normal appearing colonic mucosa and thus increase the risk for CRC.

MMR defects are known to accelerate tumor progression in LS but the early events leading to the formation and the timing and order of the molecular “hits” remain unknown. Even though aberrant CpG island methylation affecting tumor suppressor genes has been shown to occur frequently in sporadic CRC as well as in colonic adenomas, its importance in hereditary CRC remains unknown. CRCs characterized with wide-spread CpG island promoter methylation, known as CpG island methylator phenotype (CIMP), are thought to form a subtype of tumors with distinct histology compared to tumors derived from the traditional adenoma carcinoma sequence (6). In study III, the aim was to define how CpG island methylation changes throughout transformation from normal colonic mucosa to pre-cancerous and cancerous lesions from human LS mutation carriers in relation to MMR gene expression and to investigate possible carcinogenic “fields” in the normal appearing tissue. Methylation changes in pre-malignant and malignant lesions were studied by analyzing the methylation of eight CIMP maker genes and seven tumor suppressor genes

that were associated with early colon oncogenesis in a previous study from our group (7). Together these investigations emphasize the importance and early appearance of epigenetic alterations in LS-associated tumorigenesis and offer new insights to the molecular mechanisms underlying the WD-induced risk for CRC. These studies have broad implications for further studies addressing early biomarkers in CRC prevention.

REVIEW OF THE LITERATURE

COLORECTAL CANCER (CRC)

Epidemiology

Colorectal cancer (CRC) is the third most common cancer and the fourth most common cause of cancer related deaths globally (8). More than 1.2 million patients are diagnosed with CRC every year (9). In Finland, over 3000 new cases are diagnosed on average each year (10). Risk for CRC strongly increases with age and the median age at diagnosis is about 70 years. Dietary and lifestyle habits are thought to have a strong impact on CRC risk (1). Many epidemiological studies around the world demonstrate major geographical variation in CRC incidence and association with obesity, sedentary lifestyle, smoking and high consumption of alcohol and red meat (11-16). Protective effect and inverse association are suggested between CRC risk and diet rich in vegetables, fiber, dairy products and fish (17-20). Highest rates of CRC are found in North America, Western Europe, Australia and New Zealand whereas lowest rates are found in developing countries. However, rapid increase in the incidence has been reported in previous low risk countries, such as Spain, together with economic development and adaption of a so called Western lifestyle (11). Additionally, migrants moving from countries with low CRC incidence tend to acquire a higher cancer risk similar to host populations (21).

Inherited factors are involved in a substantial proportion of all CRCs. While majority of CRCs occur in sporadic manner, 15–35% have been estimated to have a heritable background (22). Epidemiologic studies have correlated increased risk with family history and a high proportion of all patients have one first to third degree relative with CRC (23). Up to 5% of CRCs occur in association with rare highly penetrant Mendelian cancer syndromes that are usually inherited in dominant manner (24). Without preventive measures the lifetime risk for CRC in these disorders is very high 10-100% (25). The hereditary CRC syndromes are discussed in greater detail in the section Inherited predisposition below.

The prognosis of CRC has steadily improved during the past two decades in many high-income countries, due to improvements in surgery, chemotherapy and screening (11, 26). Overall, 5-year survival has reached almost 65% in high-income countries, such as Australia, Canada and USA, whereas in low-income countries survival rates remain less than 50% (26, 27).

Histopathology

Colorectal tumors arise in the colonic epithelium in a multistep process called the adenoma-carcinoma sequence which is the classical sample for stepwise progression of cancer (Fig. 1). Development of CRC includes multiple well defined histological stages. It is thought to originate from hyperplastic or dysplastic colonic crypts that give rise to benign and advanced adenomas which are followed by *in situ* and finally metastatic carcinoma. Hyperplastic growth caused by a slightly increased rate of cell division results in patches of thicker epithelia with almost normal appearance. When the well-ordered layers of epithelial cells become disrupted with slight changes in the cell morphology, the lesions are described as dysplastic.

Colonic stem cells located in the base of the crypts give rise to daughter cells during mitosis which differentiate into epithelial cells that migrate towards the epithelial surface where they undergo apoptosis (28). Aberrant crypt foci (ACF), first described by Bird *et al.* (29), are clusters of hyperplastic or dysplastic colonic crypts that have aberrant appearance and growth compared to the surrounding normal colonic mucosa. The colonic epithelium is a rapidly renewing tissue which depends on homeostasis of cellular proliferation and apoptosis. The ACFs are formed when this balance is lost and therefore they are suggested to be the earliest identifiable neoplastic lesions that predispose to CRC (30, 31). A more deviant growth of dysplastic cells is called a polyp or adenoma. In the colon, several distinct types of polyp histologies are described and all of them have been considered as benign changes.

Over 90% of CRCs are thought to arise from adenomatous polyps which are the most common form of premalignant precursor lesions. A tumor is considered malignant when the dividing cells penetrate through the basement layer of the epithelium and invade to underlying tissues. Malignant transition from normal colonic epithelium into adenoma and invasive carcinoma involves accumulation of many genetic and epigenetic changes that affect genes controlling cell division, apoptosis and DNA repair within the evolving tumor cells (32, 33). However, the early molecular events predisposing to polyp formation remain unknown.

Presently, the histopathological stage of tumor at diagnosis is the most important prognostic factor and early diagnosis is therefore important. Histological classification of tumors is made according to invasion depth, lymph node involvement and presence of distant metastases (34). Although the recognition of different stages provides valuable information for prognosis and therapy, the response to treatment and outcome are not very well predicted. Therefore, early biomarkers for CRC could enable identification of patients at high risk and improve prognosis and prediction of treatment response.

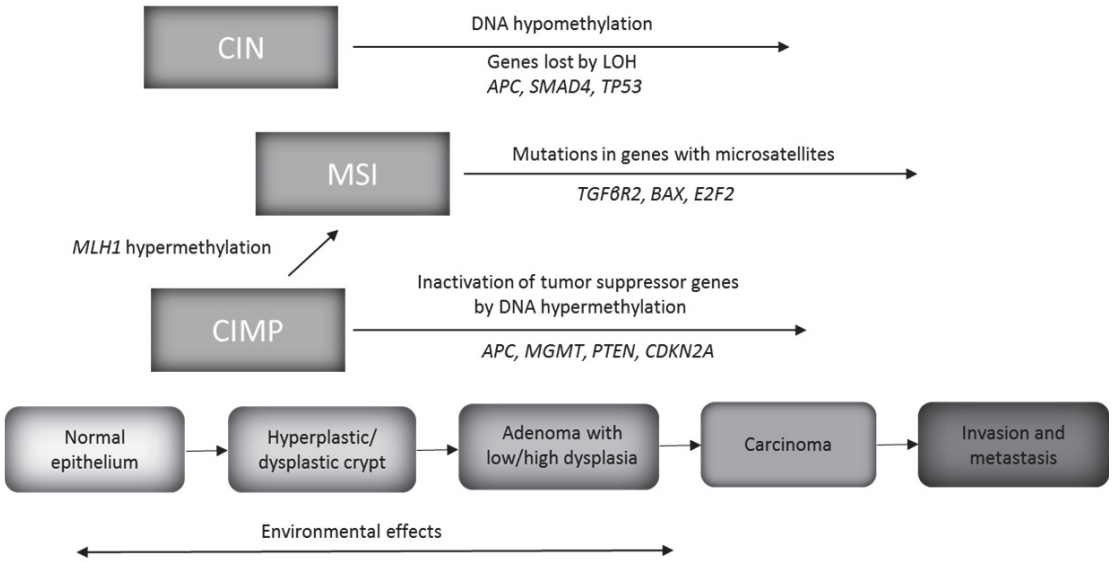

Fig. 1. Key pathways of the adenoma-carcinoma progression sequence. Modified from Boland *et al.* 2009 (35).

TUMORIGENIC PATHWAYS

Colorectal cancer is a morphologically heterogeneous disease with a variation of clinical phenotypes. Molecular and genetic studies have revealed several important signaling pathways that are involved in the development of CRC. The model for the progression sequence of genetic changes leading to colorectal cancer was originally proposed by Fearon and Vogelstein (36) and since then, the molecular genetic background of each morphological step has been relatively well defined. Recognition of molecular pathways involved in tumor development are clinically important since they contribute to prognosis and treatment response (37, 38). Moreover, the molecular subtypes of colorectal tumors can now be classified based on combination of gene expression patterns and mutations, chromosomal instability and epigenetic characteristics (39).

Chromosomal instability (CIN)

Cell cycle signaling and apoptosis of colonic epithelial cells are tightly regulated by growth limiting tumor suppressor genes and growth promoting oncogenes. Chromosomal instability (CIN) is a hallmark of tumors arising from the classical pathway described by Fearon and Vogelstein (36), where carcinomas are characterized by alterations in tumor suppressor genes such as *APC* and T*P53* and oncogenes such as *KRAS*. This pathway involves 85% of all sporadic CRCs (40). Tumors that arise via the CIN pathway are often aneuploid with high frequency of allelic imbalance and gene copy number variations (41). Events of this pathway are illustrated in Fig. 1.

The pathway was discovered through the identification of the *APC* gene which is known to play a role in familial adenomatous polyposis (FAP). Mutations of the *APC* gene are found in 80% of adenomas and carcinomas and they are considered to occur at an early stage in sporadic CRC development (42, 43). Inactivating mutations often lead to truncation of the APC protein which causes inability of APC to recognize and bind β-catenin in the cytoplasm. This increases the concentration of cytoplasmic β-catenin and results in translocation of β-catenin into the nucleus (44). Nuclear β-catenin upregulates the Wnt signaling pathway which reduces cell differentiation and apoptosis and induces proliferation and invasive growth. Even though inactivation of *APC* is a common event in colonic adenomas and carcinomas, it is seldom found in sporadic ACF. Therefore, upregulation of the Wnt signaling through mutated APC is regarded as the mechanism that drives malignant transformation from ACF to adenoma in sporadic CRC (43).

In addition to *APC* inactivation, mutations that cause activation of the *KRAS* oncogene occur at very early stages of colorectal carcinogenesis and are found in 30-50% of sporadic CRCs and advanced adenomas (45). Interestingly, *KRAS* mutations are found in up to 95% of early dysplasia including ACF and hyperplastic polyps (46, 47). However, they are considered to be insufficient to cause malignant transformation by themselves and need additional driver mutations for cancer to develop (48). KRAS is a GTP binding protein that activates a large number of signal transduction pathways which promote hyperplastic growth and suppress differentiation of colonic epithelial cells (49). It is responsible for the transduction of mitogenic signals from the cell surface epidermal growth factor receptors (EGFR). Consequently, activation of *KRAS* caused by mutations results in constant growth signaling.

Additional molecular events in the CIN phenotype include loss of function of the *TP53* tumor suppressor gene, which encodes p53 protein. Mutations in *TP53* occur in approximately 70% of all sporadic CRCs. p53 has a crucial role in controlling the cell cycle and maintaining genomic stability (50). When DNA damage occurs, p53 points out the errors to the caretaker proteins for reparation or induces apoptosis if the damage is too extensive to be repaired (51). In addition to DNA damage, it is activated by ultraviolet (UV) radiation, oxidative stress, chemicals and viruses among others (52). Inactivation of *TP53* drives carcinogenesis from non-invasive to invasive state and occurs at a later stage of tumor development during the progression from advanced adenoma to carcinoma in sporadic CRC (53).

Microsatellite instability (MSI)

Microsatellite instability represents an alternative key pathway in the development of CRC (Fig. 1). Microsatellites are repeats of short polymorphic nucleotide sequences in the DNA that are scattered throughout the genome and constitute a unique profile of individual DNA fingerprints (54, 55). Microsatellite sequences are tandemly repeated from 3 to 100 times and locate often in the non-coding regions. Microsatellite instability (MSI) results from defects in the mismatch repair (MMR) function responsible for post-replicative proofreading and editing of errors that arise during DNA replication. Failure of the MMR system causes accumulation of spontaneous point mutations and increased frequency of insertion/deletion mutations in the genome (56). Tumors characterized with MSI have 100 to 1000 fold increase in mutation rate when compared to normal cells (57). Microsatellite sequences in particular are vulnerable to accumulate mutations caused by replication errors since the polymerase complex is prone to make mistakes in repeated regions.

MMR defects in humans were originally identified in the early 1990s in relation to the discovery of the molecular genetic background of hereditary non-polyposis colon cancer (HNPCC), more recently named as Lynch syndrome (LS) (58, 59). The four main genes identified as the primary cause of MMR defects are *mutL homologue 1* (*MLH1)*, *mutS homologue 2* (*MSH2)*, *mutS homologue 6* (*MSH6)* and *postmeiotic segregation increased 2* (*PMS2)*. MSI phenotype is a hallmark of LS tumors but in addition it is detected in approximately 15-20% of sporadic CRCs. Defects in MMR are mainly caused by mutations that result in malfunction or complete loss of a MMR protein. Alternatively, silencing of *MLH1* caused by promoter hypermethylation is often seen in sporadic CRC (60). Loss of MLH1 or MSH2 is associated with complete inactivation of MMR function, whereas defects in other MMR proteins often cause only partial MMR deficiency (61). In sporadic CRC, inactivation of MMR is mainly proposed to accelerate rather than initiate tumor progression, but the exact timing of MMR inactivation during the progression sequence remains unclear (62).

Tumors with MSI show less cytogenetic abnormalities and aneuploidy compared to tumors that arise from the CIN pathway. In addition, they display induced frequency of mutations in genes such as *BRAF* and *TGF-β* and reduced frequency of mutations or allelic losses of genes typically mutated in the CIN phenotype (63, 64). MSI tumors are typically localized in the proximal region of the colon and have distinctive histological features including poor differentiation, lymphocytic infiltration and mucinous histology (65). Association of tumor-infiltrating lymphocytes is thought to result from an anti-tumoral immune response induced by MMR deficiency and high genomic mutation load, which is recognized by the immune system (66, 67). In immunohistochemically stained tumor sections, MSI tumors show loss of at least one MMR protein expression in at least 90% of lesions (68).

Knowledge about the MMR status of tumors provides valuable clinical information related to prognosis and therapy response. Overall, cancers that arise via the MSI have a better prognosis than MMR competent tumors (69). Moreover, MSI tumors do not respond to adjuvant therapy with 5-fluorouracil whereas improved response is achieved when treated with irinotecan-based chemotherapy (70, 71).

CpG island methylator phenotype (CIMP)

Altered patterns of gene expression in tumor cells are driven by many genetic and epigenetic changes (72). Epigenetic modifications are biochemical changes in the DNA and chromatin structure that regulate gene expression without altering DNA sequence itself. DNA methylation is a vital mechanism for normal cell function and it plays a key role in processes such as cell differentiation, embryonic development, X chromosome inactivation and genomic imprinting (73, 74). However, aberrant DNA methylation patterns, hypermethylation and hypomethylation, have been associated with many disease states. In normal somatic cells majority of methylation occurs in the cytosine residues of the CpG nucleotides. Hypermethylation of sequences enriched with CpG dinucleotides, called CpG islands, located around the transcription start site is associated with silencing of the adjacent gene (75).

Alterations in DNA methylation patterns are intensively studied in relation to CRC. Sporadic CRCs often show a decrease in global genomic DNA methylation content (76). However, increase in CpG island methylation affecting multiple tumor suppressor genes is observed in a subset of CRCs, giving rise to CpG island methylator phenotype (Fig. 1) (6). Hypermethylation in promoter regions of tumor suppressor genes is known to downregulate their expression and predispose to cancer (6). In addition to CRC, tumors with CIMP have been described in various cancers of other organs such as breast (77), lung (78), endometrium (79), bladder (80) and kidney (81).

Since tumors with CIMP are characterized by distinct clinical and molecular features such as proximal tumor location, poor differentiation, high rates of MSI and *BRAF*^{V600E} mutations, they are thought to represent a distinct pathway of colorectal carcinogenesis (82, 83). CIMP was originally recognized as a characteristic of non-adenomatous mucosal lesions called sessile serrated adenomas or polyps. These tumors resemble benign hyperplastic polyps but with increased dysplasia, irregular crypt architecture and a significant malignant potential. Moreover, CIMP has been associated with hypermethylation of *MLH1* promoter region which induces MMR deficiency and MSI as a consequence (84, 85), whereas large chromosomal aberrations are less frequently observed (86, 87).

Methylation markers for classification of CIMP have not been standardized. This has led to discrepancies between studies and difficulties in defining the borderline between CIMP-positive and non-CIMP tumors (88, 89). Moreover, inconsistences in different marker panels, thresholds and analytical methods used for classification have caused difficulties in assessing the value of CIMP in predicting prognosis and therapy response. Substantial effort has been made to identify accurate markers for different CIMP subtypes in CRC. Weisenberger *et al.* identified a robust panel of five CIMP marker genes (*CACNA1G, IGF2, NEUROG1, RUNX3* and *SOCS1*) that define a heavily methylated subset of CRCs with MSI and *BRAF*^{V600E} mutations (84). This panel is the most commonly used panel for CIMP and it classifies tumors with 3/5 or more methylated promoters as CIMP-positive. Ogino *et al.* (90) proposed three additional genes (*CDKN2A, CRABP1* and *MLH1*) to this panel together with three suggested categories: CIMP-high with 6/8 to 8/8, CIMP-low with 1/8 to 5/8 and non-CIMP with 0/8 hypermethylated promoters. Molecular and clinical characteristics of the intermediately methylated CIMP-low group vary among different studies and remain unclear. In comparison to the CIMP-high group, CIMP-low tumors have increased frequency of *KRAS* mutations whereas *BRAF* mutations and MSI are less frequent (82).

Aberrant methylation is known to occur at early stage of colorectal tumor development (6). Genomic DNA methylation has been suggested to accumulate in normal colonic mucosa in age-dependent manner which could be the earliest changes marking the increased risk for CRC (91). Moreover, CIMP together with *BRAF* and *KRAS* mutations has been reported in hyperplastic polyps and adenomatous lesions whereas *MLH1* hypermethylation and MSI occur at a later stage of tumor progression (92-94). The cause and molecular mechanism of aberrant DNA methylation and CIMP remain unknown, although several possible factors have been reported. For instance, increased expression of *DNMT3B* together with increased methylation has been shown to occur during progression of CRC (95). Additionally, the effect of lifestyle and dietary factors has been investigated in several epidemiological studies and consumption of a low-folate diet, smoking and high alcohol consumption have been associated with CIMP-high CRC and *BRAF* mutation (96, 97).

INHERITED PREDISPOSITION

Inherited factors are involved in a substantial proportion of all CRCs (Fig. 2). Even though CRC is often divided into sporadic and familial forms, it is noted that even in sporadic CRCs genetic factors may have an influence although currently identified genetic factors account for only up to 10% of all CRCs (98). Genomic-wide association studies (GWAS) performed in large cohorts of patients with CRC and healthy controls have identified approximately 40 common low-penetrance alleles that associate with a small increase in CRC risk (99). Consequently, genetic background can provide additional information on the cumulative cancer risk together with age and family history (100).

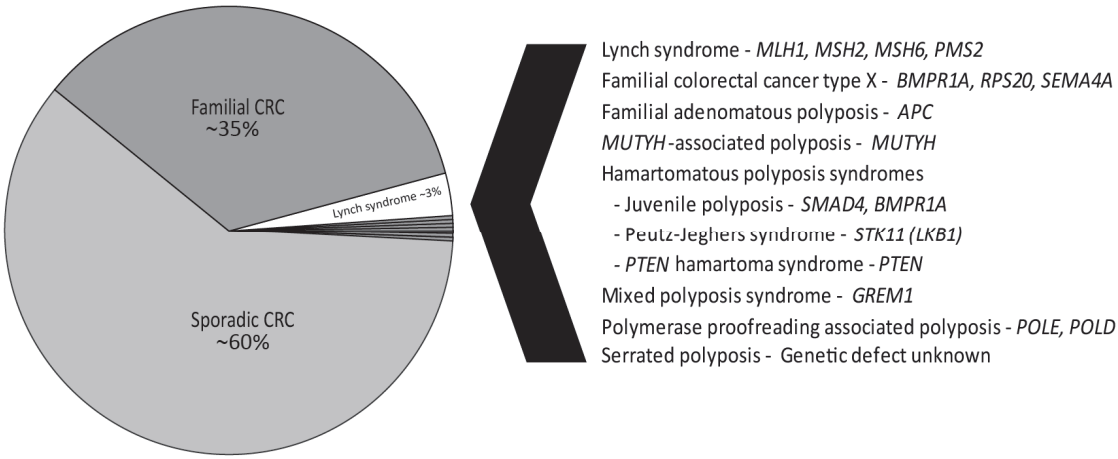

Fig.2. Genetic predisposition to colorectal cancer.

Hereditary syndromes

Colorectal cancer syndromes are divided into hereditary polyposis syndromes characterized by the development of multiple colorectal polyps and non-polyposis syndromes in which only few or no polyps occur. Each syndrome is diagnosed and classified based on genetic, pathological and clinical features. Hereditary CRC develops typically two to three decades earlier compared to sporadic CRC. Increased risk for cancer in hereditary syndromes is caused by inherited defects in genes that regulate growth regulatory processes or DNA repair in conjunction with somatic events that cause the remaining wild-type allele to become inactivated. In hereditary cancer, the inactivation of the first allele of a predisposing gene is caused by inherited germline mutations whereas the wildtype allele becomes somatically inactivated in the target tissue by genetic or epigenetic events (101). Predisposing genes for many of these syndromes have been identified, and testing for inherited mutations in families at high risk including predictive testing in asymptomatic family members has become routine. International guidelines have been established to aid the identification and diagnosis of hereditary cases and screening for precursor lesions is utilized in surveillance and early detection of tumors in familial cases.

Lynch syndrome

Lynch syndrome is the most prevalent form of hereditary CRC with a share of approximately 3% of all CRCs and autosomal dominant transmission pattern (102). The predisposed individuals have significantly increased lifetime risk for CRC (10-70%) and endometrial cancer (12-55%), depending on which gene is affected (103-105). Moreover, the syndrome is characterized by increased risk for other extracolonic manifestations such as cancers of the urinary tract, ovarian, stomach, pancreas, small bowel and brain (106). Clinical features of CRC include early onset (mean 45 years) and location in the proximal colon (70-80%) (107). Histologically tumors are characterized as mucinous and poorly differentiated with villous components and lymphocytic infiltration (108). The presence of tumor-infiltrating lymphocytes, most probably induced by anti-tumoral immune response caused by the high genomic mutation load, is thought to contribute to the improved prognosis of LS compared to sporadic CRC (66, 67).

Before the genetic basis of the syndrome became established, the Amsterdam criteria I (AC I) were set in 1991 to standardize the clinical guidelines and aid diagnosis (109) (Table 1). These criteria were amended in 1999 to take into account extracolonic manifestations (AC II) (110). After MSI was recognized as a characteristic of LS tumors, the Revised Bethesda Guidelines were established in 1996 to provide guidelines including MSI testing of tumors for identification of LS (65) (Table 1). Similarly to sporadic CRC, colorectal tumors in LS seem to evolve through the adenoma-carcinoma sequence. The risk for CRC can be managed reasonably well by repeated colonoscopies and polypectomies (111). Intensive colonoscopy surveillances are recommended for LS patients starting at the age 25 with a two-year interval (112). Since LS mutation carriers

have a relatively high risk for synchronous or metachronous tumors, total or subtotal colectomy have been suggested as the primary choice of operation for patients with CRC (113).

Majority of LS causing heterozygous germline mutations are found in the DNA mismatch repair genes *MLH1* and *MSH2* whereas mutations in *MSH6, PMS2* and *MLH3* account for minority of cases (2). MMR mechanism maintains DNA integrity by detecting and repairing nucleotide mismatches and small insertion/deletion loops that arise during DNA replication and recombination. Inactivation of the remaining wild type allele in somatic cells results in loss of the encoded protein and MMR malfunction which causes accumulation of replication errors in the genome leading to MSI (114). The progression from polyps to carcinomas is accelerated in LS (2-3 years) compared to sporadic CRC (6-10 years) due to defective MMR that results in accumulation of mutations and subsequent selection and clonal expansion of mutated cells with growth advantage (115).

The wild type allele can become somatically inactivated through deletion, point mutation or CpG island hypermethylation. Somatic inactivation caused by hypermethylation primarily occurs in the promoter region of the *MLH1* gene (116, 117). Large genomic rearrangements within *MLH1* and *MSH2* have also been reported to cause loss of the respective protein, resulting in LS phenotype in patients that lack conventional MMR gene mutations (118). Over 3000 unique sequence variants have been reported for the MMR genes in the International Society of Gastrointestinal Hereditary Tumors (InSiGHT) database (http://insight-group.org/), out of which missense mutations are the most common type of variation in addition to insertions/deletions.

Age of onset and the risk of developing various types of cancer vary depending on which MMR gene is mutated (119). Moreover, considerable differences in the clinical phenotype and age of onset exist even in patients with the same pathogenic germline mutation (3). Patients with the same mutation may express considerable differences in their clinical phenotype and age of onset or in rare cases live happily ever after without having to endure a cancer diagnosis. This raises a question about other yet unknown factors, such as dietary effects, that may together with the genotype have a significant effect on cancer risk.

Table 1. Guidelines for Lynch syndrome diagnosis.

Amsterdam I-II Criteria for diagnosis of Lynch syndrome
At least three relatives with histologically verified LS-associated cancer[1] are required of which:
One is a first degree relative of the other two
At least two successive generations are affected
At least one of the relatives with CRC is diagnosed at <50 years of age
FAP has been excluded in any CRC cases
Tumors should be verified for pathology
Bethesda Guidelines for testing MSI in colorectal tumors
MSI testing is justified when:
CRC is diagnosed at <50 years of age
Synchronous and metachronous CRC or other LS-associated cancers diagnosed at any age
CRC with MSI-high histology[2] diagnosed in a patient <60 years of age
CRC or LS-associated tumor is diagnosed <50 years of age in at least one first-degree relative
CRC or LS-associated tumor is diagnosed at any age in two first- or second-degree relatives
MSI is classified as
MSI-high when 2-5 of markers indicate MSI
MSI-low when only 1 out of 5 markers indicate MSI
MSS when none of five markers indicate MSI

[1]LS-associated cancers include CRC and cancers of endometrium, stomach, ovary, ureter or renal pelvis, small bowel, brain, hepatobiliary tract and skin

[2]MSI-high histology refers to the presence of tumor-infiltrating lymphocytes, mucinous or signet-ring differentiation or medullary growth pattern

Approximately 90% of mutations are found in *MLH1* and *MSH2* (2). Patients with *MLH1* and *MSH2* mutations have relatively early age of onset (40-50 years) and increased risk for the whole spectrum of LS associated cancers with the distinction that *MSH2* mutation carriers seem to have a moderately increased risk for extracolonic cancers (119, 120). In addition to germline mutations, constitutional epimutations have been reported in a small number of individuals meeting the clinical criteria of LS (121). Epimutations are defined as heritable alterations in gene activity which are caused by epigenetic changes, such as aberrant DNA methylation, rather than changes in the DNA sequence itself. These cases are characterized with constitutional methylation and transcriptional silencing of one of the alleles in the normal tissue which predisposes to the development of LS-like phenotype (122).

Secondary epimutations that cause hypermethylation and inactivation of *MLH1* and *MSH2* have been associated with adjacent genetic alterations and demonstrate dominant inheritance whereas constitutional hypermethylation of *MLH1* as a primary event shows non-Mendelian inheritance patterns (123). Loss of MSH2 expression is most often caused by germline mutations in *MSH2* gene, however 10% of cases are characterized with a heritable epimutations caused by deletions of the 3' end of *EPCAM* gene located upstream of *MSH2* which results in hypermethylation of the *MSH2* promoter (124-126). Deletions

result in removal of the transcription termination signal of the *EPCAM* gene which causes abnormal transcriptional elongation of the *EPCAM* transcript into *MSH2*. The clinical phenotypes of patients with *MSH2* and *EPCAM* mutations are similar with a distinction of decreased lifetime risk for extracolonic cancers in patients with *EPCAM* mutations but increased risk for endometrial cancer in cases where *EPCAM* mutation is located close to *MSH2* (127).

Patients with *MSH6* and *PMS2* mutations are less frequent and represent more distinct clinical phenotypes. Patients with *MSH6* mutations have a later age of onset and substantially lower cumulative risk for CRC (10-22%) and other cancers when compared to *MLH1* and *MSH2* mutation carriers (128). However, female *MSH6* mutation carriers have been suggested to have a higher risk for endometrial cancer compared to carriers with other MMR gene mutations (129). *MSH6* deficient tumors show lower level of MSI in general with consistent MSI only in association with mononucleotide repeat sequences (130). Clinical phenotype caused by germline mutations in *PMS2* is described as attenuated with later age of onset (131). The risk of developing CRC and other LS associated cancers is significantly lower (~20% and ~15% for CRC and endometrial cancer, respectively) in *PMS2* mutation carriers (132). In rare cases, germline mutations in *MLH3* and *MSH3* have been associated in disease phenotype but their pathogenic role remains unclear (133-135). Mutations in these genes have been suggested to behave as low-risk alleles that contribute to CRC risk in LS patients, mostly together with other low-risk variants of MMR genes.

Currently, identification of LS involves tumor analysis including MSI testing and/or immunohistochemical staining (IHC) of MMR proteins, and *BRAF* mutation testing in *MLH1* negative cases (136). $BRAF^{V600E}$ mutation is associated with *MLH1* promoter hypermethylation in sporadic MSI-CRC and therefore it is known to reduce the possibility of LS (137). Germline genetic testing involves DNA sequencing and gene arrangement analysis of all coding exons and exon-intron boundaries of MMR and the *EPCAM* genes. However, genetic testing fails to identify a germline MMR gene mutation in up to 30% of families that have LS-like phenotype with tumors that manifest MSI and absence of MMR protein (138). While a substantial proportion of such cases appears to be explained by two somatic mutations of MMR genes, it is possible that some of these patients have germline mutations affecting MMR gene expression in promoters or intronic sequences that are not detected by currently used methods. Moreover, alternative unknown mechanisms that inactivate MMR and cause similar clinical phenotypes of LS may exist.

Furthermore, ~50% of patients with family history of CRC that meet ACI but have no evidence of MSI or detectable MMR deficiency in observed tumors, are classified to have familial colorectal cancer type X (FCCX) (139). These patients have similarly increased risk for CRC with slightly higher age of onset and no association with extracolonic tumors. Consequently, “type X” refers to the largely unknown genetic etiology and it is likely that FCCX is a group of conditions with heterogeneous genetic background. Recent studies

have identified mutations associated with FCCX in several different genes which seem to affect only one or few families (e.g *BMPR1A*, *RPS20* and *SEMA4A*) (140-142).

Familial adenomatous polyposis (FAP)

Polyposis syndromes are classified based on clinical features in combination with the pathology of the polyps. Familial adenomatous polyposis (FAP) is inherited in autosomal dominant manner and it is the most common polyposis syndrome accounting for less than 1% of all CRCs (143). It is characterized by the development of hundreds of colorectal adenomatous polyps beginning in late childhood or early adolescence and extremely high risk (~100%) for early-onset CRC (144). Adenomatous polyps, most commonly described as tubular adenomas, develop throughout the colon with a slightly higher incidence in the distal parts. About half of all patients develop adenomas by the age 15 and 95% by the age 35 (145). In addition to colorectal polyps, patients are predisposed to duodenal polyps and adenomas, but the risk for duodenal cancer is substantially lower compared to CRC risk (<10-15%) (146). Moreover, the risk for cancer in other organs such as the brain, liver and thyroid is slightly increased (147).

The disease is caused by pathogenic heterozygous germline mutations in the *adenomatous polyposis coli* (*APC*) tumor suppressor gene, which was first described in 1991 (148, 149). The development of tumors follows the inactivation of the remaining wildtype allele by somatic mutations, which is considered an early event in CRC tumorgenesis. Indeed, *APC* plays a critical role in the etiology of sporadic CRC, and mutations in the *APC* gene are found in 70% of all CRCs. Tumorigenic pathway that follows inactivation of the wild type allele in FAP resembles sporadic colorectal carcinogenesis, where tumor development is often driven by mutations in *KRA*S and *TP53* (150).

APC is a large gene with 15 exons that encode a protein of 2843 amino acids that plays a significant role in the *Wnt* signaling pathway (151). The APC protein negatively regulates β-catenin oncoprotein by directing the ubiquitination and degradation of β-catenin in the cytosol. In the absence of APC, β-catenin migrates and accumulates into the nucleus and up regulates the transcription of genes involved in cell cycle entry, proliferation, differentiation and apoptosis (152). Moreover, APC has a dual role in stabilization of microtubules in the nucleus which contributes to chromosomal stability (153).

More than 1100 of likely pathogenic mutations and over 3000 *APC* variants have been reported in the InSiGHT database (http://www.lovd.nl/apc). Of all pathogenic mutations 95% are nonsense mutations and small insertions or deletions that lead to truncated protein (154). The severity of the syndrome depends on which region of the *APC* gene is mutated; germline mutations associated with the classical FAP phenotype locate in exons 5-8, 9-14 and the first half of the large exon 15 (155). The region between codons 1250 and 1464 of exon 15, called the mutation cluster region (MCR), is the most common target of both germline and somatic mutations (143). Most of the pathogenic *APC* mutations are

inherited, but 11-25% of FAP cases are sporadic with no family history of disease caused by *de novo* mutations (156).

Mutations that are localized in either end of the gene or in the alternatively spliced region of exon 9 are associated with a less severe phenotype known as attenuated FAP (AFAP) (157). Mutations that cause AFAP encode almost full-length protein which provides explanation for the milder phenotype. AFAP is characterized by a reduced number of colorectal polyps (10-100), a later age of onset and lower CRC risk (up to 70%). Currently, direct sequencing of all 15 exons of the *APC* gene is the standard method for mutation detection and accounts for >85% of all detected mutations including point mutations and small insertions and deletions (158). The remaining mutations are large deletions or duplications that can be detected by other methods such as multiplex ligation-dependent probe amplification (MLPA) (159).

Even though genotype-phenotype correlation is observed, considerable variation in clinical phenotype exists among individuals carrying the same germline mutations which suggests contribution of alternative mechanisms and/or environmental effects (160). Moreover, in up to 30% of FAP patients no germline mutations are detected (161). Regulation of the absolute gene expression and differences in allele-specific expression (ASE) may play a role in phenotypic variability in cases with and without pathogenic mutations but the exact mechanisms remain unknown (162).

Intensive surveillance is essential for patients with FAP since without surveillance and timely treatment, one or several of the adenomas will inevitably progress into cancer. The regular surveillance consists of sigmoidoscopy or colonoscopy every 1-2 years depending on the findings, starting at age 10-15 (163).
Surgery is the only effective treatment for FAP that prevents malignant changes in colorectal adenomas. Restorative proctocolectomy (RPC) is recommended as the primary choice for prophylactic surgery for FAP patients (164). Establishment of worldwide polyposis registries and surveillance programs has reduced CRC mortality substantially and other malignancies are currently the main cause of death in FAP patients (165, 166).

LIFESTYLE AND DIETARY RISK FACTORS

Environmental factors have a profound influence on the development of cancer whereas only a minority of cases are of hereditary origin (Fig. 3). Lifestyle and diet are known to contribute to cancer risk which makes them important issues to be considered by the general population and the medical community. Rates of different types of cancers varies geographically, highlighting the role of environmental factors in the development of cancer in general (167). Diet, together with age and hereditary factors, is noted to be the most important risk factor for CRC in particular, since the colorectal mucosa is in direct contact with dietary components and constantly exposed to diet-induced metabolic and physiological changes (Fig. 4) (9). However, addressing the mechanisms between diet and colorectal cancer has proven to be difficult due to the complex interactions between nutrients, genes and metabolism. Up to 50-70% of CRCs have been estimated to arise due to lifestyle and dietary effects and to be preventable by changes in these habits (168, 169). At present, recommendations for CRC prevention are based on hypotheses and epidemiological evidence linking lifestyle and dietary factors with CRC incidence since the complex network of the causes of CRC have not been completely unraveled. The main lifestyle and dietary risk factors that are thought to contribute to CRC risk are introduced in this chapter.

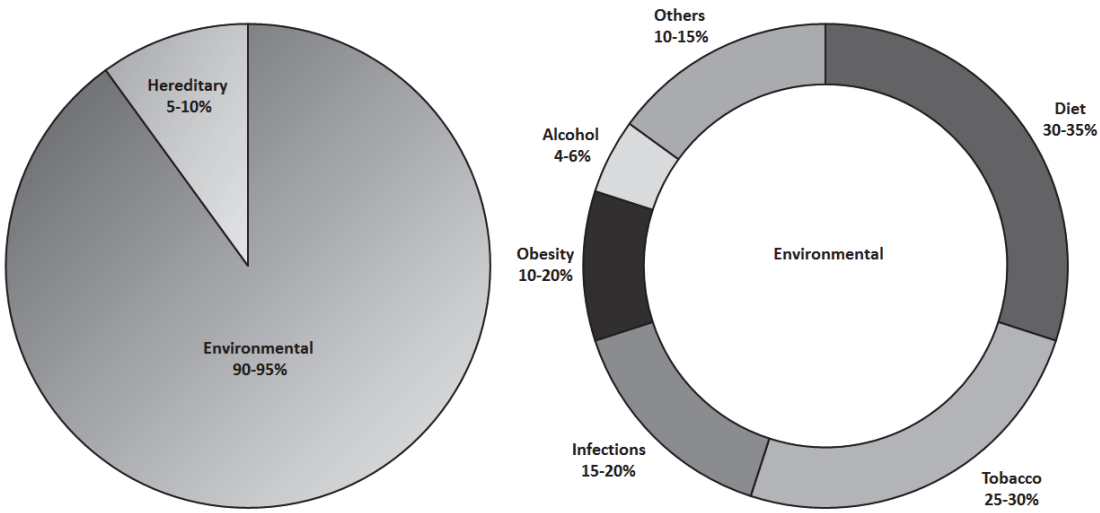

Fig. 3. Effect of hereditary and environmental risk factors in the development of cancer. Modified from Anand *et al.* 2008 (167).

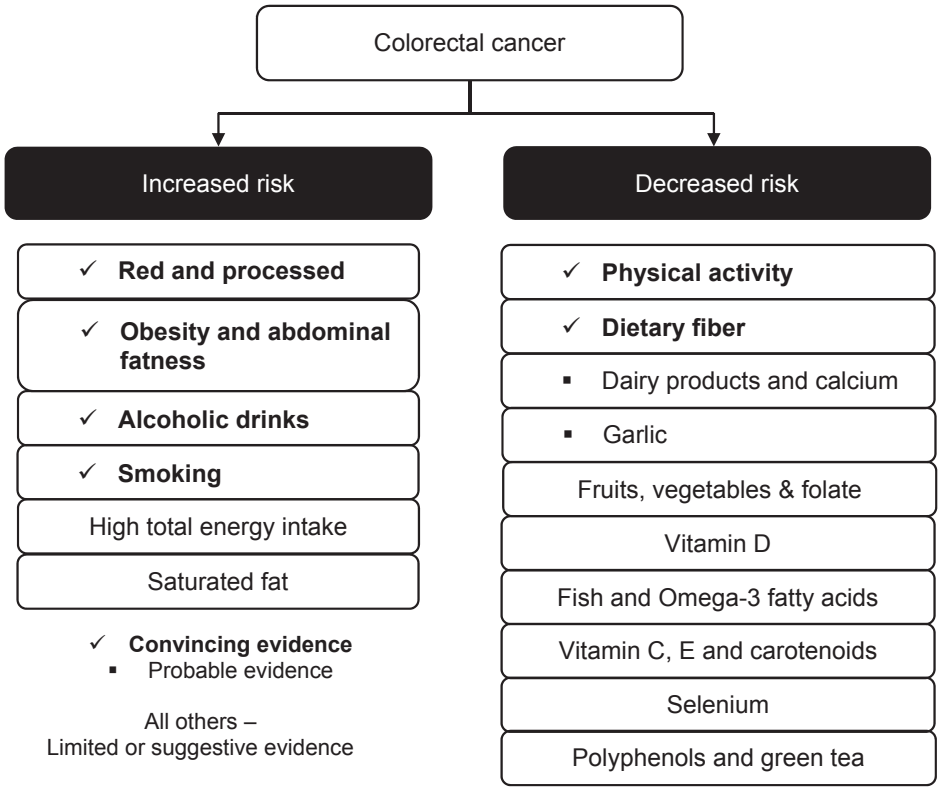

Fig. 4. Lifestyle and dietary risk factors for CRC. Modified from the AICR/WCRF Colorectal Cancer Report (1).

Smoking and alcohol consumption

High alcohol consumption appears to be a major risk factor for gastrointestinal tumors. Acetaldehyde, which is the primary metabolic product of ethanol, is a highly cytotoxic compound with carcinogenic and mutagenic activity which can damage colorectal mucosa and elevate cell proliferation (170). Alcohol is known to act as a direct inducer of cytochrome P-405 2E1 (CYP1A2) enzyme which is involved in metabolizing unknown chemical compounds in the body and increasing the production of free radicals as side products. In addition, alcohol causes reduced absorption of other B vitamins (B1, B2, B12, folate) which increases vulnerability to oxidative stress (171). Alcohol has been estimated to contribute to approximately 17% and 4% of total CRC incidence in males and females, respectively, when consumption exceeds the recommended upper limit (two drinks a day for men with about 24 g alcohol, one for women with about 12 g alcohol) (172).
In addition to alcohol consumption, cigarette smoking is thought to be another major lifestyle factor related to increased CRC risk. In a previous study, positive association was observed between smoking and CRC risk, but induction period of 30 years of smoking was

required for significant association (173). Smoking was also found to double the risk for colorectal adenomas. Tobacco smoke contains carcinogenic compounds such as acetaldehyde, benz-pyrenes, aromatic amines and N-nitrosamines that can bind DNA and disrupt normal cell functions (174).

Obesity and physical activity

Obesity, defined as body mass index (BMI, kg/m^2) greater than 30, is associated with increased CRC mortality (175). Individuals with BMI higher than 30 show 19% increased risk for CRC when compared to those with BMI between 20 and 25 (176). Increased wealth and modernization in the Western countries have been associated with the increased prevalence of overweight individuals and increased incidence of CRC. Indeed, epidemiological evidence demonstrates a higher risk for CRC in association with excess intake of total energy (177, 178).

Interestingly, exercising one hour per week was associated with a lower prevalence of colonic polyps and adenomas when compared to people who exercised less or not at all in a multiethnic screening cohort (179). A previously conducted multi-cohort study demonstrated a 24% decrease in CRC risk in physically active individuals when compared with individuals with a more sedentary lifestyle confirming the inverse association between physical activity and CRC in both men and women (180). Moreover, it has been estimated that 30-60 min of moderate to vigorous daily exercise is needed to protect against CRC (181). Physical activity is thought to improve metabolic efficiency and tissue oxygenation which leads to reduced body fat and insulin levels, whereas the lack of exercise is associated with low-grade chronic inflammation and high levels of estrogen and insulin which are known to promote excess proliferation of epithelial cells (182). Moreover, physical inactivity increases the gastrointestinal transit time and the duration of direct contact between possible carcinogens and the epithelium (167).

High consumption of dietary fat has been associated with increased prevalence of obesity due to increased intake of energy dense foods (183). Epidemiological studies concerning dietary fat consumption in relation to CRC incidence show discrepancies and the association has not been proven (184, 185). It is possible, that increased consumption of certain types of fatty acids may be more relevant for the development of CRC than the total amount of dietary fat. For example, consumption of n-3 polyunsaturated fatty acids have been suggested to associate inversely with CRC (186, 187). Polyunsaturated n-6 fatty acids act as substrates for eicosanoid production, which can activate pro-inflammatory pathways that promote colon carcinogenesis (186). Therefore, the ratio of n-6 to n-3 fatty acids has been suggested to be particularly important. Moreover, consumption of high-fat diet is known to result in significantly higher excretion of secondary bile acids in the gastrointestinal tract in order emulsify the increased level of dietary fat (188). Bile acids are implicated to be important in the etiology of CRC since they are known to induce reactive oxygen/nitrogen species (ROS/RNS) which lead to increased DNA damage and stimulation of apoptosis (189).

Multiple biological processes are known to contribute to the obesity and cancer relationship. Increased body fat is known to have a direct effect on physiological hormonal levels such as insulin, leptin, estrogen and insulin like growth factor 1 (IGF1), which can promote insulin resistance, inflammation and decreased cellular apoptosis (190). Abdominal fat, in particular, is known to increase insulin resistance and subsequent hyperinsulinemia which increases risk for CRC (1). The balance and interaction between all of these processes take part in determining the physiological cancer risk.

Western-style diet

Notable geographical variation in worldwide CRC incidence suggests significant contribution of lifestyle and dietary factors to the etiology of the disease. In addition to age and hereditary factors, dietary habits of Western population in particular are considered to promote development of CRC. Positive correlation between CRC incidence and a typical Western-style diet was first reported in the beginning of the 1980s (191). This risk was acknowledged in 2007 by the World Cancer Research Fund (WCRF) and The American Institute for Cancer Research (AICR) (192). Based on epidemiological evidence and cohort studies it was concluded that high intake of total energy, red and processed meat and high consumption of alcohol together with low intake of fiber, calcium, vitamin D, folic acid and selenium associates with increased risk for CRC. On the contrary, high intake of dietary fiber, non-starchy fruits and vegetables and fish were reported to decrease CRC risk. Moreover, consumption of a Western-style diet characterized by high intake of fat, sugar, red and processed meat was shown to decrease survival and increase risk for CRC re-occurrence when compared to diets including more fiber and less fats and sugar (193).

In 2011, a multicenter cohort study based on the European Prospective Investigation into Cancer and Nutrition (EPIC) data revealed significant inverse association between intake of cereal fiber and whole grains and CRC, strengthening the evidence that consumption of dietary fiber can prevent CRC (194). Fiber is thought to play an important role in binding and carrying waste products and cytotoxic compounds such as bile acids in the gastrointestinal tract, accelerating intestinal transit and reducing the time of exposure to luminal carcinogens (195). Butyrate, which is an intestinal microbiota metabolite of dietary fiber, has been shown to have cancer-preventive effects through its ability to modulate of cell-cycle arrest and apoptosis (196, 197). Butyrate is a short-chain fatty acid that is produced in the colonic lumen during bacterial fermentation of dietary fiber. Interestingly, butyrate producing bacteria have been shown to be underrepresented in CRC patients (198).

Considerable number of studies have identified high consumption of red and processed meat as a risk factor for CRC. Underlying mechanisms mainly thought to explain this association include increased fat intake together with meat, mutagens formed during cooking at high temperatures (heterocyclic aromatic amines, polycyclic aromatic hydrocarbons), heme iron and nitrite (199). Indeed, dietary heme has been shown to promote formation of colonic neoplastic lesions in rats (200).

Many epidemiologic studies suggest inverse effect of calcium and vitamin D intake on CRC risk, however results are inconsistent (1). Studies on animal models have shown them to contribute in regulating anti-neoplastic processes that suppress hyperproliferation and induce differentiation and apoptosis of colonic epithelial cells (201). Extensive evidence from *in vitro* studies support calcium and vitamin D to have direct anti-proliferative and apoptosis-inducing effects on normal colonic epithelial cells (202-204). Moreover, anti-proliferative effects of calcium are thought to be at least partially caused by its capability to bind dietary fatty acids and bile acids which reduces the carcinogenic effects of these compounds on colonic mucosa (205). However, in order to understand the combinational effect of calcium and vitamin D in chemoprevention, the molecular mechanisms that support their interplay and mediate protection against CRC remain to be elucidated.

Folate is a term used for a group of essential B9 vitamin compounds that maintain genomic stability by regulating DNA biosynthesis, repair and methylation. Folate is present at high concentrations in green leafy vegetables whereas folic acid (FA) is a synthetic oxidized form of folate that is primarily used in supplements. Limited folate results in decreased DNA pre-cursors purine and pyrimidine that are required for DNA synthesis and repair (206, 207). Folate deficiency accelerates carcinogenesis by causing deficiencies in these processes and is implicated in several cancers including CRC (208). In addition, deficiency causes altered cytosine methylation which can lead to global hypomethylation and/or gene-specific changes in methylation patterns that can cause inappropriate activation of proto-oncogenes (209). The role of folate and FA in CRC development remains controversial. Evidence from majority of epidemiological studies suggest high dietary intake of folate and subsequently elevated blood concentration to significantly reduce the risk of developing colorectal neoplasias (210-212). However, more recent data from clinical trials indicate that excessive intake of synthetic FA from supplements may actually increase the risk for CRC by accelerating the growth of pre-neoplastic lesions (213, 214). Since folate acts as a rate limiting factor for DNA synthesis and potential growth factor, administering high doses of FA at an inappropriate time may accelerates the growth of existing neoplastic cells (215).

MOUSE MODELS IN CRC STUDIES

The laboratory mouse (*Mus musculus*) is one of the best and most commonly used experimental model in biomedical research because of the availability of its genomic information and well-established techniques to generate transgenic and knock-out mice by targeted mutagenesis. These models are essential to study the mechanisms of pathogenesis and to establish therapeutic and preventive measures for cancer and other diseases. Different models have been developed to study alternatively expressed genes and pathways related to CRC to provide understanding of the human conditions. Genetically engineered mouse strains have been developed for hereditary colorectal cancer syndromes such as LS and FAP. Colon cancer and polyposis phenotypes studied in mice have revealed many similarities with disease phenotypes in human.

Apc mutant mice

The genetically engineered Apc^{Min} mouse was the first mouse strain generated to model human intestinal cancer (216). The *Min* (multiple intestinal neoplasia) mice carry a truncating mutation at the position 850 of the *Apc* gene (217). In contrast to human FAP, the *Apc* mice develop polyps mainly in the small intestine. Heterozygotic Apc^{Min} mice typically develop approximately 30 polyps mostly in the small intestine, by 100-120 days of age. Several additional *Apc* mutant strains with slightly varying phenotypes have been constructed since using gene knock-out technology. These include *Apc* 716, 1638 and 1309 mice strains that carry truncating mutations in the respective codons (218-220). Studies based on $Apc^{\Delta 716}$ mice showed that polyp formation is initiated by loss of heterozygozity (LOH) of the *Apc* locus in proliferative cells (219).

Mouse models have been utilized for investigating effects of dietary components on CRC risk. Studies based on especially Apc^{Min} mice have provided evidence that several bioactive food components may inhibit CRC in genetically predisposed mice while others may increase the risk. For example, a 60% calorie-restricted diet was shown to decrease the amount of intestinal polyps in Apc^{Min} mice by 60% which supports observations from human studies (221). Mechanistically, decreased amount of total energy is recognized to reduce cell proliferation and inflammation and increase apoptosis (222). Similarly, the consumption of certain fatty acids such as steraridonic acid (SDA), eicosapentaencic acid (EPA) and docosahexaenoic acid (DHA) were shown to reduce tumor number in the small intestine of Apc^{Min} mice (223). Consequently, the ratio of n-6 and n-3 fatty acids may contribute to CRC risk since low relative ratio results in decreased proinflammatory products arising from n-6 fatty acids which slows down cell proliferation and decreases the amount of detected tumors (224).

When saturated fat content was increased from 3% to 10% and 15% tumor number increased 28% and 47% in *Apc*^{Min} mice, respectively (225). Similarly, feeding high fat and low fiber diet was found to increase tumor formation in *Apc*$^{\Delta 716}$ mice (226) whereas a Western-style diet with high fat, low calcium and vitamin D was found to accelerate tumor development in *Apc*^{1638N} mice (227). In line with studies with healthy human populations, exercise was found to reduce the number of polyps in the *Apc*^{Min} mice by 30-40% (228).

Interestingly, natural compounds such as sulforaphane, derived from cruciferous vegetables, chafuroside, a derivative in oolong tea, and curcumin, which is a common plant phenolic compound, have been reported to cause a significant reduction of intestinal tumors in *Apc*^{Min} mice (229-231). Sulforaphane is known to decrease proliferation and induce apoptosis in a variety of mammalian cell lines whereas chafurosid has been shown to increase apoptosis, reduce inflammation and protect against free radicals (232, 233). Curcumin is believed to act as an antioxidant and anti-inflammatory factor through modulation of multiple signaling pathways (234).

MMR deficient mice

Mouse models with disrupted mammalian *MutL* and *MutS* homologs have been generated to study the effects of MMR defects on cancer predisposition. Analysis of the mouse models has revealed that the basic mechanisms of DNA repair are similar in mice and human and phenotypes of MMR knockout mice correlate with the DNA repair defects and the mutation frequency of the Lynch syndrome patients (235). However, unlike human *MLH1*, *MSH2* and *MSH6* mutation carriers, heterozygous mice rarely develop early-onset tumors (<1 year old) (236). Knock-out mice with homozygous mutations are predisposed to cancer with a tumor spectrum that includes gastrointestinal cancers, but unlike LS patients, majority of mice die prematurely due to T-cell lymphoma which is the predominant malignancy in mice (236). Differences in phenotype and tumor incidence between mice and human are likely explained by the smaller size and shorter life span of mice (237). Consequently, somatic loss of the wildtype allele and subsequent tumorigenesis are more likely to happen during a longer period of time in humans whereas tumorigenesis in mice is more unlikely. Differences in organ manifestation is suggested to be caused by the absence of many coding mononucleotide repeats (cMNR) in the mouse genes which makes them less vulnerable to MSI effects (237).

Heterozygous *Mlh1*$^{+/-}$ mice have been shown to have reduced longevity caused by lymphomas and to a lesser extent develop tumors in the intestinal tract (238). Of heterozygous *Mlh1*$^{+/-}$ mice half have been reported to die by the age of 18 months and approximately one third develop tumors in the intestine and other organs. Knock-out mice with homozygous mutations develop a more aggressive cancer predisposition phenotype including a spectrum of different types of tumors including intestinal cancer. MMR deficiency in *Mlh1*$^{-/-}$ mice causes a significant reduction in lifespan and a severe cancer predisposition phenotype including development of T-cell lymphomas by 6 to 8 months of age and development of small intestinal adenomas and carcinomas and skin cancer

slightly later by 6 to 12 months of age (239, 240). Similarly, rare human patients with biallelic mutations in *MLH1*, *MSH2* and *MSH6* are prone to hematological malignancies (241). Tumors arising in mice with homozygous mutations display MSI in both mono- and dinucleotide repeats at high frequency similar to human mutation carriers (242). Moreover, loss of *Msh2* in homozygous *Msh2*$^{-/-}$ mice causes MMR deficiency with a severe mutator phenotype (243). Similar to *Mlh1*$^{-/-}$ mice, tumor spectrum of these mice includes T-cell lymphomas and intestinal adenomas and carcinomas with MSI phenotype.

Msh6$^{-/-}$ mice have a longer lifespan (up to 18 months) and develop a similar cancer predisposition phenotype to *Mlh1*$^{-/-}$ and *Msh2*$^{-/-}$ mice with a delayed age of onset and lack of mononucleotide repeat instability and low level of dinucleotide instability (244). Similar to *MSH6* human mutation carriers *Msh6*$^{-/-}$ mice are predisposed to developing endometrial cancer (245, 246). *Pms2*$^{-/-}$ mice have a delayed age of onset with predisposition to hematological malignancies but not to gastrointestinal tumors (247). In *Pms2*$^{-/-}$ mice MSI occurs in both mono- and dinucleotide repeats and compared to other MMR-knockout mice increased frequency of frameshift mutations is observed (248, 249). The differences in the phenotype and tumor spectrum are most likely to be caused by the milder mutator phenotype resulting from a partial repair defect caused by *Pms2* deficiency. This is in line with observations from human *PMS2* mutation carriers that have an overall lower risk for cancer and a later age of onset (131).

To develop mouse models that more accurately mimic the cancer phenotype and tumor spectrum of LS patients, MMR knock-out mice have been intercrossed with mice carrying additional mutations in tumor suppressor or oncogenes frequently mutated in human CRC such as *APC*, *TP53* and *KRAS* (250). For example, the combination of homozygous mutations in MMR genes and heterozygous *Apc* mutations accelerates the tumor development to the intestinal tract. However, these mice display MMR deficiency in all tissue types in contrast to LS patients. More recently, mice with conditional disruption of *Msh2* were generated using mice that express transgenic intestine specific *Villin-Cre* recombinase and *LoxP* sites which together induce the deletion of exon 12 and MMR deficiency limited to the intestinal epithelium (251). In this model tumorigenesis was restricted to the intestinal tract and mice typically developed 1-2 intestinal carcinomas pathologically identical to LS tumors during the first year of their life and lacked the lymphoma phenotype completely.

AIMS OF THE STUDY

Both lifestyle and diet together with genetic and epigenetic factors are known to contribute the increased risk for CRC but the molecular mechanisms that initiate tumor development remain unknown. The main aims of this investigation were to 1) study the effects of Western-style diet on normal appearing colonic mucosa and to identify molecular mechanisms underlying the WD-induced risk for CRC, and 2) to define the timing of molecular changes occurring during transformation from normal colonic mucosa to pre-cancerous and cancerous lesions from patients with hereditary CRC.

More specific aims were:

- To study whether and how Western-style diet and genetic predisposition may separately or in interaction effect the proteome of histologically normal colonic mucosa in mouse models for Lynch syndrome and sporadic CRC (I-II).

- To define how CpG island methylation changes throughout the adenoma-carcinoma progression sequence in relation to MMR protein expression in human LS mutation carriers (III).

- To investigate possible carcinogenic field defects in the normal appearing tissue (I-III).

METHODS

MOUSE MODELS AND DIETS (I-II)

All mice included in this study were bred and treated according to the study protocol approved by the National Animal Experiment Board of Finland (ESLH-2008-06502/Ym-23). *Mlh1*$^{+/+}$ and *Mlh1*$^{+/-}$ mice with C57BL/6 background (240) were randomly assigned into two dietary groups at the age of 5 weeks. Study I included mice with both genotypes whereas study II focused on wild-type *Mlh1*$^{+/+}$ mice only (Fig. 5). Groups contained even numbers of females and males. Preceding the division to diet groups at 5 weeks (5w) of age, mice from each genotype group were sampled forming a baseline for the experiment (Fig. 5). The remaining mice were assigned to different diet groups and fed *ad libitum* with AIN-93G (AIN) control diet (252) or Western-style diet (WD) (Harlan Teklad, Madison, WI) with 12 hours light/dark circle (Fig. 5). AIN-93G control diet is a semisynthetic diet designed to meet the nutritional requirements of growing rodents whereas WD is a modified AIN-93G diet designed to reflect the nutritional content of average human Western-style diet (Table 2). Of total energy content fats comprise 39% and 17% and carbohydrates 42% and 64% in WD and AIN-93G, respectively.

Before division to diet groups genotypes were confirmed according to NCI – Mouse Repository protocol (mouse.ncifcrf.gov) using genomic DNA extracted from earmarks (DNeasy Blood & Tissue Kit, Qiagen, Germany). Briefly, PCR reaction contained primers M001 (5'-TGT CAA TAG GCT GCC CTA GG -3') (0.33μM), M002 (5'-TGG AAG GAT TGG AGC TAC GG -3') (0.33μM), and M003 (5'-TTT TCA GTG CAG CCT ATG CTC -3') (0.3μM). Amplicons M001 – M002 (500bp) and M001 – M003 (350bp) correspond to non-functional and functional *Mlh1* allele, respectively.

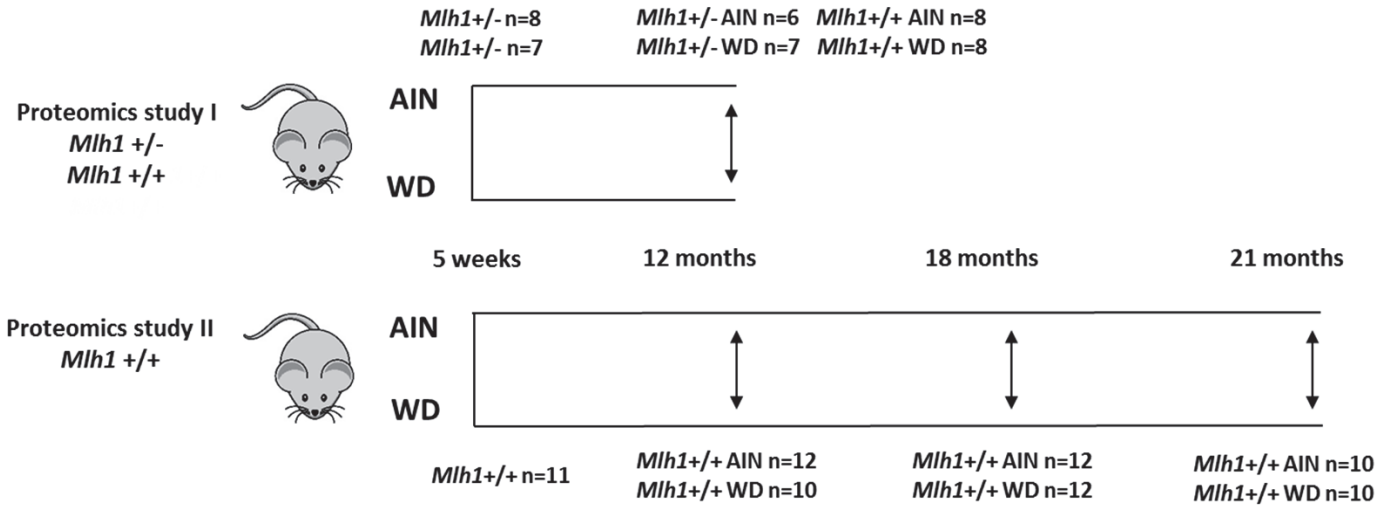

Fig. 5. Experimental design of the proteomics studies I-II

Table 2. Composition of the experimental diets.

Compound	AIN-93G[1] [g/kg][2]	WD[1] [g/kg][2]
Protein [% of energy]	**19**	**19**
Casein	200	232
L-cystine	3.0	3.0
Carbohydrates [% of energy]	**64**	**42**
Corn starch	392	305
Malodextrin	132	95
Sucrose	100	116
Cellulose	50	20
Fat [% of energy]	**17**	**39**
Anhydrous milk fat	-	133
Canola oil	-	55
Sunflower oil	-	12
Soybean oil	70	-
kcal/g	**3.8**	**4.6**
Calcium Phosphate	5.0	1.7
Folic acid	0.2	0.0003
Vitamin D3 (500 000 IU/g)	0.2	0.0003

[1]Irradiated for study II

[2]If not stated otherwise

Mice were sacrificed and sampled at the age of 12, 18 and 21 months. The colon was removed, opened, rinsed with 0.9% sodium chloride solution, inspected for neoplasia under a microscope and divided horizontally into pieces. All neoplastic/hyperplastic lesions were collected into formalin fixed paraffin (FFPE) blocks and verified at Finnish Center for Laboratory Animal Pathology, University of Helsinki. Proximal colonic mucosa (6x4 mm) for DNA, mRNA, protein and metabolite studies was separated from the underlying submucosa and muscular layer under a dissecting microscope. Since LS tumors have a tendency to arise in the proximal part of the colon, the present study concentrates on expression changes in the respective part of the mouse colon. Colonic mucosa for protein extractions was rinsed with 10 mM Tris and 5mM magnesium acetate solution (pH 8.5) (Sigma-Aldrich, Germany), snap frozen and stored at -80°C. Samples for RNA extraction were stored in RNAlater (Qiagen) at -80°C.

A more detailed description of the methodology used in this study including specifications of primers, probes, reaction conditions and MS parameters can be found in the original publications (I-III). A summary of the methods used in this study is presented in Table 3.

Table 3. Summary of methods.

Methods	Publications
DNA, RNA and protein extractions and quantification	I-III
PCR-based genotyping	I-II
Two-dimensional difference gel electrophoresis (2D DIGE)	I-II
Matrix-assisted laser desorption ionization coupled with mass spectrometry (MALDI-MS)	I-II
Liquid chromatography coupled with mass spectrometry (LC-MS)	II
Quadropole time of flight mass spectrometry (Q-TOF-MS)	II
Ingenuity Pathway Analysis (IPA)	I-II
Extraction of bile acids from tissue samples	II
Ultra performance liquid chromatography (UPLC)	II
Immunohistochemistry (IHC)	II-III
DNA bisulfite conversion	III
Bisulfite sequencing	III
Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA)	III
Microsatellite instability (MSI) analysis	III
RNA profiling by microarray	III
Statistical analyses	I-III

QUANTITATIVE PROTEOMIC ANALYSIS (I-II)

2D Difference gel electrophoresis (2D-DIGE)

2D difference gel electrophoresis (2D DIGE) was used to study proteomic changes in colonic mucosa (Fig. 6). All reagents and equipment used for this purpose were provided by GE Healthcare (UK). Half of the samples of each experimental group were labeled with 1-(5-carboxypentyl)-1'-propylindocarbocyanine halide (Cy3) N-hydroxy-succinimidyl ester and the other half with 1-(5-carboxypentyl)-1'-methylindodi-carbocyanine halide (Cy5) N-hydroxysuccinimidyl ester fluorescent dyes (400 pmol of dye for 50 µg of sample) according to manufacturer's instructions. An internal standard was created by pooling 25µg of each protein sample and labeled with 3-(4-carboxymethyl)-phenylmethyl-3'-ethyloxacarbocyanine halide (Cy2) N-hydroxysuccinimidyl ester. Proteins were separated according to their isoelectric point using 24cm long immobilized pH 3-11 non-linear gradient (IPG) strips and the Ettan IPGphor II unit. Equal amount (50µg) of internal standard labeled with Cy2 was combined with Cy3 and Cy5 labeled samples and loaded into the IPG strips using cup-loading method according to manufacturer's instructions. After isoelectric focusing IPG strips were equilibrated with 1% dithiothreitol (DTT) and 2.5% iodoacetamide. Separation of proteins according to their molecular mass was carried out by transferring the equilibrated IPG strips to 12.5% SDS-PAGE gels and running with constant power of 15 W per gel. Images were acquired by Typhoon 9400/Trio scanner with excitation/emission values 480/530 nm, 520/590 nm and 620/680 nm for Cy2, Cy3 and

Cy5, respectively. Maximum intensities between channels were optimized to differ less than 20 - 30%. Data analysis was performed using DeCyder 2D 7.0.

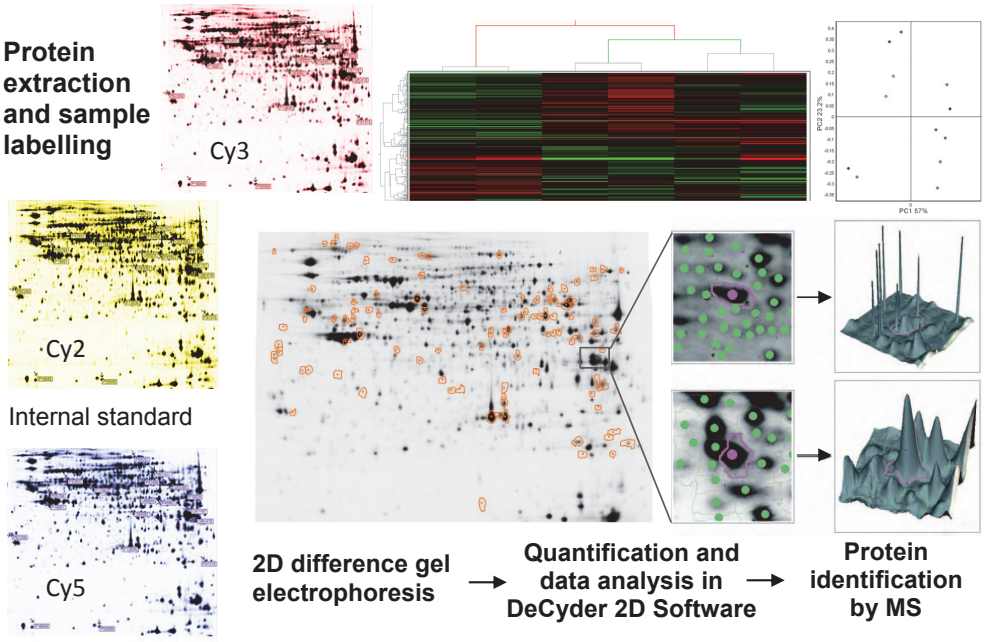

Fig. 6. Overview of the 2D DIGE method. Modified from 2-D Electrophoresis: Principles and Methods Handbook, GE Healthcare.

Protein identification and validation

Scanned 2D DIGE gels were silver-stained using PlusOne Silver staining kit (GE Healthcare). Proteins of interest were excised and in-gel digested by trypsin (Trypsin gold, Promega, Sweden). MALDI-MS and MALDI-MS/MS analyses were performed with Autoflex III and UltraFlexTreme™ (Bruker Daltonics, Germany) for studies I and II, respectively. Calibration was externally performed with peptide calibration standard (Bruker Daltonics). MALDI-MS and MS/MS spectra were acquired by accumulation of 5000 - 7000 laser shots and 10 000 – 20 000 laser shots, respectively. Processed spectra were searched against UniProt/SwissProt database (www.uniprot.org) with taxon: *Mus musculus* using Mascot server (Matrix Science, www.matrixscience.com) and FlexAnalysis and BioTools software (Bruker Daltonics).

In study II, results obtained with 2D DIGE were validated by analyzing pools of total protein extracts with quantitative liquid chromatography tandem mass spectrometry (LC-MS). Pools were created by combining 10 µg of total protein extract from individual mice of each experimental group. Of created protein pools 10 µg was used for digestion that was carried out using a modified Filter Aided Sample Prep (FASP) protocol (253). Triplicates of digested proteins (200ng) were injected for LC-MS analysis. Relative quantification between samples using precursor ion intensities was performed with TransOmics™ Informatics for Proteomics software (Nonlinear Dynamics, Waters) and ProteinLynx Global Server (PLGS V3.0). Chromatograms were aligned by the TransOmics™ software using the default settings. Alignment scores ≥ 70% were selected for further analysis. Database searches were carried out against UniProtKB-SwissProt (release 2014_04, taxon: *Mus musculus*, 32538 entries).

PATHWAY ANALYSIS (I-II)

In study I, gene ontology (GO) annotations were analyzed with the Panther Protein Classification System (http://www.pantherdb.org/) to distinguish significantly enriched functional annotations from the proteomics data. The corresponding GO terms were used to classify protein functions. GO biological process (GO BP) criteria were further applied to analyze the enriched networks and to maximize connectivity between all input genes in a given ontology class. In study II, proteomics data were analyzed with Ingenuity Pathway Analysis (IPA) (Qiagen) to identify enriched pathways and upstream regulators. Heat maps of significant z-scores were generated for upstream regulators, diseases and functions and classical pathways using R version 3.2.0 (2015-04-16) and NMF package.

HISTOLOGICAL ANALYSIS OF COLONIC CRYPTS (II)

The length was measured and the number of cells was counted from five colon crypts per mouse in blinded manner using hematoxylin and eosin (HE) stained proximal colonic tissue sections. Proliferation index (Ki67 index) was studied by immunohistochemistry (Anti-Ki67, 1:1000, ab15580, Abcam, UK). Slides were scanned with Pannoramic 250 Flash II (3D Histech, Hungary); visualization and analysis was performed with CaseViever (3D Histech). Proliferation index was calculated by dividing the number of Ki67 positive cells with the number of total cells within a crypt.

EXTRACTION AND QUANTIFICATION OF BILE ACIDS (II)

Bile acids were extracted from proximal colon mucosa in ethanol as described (254). Briefly, lyophylizated samples were homogenized in TissueLyser (Qiagen). Nordeoxycholic acid (NDCA) (Steraloids, USA) was added (0.5 µg) to the samples prior the ethanol extraction and used as an internal standard to monitor sample quality and normalization. Ultra performance liquid chromatography (UPLC) (Waters, Milford MA, USA) was utilized to measure the concertation of BAs in colonic tissue. UPLC system was interfaced with Waters Synapt G2 HDMS mass spectrometer (Waters, Milford MA, USA). Ionization was performed using an electrospray ionization (ESI) source. The ionization parameters were optimized with the internal standard, 23-nordeoxycholic acid (NDCA). The bile acids were identified by their m/z ratio and retention time and by running BA standards (NDCA, deoxycholic acid (DCA), cholic acid (CA), hyodeoxycholic acid (HDCA), chenodeoxycholic acid (CDCA), ursodeoxycholic acid (UDCA), lithocholic acid (LCA), isoLCA, alpha-muricholic acid (αMCA), βMCA, ωMCA, α-tauromuricholic acid (αTMCA), ωTMCA Steraloids, USA). The chromatograms and mass spectra were analyzed with Waters MassLynx™ Software (Waters, Milford MA, USA).

PATIENT SAMPLES (III)

Patient samples included in this study originated from the nation-wide Hereditary Colorectal Cancer Registry of Finland. Colorectal fresh frozen biopsies together with blood samples were gathered from Finnish LS mutation carriers during colonoscopy screenings and colectomies performed at Helsinki University Central Hospital and Jyväskylä Central Hospital during 10/2011–5/2013. Normal mucosa biopsies were collected from 1–4 distinct colonic regions in addition to biopsies from hyperplastic polyps, adenomas and carcinomas (Table 1/III). Half of each tumor was snap frozen in liquid nitrogen for subsequent DNA analysis while the remaining other half was submitted to histological analysis. This series formed the so called prospective series. DNA was extracted from the fresh frozen tissue biopsies using AllPrep DNA/RNA Mini Kit (Qiagen). The Institutional Ethics Board of Central Finland Health Care District approved the collection of biopsies during surveillance (K-S shp Dnro 10U/2011).

All previously diagnosed adenomas and carcinomas of LS patients included in the prospective series were gathered forming the retrospective series. Forty-three patients from the prospective series contributed to the archival specimens. The retrospective series consisted of colorectal tumors and, when available, matching normal mucosa (Table 1/III). The histology of tumors was verified by a gastrointestinal pathologist. Carcinoma sections used for DNA extraction contained 50% of tumor epithelium on average (range 30–80%). DNA was extracted from representative FFPE tissue sections according to the method of Isola *et al.* (255). The National Authority for Medicolegal Affairs (Dnro 1272/04/044/07) approved the collection of archival specimens.

MMR PROTEIN EXPRESSION (III)

Immunohistochemistry was used to measure MMR protein expression of the gene mutated in the germline in all neoplastic lesions collected from LS mutation carriers. Four-micrometer sections were cut from FFPE tissues and processed for immunohistochemistry. Specimens were immunohistochemically stained with MLH1 (1:50, clone ES05, Dako North America, Carpinteria, CA), MSH2 (1:1000, clone G219-1129, BD Pharmingen, San Diego, CA), MSH6 (1:100, clone EP49, AC00-47, Epitomics, Burlingame, CA), and PMS2 (1:1000, clone EPR3947, Abcam, UK) antibodies. MLH1, MSH2, MSH6 were visualized with Ventana BenchMark XT immunostainer using OptiView detection system and PMS2 using OptiView + Amplification detection system (Ventana Medical Systems, Tucson, AZ). Negative cancer cell immunostaining was interpreted to indicate inactivation of the respective MMR gene.

MICROSATELLITE INSTABILITY (MSI) ANALYSIS (III)

Tumor specimens were investigated for MSI using the mononucleotide repeat markers *BAT25* and *BAT26*, which are sensitive and specific indicators of the MSI-high phenotype (256, 257). Tumors with unstable *BAT25* or *BAT26* were classified as MSI, whereas those with normal *BAT25* and *BAT26* were considered microsatellite-stable (MSS).

METHYLATION ANALYSIS (III)

All methylation analyses were conducted by methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) method, the basic principle of which was described by Nygren *et al.* (258). In brief, the probes contain a recognition sequence (GCGC) for the methylation-sensitive endonuclease HhaI (Promega, Wisconsin, USA). Methylated CpG dinucleotides within a restriction site are not recognized by the HhaI enzyme. Probes that bind methylated sequences are therefore protected and amplified in PCR which generates a signal peak. All MS-MLPA reactions and analyses were performed according to the manufacturer's instructions (259) using 200–600 ng of DNA. The PCR products were separated by capillary electrophoresis (ABI 3730 Automatic DNA Sequencer, Applied Biosystems, Carlsbad, CA, USA) and analyzed by GeneMapper4.0 genotyping software (Applied Biosystems).

The methylation dosage ratio (Dm) was calculated for each normal mucosa and tumor sample as previously described (260). The Dm value of 0.15 or above (corresponding to 15% of methylated DNA) was treated as the conservative technical threshold for methylation detection (258). Since the baseline level for methylation in normal tissue may vary between probes, normal mucosa specimens were used to determine thresholds for hypermethylation in tumor tissues.

To study promoter methylation as a putative second hit, the methylation status of MMR genes was analyzed by SALSA MLPA probemix ME011 (MRC Holland, Amsterdam, The Netherlands). Analyzed *MLH1* promoter regions corresponded to regions A, B and C as described in Deng *et al.* (261). Promoter methylation of eight CIMP marker genes (*CACNA1G, IGF2, NEUROG1, RUNX3, SOCS1, CDKN2A, MLH1* and *CRABP1*) was studied by SALSA MLPA probemix ME042 (MRC Holland, Amsterdam, The Netherlands). Two alternative marker panels, proposed by Weisenberger *et al.* and Ogino *et al.* (See chapter: CpG island methylator phenotype), were used to classify tumors as CIMP-positive or non-CIMP depending on how many markers were found to be hypermethylated (3/5, 5/8 or 6/8) (84, 90).

A custom MS-MLPA assay was designed to study methylation of promoter regions of seven candidate genes (*DKK1, SFRP1, SFRP2, SFRP5, CDH1, HOXD1* and *SLC5A8)* previously associated with early colon oncogenesis (7). The methylation profile of CpG islands was investigated by bisulfite sequencing DNA from cancer cell lines (HCT15, HCT116, RKO, SW480, T84, AN3CA and HEC59) and normal tissue (Genomic DNA – Human Adult Normal Tissue: Uterus and Colon, from a single donor, Biochain, CA, USA). Bisulfite conversion of DNA (600ng) was carried out using EZ DNA Methylation-Direct™ Kit (Zymo Research, Orange, CA, USA). The promoter areas under investigation were amplified and sequenced from bisulfite-modified DNA with methylation-unbiased primers. Custom MS-MLPA probes were subsequently designed to target representative methylation-sensitive restriction enzyme HhaI restriction sites (GCGC). To complete the MS-MLPA assay, Salsa MLPA P300-A2 Human DNA Reference-2 (MRC Holland, Amsterdam, The Netherlands) was added to the custom designed MS-MLPA probe mix. The custom assay was optimized against bisulfite sequencing as described (262).

To determine the sensitivity of the MS-MLPA method a gradient with decreasing amount (100%, 50%, 25%, 15%, 5%, 0%) of methylated control DNA diluted into a solution of unmethylated control DNA (Human Methylated & Non-methylated DNA Set, Zymo Research, Orange, CA, USA) was analyzed.

DEMETHYLATION TREATMENT AND RNA EXPRESSION PROFILING OF CELL LINES (III)

Cancer cell lines were treated with demethylation agents according to Derks *et al.* (263). RNA profiling of treated and untreated cell lines was performed using Affymetrix Human Genome U133 Plus 2.0 GeneChip® microarrays (Affymetrix, Santa Clara, CA). Samples were amplified, labeled and hybridized as described (264). Microarray data analysis was performed by GeneSpring GX software, version 12 (Agilent Technologies, Santa Clara, CA) using RMA normalization. Statistically significant gene expression changes were identified by moderated *t* test combined with the Benjamini and Hochberg correction for multiple testing and by using filters based on p-value cut-off 0.05 and fold change cut-off +/-1.5.

STATISTICAL ANALYSES (I-III)

Statistical significance of observed changes between experimental groups in mouse weight (I-II), amount of tumors (I-II), length of colon crypts (II), number of cells (II), proliferation index (II) and bile acids concentrations (II) were tested with Mann-Whitney U test. The amount of tumors in study II follow negative binomial distribution which was utilized to analyze the effect of age and diet on tumor development (265). Calculations were performed in R version 3.2.0 (2015-04-16).

Protein expression changes detected by 2D-DIGE (I-II) were analyzed by DeCyder 2D version 7.0 (GE Healthcare). Statistical significance of expression changes observed between the experimental groups were tested with the Student's *t*-test with significance threshold $p \leq 0.01$. Proteins with $p \leq 0.01$ were considered as proteins of interest (POI). Mice fed with WD were compared with control diet groups (AIN) in order to detect diet effects at different time points. Combined effect of diet and aging was studied by comparing different time points. Hierarchical clustering and principal component analysis (PCA) were performed on filtered protein set using DeCyder 2D Extended Data Analysis for normalized protein abundances in order to demonstrate differences between experimental groups. Statistical data was utilized to detect the protein spots that contributed to the highest differences between experimental groups. These protein spots were then selected and processed for protein identification (Fig. 5).

For LC-MS proteomic analysis (II) the between-subject ANOVA design scheme of TransOmicsTM software was utilized. The thresholds to accept protein leads based on differential intensities between experimental groups were: absolute fold-change ≥ 2 computed from averaged and normalized protein intensities, and p value ≤ 0.05 for ANOVA in all comparisons.

Statistical analysis of methylation data (III) was performed using the SPSS software, version 20.0 (IBM SPSS Inc. Chicago, IL, USA). Frequency of methylated target sites in each type of tissue specimen was calculated separately for each gene using the probe-specific threshold values. Fisher's exact test was used to calculate 2-sided *p* values for each pairwise comparison and Bonferroni correction was used to adjust *p* values for multiple comparisons. Pearson's correlation was used to study the association between age at biopsy and normal colonic mucosa methylation in the prospective series. Statistical significance of methylation changes detected in normal colonic mucosa (III) between study groups was tested by One-way ANOVA and Tukey's post hoc test was used for pairwise comparisons. Alternatively, the non-parametric test Kruskal-Wallis (k samples, pairwise comparisons) was utilized for series that were not normally distributed. Homogeneity of variances was tested by Levene's test and normality by Shapiro-Wilk test. *P* values ≤ 0.05 were considered significant.

RESULTS AND DISCUSSION

I. EFFECTS OF WESTERN-STYLE DIET ON NORMAL COLONIC MUCOSA OF MOUSE MODELS FOR LS AND SPORADIC CRC (I-II)

Western diet increases weight and induces neoplastic changes in the colon

Overall in both studies I and II, mice fed with WD gained significantly more weight when compared to the control diet group (Fig. 6). Weight gain was defined as the increase in weight between the experiment start point (5 weeks) and different time points of experiment (12m, 18m and 21m). No significant differences were observed in average weight between experimental groups at the start point of the experiment. Experimental groups consisted of equal numbers of female and male mice. In both studies male mice tended to gain more weight compared to females. When sexes were combined, the average weight gain was 15.13g ± 4.85 and 26.97g ± 7.77 in study I for mice fed with AIN and WD, respectively, indicating significant effect of diet (p=0. 0005). No differences in weight gain was observed between the two different genotypes (*Mlh1*$^{+/+}$ and *Mlh1*$^{+/-}$). Study II focused on wild-type *Mlh1*$^{+/+}$ mice only. In study II, the average weight gain was 20.8g ±5.2 and 24.2g ±6.0 (12m); 23.1g ±6.1 and 29.6g ±7.7 (18m) and 24.3g ±6.0 and 29.0g ± 6.5 (21m) for mice fed with AIN and WD, respectively (Fig. 7). In study II, the differences in average weight and weight gain for mice were smaller between the two different diet groups. This was most probably caused by the different environments of the two animal facilities utilized in the two studies. In addition, the fact that irradiated diet was required for study II had most likely significant effects on the diversity of gut microbiota, which was not addressed in this study. Interestingly in study II, mice fed with AIN continued to gain weight until 21 months of age, while WD fed mice started to lose weight after 18 months most probably due to deterioration of health.

All macroscopic findings were histologically verified by an animal pathologist. In study I, 2 colonic hyperplasias, 3 adenomas and 1 adenocarcinoma were observed among the 29 mice at 12m (Fig. 8). Although differences remained statistically non-significant due to low number of mice, five out of the six tumors were detected in mice fed with WD including both genotypes, indicating that WD increases the risk of colonic tumors also in mice with no genetic predisposition. The single exception, a mouse with hyperplasia, was a carrier of the *Mlh1* mutation. In study II, 7 adenocarcinomas, 1 adenoma and 12 hyperplasias were detected in 77 mice included in the study (Fig. 8). Of all tumors in study II, 16 out of 20 were detected in mice fed with WD when compared to AIN (p=0.006). Both aging and diet increased significantly the likelihood of developing a tumor (Fig. 8). Coefficients for aging and WD were 1.91 (p=0.012) and 1.41 (p=0.012), respectively. Compared to study I, mice developed less colonic lesions and only three tumors were detected at 12m, all of them in WD fed mice. The majority of tumors were detected at 18m and 21m. Overall, in studies I-II 8 colonic adenocarcinomas, 4 adenomas and 14 hyperplasias were detected altogether.

Of all colonic lesions 21 out of 26 were detected in mice fed with WD indicating that WD feeding promotes the development of colonic tumors (p=0.001).

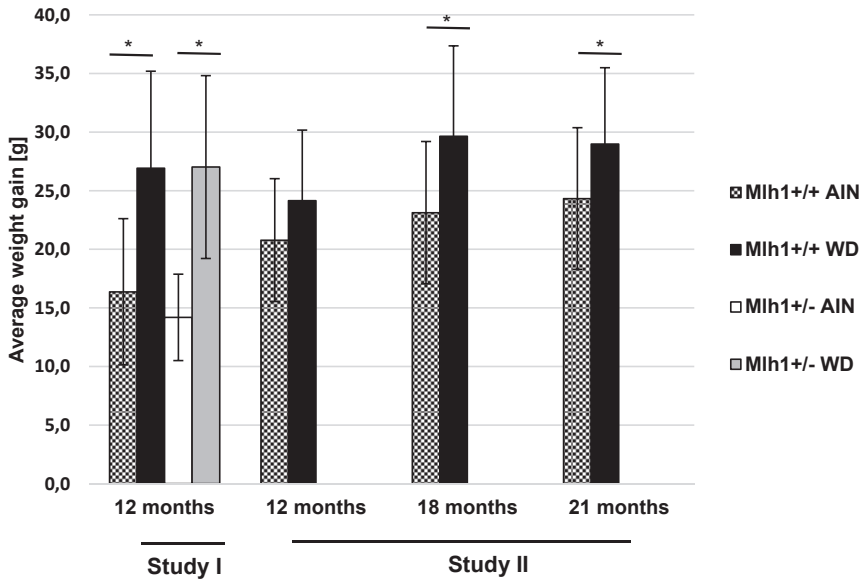

Fig.7. Increased average weight gain in mice fed with Western diet. Statistical significance (*$p \leq 0.05$) of pairwise comparisons were tested by Mann-Whitney U test. Error bars represent standard error of the mean.

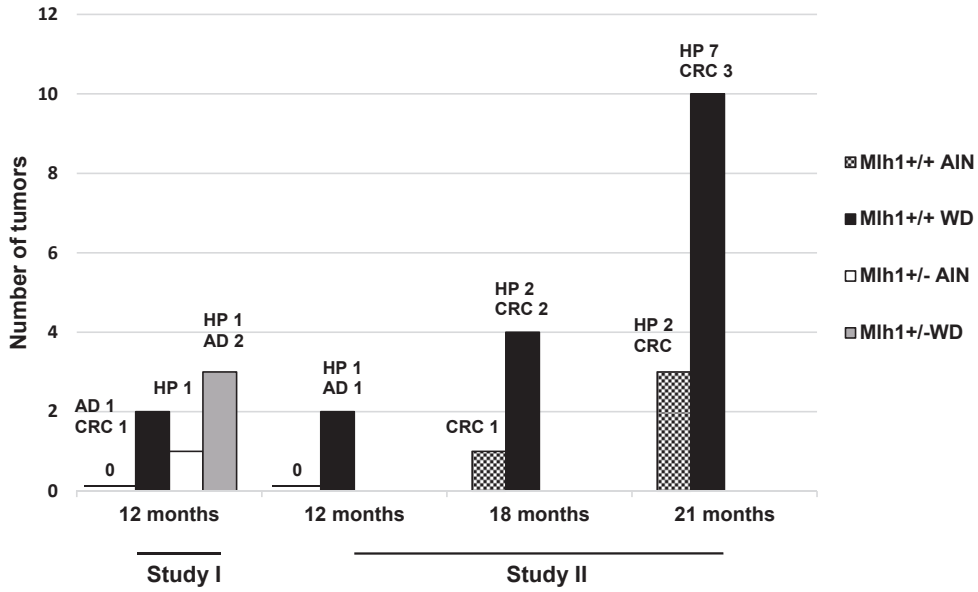

Fig.8. Colonic tumor number in different experimental groups in studies I-II. Tumor histologies are specified as hyperplasia (HP), adenoma (AD) or carcinoma (CRC).

Our observations are in line with Newmark *et al.* who reported for the first time that long-term feeding of WD by itself was able to induce tumors in the colon of wild-type C57Bl/6 mice without any other chemical carcinogen exposure or targeted mutations (266, 267). Similarly, they reported increased body weight after one month of WD feeding which gradually increased during the study. The nutritional content of the Western-style diet used by Newmark *et al.* was comparable to the diet used in our investigation. However, Newmark *et al.* used corn oil as a fat source, whereas our modification of the diet contained substantially increased amount of saturated fat from anhydrous milk, which may affect tumor susceptibility.

Significant changes in the colon proteome caused by aging and diet

Increased number of tumors observed in mice fed with WD is most probably caused by accumulation of alterations in the colonic epithelium which precede tumor development and act as drivers for colonic tumorigenesis. To determine proteomic alterations in normal appearing colonic mucosa associated with WD and aging, colonic proteomes of mice were studied at different time points (5w, 12m, 18m and 21m). Proteome differences between the experimental groups were studied with 2D difference gel electrophoresis (2D DIGE) and analyzed with DeCyder2D Software 7.0. In studies I and II, 1383 and 1974 differentially expressed proteins were detected out of which 394 and 368, respectively, were regarded as statistically significant ($p \leq 0.01$). In both studies, most protein expression changes were detected between the experimental start point and the three different time points, indicating that aging had the most prominent effect on the overall protein expression.

In study I, based on the significant protein expression changes detected by 2D DIGE the 5-week-old mice clustered separately from the other experimental groups (Fig. 9). The two diet groups containing mice with both genotypes clustered separately according to diet, resulting in two additional clusters. This indicates that overall proteomes were more consistently changed by diet than by *Mlh1* genotype. Protein expression changes often showed similar trends in the wild-type *Mlh1*$^{+/+}$ and genetically predisposed *Mlh1*$^{+/-}$ mice. However, most significant expression changes were detected in *Mlh1* mutation carriers fed with WD when compared to AIN control diet groups whereas the proteomes of wildtype *Mlh1*$^{+/+}$ mice expressed more variability. Less statistically significant protein expression changes were observed in *Mlh1*$^{+/+}$ mice in association with diet. This may indicate that *Mlh1* deficiency contributes to the overall expression changes but the robust effect of diet *per se* on proteomes may have impeded the recognition of possible genotype effects. No significant genotype effects were observed in proteomes between *Mlh1*$^{+/+}$ and *Mlh1*$^{+/-}$ mice within the same diet group. However, further studies are required to elucidate the interaction of *Mlh1*$^{+/-}$ genotype and diet.

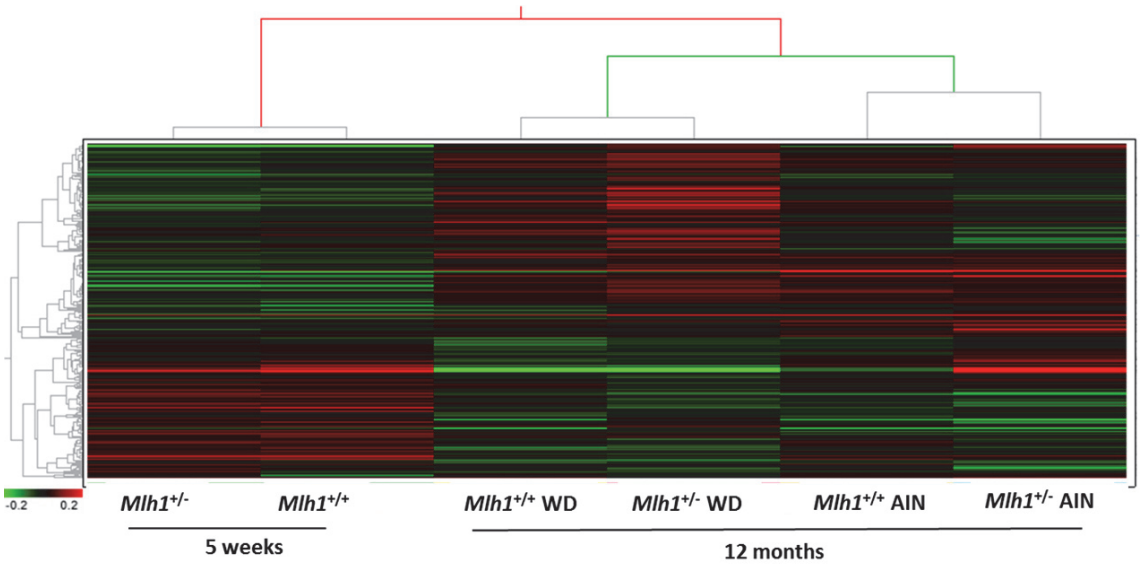

Fig. 9. Hierarchical clustering of experimental groups in study I based on average values of significant protein expression changes detected by 2D DIGE. Color scale represents logarithmic values of expression fold changes. Proteomes were more consistently changed by diet and aging than by genotype.

Since dietary habits are known to predispose to sporadic CRC and the effect of WD on colonic proteome was evident in study I, study II focused on wild-type *Mlh1*$^{+/+}$ mice only. In order to further investigate effects of WD and aging on the colonic proteome and CRC risk, extended number of mice and two additional time points (18m and 21m) were analyzed by 2D DIGE (Fig. 5). Principal component analysis (PCA) based on 2D DIGE proteomic data was utilized to demonstrate differences between experimental groups (Fig. 10A). Similar to study I, colonic proteomes of mice at experimental start point (5w) differed the most from other experimental groups whereas WD fed mice diverged from AIN fed mice over time due to accumulation of protein expression changes caused by diet (Fig. 10A). Principal component (PC) 1 mainly associated with diet and described up to 26% variability between individuals, while PC2 described additional 11% of variability and associated mainly with age. Interestingly, in diet group comparisons most of significant protein expression changes were detected at 12m between WD and AIN control diet group. Moreover, proteomes of mice at 18 months did not differ significantly from proteomes of mice at 21 months among the same diet groups (Fig. 10A). Therefore, validation of results and downstream analysis focused on differences detected at 12 and 18 months. Since tumorigenesis is a multistep process, we reasoned that accumulation of alterations that occurred at earlier time points may have predisposed to colonic tumorigenesis at later stages which was observed as increased tumor number in 21m WD mice.

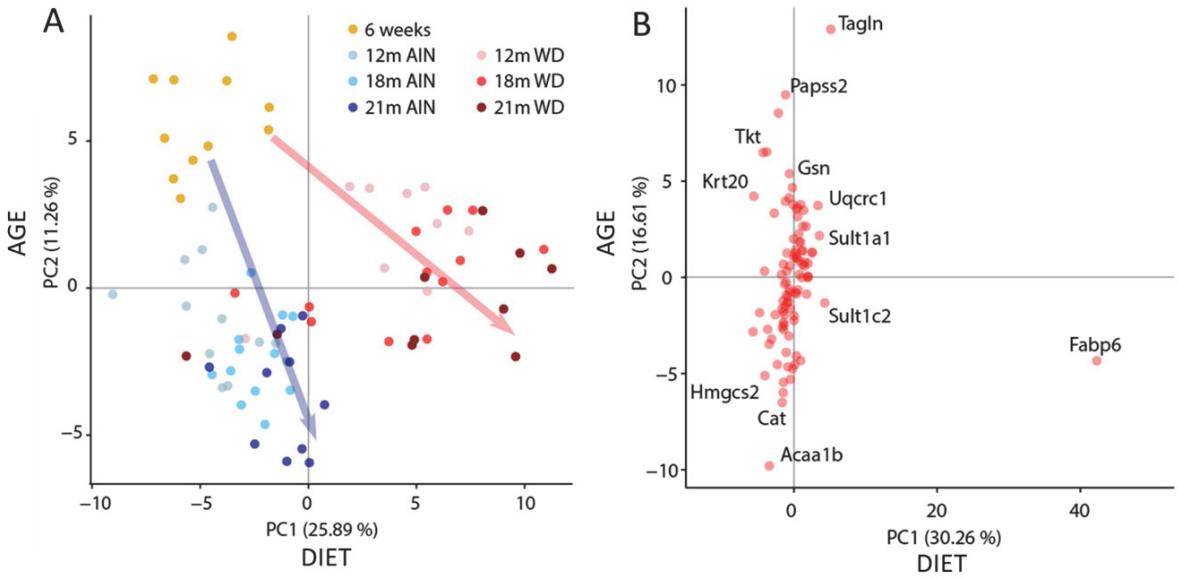

Fig. 10. PCA based on the expression profiles of 96 proteins identified by 2D DIGE (study II). **A.** PCA with colored clusters of experimental groups. Each dot represents the protein expression profile of an individual mouse. Protein expression profiles separate experimental groups according to diet (PC1) and age (PC2). Arrows indicate a shift in protein expression profiles of mice fed with WD and AIN control diet from the mice at experiment start point. **B.** PCA based on the expression of the 96 identified proteins indicates the ones that contribute to the highest differences between experimental groups. PC1 and PC2 indicate the proteins that show most prominent expression change between diet and age group comparisons, respectively.

Proteins involved in energy metabolism and cellular toxicity affected by diet

Since most significant protein expression changes in study I were detected between *Mlh1*$^{+/-}$ WD and AIN control diet groups, proteins for identifications were strictly selected from these comparisons. Altogether, 18 proteins were successfully identified by peptide mass fingerprinting (MALDI-TOF) (Table 4). Many of the detected expression changes associated to metabolic processes and neutralization of cell toxicity. *Mlh1*$^{+/-}$ WD mice showed a remarkable 48% (p=0.008) increase in fatty acid-binding protein 2 (FABP2) expression when compared to *Mlh1*$^{+/-}$ AIN control diet group indicating a response to the high dietary fat content of WD (20%) compared to AIN (7%). FABP2 is known to bind a variety of fatty-acids and elimination of its function in knock-out mice leads to disturbances in lipid metabolism and high-fat diet induced fatty liver (268). Similarly, a 24% (p=0.004) increase was observed in *Mlh1*$^{+/-}$ WD mice when compared to *Mlh1*$^{+/-}$ AIN for medium-

chain specific acyl-CoA dehydrogenase (ACADM) expression, involved in fat digestion and fatty acid oxidation (FAO). Most probably, increased amount of dietary fat shifts cellular metabolism to utilize fatty acids as a primary source of energy instead of glucose. Comparisons between *Mlh1*$^{+/+}$ WD and control diet mice showed similar trends in expression, although they were not statistically significant.

Consumption of Western-style diet has been previously associated with increased toxicity caused by reactive oxygen species (ROS) in mice (269). Peroxisomal FAO is the main metabolic processes responsible for the generation of hydrogen peroxide (H_2O_2) that belongs to ROS. Increased FAO and oxidative phosphorylation, indicated by 14% (p=0.005) increase in ATP synthase subunit α (ATP5A1) in WD fed mice, suggests WD feeding to cause metabolic excess of ROS (270). High levels of ROS production may lead to induction of apoptosis or necrosis whereas continuous exposure to low levels of ROS increases cell proliferation, likely through altered expression of growth factors and oncogenes, which can eventually lead to cancer (271).

The cellular response to protect cells from increased reactive oxygen species is to up-regulate enzymes involved in the production of electron donor NADPH. This maintains a reducing atmosphere in the cells that prevents oxidative damage. Elevated cellular response against ROS toxicity was indicated in *Mlh1*$^{+/-}$ WD when compared to *Mlh1*$^{+/-}$ AIN mice by increased expression of transaldolase (TALDO) (16%, p=0.015) and transketolase (TKT) (7%, p=0.013) involved in the pentose phosphate pathway (PPP) which generates NADPH. Moreover, isocitrate dehydrogenase [NADP] cytoplasmic (IDH1) enzyme, which catalyses synthesis of 2-oxoglutarate from isocitrate with simultaneous reduction of one NADP+ molecule to NADPH, showed 18% (p=0.001) increased expression in *Mlh1*$^{+/-}$ WD mice when compared to *Mlh1*$^{+/-}$ AIN. IDH1 has been shown to act as a tumor suppressor with an important role in antioxidant cellular processes (272). In addition, a remarkable 55% (p=0.0005) up-regulation of selenium-binding protein 1 (SELENBP1) was observed in *Mlh1*$^{+/-}$ WD mice when compared to *Mlh1*$^{+/-}$ AIN. SELENBP1 is thought to play a role in selenium-dependent ubiquitination/deubiquitination mediated degradation of proteins and to have tumor suppressor function through inhibiting cell proliferation in low concentrations of the ROS such as H_2O_2 (273). In summary, increased production of NADPH in normal colonic mucosa of mice fed WD indicates up-regulation of cellular processes fighting against toxicity of ROS to prevent oxidative damage.

Table 4. Identified proteins by 2D DIGE in study I.

UniProt ID	NCBI Gene ID	NCBI Gene Symbol	UniProt Protein Name	MW (kDa)	pI	*Mlh1*$^{+/-}$ AIN / *Mlh1*$^{+/-}$ WD	
						Av.Ratio	[1]*T*-test
P13634	12346	[3]*Car1*	Carbonic anhydrase 1	28,4	6,44	1,94	0,015
P17563	20341	[3]*Selenbp1*	Selenium-binding protein 1	53,1	5,87	1,55	0
P55050	14079	[2,3]*Fabp2*	Fatty acid-binding protein, intestinal	15,1	6,62	1,48	0,008
P45952	11364	[2,3]*Acadm*	Medium-chain specific acyl-CoA dehydrogenase, mitochondrial	46,9	8,6	1,24	0,004
Q91Y97	230163	[3]*Aldob*	Fructose-bisphosphate aldolase B	39,9	8,52	1,19	0,014
O88844	15926	[2,3]*Idh1*	Isocitrate dehydrogenase [NADP] cytoplasmic	47	6,73	1,18	0,001
Q8K419	16855	[3]*Lgals4*	Galectin-4 (Lactose-binding lectin 4)	36,4	9,13	1,17	0,019
Q93092	21351	[3]*Taldo1*	Transaldolase	37,5	6,57	1,16	0,015
Q03265	11946	[3]*Atp5a1*	ATP synthase subunit alpha, mitochondrial	59,8	9,22	1,14	0,005
P19001	16669	[2,3]*Krt19*	Keratin, type I cytoskeletal 19	44,5	5,28	1,13	0,011
P40142	21881	[2,3]*Tkt*	Transketolase	68,3	7,23	1,07	0,013
P42932	12469	[3]*Cct8*	T-complex protein 1 subunit theta	60,1	5,44	-1,09	0,002
P62259	22627	[2,3]*Ywhae*	14-3-3 protein epsilon	29,3	4,63	-1,09	0,014
P05784	16668	[3]*Krt18*	Keratin, type I cytoskeletal 18	47,5	5,22	-1,19	0
P52480	18746	[3]*Pkm2*	Pyruvate kinase isozymes M1/M2	58,4	7,18	-1,3	0,016
P19324	12406	[3]*Serpinh1*	Serpin H1	46,6	8,88	-1,33	0
P16045	16852	[3]*Lgals1*	Galectin-1	15,2	5,32	-1,33	0,001
P97315	13007	[3]*Csrp1*	Cysteine and glycine-rich protein 1	20,6	8,9	-1,59	0,005

[1] *T*-test was calculated using DeCyder 2D Software

[2] Identified in study II by 2D DIGE

[3] Identified in study II by LC-MS

Induced cell proliferation and deregulation of apoptosis pathways

In study II, proteins with significant expression changes between WD and AIN diet groups and different time points were chosen for identification by MALDI-MS. Altogether 96 proteins were successfully identified, out of which 48, 35 and 28 unique proteins showed significant difference in expression when comparing mice fed with WD to AIN at 12, 18 and 21 months, respectively (Table S2 and S3/II). PCA was utilized to identify proteins with highest variability between experimental groups in the 2D DIGE dataset (Fig. 10B). The proteins with highest variability between experimental groups were: catalase (CAT), cytochrome b-c1 complex subunit 1, mitochondrial (UQCRC1), fatty acid binding protein 6 (FABP6, also known as ileal bile acid binding protein (ILBP)), gelsolin (GSN), 3'-phosphoadenosine 5'-phosphosulfate synthase 2 (PAPSS2), sulfotransferase family, cytosolic 1A, phenol-preferring, member 1 (SULT1A1), sulfotransferase family, cytosolic 1C, member 2 (SULT1C2) and transgelin (TAGLN). 2D DIGE results were validated by LC-MS which is a platform for high-throughput proteome analysis (Table S5/II). Validation was performed using triplicates of pooled total protein extracts for each experimental group at 12m and 18m. In 2D DIGE, only proteins with significant expression changes between experimental groups were selected for identification by MALDI-MS, whereas validation by LC-MS was conducted in a high-throughput manner (Fig. 11). A remarkably high proportion (68%) of proteins identified by 2D-DIGE were successfully detected with LC-MS (Fig. 11).

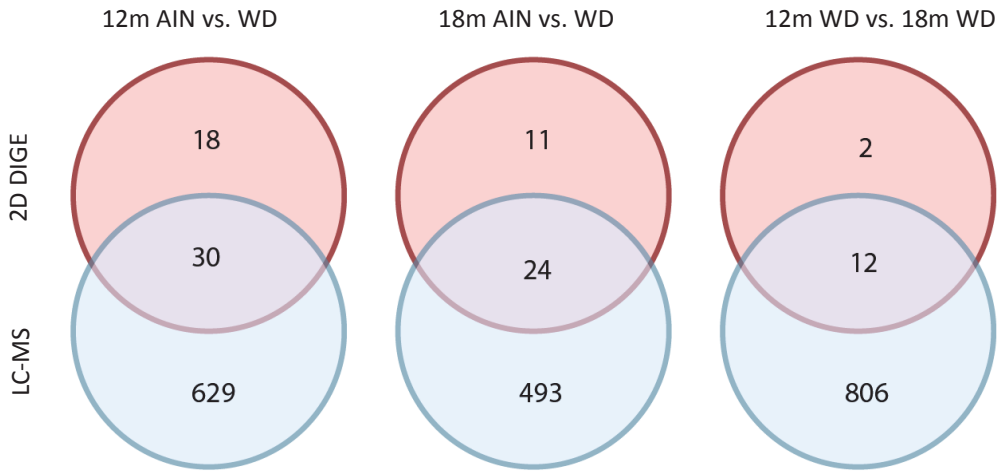

Fig. 11. Comparison of proteins detected by 2D DIGE and LC-MS (II).

The Ingenuity Pathway Analysis (IPA) software for systems biology analysis was utilized to identify upstream activators and enriched pathways in the 2D DIGE and LC-MS proteomics datasets. In 2D DIGE dataset, activation of peroxisome proliferator-activated receptor (PPAR) α, γ and δ and insulin receptor networks together with inactivation of acyl-CoA oxidase 1, palmitoyl (ACOX1), mitogen-activated protein kinase kinase kinase kinase

4 (MAP4K4) and tumor necrosis factor (TNF) pathways all pointed to increased proliferation, deregulation of apoptosis and cell death in the colonic mucosa of 12m and 18m WD mice in particular (Fig. 12A). Interestingly, pathway analysis of LC-MS proteomic data confirmed the changes detected by 2D DIGE in PPAR and ACOX1 pathways and in addition indicated inactivation of bile acid metabolism and farnesoid X receptor (FXR, also known as NR1H4), the main nuclear receptor for bile acids (274) (Fig. 12B).

Concurrent increase of proliferation and avoidance of apoptosis in normal tissue is the most important oncogenic driver event when considering hallmarks of cancer (275). Previous studies performed with mice have reported WD to cause colonic hyperproliferation and increased risk for neoplasia (276-278). Crypt length and cell number of colonic crypts were analyzed to study the morphological effects of increased proliferation and decreased apoptosis indicated by the proteomic data. Indeed, colonic crypts of 12m and 18m WD mice were 24 µm (p=3.15e-9) and 24 µm (p=1.32e-8) longer when compared to AIN control diet groups, respectively (Fig. 13 A-B). Similarly, increased amount of cells per crypt was observed in WD mice compared to AIN with the highest number of cells observed in the crypts of 12m WD mice which indicated that differences in crypt length were caused by increased number of cells and not by increased cell size. Difference in crypt length and number of cells between diet groups at 21 months was not significant.

The intestinal epithelium is renewed due to proliferation at the base of the crypts and apoptosis at the crypt surface. Previous studies have demonstrated an increased proliferative activity together with concurrent reduction in apoptosis in normal appearing colonic mucosa in patients with advanced colorectal adenomas or carcinomas (279, 280). The expansion of proliferative zone from the base of the crypts to the upper portion has been associated with increased risk for CRC (281, 282). The positive staining of nuclear protein Ki67 is associated with cellular proliferation. Here, the proliferation index, defined as a ratio between Ki67 positive cells and total cells within the crypt, was observed to be 40% higher in crypts of 12m WD compared to 12m AIN mice (p = 3.228e-6) (Fig. 13 C-D). Control diet did not result in significant changes in proliferation between different time points, indicating that significant increase in length of crypts observed between 12m and 18m AIN mice was mainly due to increased cell size. These results together with the proteomic data indicate that increased crypt length in mice fed with WD and association with up-regulated cell proliferation in 12m WD mice in particular may act as a driver for colonic tumorigenesis.

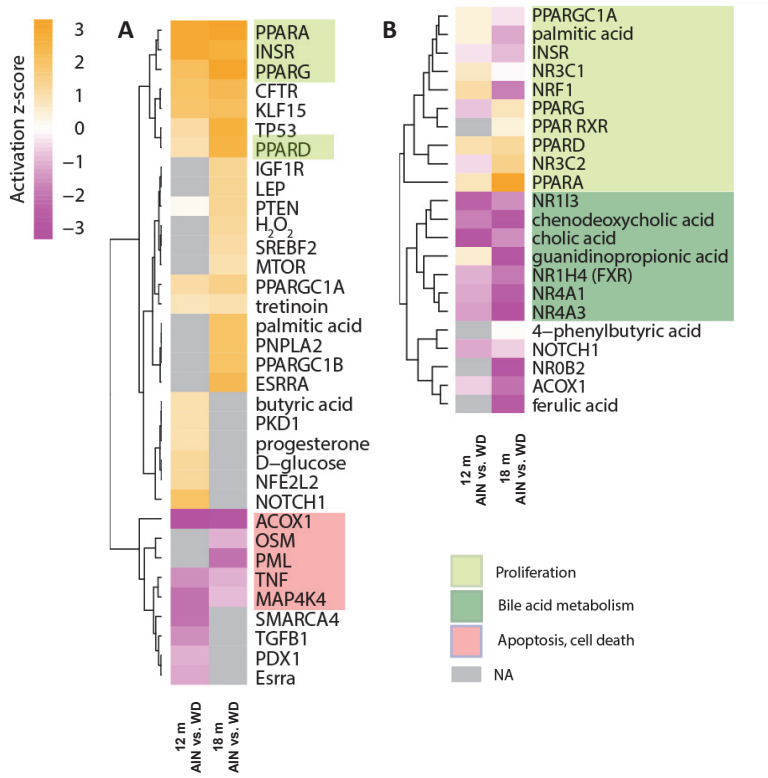

Fig.12. Enriched pathways and upstream activators based on **A.** 2D DIGE **B.** LC-MS proteomics dataset (II). Ingenuity Pathway Analysis (IPA) based on protein expression changes identified by 2D-DIGE and LC-MS revealed activation of pathways involved in proliferation and inactivation of pathways involved in apoptosis and bile acid sensing and transport when comparing the different diet groups at 12m and 18m.

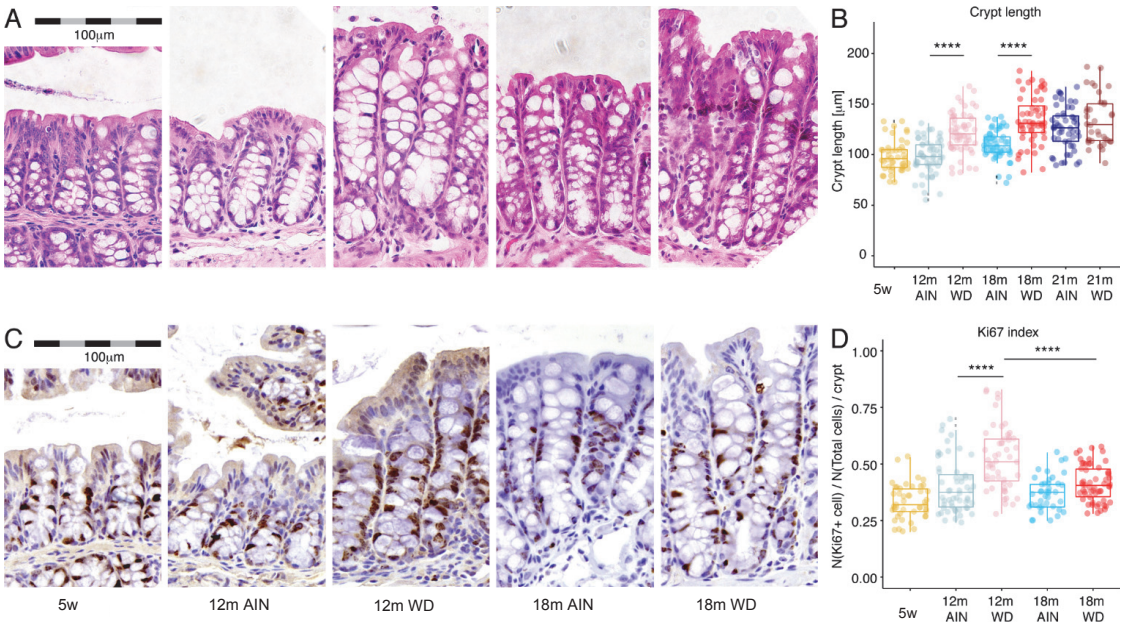

Fig. 13. Analysis of colonic crypt length and proliferation index in different experimental groups (II). **A.** Representative images of hematoxylin and eosin stained colonic crypts from different experimental groups (scale 100 μm). **B.** Increased crypt length detected in 12m and 18m WD mice when compared to control groups. **C.** Representative images of IHC stainings for Ki67 proliferation marker in different experimental groups. **D.** Extended proliferation zone (Ki67 index) in colon crypts of 12m WD mice when compared to control diet and to 18m WD mice. **** $p < 0.0001$.

Altered bile acid transport induced by diet

Our proteomic data indicated increased cellular toxicity due to increased amount of ROS (I, II) together with disruption of lipid metabolism (I, II) and bile acid sensing and transport (II) in mice fed by WD. In study II, FABP6, a cytosolic transporter of bile acids, showed the most prominent difference in expression between diet groups at all time points (Fig. 14). Based on individual expression data from 2D DIGE, the expression of FABP6 increased significantly in both groups during the first 12 months regardless of the diet. However, a remarkably lower expression was consistently detected in mice fed with WD when compared to AIN at all time points. The most significant difference in expression between diet groups was observed at 12m (-4.52, p = 1.81e-7). Results were confirmed by LC-MS (Fig. 14). In LC-MS, organic solute transporter β subunit (OSTβ), also known as solute carrier family 51, beta subunit (SLC51B) showed a similar expression pattern as FABP6, with significant reduction in expression in 12m WD when compared to 12m AIN mice (-2.16, p = 0.018). OSTβ is exclusively expressed within the basal membrane of colonic epithelial cells and responsible for transport of bile acids into the extracellular space (283).

Fatty acid binding proteins are small (15 kDa) cytosolic proteins that bind long-chain fatty acids and show tissue specificity. Through their ability to control fatty-acid transport and metabolism FABPs are proposed to be central regulators of lipid metabolism, inflammation and energy homeostasis (284). FABP6, mainly expressed in the intestinal cells of distal parts of small intestine and proximal colon, has a high affinity for binding bile acids and is essential for the enterohepatic circulation of bile acids (285, 286). Enhanced fecal excretion and disrupted apical-basal transport of bile acids in the intestinal cells of FABP6 knock-out mice indicates that intracellular transport of bile acids is tightly dependent on FABP6 (287). Consequently, binding and transport of bile acids from lumen to portal circulation is reduced indicating that colonic tissue of mice fed with WD might have a defected intestinal apical-basal bile acid transport similar to *Fabp6*$^{-/-}$ mice.

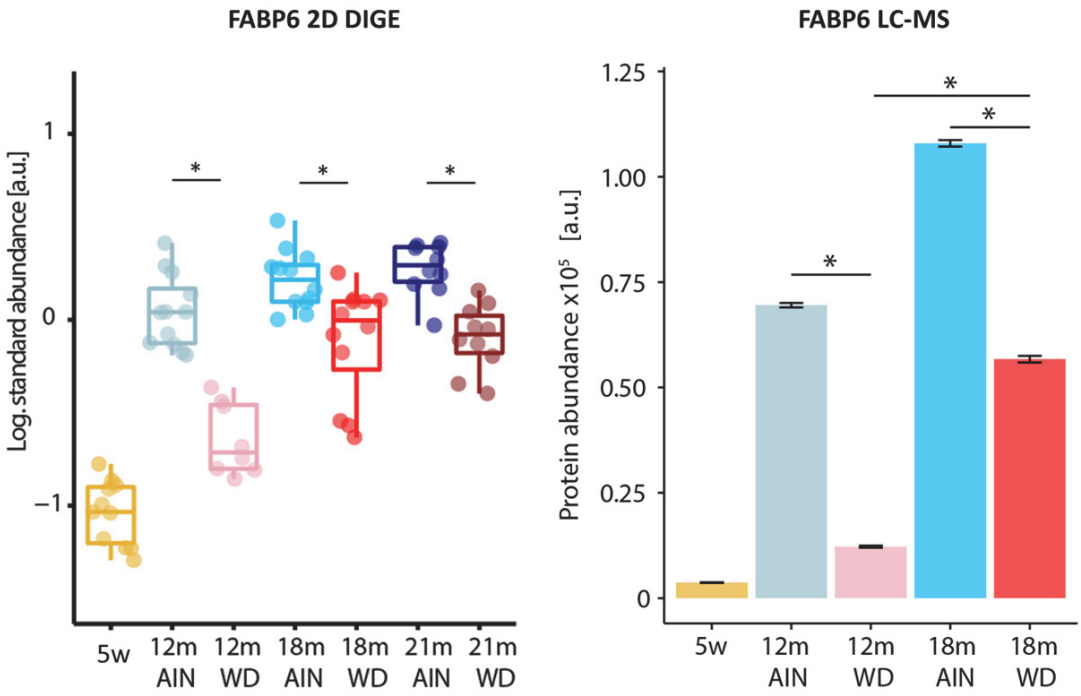

Fig.14. Protein expression of FABP6 measured by 2D DIGE and LC-MS (II).

Bile acids are derived from cholesterol in the hepatocytes as a part of enterohepatic circulation which is regulated by dietary and hormonal signals. Cholic acid (CA) and chenodeoxycholic acid (CDA) are the two most common primary bile acids produced by liver. Most of the bile acids are efficiently absorbed in the ileum and proximal colon by the apical sodium-dependent bile acid receptor (ASBT; gene symbol S*LC10A2*) (288). In the cytosol, bile acids bind to FABP6 and are exported across the basolateral membrane and back into the portal circulation by the heterodimeric transporter OSTα-OSTβ (283). Bile acids that escape the absorption in the ileum are transferred into the colon where they are deconjugated by colonic microbiota and converted into secondary bile acids.

Cells of the gastrointestinal tract are constantly exposed to high physiologic level of bile acids. Consumption of high-fat and high-beef diet is known to result in significantly higher excretion of fecal secondary bile acids, mainly deoxycholic acid (DCA) and lithocholic acid (LCA) (188). Consequently, exposure to bile acids is most prevalent among individuals who have a high dietary fat intake (289). Elevated secretion of bile acids reflects increased production of bile acids in the liver needed in order to emulsify the high level of dietary fat. Therefore, notable reduction in bile acid transporters observed in the colonic epithelium of 12m WD mice directed us to address the intracellular bile acid concentrations. Indeed, UPLC-MS analysis of separate bile acids revealed a significant decrease in the total concentration of bile acids and secondary bile acids DCA (30%) and LCA (36%) in particular in the colonic mucosa tissue of 12m WD mice (Fig. 15). No significant differences were observed in BA concentrations when comparing diet groups at 18 months.

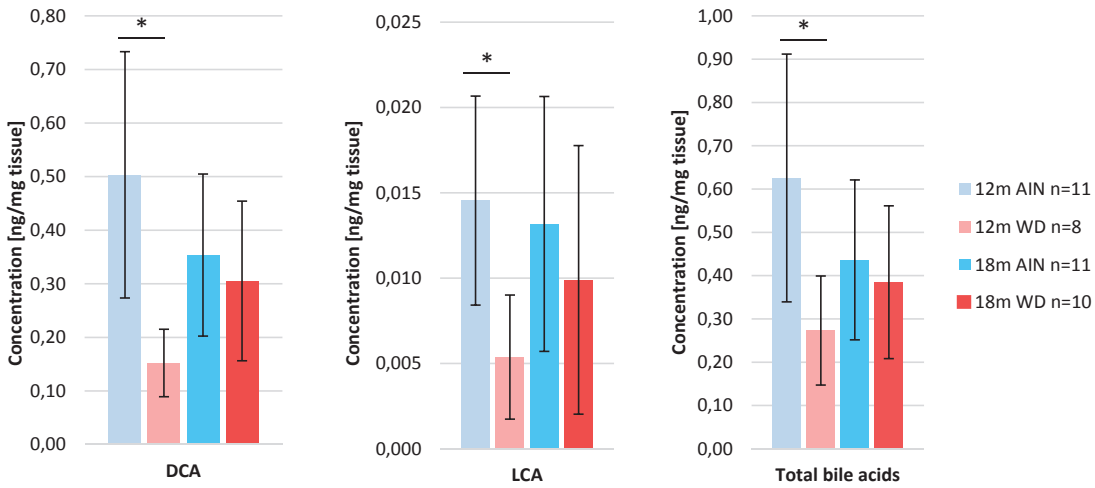

Fig. 15. UPLC-MS measurement of bile acid concentrations in colonic mucosa. Statistical significance (*$p \leq 0.05$) of pairwise comparisons were tested by Mann-Whitney U test.

Even though consumption of high-fat diet, such as WD, is known to result in elevated secretion of bile acids in colon lumen, we detected decreased intracellular concentration of bile acids in the colonic mucosa tissue. Bile acids are known to act as endogenous ligands for their nuclear receptor FXR which directly induces the expression of proteins, such as FABP6, involved in bile acid binding and transport (274). Consequently, reduction in intracellular bile acid ligands observed in 12m WD mice most probably causes decreased activity of FXR, which was predicted by pathway analysis (Fig. 12B). Decreased activity of FXR, followed by down-regulation of FABP6 and other bile acid transporters such as OSTβ, may cause further accumulation of bile acids in the colon lumen and increased toxicity on the luminal side of the epithelium.

Bile acids are implicated to be important in the etiology of cancer of the gastrointestinal tract, including cancer of the esophagus, stomach, small intestine, liver, biliary tract, pancreas and colorectum (290). Moreover, previous epidemiologic studies have reported increased fecal bile acid concentrations in populations with a high CRC incidence (291). Based on previous studies, secondary bile acids DCA and LCA in particular appear to be correlated with increased incidence of CRC (292). However, previous studies on bile acids and CRC have concentrated on quantifying fecal bile acids whereas this study is to our knowledge the first to address the intracellular concentrations in association with diet.

Bile acids are likely to be related to carcinogenesis since they are known to induce ROS in the colonic epithelium (189) which is in line with our proteomic data that suggested increased oxidative stress in mice fed with WD. Frequently repeated long-term exposure to high concentration of bile acids can cause cells to acquire mutations and retain unrepaired DNA damage that may offer growth advantage and resistance to apoptosis. Such apoptosis-resistant cells may clonally expand due to natural selection and give rise to altered subpopulations which can eventually lead to tumor development. Several mechanistic studies have shown increased resistance to DCA-induced apoptosis in “normal” colonic epithelial cells from CRC patients (293-295). Moreover, increased bile acid levels have been shown to induce cell proliferation (296). Consistent with previous studies, our data implicated concurrent increase of proliferation and decreased apoptotic processes in the colonic epithelium exposed to WD. On the contrary, we observed remarkably decreased expression of proteins involved in the cellular transport of bile acids and consequently decreased intracellular concentrations of bile acids even though concentrations in the lumen and feces are expected to be increased due to the high fat content of WD. Disrupted bile acid transport caused by WD provides a possible novel mechanism for initiative tumorigenesis underlying the WD-induced risk for CRC.

II. HYPERMETHYLATION IN LS ASSOCIATED TUMORIGENESIS (III)

Disease phenotype, such as age of onset and tumor spectrum, vary considerably in human Lynch syndrome mutation carriers even among individuals that carry the same germline mutation. Phenotypical variation suggests the existence of other somatic driver events and implication of lifestyle factors on risk accumulation. Apart from genetic alterations, epigenetic changes play a major role in the initiation and progression of CRC. Epigenetic changes are particularly interesting since they are potentially reversible and can be modified by the environment. These changes can accumulate over time due to aging and many environmental factors such as diet.

We utilized the unique features of LS, including increased tumor incidence and availability of tissue specimens gathered during colonoscopy surveillances, to study the temporal sequence of molecular changes that occur during different steps of tumorigenesis. Biopsies from normal colorectal mucosa together with blood samples were gathered from 55 Finnish Lynch syndrome mutation carriers during colonoscopy screenings and colectomies performed in Helsinki University Central Hospital and Jyväskylä Central Hospital during 2011–2013 (Table 1/III). Out of these patients 39 (71%), 13 (24%) and 3 (5%) were *MLH1, MSH2* and *MSH6* mutation carriers, respectively (Table 5). The prospective series was utilized to study methylation changes in the normal appearing colonic mucosa in association with age and previously diagnosed cancer. Furthermore, to study the methylation changes occurring during the adenoma-carcinoma progression sequence, all archival tubular and villous adenomas and carcinomas previously diagnosed for the LS patients were gathered from the nation-wide registry forming the retrospective series.

Table 5. Mutation specifications for patients included in study.

Mutation	Transcript	HVGS protein	No. of individuals with mutation
MLH1 c.1732-2941_1896+432del	NM_000249.3	p.Pro579_Glu633del	25
MLH1 c.1975C>T	NM_000249.3	p.Arg659*	2
MLH1 c.320T>G	NM_000249.3	p.Ile107Arg	3
MLH1 c.454-1G>A	NM_000249.3	p.Glu153Phefs*8	6
MLH1 c.543C>G	NM_000249.3	p.Gly181Gly, p.(Glu153Phefs*8)	1
MLH1 c.546-2A>G	NM_000249.3	p.Arg182Serfs*6	1
MLH1 c.1039-1G>A	NM_000249.3	p.Thr347Lysfs*8	1
MSH2 c.1553_1554_delCA	NM_000251.1	p.Gln518Leufs*10	3
MSH2 c.1667_1671delGACTT	NM_000251.1	p.Leu556Phefs*4	2
MSH2 c.1737dupA	NM_000251.1	p.Glu580Argfs*18	1
MSH2 c.1777C>T	NM_000251.1	p.Gln593*	2
MSH2 c.1807 G>A	NM_000251.1	p.Asp603Asn	2
MSH2 c.696_697delTT	NM_000251.1	p.Ser233Hisfs*22	2
MSH2 c.2459-?_2802+?del	NM_000251.1	p.?	1
MSH6 c.2735G>A	NM_000179.2	p.Trp912*	1
MSH6 c.900dupG	NM_000179.2	p.ys301Glufs*11	1
MSH6 c.3013C>T	NM_000179.2	p.Arg1005*	1

Accumulation of molecular genetic defects during tumorigenesis

MMR deficiency is proposed to accelerate rather that initiate tumor progression (62). The timing of somatic inactivation of the remaining wild-type allele was evaluated from colorectal specimens by studying MMR protein expression by IHC. All studied colorectal adenomas with high dysplasia and carcinomas from LS patients showed loss of MMR protein expression corresponding to the gene mutated in the germline (Table 6). Overall, loss of MMR protein expression increased along with dysplasia level (0% in normal mucosa, 50 – 68% in low dysplasia adenomas, and 100% in high dysplasia adenomas and carcinomas) suggesting that inactivation of MMR protein expression is a relatively late event in LS tumorigenesis. Of low dysplasia adenomas, only 68%, 67% and 50% showed loss of expression of *MLH1*, *MSH2* and *MSH6* mutation carriers, respectively (Table 6). Corresponding frequencies of adenomas with low dysplasia showed MSI (Table 6). Specifically, MSI analysis performed with mononucleotide repeat markers BAT25 and BAT26 showed that all MMR deficient adenomas and carcinomas were microsatellite unstable with one exception (adenoma with low dysplasia). All low dysplasia adenomas that retained MMR protein expression were microsatellite-stable. The fact that almost half of the low dysplasia adenomas were microsatellite-stable and retained MMR expression suggests the existence of other somatic driver events that precede inactivation of wild-type allele and induce polyp formation in LS.

Our findings are consistent with a previous study by Yurgelun *et al.* that demonstrate a significant association between the likelihood of an MMR deficient phenotype and increased size in LS-associated adenomas (297). Moreover, frequent occurrence of MMR deficient crypt foci observed in non-tumorous mucosa of LS mutations carriers indicates a high frequency of biallelic MMR gene inactivation, which is in sharp contrast with the relatively low number of clinically manifest adenomas and carcinomas in LS (298). These observations together highlight the necessity of other tumorigenic events that may arise before or after MMR gene activation independently of MMR status.

Promoter methylation of MMR genes was studied as a possible cause of the “second hit” in MMR deficient tumors. A previous study by Auclair *et al.* suggested that *MLH1* promoter methylation induces gene inactivation in a density-dependent pattern, whereas low-level methylation in unlikely to reduce gene expression (299). In our investigation, methylation of *MLH1* was mostly detected in the distal promoter (region A), which has no direct association with protein loss (261). This region was found to be methylated in 30%, 31% and 60% of low-grade adenomas, high-grade adenomas and carcinomas, respectively. Methylation of region C, which is most commonly associated with protein loss and MSI, was detected in one high dysplasia adenoma (1/13), indicating that the corresponding allele may be silenced by hypermethylation. In summary, hypermethylation of *MLH1* allele region C was detected in 1/42 (2%) of all tumors with silenced MLH1 protein. These results are in line with previous investigations that have reported inactivation of *MLH1* in association with hypermethylation of region C in a small but definitive proportion of LS tumors (2-15%) (64, 116, 117). No methylation was detected in the promoter regions of *MSH2* and *MSH6*. *KRAS* activating mutations are considered to occur during the early stages of the adenoma-carcinoma progression sequence in association with CIN phenotype in particular (45). Frequency of *KRAS* mutations was 8%, 25% and 30% in low dysplasia adenomas, high dysplasia adenomas and carcinomas, respectively, which indicates increased frequency of mutations along with dysplasia, complying with observations from sporadic adenomas (300).

Table 6. Molecular characteristics of tumors included in study III.

		Adenoma low dysplasia	Adenoma high dysplasia	Carcinoma
Age at diagnosis		54 (32-69)	49 (25-74)	52 (31-74)
Decreased expression of MMR protein	MLH1	17/25 (68%)	13/13 (100%)	12/12 (100%)
	MSH2	4/6 (67%)	1/1 (100%)	3/3 (100%)
	MSH6	1/2 (50%)	-	5/5 (100%)
	Total	22/33 (67%)	14/14 (100%)	20/20 (100%)
MSI	MLH1	13/21 (62%)	12/12 (100%)	12/12 (100%)
	MSH2	2/4 (50%)	1/1 (100%)	3/3 (100%)
	MSH6	1/2 (50%)	-	5/5 (100%)
	Total	16/27 (59%)	13/13 (100%)	20/20 (100%)
[1]CIMP	MLH1	4/21 (19%)	2/12 (25%)	6/12 (50%)
	MSH2	0/4 (0%)	0/1 (0%)	0/3 (0%)
	MSH6	0/2 (0%)	-	4/5 (80%)
	Total	4/27 (15%)	2/13 (15%)	10/20 (50%)
Mutated *KRAS*	MLH1	1/19 (5%)	3/11 (27%)	3/12 (25%)
	MSH2	1/4 (25%)	0/1 (0%)	1/3 (33%)
	MSH6	0/2 (0%)	-	2/5 (40%)
	Total	2/25 (8%)	3/12 (25%)	6/20 (30%)

[1] CIMP classification was performed according to the Weisenberger criteria

Hypermethylation in pre-malignant and malignant lesions

Colorectal cancer is known to develop as a result of accumulation of genetic and epigenetic defects. Previous studies in sporadic CRC have demonstrated that promoter hypermethylation of tumorsuppressor genes can act as an alternative mechanism to mutations that causes transcriptional silencing (301, 302). Promoter methylation has been detected in CRC associated normal colonic mucosa and ACF which suggests it to occur as an early event in the colonic adenoma-carcinoma progression sequence (303, 304). The importance of CpG island hypermethylation in hereditary colorectal cancer syndromes such as Lynch syndrome remains unknown.

Aberrant CpG island methylation, previously associated mainly with sporadic CRC, gives rise to CpG island methylator phenotype (CIMP) (6). CIMP tumors are thought to form a distinct subtype of colorectal tumors that may be more responsive to demethylating agents used for chemoprevention (305). Eight genes previously associated with CIMP (*CACNA1G, IGF2, NEUROG1, RUNX3, SOCS1, CDKN2A, MLH1* and *CRABP1*) were used to study methylation changes in pre-malignant and malignant colonic lesions gathered from LS patients. Frequency of hypermethylation in different tissue specimens was calculated separately for each gene (Fig.16). Hypermethylation in tumor tissues was evaluated against probe-specific thresholds derived from normal colonic mucosa. As no consensus criteria exists for scoring CIMP, tumors were classified as CIMP-positive and non-CIMP according to two alternative CIMP marker panels from literature (84, 90). The frequency of CIMP-positive tumors increased from normal colonic mucosa to adenomas with low dysplasia to adenomas with high dysplasia and carcinomas regardless of which criteria were used. In summary, 4–15% of LS associated low dysplasia adenomas, 23% of high dysplasia adenomas and 25–50% of LS colorectal carcinomas could be classified as CIMP-positive depending on which markers and criteria were used (Fig.2/III). These results are consistent with previous investigations that have addressed hypermethylation of tumor suppressor genes in LS and sporadic colorectal adenomas (306, 307).

Highest frequency of hypermethylation was observed in the high dysplasia adenomas and carcinomas (Fig.13). Out of the eight studied CIMP markers the highest frequencies of hypermethylation in tumors were observed in *IGF2*, *NEUROG1*, *MLH1* and *CRABP1*. Hypermetylation frequency of *IGF2* and *NEUROG1* was significantly increased in all tumor types when compared to normal colonic tissue whereas the frequencies of methylated *MLH1* and *CRABP1* were only increased in carcinomas. Interestingly, a significant increase in *IGF2* (68%, $p \leq 0.001$) and *NEUROG1* (48%, $p \leq 0.001$) methylation was observed already in the low dysplasia adenomas (Fig.16), which indicates that methylation of these genes is an early event and could be further studied as potential early marker for CIMP and colonic oncogenesis.

Additionally, a custom-designed MS-MLPA kit was compiled to study methylation changes in seven additional candidate genes (*DKK1, SFRP1, SFRP2, SFRP5, CDH1, HOXD1* and *SLC5A8*) previously associated with early colonic oncogenesis. Reduced mRNA expression of these genes accompanied by increased promoter methylation was detected in a previous study performed in our group using the normal colonic mucosa of *Mlh1*$^{+/-}$ mice fed by Western-style diet (7). Methylation of two secreted frizzled-related proteins *SFRP1* and *SFRP2* were found to be significantly increased in high dysplasia adenomas and carcinomas compared to normal colon (Fig.13). Hypermethylation of *SFRP2* was found to be significantly increased in all tumor types when compared to normal tissue, including adenomas with low dysplasia (37%, $p=0.042$). Significantly increased hypermethylation frequencies for *SFRP1* were only observed in high dysplasia adenomas (67%, $p=0.001$) and carcinomas (67%, $p=0.001$) (Fig.13).

To evaluate the functional significance of methylation on gene expression, mRNA profiling of colorectal, endometrial and ovarian cancer cell lines treated with demethylating agent 5-aza-CdR and histone deacetylase inhibitor TSA. Microarray analysis revealed consistent treatment-induced upregulation of *SFRP1* (1.8 - 7.2 fold) in the MMR deficient cell lines HCT15, HCT116 and HEC59 analogous to LS associated CRC and endometrial cancer whereas *SFRP2* was found to be significantly upregulated (1.9 fold) mainly in HCT116. Moreover, methylation and expression of *SFPR1* (r= -0.69, *p*=0.001) and *SFRP2* (r= -0.66, *p*=0.002) were found to be inversely correlated in cancer cell lines (treated and untreated) and corresponding normal tissues which suggests methylation to have a significant relevance in the expression of these genes.

In the normal colonic epithelial cells SFRPs function as negative regulators of Wnt signalling pathway that inhibit binding of WNT proteins to their receptor frizzled (FRZ) (308-310). Loss of SFRP protein expression during early stages of tumorigenesis results in abnormal activation of Wnt pathway (311). Transcriptional silencing of *SFRP*s caused by promoter hypermethylation promotes Wnt signalling through activation of the APC protein complex which induces cell proliferation and formation of ACF (312). Frequent promoter hypermethylation and gene silencing of the *SFRP* gene family members have been detected in ACF and primary colon cancer (313). Interestingly, promoter hypermethylation together with simultaneous reduction in gene expression has been observed in both CIMP-H and non-CIMP tumors, which indicates that aberrant methylation and silencing of *SFRP*s genes can occur in colorectal tumors irrespective of their molecular subtype (85).

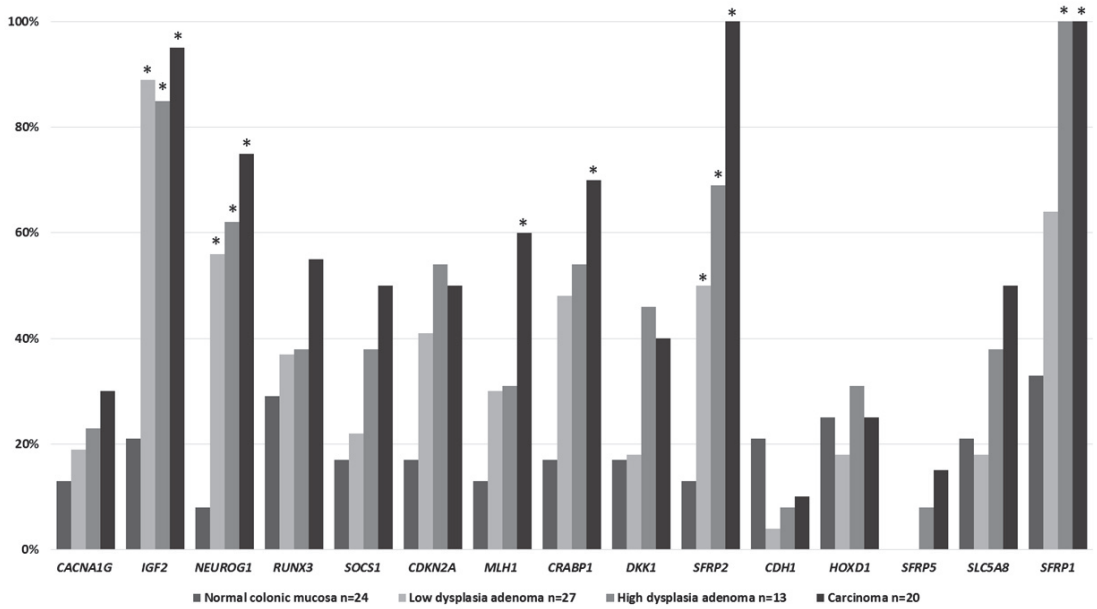

Fig.16. Frequency of hypermethylated genes in different colorectal specimens. Pairwise comparisons between hypermethylation frequency in tumors against colonic mucosa were tested for statistical significance (*$p \leq 0.05$) by Fisher's exact test.

Field defects in normal appearing colonic mucosa

Field defects are described as clonal abnormalities in the epithelial gene expression that precede and predispose to development of cancer and may cause simultaneous occurrence of multiple tumors (314). Previous studies have demonstrated genetic and epigenetic alterations in the macroscopically normal colonic mucosa that do not produce a morphological change but predispose to malignant transformation (315, 316). Field defects are suggested to occur in the immediate vicinity of the tumor or alternatively in a more widespread manner in a form of discrete patches (294, 304). Widespread changes have been suggested to be caused by dietary exposure as a consequence of interplay between underlying genetic defects and differences in luminal factors such as bile acids along the colon (294). Hence, some areas would be more susceptible to carcinogens found in the colonic lumen. Identification of such fields would be of clinical relevance since they provide insight to the earliest stages of CRC development and potential markers for risk assessment.

Colorectal cancer risk increases with age and aging itself is known to associate with accumulation of DNA methylation in human normal colonic mucosa (317-320). Increased methylation in association with aging has been detected in normal appearing colonic tissue in a subset of tumor suppressor genes which often become more substantially methylated

during neoplastic progression (321-323). Even though causes of age-related methylation are unknown, it is proposed that environmental exposure and diet may play a role together with genetic predisposing factors (324). In our investigation, a moderate to strong positive correlation between normal colonic mucosa DNA methylation and age at biopsy was observed in CpG islands of *IGF2*, *NEUROG1*, *SFRP1*, *SFRP2* and *SLC5A8* (Fig.S3/III). No age-related correlation was observed in *MLH1* region C methylation.

Possible carcinogenic fields were investigated in the normal appearing colonic mucosa in association with age and previously diagnosed cancer. Methylation of CIMP markers and candidate genes were analyzed from normal colonic mucosa biopsies by MS-MLPA. Patients were divided into four groups depending on age at biopsy and absence vs. presence of (previous or concurrent) CRC (groups 1-4). The first two groups (groups 1-2) included patients that were 50 years old or below and consisted of 22 patients that had not been diagnosed with CRC (low-risk mucosa) and 6 individuals with previouly or concurrently diagnosed CRC (high-risk mucosa). The last two groups (groups 3-4) consisted of patients above 50 years old and included 17 individuals that had not been diagnosed with CRC and 10 individuals with CRC, respectively. Duration between CRC diagnosis and time of biopsy was 5,1 years (range 0-11,5) in group 2 and 5,5 years (range 0-12,4) in group 4. Average age at biopsy was comparable in each group: 35 (range 26-50), 43 (range 39-48), 61 (range 51-75) and 63 (range 51-74), respectively.

When the effect of CRC on CIMP marker methylation in normal colonic mucosa was studied within the age two groups (≤50 and above 50), no significant differences were observed between low-risk mucosa vs. high-risk mucosa methylation. However, a significant increase in *IGF2* and *NEUROG1* methylation was detected when comparing the age groups with each other (≤50 vs. above 50) indicating age-dependent methylation (Fig.S4/III). In mutation carriers above 50 years old, two markers from the candidate gene panel *SFRP1* ($p < 0.0001$) and *SLC5A8* ($p= 0.007$) revealed a significantly elevated average degree of methylation in high-risk mucosa group when compared to all other groups which indicated a potential field defect (Fig.4/III). Methylation and silencing of *SLC5A8* is frequently detected in ACF and CRCs (325). *SLC5A8*, that encodes a sodium transporter and is a member of the sodium solute symporter family (SLC5), mediates directly the absorption of short-chain fatty acids (e.g butyrate) from the apical membrane of the intestinal tract into the colon (326, 327). Butyrate is a by-product of bacterial fermentation and has been proven to have anti-proliferative control of the cell cycle through its effect on histone acetylation (328).

Our findings are consistent with a recent study by Luo *et al.* that reported increased genome-wide DNA methylation content in (sporadic) CRC-associated normal colonic mucosa compared to normal mucosa from cancer-free individuals (329). These results together with our data urge further studies with prospective study settings to confirm whether the methylation state of normal appearing colonic mucosa could predispose to development of CIMP-positive tumors and predict the risk for CRC.

Differences between low-risk and high-risk mucosa were small although statistically significant. Since observed differencies in methylation between groups were small, sensitivity of MS-MLPA method was tested by analysing a dilution series with a decreasing amount of methylated control DNA (Fig.17). Results indicate that even 5% increase in methylation could reliably be detected in most cases indicating that observed differences were unlikely to be explained by e.g technical variation. *SLC5A8* was discarded from analysis, since its methylation levels were not affected in the methylated and non-methylated controls.

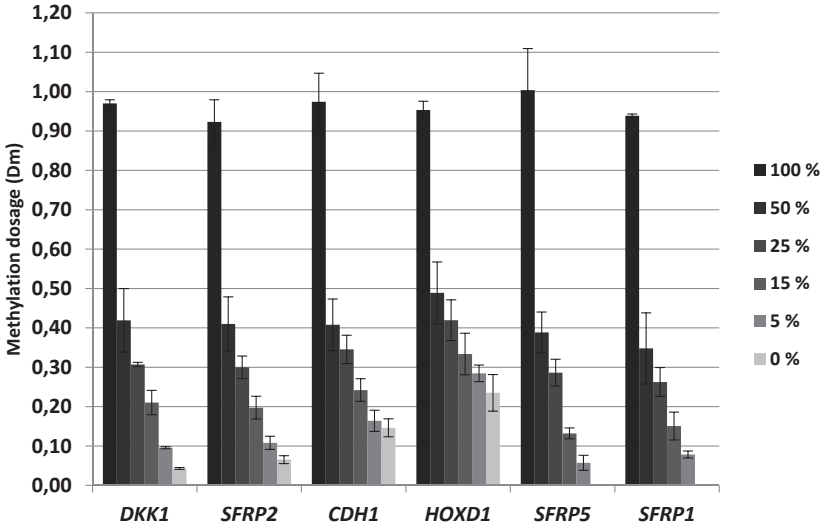

Fig.17. MS-MLPA sensitivity. Sensitivity of the method was evaluated against a gradient of methylated DNA.

It is difficult to discrimminate the molecular changes that are integral in driving cancer from innocent bystanders changes induced by aberrant DNA repair or defective apoptosis. Therefore, mechanistic studies are needed in order to elucidate early molecular drivers of CRC. Moreover, it has been concluded that malignant transformation is a results of accumulation of defects in several genes that have low individual effect by themselves (330). However, changes that occur during early stages of tumorigenesis are more likely to act as driver events whereas later changes may simply reflect the transformed phenotype. Epigenetic changes are particularly interesting, since they are potentially reversible and can be modified by pharmacological agents and diet unlike germline mutations. Idenfication of epigenetic changes underlying field defects could be utilized in chemoprevention prior the development of malignant lesions (331).

CONCLUSIONS

The study on the effects of Western-style diet on the normal appearing colonic mucosa of mouse models for Lynch syndrome (*Mlh1*$^{+/-}$) and sporadic CRC (*Mlh1*$^{+/+}$) provided the following conclusions (I-II):

- Quantitative proteomic analysis of histologically normal proximal colon mucosa revealed significant protein expression changes caused by Western-style diet and aging.
- Proteomes were more consistently changed by diet and aging than by *Mlh1* genotype.
- Significant up-regulation of genes associated with fatty acid catabolism reflected increased oxidative stress after 12 months of WD feeding which may induce DNA damage and eventually lead to cancer.
- Analysis of the proteomic data of the *Mlh1*$^{+/+}$ wild type mice indicated strong activation of proliferative processes and inactivation of apoptotic processes, two important driver events of cancer, in 12m and 18m WD mice in particular.
- Expansion of proliferation zone was detected in the colonic crypts of 12m WD mice implicating increased risk for tumorigenesis.
- Proteomics data coupled with measurement of bile acids in tissue specimens indicated down-regulation of bile acid sensing and transport together with decreased intracellular secondary bile acids in mice fed with WD, suggesting disrupted bile acid homeostasis.

The analysis of molecular genetic changes in normal colonic mucosa biopsies and tumors of different stages of the adenoma-carcinoma progression sequence in human Lynch syndrome provided the following conclusions (III):

- Expression of MMR protein corresponding to the gene mutated in the germline decreased along with dysplasia in tumors but occurred as a relatively late event in the tumor progression sequence, suggesting the presence of other somatic events that drive neoplastic transformation.
- Methylation of CIMP marker loci and selected candidate genes increased together with dysplasia and preceded inactivation of MMR in some low dysplasia adenomas which indicated that methylation occurs as an early event in LS-associated tumorigenesis.
- A proportion of low dysplasia adenomas could already be classified CIMP-positive and the frequency of CIMP further increased in high dysplasia adenomas and carcinomas which highlights the importance and early appearance of epigenetic alterations in LS in general.

- Significant increase in the average degree of methylation of two candidate genes (*SFRP1* and *SLC5A8*) was observed in normal colonic mucosa biopsies from patients with CRC (high-risk mucosa) compared to those without (low-risk mucosa) indicating a possible carcinogenic “field”.

FUTURE PROSPECTS

In light of the presented thesis, future prospects for the studies regarding the diet experiment may include:

- DNA methylome analysis of Western-style diet effects in normal colonic mucosa of mice to address possible epigenome-wide effects of the diet.
- Metabolite analysis of the normal colonic tissue to provide a possible mechanistic link for the changes detected in the proteome.
- Analysis of apoptotic processes in normal colonic mucosa in relation to diet.
- In depth analysis of the possible oncogenic effects of the disrupted bile acid transport and the activity of nuclear bile acid receptor FXR.

The gathering of clinical samples from Lynch syndrome patients continues in collaboration with surgeons. On-going studies and future prospects for this project include:

- Validation of preliminary results of possible field changes regarding normal colonic mucosa methylation in an extended sample set including tissue specimens gathered from the same individuals in follow-up colonoscopies.
- Targeted sequencing of key oncogenes and tumor suppressor genes from tumor-specimens in aim to detect possible driver mutations in genes encoding for epigenetic modifiers that may contribute to observed methylation changes.
- Utilization of clinical specimens to validate markers for disrupted bile acid pathway in normal colonic mucosa.

ACKNOWLEDGEMENTS

The work for this thesis was carried out under the supervision of Professors Minna Nyström and Päivi Peltomäki at the Division of Genetics, Department of Biosciences and the Department of Medical and Clinical Genetics at University of Helsinki, respectively. The work was primarily funded by the Integrative Life Science Doctoral Program (ILS; former Viikki Doctoral Program in Molecular Biosciences, VGSB) and the European Research Council (ERC). Cancer Society of Finland and Sigrid Juselius Foundation are also acknowledged for providing financial support for this work.

I want to express my sincere gratitude to both of my supervisors for the wonderful learning experience and for enabling this work. I truly admire and value the close collaboration between the two research groups that I have had the pleasure to work with, it has made my experience exceptional and fascinating. I would like to thank Professor Minna Nyström for her support and supervision during my MSc thesis work and later on during my PhD studies. I am truly grateful for your guidance during these years. I am truly grateful for Professor Päivi Peltomäki for her supervision and expertise and for the opportunity to join her group during my PhD research and for the chance to work with a project that I am passionate about.

I am grateful to Docents Anne-Maria Pajari and Markku Aarnio for their detailed revision of the thesis. Thank you for your time and efforts, I truly value the comments and feedback you gave me regarding the thesis. I would also like to express my gratitude for both members of my thesis committee Associate Professor Ville Hietakangas and Docent Nina Horelli-Kuitunen for their time and expertise during these years. I was lucky to have both of you as a part of the committee from the very beginning and enjoyed our meetings together every year.

I would like to thank Division of Genetics at the Department of Biosciences in Viikki for providing great working environment and facilities. Special thanks for Docents Katarina Pelin and Pekka Heino from the Division of Genetics for their kind help and guidance in all study related matters during my Helsinki University years. I appreciate your sincere efforts to organize courses and support the students, both of you have taught me a lot.

I wish to thank warmly all previous and current members of Minna's research group including Laura, Saara, Denis, Marjaana and Pauliina from the "WD team" along with Jukka, Minttu and Mariann from the functional MMR team. I am grateful for your help, support and friendship. A special thanks to the WD team for your contribution and efforts regarding the diet study. Denis, thank you for constantly making me think out of the "genetics box" and pushing me out of my comfort zone. We had such amazing times working together in the lab, some early mornings and late nights included! I value your drive, intelligence and passion.

I would also like to thank the Department of Medical and Clinical Genetics for providing excellent staff and working facilities in Biomedicum Helsinki. I cannot thank enough all previous and current members of Päivi's group including Annette, Sippy, Taina, Johanna, Emmi, Anni, Saila, Noora, Soheila and Aliisa. Thank you for your help and encouragement and for making each day coming to work a pleasure. A huge thank you to Saila for the valuable help and efforts with managing the clinical samples and lab work regarding the methylation study.

Professors Heikki Järvinen and Jukka-Pekka Mecklin are thanked for leading the important collaboration between the clinics and HNPCC research groups in Finland and for providing the invaluable patient samples for our projects. I would also like to thank all other collaborators that have contributed to this work. I want to especially thank Professor Ari Ristimäki for the histopathological evaluation of LS tumors as well as Professor Marc Baumann, Rabah Solimany, Maciej Lalowski and Nina Sipari for their expertise in the fields of proteomics and metabolomics. I am grateful for Tuula Lehtinen, Kirsi Pylvänäinen and Beatriz Alcalá-Repo for managing the database and for helping with the sample collections as well as for all patients who have participated in this research.

I want to thank my dear friends from my time at University of Turku including Johanna, Minna, Anni, Elina, Jenni, Riina, Janina, Maija and Hanna. Thank you for your friendship and support. It truly inspires me to see each one of you following your dreams and overcoming challenges. Especial thanks to Johanna for giving me my wonderful and at times very therapeutic dog Misty!

Above all I would like to thank my family for their endless amount of support and encouragement. Thank you äiti, daddy, Edmund, Godfrey, Ida and Wilfrid, I miss you every day! Thank you Grannie and Grampi for your love and support. I would also like to extend my gratitude to the Lowndes-Fraser branch of the family, your support means a lot to me. Lämpimät kiitokset myös Raijalle, Veijolle ja koko Valon perheelle avusta ja tuesta. Viikonloppureissut teidän luona Pajutiellä ovat olleet tärkeitä.

Last but not least, thank you Raimo for your love and understanding. Thank you for being there for me throughout this journey and for putting a smile on my face every day no matter what.

Helsinki, May 2016

Satu Valo

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Helsinki 2016 ISSN 2342-3161 ISBN 978-951-51-2235-3