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Cloudberry (*Rubus chamaemorus*) and Its Components as Chemopreventive Constituents in *Apc*^{Min} Mice and Human Colon Adenocarcinoma Cells

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Cloudberry *(Rubus chamaemorus)* and its components as chemopreventive constituents in *Apc*^{Min} mice and human colon adenocarcinoma cells

Essi Päivärinta

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Agriculture and Forestry of the University of Helsinki, for public examination in Walter Hall, EE Building, on 12th May 2017, at 12 o'clock

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To my family

ABSTRACT

A diet rich in fruits and vegetables has been suggested to decrease the risk of colorectal cancer and the protective effects of these foods could probably be at least partly mediated by their polyphenolic compounds. The number of different polyphenols is huge and their effects on cells and tissues may differ. The promising results on chemopreventive effects of ellagitannin-rich pomegranate suggest that the other dietary sources of ellagitannins may also be effective in cancer prevention.

The overall aim of the research reported in this thesis was to study the effects of cloudberry *(Rubus chamaemorus*), which is rich in ellagitannins, on all phases of intestinal tumour development. Specifically, the effects of cloudberry on the first two phases of carcinogenesis, initiation and promotion, were studied using the *Apc*^{Min} mouse model. In addition, the effects of cloudberry extract on the indicators of the last phase of cancer development, progression and metastasis, and particularly cancer cell migration that is essential for this phase, were studied using scattering and wound healing assays in hepatocyte growth factor-induced HT29 and HCA7 human colon adenocarcinoma cell lines.

A whole-cloudberry diet containing 10% (w/w) freeze-dried cloudberries significantly decreased the number and size of intestinal adenomas in *Apc*^{Min} mice. In contrast, cloudberry seeds, cloudberry pulp, or pure ellagic acid, when incorporated into the diets in concentrations that corresponded to the whole-cloudberry diet, had no apparent effect. The effects of cloudberry on the development of adenomas were also compared with the effects of bilberry *(Vaccinium myrtillus),* which is rich in anthocyanins. Both berries decreased the number of adenomas, but their effects of adenoma size were different: thus, the adenoma size was decreased by cloudberry and increased by bilberry in comparison to the control diet. The opposite effects of the berries on tumour growth were associated with the changes in the gut microbiota, intestinal immunity, and the expression of energy metabolism-related genes.

The activation of Met, which is also known as hepatocyte growth factor receptor induces cell migration and is considered to play an important role in tumour metastasis. Cloudberry extract inhibited cell migration by inhibiting the activation of Met in hepatocyte growth factor-induced human HT29 and HCA7 colon adenocarcinoma cells, and thus Met signalling and consequent activation of the phosphatidylinositol 3-kinase/AKT pathway. The activation of Met signalling in the tumours of cloudberry-fed *Apc*^{Min} mice was also inhibited.

The results of the research presented in this thesis suggest that consumption of cloudberry or cloudberry preparations may reduce the risk of colon cancer, slow down the growth of colon adenomas, and have therapeutic value in reducing cancer progression and metastasis. Long-term studies in human subjects are needed to confirm these results.

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And finally, I don't have the adequate words to express my gratitude to you, Jyrki, but I know that you understand without any words.

Järvenpää, April 2017

Essi Päivärinta

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following published articles, which are referred to by their Roman numerals (I-IV) in the text.

I Misikangas M[‡], Pajari AM[‡], Päivärinta E[‡], Oikarinen SI, Rajakangas J, Marttinen M, Tanayama H, Törrönen R, Mutanen M. Three Nordic berries inhibit intestinal tumorigenesis in multiple intestinal neoplasia/+ mice by modulating β-catenin signaling in the tumor and transcription in the mucosa. J Nutr 2007;137:2285-90. ([‡]contributed equally)

II Päivärinta E, Niku M, Maukonen J, Storvik M, Heiman-Lindh A, Saarela M, Pajari AM, Mutanen M. Changes in intestinal immunity, gut microbiota and expression of energy metabolism-related genes explain adenoma growth in bilberry and cloudberry-fed *Apc*^{Min} mice. Nutr Res 2016;36:1285-1297.

III Päivärinta E, Pajari AM, Törrönen R, Mutanen M. Ellagic acid and natural sources of ellagitannins as possible chemopreventive agents against intestinal tumorigenesis in the Min mouse. Nutr Cancer 2006;54:79-83.

IV Pajari AM, Päivärinta E, Paavolainen L, Vaara E, Koivumäki T, Garg R, Heiman-Lindh A, Mutanen M, Marjomäki V, Ridley AJ. Ellagitannin-rich cloudberry inhibits hepatocyte growth factor-induced cell migration and phosphatidylinositol 3-kinase/AKT activation in colon carcinoma cells and tumors in Min mice. Oncotarget 2016;7:43907-43923. doi: 10.18632/oncotarget.9724

ABBREVIATIONS

ACF	aberrant crypt focus
AOM	azoxymethane
Apc	murine adenomatous polyposis coli gene
APC	human adenomatous polyposis coli gene
COX-2	cyclooxygenase-2
DMBA	7,12-dimethylbenz(a)anthracene
ERK	extracellular signal-regulated kinase
FAP	familial adenomatous polyposis
GAE	gallic acid equivalent
HGF	hepatocyte growth factor
IL	interleukin
iNOS	inducible nitric oxide synthase
JNK	Jun amino-terminal kinase
LOH	loss of heterozygosity
Min	multiple intestinal neoplasia
NF-κB	nuclear factor κB
NMBA	*N*-nitrosomethylbenzylamine
PCNA	proliferating cell nuclear antigen
PI3K	phosphatidylinositol 3-kinase
TNF-α	tumour necrosis factor-α
VEGF	vascular endothelial growth factor

1 Introduction

Colorectal cancer is the third most common cancer type worldwide, the second most common after breast cancer in women and the third most common after lung and prostate cancers in men. Regional differences are enormous: in 2012, the highest age-standardised incidence rates for colorectal cancer were in Oceania (34.8/100 000/year) and in Europe (29.5), and the lowest (4.1) in Western Africa (IARC 2012). The incidence rate for colorectal cancer in Finland was 17.0/100 000/year for men and 13.4/100 000/year for women in 2014. However, the incidences for both genders were higher in Southern Finland than in Northern Finland (Pukkala and Patama 2010). The regional differences, both worldwide and in Finland, are assumed to be the result from the differences in diet and other lifestyle factors.

Since dietary constituents and their metabolites are in direct contact with the digestive tract, the tissues of the digestive tract are susceptible to both cancer-preventive and cancer-promoting effects of the diet. Furthermore, diets rich in fruits and vegetables are suggested to decrease the risk of colorectal cancer (WCRF/AICR 2011). The beneficial effects of these foods may at least partly be explained by their various polyphenolic compounds, and the mechanisms that mediate their effects on the intestinal mucosa have been studied intensively since the beginning of 2000s (Brown *et al.* 2012, Little *et al.* 2015, Macdonald and Wagner 2012). However, the molecular mechanisms are numerous and complex, and are still not known in detail. Since many of the polyphenols are antioxidants *in vitro* (Manganaris *et al.* 2014), they were initially thought to protect cells from mutations that lead to malignant transformation by inhibiting oxidative reactions (Stevenson and Hurst 2007). Today it is known that they probably prevent carcinogenesis through several mechanisms including antiproliferative, proapoptotic, antiangiogenic and anti-inflammatory effects (Afrin *et al.* 2016, Fresco *et al.* 2010). Furthermore, due to their limited absorption in the upper gastrointestinal tract, many polyphenols end up to the colon and are metabolized by gut microbiota (Tomás-Barberán *et al.* 2016). The metabolites of polyphenols formed may be more bioavailable than the original polyphenols they were derived from and probably mediate some of the beneficial effects of polyphenols (Williamson and Clifford 2010). This possible mechanism therefore makes the entire cancer reducing effect of polyphenols even more complex to describe. Thus, polyphenols may affect gut health in the form they were ingested, as intermediate metabolites and/or degradation products produced in the stomach and/or in the small intestine, and/or as metabolites produced by colonic microbiota. In addition, colorectal cancer is known to develop through several phases (Fodde *et al.* 2001), and the polyphenols are likely to affect all of them (Surh 2003).

Many epidemiological studies suggest that diets rich in fruits and vegetables reduce the risk of colorectal cancer (Song *et al.* 2015). Experimental studies on the anti-carcinogenic effects of some berries, such as black raspberry and strawberry, have been published so far, but the effects of Finnish wild berries have not been studied in *in vivo* cancer models. In addition, pomegranate, which is rich in ellagitannins has been shown to exhibit anti-carcinogenic and anti-inflammatory properties both in *in vitro* and *in vivo* models, but chemopreventive effects of ellagitannin-rich cloudberry have so far been studied in only two *in vitro* studies (Wu *et al.* 2007, McDougall *et al.* 2008).

The research done for this thesis focuses on the chemopreventive effects of cloudberry, a rich source of ellagitannins, in the experimental models of colon cancer, and the effects of anthocyanin-rich bilberry are used as a reference for comparison purposes. The aims of the studies I–III were to investigate the effects of cloudberry and its components on the first two steps of carcinogenesis, initiation and promotion, in the *Apc*^{Min} mouse. Study IV aimed to explore the third step of carcinogenesis, progression, in hepatocyte growth factor-induced colon adenocarcinoma cells. Inhibiting all three steps of cancer development by dietary means may help to decrease both the incidence and mortality of colon cancer.

2 Colon carcinogenesis and the *Apc*^{Min} mouse as an experimental model

2.1 Colon carcinogenesis

A majority (70%) of colorectal cancers develop sporadically, *i.e.* without any inherited predisposition to cancer. However, 20-30% of cases have a familial background, and approximately 5% are explained by highly penetrant inherited mutations (Jasperson *et al.* 2010, Hahn *et al.* 2016). Lynch syndrome is characterised by defects in DNA mismatch repair genes and accounts for 2-4% of all colorectal cancers (Jasperson *et al.* 2010), whereas *familial adenomatous polyposis* (FAP) with a germline mutation in *APC* tumour-suppressor gene accounts for 0.5% of all colorectal cancers (Hahn *et al.* 2016, Fearon *et al.* 2011). However, it has been estimated that 70-80% of sporadic colorectal tumours also have inactivating somatic mutations in the *APC* gene. The gene is referred to as a gatekeeper gene and the inactivation of this gene, which probably occurs in the intestinal stem cells, leads to genetic instability and thus the accumulation of additional mutations, activating oncogenes and inactivating tumour-suppressor genes. These all act in concert and result in tumour development (Fodde *et al.* 2001, Vermeulen and Snippert 2014). The development of colorectal cancer proceeds through a well-defined adenoma-carcinoma sequence and it is unlikely to start without the initial *APC* mutation (Fearon and Vogelstein 1990, Fearon 2011).

A normal cell first becomes mutated and changed to an initiated cell in the initiation phase. In the case of the *APC* gene, heterozygous mutation with one mutated and the other wild-type allele will still maintain histologically normal tissue, whereas a mutation in the other allele leads to the loss of heterozygosity (LOH), which in turn leads to the activation of the Wnt signalling pathway, increased cell division (proliferation) and the development of early adenoma (Fodde *et al.* 2001). In the promotion phase, the adenoma grows bigger and becomes more malignant as a result of accumulating mutations in tumour-suppressor genes and proto-oncogenes. This in turn, leads to the activation of various signalling pathways including phosphatidylinositol 3-kinase (PI3K)/AKT, mitogen-activated kinase (MAPK), and nuclear factor κB (NF-κB) pathways (Dammann *et al.* 2014). In addition to increased proliferation, programmed cell death (apoptosis) in tumours is decreased and the imbalance between proliferation and apoptosis promotes tumour growth. Blood supply in growing tumours is guaranteed by increased formation of new blood vessels (angiogenesis). In the last phase of tumourigenesis, progression, the carcinoma invades to the surrounding tissues and forms metastases due to the migration of cancer cells and the breakdown of the extracellular matrix

(Fearon 2011). The steps of colon cancer development with the most essential molecular changes and signalling pathways studied in this thesis are presented in Figure 1.

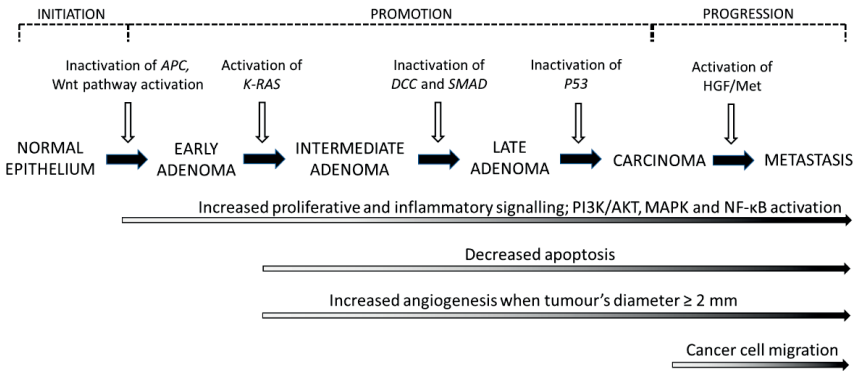

Figure 1. The development of colorectal cancer through a multistep process. The adenoma-carcinoma sequence proceeds from normal epithelium and ends in carcinoma and metastases. This process is mediated by the inactivation of tumour-suppressor genes such as *APC (adenomatous polyposis coli), DCC (deleted in colorectal carcinoma)* gene*, SMAD,* and *P53,* and by the activation of proto-oncogenes, *e.g. K-RAS*. The activation of HGF/Met signalling is characteristic for the last phase of carcinogenesis but may also be present in the previous phases (Modified based on Fearon 2011; Dammann *et al.* 2014, Trusolino *et al.* 2010).

2.2 The *Apc*^{Min} mouse

The *Apc*^{Min} mouse strain (Min, multiple intestinal neoplasia) is the most widely used animal model for studying human familial adenomatous polyposis (FAP). Because the molecular mechanisms of tumour development are similar in the *Apc*^{Min} mouse and in human sporadic colon tumourigenesis, it is also used as a model for sporadic colon cancer. This mouse strain was originally established by exposing C57BL/6J mice to the chemical carcinogen, ethylnitrosourea, after which some of the progeny of these mice were found to develop multiple adenomas in the small intestine and to carry a heterozygous dominant mutation in the *Apc* gene, which is the mouse homolog for the human *APC* gene (Moser *et al.* 1990). The nonsense mutation at codon 850 causes the mutated *Apc* allele, which results in the formation of a truncated form of the Apc protein. However, the full-length protein coded by the non-mutated, wild-type allele will still prevent the activation of Wnt signalling and dysregulated cell proliferation. Mutation in the *Apc* gatekeeper gene makes the

cell susceptible to additional mutations, and a mutation of the wild-type *Apc* allele leads to tumour development (Moser *et al.* 1995). *Apc*^{Min} mice on a C57BL/6J background usually develop between 30 to 50 adenomas in the small intestine, and less than one tumour per animal in the colon. The tumours are benign in the mouse model and do not become invasive, presumably due to the short lifespan of *Apc*^{Min} mice, which is 150 days at the most (Moser *et al.* 1995, Bilger *et al.* 1996).

Tumour development in *Apc*^{Min} mice results from the accumulation of β-catenin in the cytoplasm and the nucleus of intestinal epithelial cells, which leads to an aberrant activation of β-catenin-dependent Wnt signalling pathway. The amount of free β-catenin in the cytoplasm of normal cells is tightly regulated by the destruction complex, which is formed by Apc and other proteins, such as glycogen synthase kinase 3β, axin, and casein kinase 1α. The complex phosphorylates β-catenin and thereby targets it for ubiquitination and subsequent proteasomal degradation. The coordinated phosphorylation and destruction of β-catenin are disrupted in the cells that have a homozygous *Apc* mutation. Thus, β-catenin accumulates in the cytoplasm and translocates to the nucleus, where it participates in activating the expression of genes that enhance cell proliferation, *e.g. c-myc* and *cyclin D1,* and thus leads to the tumour development (Aoki and Taketo 2007).

3 Cloudberry, bilberry, their compounds and metabolites

3.1 Cloudberry

Cloudberry *(Rubus chamaemorus)* belongs to the *Rubus* genus and is closely related to raspberry *(R. idaeus)*, blackberry *(R. fruticosus),* black raspberry (common name for *R. leucodermis, R. occidentalis* and *R. coreanus),* and arctic bramble *(R. arcticus)*. As a member of the *Rosaceae* family it is also related to strawberry *(Fragaria x ananassa)*. The *Rubus* and *Fragaria* berries are, in addition to pomegranate, certain nuts, and oak-aged wines, the most important dietary sources of ellagitannins, polyphenols that belong to the group of hydrolysable tannins (Koponen *et al.* 2007, Ovaskainen *et al.* 2008, Landete 2011). The content of ellagitannins in fresh cloudberries is more than 300 mg/100 g (Koponen *et al.* 2007) and consists mainly of the dimeric sanguiin H-6 and the trimeric lambertianin C, but the monomeric pedunculagin also exists (Kähkönen *et al.* 2012; Fig. 2). In addition, cloudberry contains some free ellagic acid, hydroxycinnamic acids (*p*-coumaric acid, caffeic acid, ferulic acid, sinapic acid), hydroxybenzoic acids (gallic acid, vanillic acid), flavan-3-ols, flavonols (quercetin), and proanthocyanidins (cyanidin) (Määttä-Riihinen *et al.* 2004, Mattila *et al.* 2006). Lignans and small amounts of carotenoids, mainly β-carotene, also exist in cloudberries (Smeds *et al.* 2012, Lashmanova *et al.* 2012). However, ripe cloudberries contain no or negligible amounts of anthocyanins (Jaakkola *et al.* 2012, Koponen *et al.* 2007).

Cloudberry contains large seeds, which make up 12% of its fresh weight (Johansson *et al.* 1997) and 47% of dry weight (Päivärinta *et al.* 2006). Cloudberry seeds contain large amounts of lignans, mainly medioresinol, lariciresinol, and syringaresinol (Smeds *et al.* 2012). The oil content of seeds is 12% of dry weight, whereas that of the fresh berries is 1.4% (Johansson *et al.* 1997). The dominating fatty acids are linoleic acid (41%), α-linolenic acid (36%), and oleic acid (14%) (Johansson *et al.* 1997).

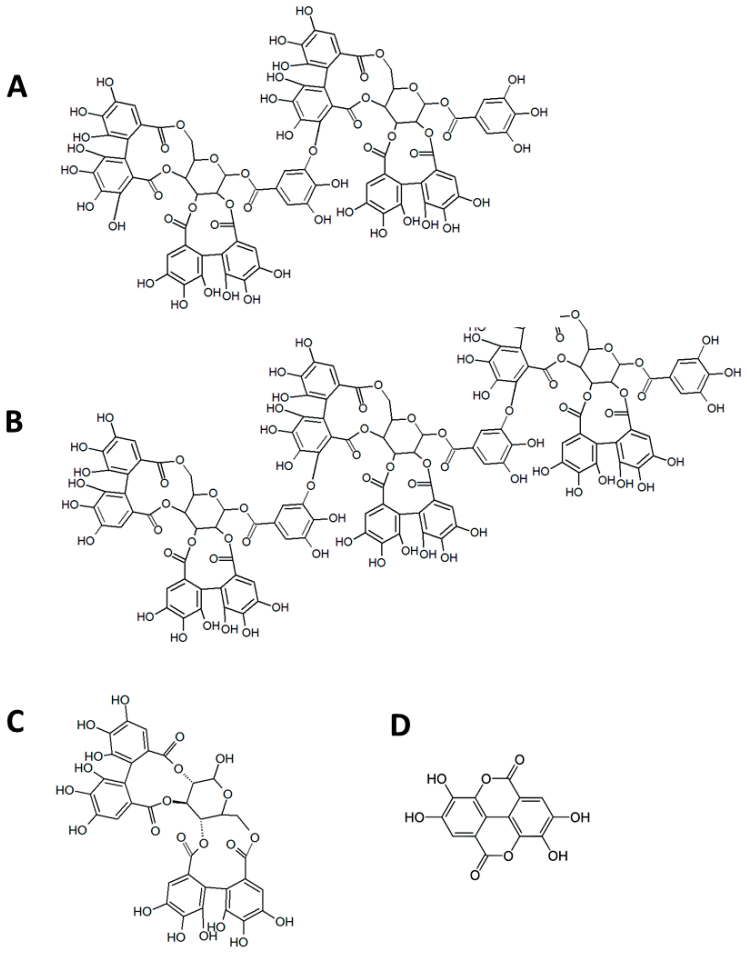

Figure 2. Structures of the main ellagitannins of cloudberry: dimeric sanguiin H-6 (A), trimeric lambertianin C (B), and monomeric pedunculagin (C). Small amounts of free ellagic acid (D) are also found in cloudberries.

Ellagitannins constitute a complex group of polyphenols with an enormous structural variability (Landete 2011). As hydrolysable tannins some of them release ellagic acid in the human stomach and/or the small intestine, but the efficiency of ellagic acid release may be different depending on the structure of ellagitannin. Pharmacokinetic studies have shown that raspberry ellagitannins (sanguiin H-10, sanguiin H-6, and lambertianin C) released ellagic acid more efficiently than pomegranate ellagitannin punicalagin (González-Barrio *et al.* 2010, González-Sarrías *et al.* 2015). Although small amounts of ellagic acid are absorbed in the stomach and in the proximal small intestine, and quickly eliminated from the blood (González-Barrio *et al.* 2010,

Seeram *et al.* 2006b), ellagitannins are not absorbed intact (Espín *et al.* 2007). The majority of ellagitannins and ellagic acid reach the distal small intestine and colon, where they are metabolised to more bioavailable urolithins by gut microbiota (Fig. 3). Urolithins are absorbed, then metabolised in the liver and excreted in urine and bile. They also undergo active enterohepatic circulation along with the bile acids (Espín *et al.* 2007). Urolithins in mice accumulate in the colon, the small intestine, and the prostate (Seeram *et al.* 2007), and they have also been found in the prostate in humans (González-Sarrías *et al.* 2010). *In vitro* urolithins have been shown to inhibit cell proliferation in colon and prostate cancer cells (Kasimsetty *et al.* 2010, Cho *et al.* 2015, Seeram *et al.* 2007).

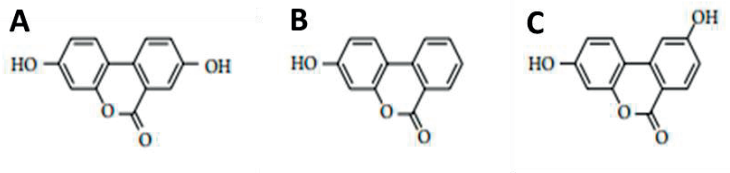

Figure 3. Structures of the main microbial metabolites of ellagitannins and ellagic acid: urolithin A (A), urolithin B (B), and isourolithin A (C).

3.2 Bilberry

Bilberry, *Vaccinium myrtillus,* was used as the reference berry in the studies conducted for this thesis, due to its very different total polyphenol content in comparison to cloudberry: bilberry is rich in anthocyanins but contains no ellagitannins (Koponen *et al.* 2007). The total anthocyanin content in bilberries is approximately 610–800 mg/100 g of fresh weight (expressed as weight of aglycone moieties), of which around 35% delphinidin, 33% cyanidin, 16% malvidin, 12% petunidin, and 4% peonidin (Määttä-Riihinen *et al.* 2004, Koponen *et al.* 2007). Bilberries also contain some hydroxycinnamic (*p*-coumaric acid and caffeic or ferulic acids), flavonols (myricetin and quercetin), flavan-3-ols (mostly (-)-epicatechin), and proanthocyanidins (Määttä-Riihinen *et al.* 2004).

A proportion of anthocyanins is taken intact into the epithelial cells of the stomach and the small intestine, and metabolised in the cells before they enter the circulation, from which they are rapidly cleared (Fang 2014). However, the

total absorption of the anthocyanins is limited and thus most of anthocyanins reach the distal small intestine and the colon, where they are mainly metabolised to protocatechuic acid. In addition, smaller quantities of ferulic, syringic, gallic, vanillic, *p*-coumaric and caffeic acid have also been detected (Aura *et al.* 2005, de Ferrars *et al.* 2014, Fleschhut *et al.* 2006, Hidalgo *et al.* 2012, Vitaglione *et al.* 2007).

The nutritional compositions of cloudberry and bilberry are similar except for the concentration of insoluble fibre, which in cloudberries is 5.8 g/100 g of fresh weight and in bilberries 2.6 g/100 g. The concentration of soluble fibre in both berries is 0.5 g/100 g of fresh weight (National Institute for Health and Welfare 2017, www.fineli.fi).

4 Experimental evidence of ellagitannin and anthocyanin-containing berries in gastrointestinal cancer chemoprevention

4.1 *In vitro* studies

The effects of ellagitannin-containing berries and ellagic acid have been investigated in many cell lines, and the results of *in vitro* studies using gastrointestinal cells are reviewed in Table 1. The studies are presented chronologically in order to show the progress of the research. Most studies have focused on berries containing both ellagitannins and anthocyanins, *e.g.* black raspberry, strawberry, and (red) raspberry, and there are only two studies that show the effects of cloudberry in cell cultures (McDougall *et al.* 2008, Wu *et al.* 2007). All studies are not fully comparable due to the differences in the composition of berry extracts and their concentrations in cell culture medium. For example, a study on cloudberries by Wu *et al.* (2007) used whole-berry extracts, whereas McDougall *et al.* (2008) used polyphenol-rich extracts from which sugars, organic acids, carotenoids, and vitamin C had been removed. Whole-cloudberry extract inhibited cell growth in HT-29 colon carcinoma cells, and increased the mRNA expression of $p21^{WAF1}$, an inhibitor of cell proliferation, as well as Bax, a promoter of apoptosis (Wu *et al.* 2007). Polyphenol-rich extracts of strawberry, arctic bramble and cloudberry reduced the viability of Caco-2 colon adenocarcinoma cells (McDougall *et al.* 2008). In conclusion, study results presented in Table 1 suggest that ellagitannin-containing berry extracts and ellagic acid may inhibit cell proliferation and migration, induce apoptosis, attenuate inflammatory signalling and reduce oxidative damage in tumour cell cultures. However, since most berries contain both ellagitannins and anthocyanins, it remains unclear to what extent the effects are specific for ellagitannins *per se*.

Table 1. Summary of reviewed *in vitro* studies on the effects of ellagitannin and anthocyanin-containing berry extracts or ellagic acid (EA) in human gastrointestinal* tumour cell cultures.

Reference	Cell line(s)	Berries or compounds studied / Study design	Main findings: the effects of berry extracts and EA on carcinogenesis
Katsube *et al.* 2003	HCT116 colon carcinoma cells HL60 leukemia cells	Extracts from raspberry, blackberry and strawberry at 2–6 mg dry wt/mL for 48 hrs	Cell growth ↓ by all the berries in both cell lines; the mechanisms were not studied.
Losso *et al.* 2004	Caco-2 colon adenocarcinoma cells HUVEC[1] cells HEL 299 normal lung fibroblasts MCF-7 and Hs 578T breast cancer cells DU 145 prostate cancer cells	Ellagic acid at 1–100 µmol/L for six hours	No effect on the viability of normal fibroblasts. Caco-2 were the most sensitive cells. Cell proliferation ↓, migration ↓ in all cancer cells. Apoptosis ↑, the activities of pro-matrix metalloproteinases MMP-2 and MMP-9 ↓, the levels of secreted vascular endothelial growth factor VEGF165 ↓. Tube formation in HUVEC cells ↓.
Olsson *et al.* 2004	HT-29 colon adenocarcinoma cells MCF-7 breast cancer cells	Raspberry extract at 0.025, 0.05, 0.25, and 0.5% of plant dry wt/total wt for 24 hrs	Proliferation dose-dependently ↓ in both cell lines. Correlation between the inhibitory effect and vitamin C levels in extracts; probably synergism between vitamin C and other compounds.
Larrosa *et al.* 2006	Caco-2 colon adenocarcinoma cells CCD-112CoN normal colon cells (fibroblasts)	Caco-2: EA at 1-30 µM, punicalagin (PUNI) at 1-100 µM, and their mixtures (1 + 1 µM, 10 + 10 µM) for 72 hrs; CCD-112CoN: EA at 30 µM and PUNI at 100 µM for 12 days (media incl. EA and PUNI were replaced every 3 days)	Caco-2: Proliferation dose- and time-dependently ↓ by EA, punicalagin and their mixture (1 + 1 µM, 10 + 10 µM); apoptosis ↑ (Bcl-xL ↓, activation of caspases 3 and 9); cyclin A and B1 ↓, cyclin E ↑; cell-cycle arrest in S phase. CCD-112CoN cells; no effect on apoptosis.
Olsson *et al.* 2006	HT-29 colon adenocarcinoma cells MCF-7 breast cancer cells	Extracts from organically or conventionally cultivated STR at 0.025%, 0.05%, 0.25%, 0.5% (plant dry wt/total wt) for 24 hrs	Proliferation ↓ by all STR extracts in both cell lines. For HT29 cells, a negative correlation between the content of ellagic acid and the extent of antiproliferation was found at 0.05% STR extract.
Seeram *et al.* 2006a	HCT116 colon carcinoma cells HT-29 colon adenocarcinoma cells KB and CAL-27 oral tumour cells MCF-7 breast cancer cells LNCaP prostate carcinoma cells	Blackberry (BlackB), BRB, BB, red raspberry (RRB) and strawberry (STR) extracts at 25-200 µg/mL for 48 hrs	Proliferation dose-dependently ↓ in all each cell lines. In HT-29 cells, apoptosis ↑ by BRB and STR at 200 µg/mL; the pro-apoptotic effect of other berries was smaller. No effect on apoptosis.

to be continued

Table 1 continues: Summary of reviewed *in vitro* studies on the effects of ellagitannin and anthocyanin-containing berry extracts or ellagic acid (EA) in human gastrointestinal* tumour cell cultures.

Reference	Cell line(s)	Berries or compounds studied / Study design	Main findings: the effects of berry extracts and EA on carcinogenesis
Coates *et al*. 2007	HT-29 colon adenocarcinoma cells Caco-2 colon adenocarcinoma cells HT115 colon carcinoma cells	Colon-available raspberry extract (CARE) prepared by subjecting the original extract to an *in vitro* digestive process; at several concentrations ranging between 0–50 µg/ml for 24–48 hrs	HT-29: H_2O_2-induced DNA damage ↓, the number of cells in G_0/G_1 phase of cell cycle ↓ HT115: invasion in Matrigel invasion assay ↓ Caco-2: no effect on barrier function of the monolayer.
Wu *et al*. 2007	HT-29 colon carcinoma cells	Cloudberry (CB), STR, and RB extracts at 0-60 mg/mL for 24 hrs for the proliferation assay and RNA extraction, and for 48 hrs for the DNA fragmentation assay	CB extract: 40% ↓ in cell growth, 14-fold ↑ in $p21^{WAF1}$ and 1.3-fold ↑ in Bax mRNA expression at 40 mg/mL for 24 h. DNA fragmentation by CB extract at 40-60 mg/mL. RB and STR extracts: 20-30% ↓ in cell growth, 5.8-fold ↑ by RB and 3-fold ↑ by STR in $p21^{WAF1}$ mRNA expression at 60 mg/mL.
McDougall *et al*. 2008	Caco-2 colon adenocarcinoma cells HeLa cervical cancer cells	Raspberry (RB), cloudberry (CB), arctic bramble (AB), strawberry (STR), blueberry (BB), and pomegranate (PG) extracts enriched in polyphenols (without sugars, organic acids, vitamin C and carotenoids) at 25, 50, and 75 µg of GAE/mL for 72 hrs	STR, AB, CB and RB: cell viability of HeLa cells ↓ (to ≤ 50% of control at 50 µg gallic acid equivalents (GAE)/mL). STR, AB, and CB: viability of CaCo-2 cells ↓ (25 µg of GAE/mL more effective than higher concentrations in Caco-2 cells). Mechanisms of antiproliferation were not studied.
Romier *et al*. 2008	Caco-2 colon adenocarcinoma cells	EA at 50 µmol/l for 24 hrs for the proliferation, cytotoxicity and nuclear factor κB activation assays, for 48 hrs for the interleukin-8 secretion assay	No antiproliferative or cytotoxic effects at 50 µM EA. Lipopolysaccharide-induced nuclear factor κB activation ↓, interleukin-1β induced interleukin-8 secretion ↓; no effect on IkB-α phosphorylation.
Zhang *et al*. 2008	HT-29 colon adenocarcinoma cells HCT116 colon carcinoma cells KB and CAL-27 oral tumour cells LNCaP and DU145 prostate carcinoma cells	STR extracts including ET-rich extract and purified STR phenolic compounds including EA; extracts at 250 µg/mL for 48 hrs, EA at 100 µg/mL for 48 hrs	ET-rich extract: proliferation ↓ in all cell lines by 30-95%. KB cells were the most and DU145 cells the least sensitive to the extract. EA: proliferation ↓ in all cells by 30–80%. HCT-116 were the most and HT-29 the least sensitive cells.

to be continued

Table 1 continues

Reference	Cell line(s)	Berries or compounds studied / Study design	Main findings: the effects of berry extracts and EA on carcinogenesis
God *et al*. 2010	LoVo colon cancer cells AGS stomach cancer cells MCF-7 breast cancer cells	Red raspberry extract, ascorbic acid only (the same pH and antioxidant activity), or HCl only (the same pH) at 5%, 7.5%, and 10% for 48 hrs	LoVo and AGS: cell growth ↓; more effective than ascorbic acid only. MCF-7: antioxidant effect had a larger role in apoptosis-independent cell killing than for other cells.
Johnson *et al*. 2011	HT29 colon adenocarcinoma cells	75 black raspberry (BRB) extracts from different cultivars, production sites and fruit maturity stages at 0.6 and 1.2 mg/mL for 48 hrs	Cell growth ↓ in a dose-dependent manner; the higher concentration had cytotoxic effects. The antiproliferative effect was influenced by the horticultural parameters.
Brown *et al*. 2012	HT-29 colon adenocarcinoma cells HT115 colon carcinoma cells	Raspberry, strawberry, and blackcurrant extracts subjected to an *in vitro* digestion and fermentation process to simulate conditions *in vivo* at 0–50 μg/ml GAE for 24–48 hrs	HT-29: H_2O_2- induced DNA damage ↓, faecal water-induced mutations ↓ HT115: invasion of HT115 cells in Matrigel invasion assay ↓
Umesalma *et al*. 2015	HCT-15 colon adenocarcinoma cells	EA at 20–120 μM for 0–48 h	Cell viability ↓; cell cycle arrest; expressions of proliferating cell nuclear antigen, cyclin D1, phosphatidylinositol 3-kinase, pAKt, and Bcl-2 ↓; expressions of Bax, cyt *c* and caspase-3 ↑; DNA fragmentation ↑; all in a dose- and/or time-dependent manner.

BERRIES: AB, arctic bramble; BB, blueberry; BlackB, blackberry; BRB, black raspberry; CB, cloudberry; PG, pomegranate; RB, raspberry; RRB red raspberry; STR strawberry.

OTHER ABBREVIATIONS: EA, ellagic acid; GAE, gallic acid equivalents; HUVEC, human umbilical vein endothelial cells. The ↑ ↓ arrows signify the direction of the change in comparison to the respective controls.

* If cells of tissues and organs other than gastrointestinal cells were used in a study, they have also been included in the table.

4.2 Studies in animal models

Hitherto *in vivo* studies on the effects of ellagitannin-containing berries on experimental tumourigenesis have used black raspberries *(Rubus occidentalis)* and strawberries *(Fragaria × ananassa)* (reviewed in Table 2). Black raspberry contains moderate amounts of ellagitannins (175 mg/100 g dry weight; Stoner *et al*. 2006) but it is also a rich source of anthocyanins (381 mg/100 g fresh weight; Scalzo *et al*. 2008). Strawberry is a moderate source of both ellagitannins and anthocyanins (77 mg and 38 mg/100 g fresh weight; Koponen *et al*. 2007). Most of these studies have focused on oesophageal tumourigenesis and there are only three published studies that show the effects of black raspberry on intestinal (Bi *et al*. 2010) or colon carcinogenesis (Harris *et al*. 2001, Pan *et al*. 2015). These studies showed that black raspberry-feeding inhibited tumour development in *Apc*1638$^{+/-}$ mice by suppressing β-catenin signalling and in *Muc2*$^{-/-}$ mice by reducing chronic inflammation (Bi *et al*. 2010). Furthermore, black raspberry reduced the number and size of colonic tumours and reversed the levels of *Apc*-regulated metabolites in *Apc*^{Min} mice (Pan *et al*. 2015). Black raspberry also reduced AOM-induced tumourigenesis and the concentration of urinary 8- hydroxy-2'-deoxyguanosine, a marker of oxidative stress, in Fischer 344 rats (Harris *et al*. 2001). An earlier study reported that a high concentration of ellagic acid reduced the number of colon adenomas in AOM-induced Fischer 344 rats (Rao *et al*. 1991).

In summary, the study results reviewed in Table 2 suggest that ellagic acid and ellagitannin-containing berries, particularly strawberries and black raspberries, inhibit tumour development in the oral cavity, the oesophagus and the colon both in carginogen-induced and genetically modified animal models. The mechanisms beyond the inhibitory effects include decreased formation of DNA adducts, attenuated inflammatory signalling and angiogenesis, and restoration of gene expression dysregulated either by carcinogen-treatment or genetic modification.

Table 2. Summary of reviewed animal studies on the effects of ellagitannin and anthocyanin-containing berries or ellagic acid (EA) on gastrointestinal carcinogenesis. All studies contained a control group fed a similar diet without berries or phenolic compounds. In carcinogen-induced models, the results are expressed in relation to the respective carcinogen-treated control group. All berries used were freeze-dried and powdered, and the quantities of berries in the diets are expressed as weight/weight (w/w) percentages.

Reference	Animal model	Diets and duration of the feeding period in days and weeks	Main findings: the effects of berries and EA on carcinogenesis
Rao *et al*. 1991	F344 rats, ♂ AOM-induced colon carcinogenesis	AIN-76A + 4,000 or 8,000 mg EA/kg diet Before, during and after AOM treatment 56 wks	8,000 mg EA/kg: number of colon adenomas ↓, no effect on the number of adenocarcinomas. Long-term dietary administration of EA at 2,000–8,000 mg/kg diet individually or in combination with other compounds was not toxic.
Ahn *et al*. 1996	Fischer 344 (F344) rats, ♂ Isolated oesophageal and hepatic microsomes were used for enzyme activity assays	AIN-76 A + 0.4 g or 4.0 g EA/kg diet 23 days	Total P450 content in liver ↓, activities of certain hepatic phase II enzymes ↑. No remarkable changes in the expression or activities of oesophageal enzymes.
Stoner *et al*. 1999	F344 rats, ♂ NMBA-induced oesophageal tumours	AIN-76A + 5% or 10% STR (containing EA 0.34 and 0.67 mg/kg diet) Before, during and after NMBA treatment 24 wks	Tumour number and formation of O^6-methylguanine adducts in oesophageal DNA ↓ in a dose-dependent manner. The effect of STR was not explained by its EA alone.
Carlton *et al*. 2001	F344 rats, ♂ Nitrosamine carcinogen (NMBA)-induced oesophageal tumours	AIN-76A + 5% or 10% STR Before, during and after the NMBA treatment (complete carcinogenesis study; 32 wks) or starting after the 5 wks' NMBA treatment (post-initiation study; 20 wks) or short-term study for DNA adduct analyses	5% and 10% STR: tumour number ↓ both in the complete carcinogenesis study and in the post-initiation study; in the latter, 5% was more effective than 10%. Formation of O^6-methylguanine adducts in oesophageal DNA ↓by both STR concentrations.
Harris *et al*. 2001	F344 rats, ♂ Azoxymethane (AOM)-induced aberrant crypt foci and colon tumours	AIN-76A + 0, 2.5, 5, or 10% BRB 9 or 33 wks after AOM treatment	2.5, 5, and 10% BRB: ACF number ↓, tumour number ↓, concentration of urinary 8- hydroxy-2'-deoxyguanosine ↓. 10% BRB: number of adenocarcinomas ↓.
Kresty *et al*. 2001	F344 rats, ♂ NMBA-induced oesophageal tumours	AIN-76A + 5 or 10% BRB Anti-initiation: before, during and after NMBA treatment for 30 wks Post-initiation: after NMBA treatment for 15, 25 and 35 wks	5 and 10% BRB in anti-initiation assay: tumour number ↓. 5 and 10% BRB in post-initiation assay: at 25 wks, tumour incidence ↓, tumour number ↓, proliferation rates and preneoplastic lesion development ↓. At 35 wks: 5% BRB only reduced these parameters.

to be continued

Table 2 continues: Summary of reviewed animal studies on the effects of ellagitannin and anthocyanin-containing berries or ellagic acid (EA) on gastrointestinal carcinogenesis.

Reference	Animal model	Diets and duration of the feeding period in days and weeks	Main findings: the effects of berries and EA on carcinogenesis
Chen *et al*. 2006a	F344 rats, ♂ NMBA-induced oesophageal tumours	AIN-76A + 5% BRB 9, 15 and 25 wks after NMBA treatment	Tumour number ↓, mRNA and protein expressions of iNOS, COX-2, and c-*Jun* ↓ at 25 wks. Activities of iNOS and COX-2 ↓.
Chen *et al*. 2006b	F344 rats, ♂ NMBA-induced tumours from the study of Chen *et al*. (2006a) above	AIN-76A + 5% BRB 9, 15 and 25 wks after NMBA treatment (Chen *et al*. 2006a)	Vascular endothelial growth factor (VEGF) mRNA expression ↓, microvessel density of oesophagus ↓. Correlation between the expression levels of VEGF, COX-2 and iNOS.
Lechner *et al*. 2008	F344 rats, ♂ No carcinogenic or genetic modification	AIN76-A + 5% BRB 3 wks	Modification of the expression of 36 genes in the rat oesophagus; some of them were associated with cancer mechanisms.
Stoner *et al*. 2008	F344 rats, ♂ Short-term NMBA-induction of oesophageal carcinogenesis Sacrificed 24 h after the last injection	AIN-76A + 5% BRB / 5 µmol phenylethyl isothiocyanate (PEITC) 2 wks before and during the one week's NMBA treatment	Dysregulation of expression of 2,261 genes by NMBA. Restoration of expression of 1,323 genes by PEITC and 462 genes by BRB to near-normal level. The 53 genes common to PEITC and BRB were involved in phase I and II metabolism, oxidative damage, apoptosis, cell cycling, and angiogenesis.
Bi *et al*. 2010	*Apc* $1638^{+/-}$ and *Muc2*$^{-/-}$ mice Intestinal tumourigenesis	Western-style diet (WD) + 10% BRB 12 wks	Tumour incidence ↓, tumour number ↓, cell proliferation ↓ in both mouse strains; β-catenin expression ↓ in *Apc* $1638^{+/-}$ mice, expression of COX-2 and inflammatory cytokines ↓ in *Muc2*$^{-/-}$ mice
Stoner *et al*. 2010	F344 rats, ♂ NMBA-induced oesophageal tumours	AIN-76A + 5% BRB / red raspberry / blueberry / STR 30 wks after NMBA treatment	All berries: tumour incidence and number equally ↓, serum interleukin-5 and GRO/KC (rat homologue for human interleukin-8) ↓. Serum IFNγ ↑ by BRB (richest in ETs).
Umesalma and Sudhandiran 2010a, 2010b, 2011; Umesalma *et al*. 2014	Wistar rats, ♂ DMH-induced colon carcinogenesis	EA 60 mg/kg body weight once daily; orally in water, and commercial pellet diet After DMH treatment, for 15 wks	2010a: Number of ACFs ↓, number of crypts/ACF ↓, level of lipid peroxidation in colon tissue and plasma ↓, activities of antioxidant enzymes, reduced glutathione, vitamin E, and vitamin C ↑ in colon. 2010b: Expression of inflammatory mediators NFκB, iNOS, COX-2, TNF-α, and interleukin-6 ↓. 2011: Apoptosis ↑: expressions of PI3K, pAKT, and Bcl-2 ↓, and expressions of Bax, caspase-3 and cytochrome c ↑. 2014: Proliferation ↓ (argyrophilic nucleolar organizing regions, AgNOR ↓, PCNA positive nuclei ↓), cyclin D1 expression ↓, mast cells ↓, glycoproteins in colon tissue ↓, expression of matrix metalloproteins MMP-2 and MMP-9 ↓, p53 expression ↑, activities of phase I enzymes ↓ and phase II enzymes ↑

to be continued

Table 2 continues

Reference	Animal model	Diets and duration of the feeding period in days and weeks	Main findings: the effects of berries and EA on carcinogenesis
Wang *et al*. 2010	F344 rats, ♂ NMBA-induced oesophageal tumours	AIN-76A + 5% or 10% BRB / STR / BB ET-rich residues from equal amounts of berries Before, during and after NMBA treatment 30 wks	Berries and berry residues: tumour number ↓ equally. 10% berry diets: tumour volume slightly more ↓ than diets with berry residues or 5% berries. ET concentration of diets was not associated with chemopreventive effects.
Wang *et al*. 2011b	F344 rats, ♂ NMBA-induced oesophageal tumours	AIN-76A + 5% BRB 35 wks after NMBA treatment	Tumour number and size ↓. Dysregulation of expression of numerous genes by NMBA; restoration of expression levels of many genes towards control levels by BRB in preneoplastic oesophagus and tumours. The NMBA-induced upregulation of Ki-67, CD45, CD34, and MMP10 ↓ by BRB. Bcl-2/Bax ratio, COX-2, and VEGF expressions ↓, expression of p27 ↑.
Casto *et al*. 2013	Syrian Golden Hamsters, ♂ 7,12-dimethylbenz(a)-anthracene (DMBA) induced oral tumourigenesis	AIN-76A + 5% or 10% STR Before, during, and after (complete chemoprevention bioassay) DMBA treatment or after DMBA treatment only (post-initiation chemoprevention) All feeding periods for 12 wks after DMBA treatment	5% and 10% STR: number of oral lesions ↓ in complete and post-initiation chemoprevention bioassays. 5% STR modulated the expression of genes related to tumour development in post-initiation study.
Pan *et al*. 2015	*Apc*^{Min} and wild-type (WT) mice	*Apc*^{Min} mice fed with AIN-76A with or without 5% BRB WT-mice fed with AIN-76A for 8 wks	Number and size of intestinal and colonic tumours ↓. The *Apc* gene mutation changed and BRB-feeding reversed the levels of several metabolites involved in amino acid, glutathione, lipid and nucleotide metabolism in the colonic mucosa, liver and faeces.

BERRIES: BB, blueberry; BRB, black raspberry; STR strawberry.

OTHER ABBREVIATIONS: ACF, aberrant crypt foci; AOM, azoxymethane; COX-2, cyclooxygenase; DMBA, 7,12-dimethylbenz(a)anthracene; IFNγ, interferon gamma; iNOS, inducible nitric oxide synthase; NMBA, *N*-nitrosomethylbenzylamine; TNF-α, tumour necrosis factor α; PEITC, phenylethyl isothiocyanate; PI3K, phosphatidylinositol 3-kinase; VEGF, vascular endothelial growth factor. The ↑ ↓ arrows signify the direction of the change in comparison to the respective controls.

4.3 Human studies

Only a few clinical studies on the effects of ellagitannin-containing berries on human gastrointestinal cancers have been published. In colorectal cancer patients, 60 g of freeze-dried black raspberries per day for four weeks decreased the methylation of tumour suppressor genes and protectively changed the expression of proteins related to cell proliferation, apoptosis, angiogenesis, and Wnt pathway (Wang *et al.* 2011a). The same study found that black raspberry treatment increased the plasma concentration of granulocyte macrophage colony stimulating factor (GM-CSF), which can activate the immune response against tumours (Jinushi *et al.* 2009). Furthermore, black raspberry decreased the concentration of pro-inflammatory IL-8, and modulated the markers of proliferation, apoptosis, and angiogenesis in adenocarcinomas and adjacent normal-appearing tissue into the protective direction (Mentor-Marcel *et al.* 2012). Two daily rectal suppositories of black raspberry (each containing 720 mg black raspberry powder) administered to familial adenomatous polyposis (FAP) patients for nine months significantly decreased the burden but not the number of rectal polyps. However, no additional benefit was seen when the rectal suppositories were combined with 60 g of orally administered freeze-dried black raspberries per day for nine months. Black raspberry also decreased the expression of DNA methyltransferase 1 expression and *p16* promoter methylation in rectal polyps (Wang *et al.* 2014a).

The protective effects of black raspberries and strawberries on preneoplastic lesions of the oesophagus and oral cavity have also been reported. Freeze-dried black raspberries, 32 g for females or 45 g for males, administered to Barrett's oesophagus patients once a day for 26 weeks reduced urinary excretion of 8-iso-prostaglandin F2α, a marker of lipid peroxidation, and increased the expression of GST-pi, a marker of detoxification, in Barrett´s oesophagus epithelium (Kresty *et al.* 2016). In patients with dysplastic lesions of the oesophagus, 60 g of freeze-dried strawberries daily for six months inhibited the progression of precancerous growth. The berry treatment reduced the histological grade of lesions, inhibited the protein expression of iNOS, COX-2, NF-κB, pS6, and inhibited cell proliferation. However, the smaller dose (30 g/day) did not affect the grade of lesions or any of the measured parameters (Chen *et al.* 2012). A 10% (w/w) freeze-dried black raspberry gel applied four times daily for six weeks modified gene expression, decreased the level of COX-2 protein and reduced loss of heterozygosity (LOH) in the oral intraepithelial neoplasia in patients with premalignant oral intraepithelial lesions (Mallery *et al.* 2008, Shumway *et al.* 2008). The same dosage of gel for three months decreased the lesion size and histopathological grade, and reduced the LOH events in the oral intraepithelial lesions (Mallery *et al.* 2014). In summary, black raspberries and strawberries are suggested to inhibit carcinogenesis in the colon, the oesophagus, and the oral cavity in human

subjects by regulating the expression of genes and proteins related to cell proliferation, apoptosis, angiogenesis, and inflammatory response both in the tumours and the adjacent normal-appearing tissue.

4.4 Pomegranate, its ellagitannins and their microbial metabolites urolithins

Pomegranate *(Punica granatum)* is the most familiar and widely studied source of ellagitannins, and the interested readers are directed to a recent review on its effects on colorectal cancer chemoprevention (Núñez-Sánchez *et al.* 2015a). Briefly, both pomegranate ellagitannins and their metabolites, the urolithins, inhibit cell proliferation and induce apoptosis in several colorectal cancer cell lines (Larrosa *et al.* 2006, Seeram *et al.* 2005, González-Sarrías *et al.* 2009, González-Sarrías *et al.* 2014, Kasimsetty *et al.* 2010). Pomegranate extract, juice, and peel extract inhibit carcinogen-induced colon tumourigenesis in rodent models (Sadik *et al.* 2013, Banerjee *et al.* 2013, Boateng *et al.* 2007, Waly *et al.* 2012, Waly *et al.* 2014). Free ellagic acid and its conjugates, gallic acid and 12 urolithin derivatives were found in colon tissues of colorectal cancer patients that had consumed 900 mg pomegranate extracts per day for 15 days before surgical resection. Interestingly, the concentrations of all metabolites were higher in the normal colonic tissue than in the malignant tissue (Nuñez-Sánchez *et al.* 2014). The same trial found that the intake of pomegranate extract changed the expression of micro-RNAs (miRNA) both in malignant and normal colonic tissues. Even though the regulated miRNAs targeted cancer-related genes, no association was found between the tissue urolithins and the changes in the miRNA expression (Nuñez-Sánchez *et al.* 2015b).

5 Proposed mechanisms of ellagitannin-containing berries and their compounds in colon cancer prevention

The mechanisms that mediate the chemopreventive effects of berries, berry extracts, and their compounds have been studied for decades, but they are still not known in detail. The effects of ellagic acid have been investigated since 1982, when its antimutagenic activity was shown (Wood *et al.* 1982). The early studies that followed the Wood group's study were focused on the ability of ellagic acid to inhibit metabolic activation of carcinogens by inhibiting cytochrome P450 enzymes, inducing certain phase II enzymes, and interfering in the binding of carcinogen metabolites to DNA (Ahn *et al.* 1996). Thereafter, berries, berry extracts and their compounds have been shown to inhibit carcinogenesis through numerous mechanisms and the role of gut microbiota in mediating their effects has also been found to be important. Both human and animal studies have shown that the composition of gut microbiota is associated with the colorectal carcinogenesis (Borges-Canha *et al.* 2015, Irrazábal *et al.* 2014, Schwabe and Jobin 2013). Furthermore, diet is known to modulate the composition of gut microbiota (David *et al.* 2014, Salonen and de Vos 2014, Walker *et al.* 2011), and dietary sources of ellagitannins have also been found to affect gut microbes both *in vitro* and *in vivo* (Li *et al.* 2015a and 2015b, Puupponen Pimiä *et al.* 2013). The effects of ellagitannins and their dietary sources on the gut microbiota are described here first, followed by other effects.

5.1 Gut microbiota

Cloudberry, raspberry and their ellagitannins have antimicrobial activities and they selectively inhibited the growth of Gram-negative bacteria, such as the intestinal pathogen *Salmonella enterica,* but not the Gram-positive probiotic lactic acid bacteria *in vitro* (Puupponen-Pimiä *et al.* 2001, 2005). Pomegranate juice by-product and punicalagins inhibited the growth *in vitro* of the pathogenic *Clostridium* species and *S. aureus,* but had no or only a slightly inhibiting effect on the growth of lactobacilli, and even enhanced the growth of some bifidobacteria (Bialonska *et al.* 2009). Another study showed that pomegranate juice and extract increased the growth of lactobacilli and bifidobacteria, but inhibited the growth of *B. fragilis* group, clostridia, and *Enterobacteriaceae* in a dose-response manner *in vitro,* and may thus be able to act as potential prebiotics (Li *et al.* 2015).

Pomegranate juice by-product rich in gallic acid oligomers, ellagic acid, and glucose increased the total number of bacteria, the number of *Bifidobacterium* ssp. and the *Lactobacillus-Enterococcus* group, and the concentrations of short chain fatty acids in a fermentation system that simulated the human colon and was inoculated with samples of human faeces. However, the same treatment in the same set up did not affect the growth of the *Clostridium coccoides-Eubacterium rectale* group and the potentially harmful *C. histolyticum* group. In contrast, punicalagins alone had no effect on the growth of bacteria or production of short chain fatty acids (Bialonska *et al.* 2010). Pomegranate extract (250 mg/kg body weight/day) or its main metabolite urolithin A (15 mg/kg body weight/day) in diet of Fisher rats increased the number of faecal bifidobacteria, lactobacilli and *Clostridium spp*. (Larrosa *et al.* 2010). The number of caecal *Bifidobacterium* ssp. was also increased in Balb/c mice fed with a high-fat diet supplemented with pomegranate peel extract (6 mg/day/mouse) in comparison to control mice (Neyrinck *et al.* 2013).

Human subjects can be stratified into urolithin producers and non-producers as indicated by the findings of intervention studies. Of healthy subjects, 60–80% belong to phenotype A that produce only urolithin A, 10 – 30% to phenotype B that produce isourolithin A and/or urolithin B in addition to urolithin A, and 5 – 15% to phenotype 0 that have no urolithin production (Tomás-Barberan *et al.* 2014). This inter-individual variability can affect the outcome of intervention studies and should be considered when analyzing the results. Li *et al.* (2015) found that both the baseline gut microbiota and the changes induced by daily intake of 1000 mg pomegranate extract for four weeks were different in urolithin producers and non-producers; *e.g.* the phylum *Firmicutes* was decreased and *Proteobacteria* increased in urolithin producers but not in non-producers after the four weeks' intervention. In addition, pomegranate extract consumption enhanced urolithin A production in producers and induced production in some but not in all non-producers (Li *et al.* 2015). Another study found that an intake of 200 ml/day of pomegranate juice (containing 880 mg ellagic acid and ellagitannins) for four weeks increased the faecal concentrations of urolithin A and other phenolic metabolites, but did not change the composition of gut microbiota (Mosele *et al.* 2015). Furthermore, no differences were found in the diversity of faecal predominant bacterial populations of human subjects after a daily intake of strawberries, raspberries and cloudberries containing 790 mg ellagitannins or a diet with practically no ellagitannins for eight weeks. However, predominant bacterial profiles changed in four out of 20 subjects in the berry group, and two of these subjects did not produce urolithins at baseline but the production of urolithins was induced during the period of berry supplementation (Puupponen-Pimiä *et al.* 2013).

In summary, dietary ellagitannins modulate the metabolism of phenolic compounds in the gut and can alter the composition of gut microbiota. The latter occurs as a consequence of antimicrobial and prebiotic effects. Ellagitannins, and other polyphenols, promote the growth of lactobacilli and bifidobacteria, and thus act as prebiotics (Dueñas *et al.* 2015). These changes in gut microbiota are suggested to decrease the production of genotoxic compounds and increase the concentrations of bioavailable phenolic metabolites.

5.2 Anti-genotoxic effects

Carcinogenesis is initiated by mutations in genes that are associated with cell proliferation, differentiation and apoptosis, and ellagitannin-containing berry extracts have been found to protect cells from genotoxic effects *in vitro*. Since berry extracts and single berry polyphenols are potent antioxidants *in vitro* (Skrovankova *et al.* 2015), their anti-genotoxic effects may be at least partly due to their antioxidant activity. Raspberry polyphenols that were digested in *in vitro* conditions inhibited H_2O_2-induced DNA damage in HT-29 cells (Coates *et al.* 2007). Moreover, digested and then fermented raspberry and strawberry extracts decreased both faecal water and H_2O_2-induced genotoxic effects in the same cell line (Brown *et al.* 2012). The feeding of black raspberry to azoxymethane-treated Fischer 344 rats decreased the number of aberrant crypt foci and total number of tumours, and it also reduced the concentration of urinary 8-hydroxy-2'-deoxyguanosine (Harris *et al.* 2001), which is a widely used biomarker for endogenous oxidative DNA damage (Valavanidis *et al.* 2009). However, the antioxidant capacity of a compound or an extract does not always correlate with its anti-carcinogenic or antiproliferative activity (Liu *et al.* 2002, Meyers *et al.* 2003); and not all the strong antioxidants are strong anti-carcinogenic agents, and *vice versa* (Wang *et al.* 2013).

Berry compounds have also been suggested to selectively bind to DNA and thus block DNA methylation and enhance the repair of pro-mutagenic adducts. For example, black raspberry feeding before the treatment with *N*-nitrosomethylbenzylamine carcinogen inhibited the formation of the pro-mutagenic O^6-methylguanine adducts in the rat oesophagus, even though it did not affect the metabolism of *N*-nitrosomethylbenzylamine (Kresty *et al.* 2001).

5.3 Cell proliferation, apoptosis and invasive growth

All functions in normal cells are strictly regulated by numerous signalling pathways, whereas this control is lost in cancer cells as the result of mutations and/or epigenetic modifications in DNA. Dysregulated expression and

activation of proteins that regulate cell proliferation, differentiation, and apoptosis lead to the uncontrolled growth and formation of neoplastic tissue (Hanahan and Weinberg 2000, 2011). Proliferation, and differentiation, and all other steps in the life cycle of cell, are regulated by many factors including cell cycle proteins, signal transduction pathways, conditions in the cell microenvironment, and cell metabolism (Hanahan and Weinberg 2000, 2011). Apoptosis can be induced or blocked by factors that regulate either the extrinsic or intrinsic apoptosis pathway (Kiraz *et al.* 2016). Alterations in some or all of these factors accumulate in cells as they change from normal to malignant phenotype.

Polyphenolic compounds including ellagitannins have been suggested to prevent carcinogenesis by inhibiting the alterations associated with uncontrolled proliferation (Feitelson *et al.* 2015) and by inducing apoptosis (Cho *et al.* 2015, González-Sarrías *et al.* 2016, Kasimsetty *et al.* 2010, Larrosa *et al.* 2006, Seeram *et al.* 2005 and 2006a). The antiproliferative effects of ellagitannins may also be due to their anti-inflammatory effects on the colon mucosa (Adams *et al.* 2006, Banerjee *et al.* 2013, Kim *et al.* 2016, Marín *et al.* 2013). In addition, a few studies have shown that ellagic acid and ellagitannins may prevent invasive growth by inhibiting angiogenesis (Ceci *et al.* 2016, Kowshik *et al.* 2014, Labrecque *et al.* 2005, Lee and Lee 2005, Sartippour *et al.* 2008, Wang *et al.* 2012b, Wang *et al.* 2014b, Zhao *et al.* 2013) and cancer cell migration (Ceci *et al.* 2016, Gu *et al.* 2016, Ko 2015, Ko *et al.* 2015, Rocha *et al.* 2012, Wang *et al.* 2012a, Pitchakarn *et al.* 2013) in several cancer types. However, there is only one study that reports indirect effects of ellagic acid on angiogenesis in an *ex vivo* colon cancer model (Haraguchi *et al.* 2014) and another study investigated the effects of ellagic acid on colon cancer cell migration (Losso *et al.* 2004). The hallmarks of cancer presented by Hanahan and Weinberg (2000, 2011) that were suggested to be influenced by ellagitannin-containing berry extracts, ellagic acid, ellagitannins, and their metabolites in colon cancer models are summarized in Figure 4.

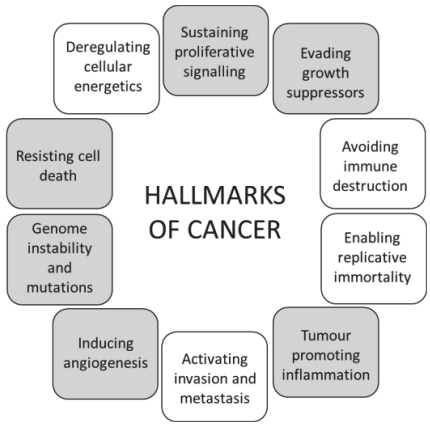

Figure 4. Hallmarks of cancer, 10 biological capabilities acquired during the tumour development, are represented by the rectangles. The hallmarks that are reported to be inhibited by ellagitannin-containing berry extracts, ellagic acid, ellagitannins, and their metabolites in colon cancer models according to the literature published so far are shown in grey shading. The figure is modified from that shown in the article by Hanahan and Weinberg (2011).

Anti-proliferative effects

Cell cycle, a process including replication of DNA in the S (synthesis) phase and cell division in the M (mitosis) phase, is driven by complexes of cyclin-dependent kinases (CDK) and cyclins. Its normal progress is assessed at checkpoints G1 before the S phase, G2 before the M phase, and at spindle assembly checkpoint at the M phase. In normal cells, the cell cycle arrest at the abovementioned checkpoints allows cells to repair possible defects in DNA synthesis and chromosome segregation, whereas in mutated and tumour cells a resulting dysregulated cell cycle leads to continuous and uncontrolled cell proliferation (Malumbres and Barbacid 2009). However, several studies have shown that ellagitannin-containing berry extracts, ellagitannins, and ellagic acid inhibit cell proliferation in colon tumour cells (Johnson *et al.* 2011, God *et al.* 2010, Katsube *et al.* 2003, Larrosa *et al.* 2006, Losso *et al.* 2004, Olsson *et al.* 2004, Olsson *et al.* 2006, Seeram *et al.* 2006, Wu *et al.* 2007, Zhang *et al.* 2008). A few studies that investigated the underlying mechanisms suggest that the antiproliferative effects were due to the cell cycle arrest and changes in the expression of cell cycle proteins (Coates *et al.* 2007, Larrosa *et al.* 2006, Umesalma *et al.* 2015). Raspberry polyphenols that had been digested in *in vitro* conditions induced cell cycle arrest in the G1 phase in HT-29 colon adenocarcinoma cells (Coates *et al.* 2007), whereas ellagic acid, punicalagin, alone, or in any combination induced cell cycle arrest in the S phase and decreased the expression of cyclins A and B1 in Caco-2 cells. However, the

increase in the expression of cyclin E was not in line with the findings mentioned above (Larrosa *et al.* 2006). In another study, ellagic acid induced cell cycle arrest in the G2/M phase and downregulated the expression of cyclin D1 and proliferating cell nuclear antigen (PCNA), a widely recognized proliferation marker, in HCT-15 colon adenocarcinoma cells (Umesalma *et al.* 2015). Ellagic acid (60 mg/kg body weight orally in water) also decreased the cyclin D1 expression and the number of PCNA-positive nuclei in the colon tissue of DMH-treated Wistar rats (Umesalma *et al.* 2014). The antiproliferative effects of cloudberry, strawberry, and raspberry extracts in HT-29 colon adenocarcinoma cells were associated with increased mRNA expression of CDK1/2 inhibitor $p21^{Cip1/Waf1}$, but the concentrations of berry extracts in cell culture medium were not physiologically relevant (Wu *et al.* 2007).

Pro-apoptotic effects

The chemopreventive effects of ellagitannin-containing berries, ellagitannins, and ellagic acid have also been suggested to be due to the induction of programmed cell death, apoptosis. In Caco-2 cells, ellagic acid, punicalagin, and mixture of them decreased the expression of anti-apoptotic Bcl-xL and activated caspase-9 and caspase-3, thus inducing apoptosis by intrinsic pathway. The observed effect on apoptosis was specific to Caco-2 cells and was not detected in CCD-112CoN normal colon fibroblasts (Larrosa *et al.* 2006). Umesalma *et al.* (2011, 2015) found similar pro-apoptotic effects by ellagic acid both in HCT-15 colon adenocarcinoma cells *in vitro* and in the colon tissue of DMH-induced Wistar rats *in vivo,* which showed increased expression of Bax, caspase-3 and cytochrome c, and decreased expression of Bcl-2. All these changes indicated activation of the intrinsic apoptotic pathway, which was suggested to be a result of inhibited PI3K/Akt pathway (Umesalma *et al.* 2011, 2015). Increased Bax expression and DNA fragmentation were also found in Caco-2 cells treated with cloudberry extract, but the concentration of extract (40–60 mg/mL) was far from a physiologically relevant concentration (Wu *et al.* 2007).

Invasive growth

Invasive growth is characterised by angiogenesis, tumour-promoting inflammation and cancer cell migration. Angiogenesis, the formation of new capillaries, is essential for tumour growth. When the diameter of a solid tumour exceeds two millimetres, new vasculature is required to provide the tumour with oxygen and nutrients (Bergers and Benjamin 2003). Vascular endothelial growth factor (VEGF) is regarded an important angiogenesis factor during carcinogenesis and tumour metastasis (Goel and Mercurio 2013). Ellagic acid has been found to inhibit angiogenesis by the VEGF pathway in several cancer types both *in vivo* and *in vitro* (Ceci *et al.* 2016, Kowshik *et al.* 2014, Labrecque *et al.* 2005, Wang *et al.* 2012b, Zhao *et al.*

2013). However, there is only one published study on the effects of ellagic acid on angiogenesis in colon cancer, in that study Haraguchi *et al.* (2014) reported that dietary ellagic acid elevated caecal succinate level, which in turn inihibited angiogenesis in an *ex vivo* angiogenesis assay. Moreover, a black raspberry diet (5% w/w) was found to downregulate VEGF mRNA expression and microvessel density in the oesophagus of NMBA-treated rats, which probably resulted from decreased expressions of COX-2 and iNOS (Chen *et al.* 2006a,2006b).

Inflammation is characteristic of invasive growth, but it is already present in the initiation phase of carcinogenesis (Elinav *et al.* 2013) and is associated with increased colorectal cancer risk, such as in individuals with inflammatory bowel disease (Sebastian *et al.* 2014) and obesity (Divella *et al.* 2016). Compounds with immunomodulatory and anti-inflammatory properties, including ellagitannins, can therefore inhibit carcinogenesis by attenuating pro-inflammatory signalling (Adams *et al.* 2006, Banerjee *et al.* 2013, Marín *et al.* 2013, Shi *et al.* 2015, Umesalma *et al.* 2010). Anti-inflammatory effects of ellagic acid and ellagitannins may be mediated by inhibition of transcription factor nuclear kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) signalling pathways, but also through the mammalian target of rapamycin (mTOR) pathway, which all have key roles in inflammatory response (Kim *et al.* 2016, Romier *et al.* 2009).

Ellagic acid inhibited lipopolysaccharide-stimulated NF-κB activity in Caco-2 cells, and also reduced interleukin (IL) 1β-induced secretion of IL-8 (Romier *et al.* 2008). Pomegranate extract also inhibited cytokine-stimulated NF-κB and ERK1/2 activities in Caco-2 cells, and decreased the synthesis of nitric oxide, IL-8, and prostaglandin E_2, probably through the NF-κB pathway (Romier-Crouzet *et al.* 2009). The feeding of black raspberry decreased the number and incidence of intestinal tumours in addition to the mRNA expression of COX-2 and proinflammatory cytokines in *Muc2*$^{-/-}$ mice, a widely used rodent model of chronic intestinal inflammation (Bi *et al.* 2010).

Cancer cell migration is one of the essential prerequisites to tumour progression and metastasis, and there are a few studies, which show that ellagic acid and ellagitannins inhibit migration in prostate cancer cells both *in vitro* (Pitchakarn *et al.* 2013, Wang *et al.* 2012a) and *in vivo* (Wang *et al.* 2014b), and in breast cancer cells *in vitro* (Rocha *et al.* 2012). There are hitherto no published studies that investigate the migration of colon cancer cells. However, the hepatocyte growth factor (HGF)-Met signalling pathway essential for cancer cell migration and an important target in the research for cancer treatments has been suggested to be involved in cancer cell migration in intestinal epithelial tissues.

6 Objectives of this study

The overall aim of this thesis was to study the chemopreventive effects of cloudberry on intestinal tumourigenesis in experimental models. The specific objectives were set during the progression of the studies as follows:

1) The first objective was to study, whether cloudberry and bilberry prevent tumour formation in *Apc*^{Min} mice.

2) The cloudberry effectively reduced both the number and size of intestinal tumours in *Apc*^{Min} mice. The next aim, therefore, was to reveal whether cloudberry pulp and seeds have distinct effects on tumour formation in *Apc*^{Min} mice. The effects of pure ellagic acid, which mimics the content of ellagitannins and ellagic acid in the cloudberry diet were also studied.

3) Both cloudberry and bilberry decreased the number of intestinal tumours in *Apc*^{Min} mice, but only cloudberry reduced the tumour size. The next objective was to study the molecular mechanisms behind the different effects of cloudberry and bilberry. The research focused on the effects of the berries on the gut microbiota, intestinal immunity, and mucosal global gene expression.

4) Cloudberry inhibited the first two steps of carcinogenesis in *Apc*^{Min} mice, thus the following objective was to study the effects of cloudberry on the indicators of cancer progression, particularly cancer cell migration and underlying cell signalling in human adenocarcinoma cell lines.

5) Because cloudberry inhibited Met signalling in human adenocarcinoma cells, the last aim in this research series was to ascertain whether cloudberry feeding had affected Met signalling in tumours of *Apc*^{Min} mice.

7 Study designs, materials and methods

7.1 Study designs and diets in animal studies (I-IV)

C57BL/6J *Apc*^{Min} mice, which carry a heterozygous *Apc* mutation and spontaneously develop intestinal tumours after spontaneous loss of the wild-type allele (Moser *et al.* 1995), were used to study the effects of berries on intestinal carcinogenesis. The mice (n=10-12/group) were fed experimental diets for 10 weeks. All diets were modified high-fat, non-fibre AIN93-G diets (Reeves *et al.* 1993) which had 41% of their energy from fat, 39% from carbohydrates, and 19% from protein. The composition and content of fat approximated to that in a typical Western-type diet and provided the intake of saturated, monounsaturated and polyunsaturated fatty acids in the ratio of 3:2:1. The compositions of diets in Studies I-IV relevant for this thesis are presented in Tables 3 and 4.

Studies I, II and IV

Experimental diets were prepared by adding 10% (w:w) freeze-dried berries to the modified high-fat AIN93-G diet, and the control diet was of a similar formulation to the berry treatment diet but without the berries (Table 3). The compositions of diets were designed so that the contents of carbohydrate, protein and fat were equal in both berry diets and in the control diet. Berries were obtained from Kiantama Oy (Suomussalmi, Finland), freeze-dried and ground. The texture of the diets was homogeneous apart from the cloudberry diet that contained whole cloudberry seeds. The polyphenol concentrations in the diets were calculated using the values that were obtained from the freeze-dried berries and are shown in Table 5. The methods used were described earlier (Määttä-Riihinen *et al.* 2004a, 2004b). The daily food consumption of a mouse was estimated to be 2.5 g/mouse/day, thus the calculated total intake of ellagitannins and free ellagic acid in the cloudberry group was 3.9 mg ellagic acid equivalents/mouse/day. Correspondingly, the total intake of anthocyanins was 13.8 mg/mouse/day and the intake of flavonols 0.26 mg/mouse/day in the bilberry group.

Study III

Cloudberry was found to be by far the most chemopreventive berry in Study I and the seeds made up nearly half of the freeze-dried cloudberries, therefore the effects of the components of cloudberry on adenoma formation were studied separately. The diets in Study III contained either cloudberry seeds, cloudberry pulp or pure ellagic acid (Table 5). The control diet without the berry components was similar to that formulated and used in Study I.

Cloudberries were obtained from Kiantama Oy (Suomussalmi, Finland), freeze-dried and ground before the seeds and pulp were separated from each other by sieving. The freeze-dried cloudberries were found to contain 47% (w/w) seeds and 53% (w/w) pulp. The same concentrations of seeds and pulp in the separate diets in comparison to the whole-cloudberry diet in Study I were formulated by adding 4.7% (w/w) seeds and 5.3% (w/w) pulp to the corresponding diets. Contents of ellagitannins and free ellagic acid in the cloudberry seeds and pulp were analysed as described previously (Häkkinen *et al.* 2000, Määttä-Riihinen *et al.* 2004b) and their concentrations in the diets were calculated based on the analysed values. The concentrations of ellagitannins and free ellagic acid in the seed diet were 807 and 42 mg/kg, and in the pulp diet 820 and 34 mg/kg, respectively. The calculated intakes of ellagitannins and free ellagic acid were 2.0 and 0.11 mg/mouse/day in the seed group and 2.1 and 0.08 mg/mouse/day in the pulp group and they were based on the estimated food consumption of 2.5 g/mouse/day. Pure ellagic acid (Sigma E-2250; Sigma-Aldrich Co., St. Louis, MO, USA) was added to the diet at 1565 mg/kg of diet, equal to its concentration in the 10% (w/w) whole-cloudberry diet in Study I, and the mean intake of pure ellagic acid was 3.9 mg/mouse/day. The composition of diets in Study III are presented in Table 4.

Table 3. Composition of treatment and control diets (g/kg diet) fed in Studies I, II and IV.

Ingredient	Control diet [a] g/kg	Cloudberry diet [b] g/kg	Bilberry diet [c] g/kg
Freeze-dried berry, containing		100	100
*Anthocyanins (mg)**		*0*	*5532*
*Flavonols (mg)***		*2*	*104*
*Total ellagic acid (mg)****		*1564*	*0*
Casein	236.2	217.8	220.7
Dextrose	479	412.6	413.2
Butter	148.9	142.5	141.1
Sunflower oil	13.3	9.8	8.8
Rapeseed oil	62.2	59.5	59.0
Mineral mix AIN-93-MX	41.6	39.8	39.4
Vitamin mix AIN-93-VX	11.8	11.3	11.2
L-cystine	3.6	3.4	3.4
Cholinechloride	3.6	3.4	3.4
Tert-butylhydroxyquinone	0.014	0.014	0.014

[a] Control diet was a high-fat, non-fibre modification of the AIN-93G diet (Reeves *et al.* 1993).

The [b] cloudberry and [c] bilberry diets were modified from the [a] control diet by the addition of cloudberry, or the reference bilberry. Addition of berries was compensated for by decreasing the amounts of other ingredients in the diets and taking into account the amounts of energy nutrients in berries.

*, **, *** mg/100 g freeze-dried berries and thus in a kg of diet

Table 4. Composition of treatment and control diets (g/kg diet) in Study III (CB, cloudberry; EA, ellagic acid).

Ingredient	Control diet [a] g/kg	CB seed diet [b] g/kg	CB pulp diet [c] g/kg	EA diet [d] g/kg
Cloudberry seeds		46.6		
Cloudberry pulp			53.4	
Ellagitannins from berry components *(mg)**		*807*	*820*	
Free ellagic acid from berry components *(mg)***		*42*	*34*	
Ellagic acid, *mg* (Sigma E-2250)				*1564*
Casein	236.2	222.6	229.4	235.8
Dextrose	479.0	459.2	440.7	478.3
Butter	148.9	142.7	144.6	148.7
Sunflower oil	13.3	11.4	12.9	13.3
Rapeseed oil	62.2	59.6	60.4	62.1
Mineral mix AIN-93-MX	41.6	39.9	40.4	41.5
Vitamin mix AIN-93-VX	11.8	11.3	11.5	11.8
L-cystine	3.6	3.5	3.5	3.6
Cholinechloride	3.6	3.5	3.5	3.6
Tert-butylhydroxyquinone	0.014	0.013	0.013	0.014

[a] Control diet was a high-fat, non-fibre modification of the AIN-93G diet (Reeves *et al.* 1993).

The [b] cloudberry (CB) seed, [c] cloudberry pulp and [d] ellagic acid diets were modified from the [a] control diet. Addition of cloudberry seeds or pulp was compensated for by decreasing the amounts of other ingredients and taking into account the amounts of energy nutrients in cloudberry components.

[d] Ellagic acid diet was modified from the [a] control diet. The addition of ellagic acid was compensated for by decreasing the amounts of other ingredients.

*, ** mg from the amount of cloudberry seeds or pulp added to a kg of diet

Table 5. Concentrations (mg/100g) of phenolic compounds in freeze-dried cloudberries and bilberries incorporated into the diets fed in Studies I, II and IV.

Phenolic compound	Cloudberry mg/100g	Bilberry mg/100g
Total anthocyanins, including	-	*5532*
Delphinidin glycosides	-	1717
Cyanidin glycosides	-	1818
Petunidin glycosides	-	840
Peonidin glycosides	-	462
Malvidin glycosides	-	695
Total flavonols, including	*2*	*104*
Quercetin glycosides	2	90
Myricetin glycosides	-	14
Total ellagic acid [b]	*1564*	*0*

* ellagitannins and unconjugated (free) ellagic acid

7.1.1 *Apc*^{Min} mice

The study protocols were approved by the Laboratory Animal Ethics Committee of the University of Helsinki. Male and female C57BL/6J Min/+ (*Apc*^{Min}) mice were bred at the Experimental Animal Unit of the University of Helsinki from inbred mice that had been originally obtained from the Jackson Laboratory (Bar Harbor, ME, USA). The mice were genotyped by using a PCR assay for the *Apc* alleles (Dietrich *et al.* 1993). At the age of five weeks, the mice were stratified by litter and sex and assigned randomly to the control or experimental diets in 10–12 mice per group. The duration of the feeding period was 10 weeks. The mice were housed in plastic cages in a temperature and humidity controlled animal facility, with 12-h light/dark cycle, and they had free access to a semisynthetic diet and tap water. Body weights and general welfare of the animals were recorded weekly. Mice that had a considerable loss of weight were sacrificed before the end of the feeding period and excluded from the analysis. The mice that were originally fed for Study I were also used in Studies II and IV. The final number of mice that were used in the analyses were 10 in the control group, 11 in the cloudberry group, and 12 in the bilberry group. Only the data and tissues from the control and cloudberry-fed mice were used in Study IV. The total number of mice in Study III was 47 (control 11, cloudberry seed 12, cloudberry pulp 12, and ellagic acid 12 mice).

7.1.2 Tumour scoring and sample collection

At the end of the feeding period mice were killed by CO_2 asphyxiation. The small intestine, caecum and colon were removed immediately. The small intestine was divided into five sections, opened longitudinally, rinsed with ice-cold isotonic saline and placed flat on objective glasses. The colon and caecum were kept together but were otherwise treated identically to the small intestine. The number, diameter, and location of adenomas were determined microscopically at a magnification of x 67.

Samples (7 mm) were taken from the distal end of the middle section of the small intestine, and fixed in buffered 4% formaldehyde solution overnight and embedded in paraffin for immunohistochemical analyses. The mucosa samples for RNA extraction were taken from the most distal section of the small intestine, namely the ileum. Sampling took place immediately after the tissue was confirmed to be free of any tumours and kept in RNA stabilization reagent (Qiagen) until isolation. Small diameter (≤1 mm) and large diameter (≥1.1 mm) adenomas were excised, collected separately according to the size category, snap frozen in liquid nitrogen and stored in −70°C for protein extraction and Western blot analyses in Study IV. Caecum contents for PCR-DGGE analysis in Study II were collected and frozen immediately at −70°C.

7.2 Study design and cloudberry extract in cell culture studies (IV)

Two human colon adenocarcinoma cell lines, HT29 cells that carry two mutated *APC* alleles and HCA7 cells with wild type *APC* alleles, were used to study the effects of cloudberry extract on cell migration and cell signalling *in vitro*. Cell migration was studied in scattering and wound healing assays both with and without hepatocyte growth factor (HGF) induction (Potempa *et al.* 1998, Mataraza *et al.* 2003). Cells were cultured at 37°C in a 5% CO_2 atmosphere in Dulbecco's Modified Eagle's medium (Gibco-Invitrogen) supplemented with 10% foetal calf serum, penicillin and streptomycin. For all experiments in Study IV, cells were seeded on fibronectin-coated (10 µg/mL) tissue culture dishes. Cells were incubated for 48−72 h before time-lapse microscopy for the cell scattering experiments, and allowed to grow to 100% confluence for the wound healing experiments. The concentration of cloudberry extract (5 µL/mL cell culture medium) was chosen based on preliminary experiments and it did not result in a reduction in cell numbers nor did it induce cell necrosis. Two independent preparations of cloudberry extract were used in Study IV.

7.2.1 Composition of cloudberry extract

Cloudberry extract was prepared by crushing six grams of freeze-dried cloudberries (including the seeds and peels) and extracting it with methanol that was removed by rotary evaporation after centrifugation at 3500g for 10 min. The dried sample was then reconstituted in 4.5 mL of Milli-Q water and frozen. The phenolic profile of cloudberry extract was determined using Waters ACQUITY ultra performance liquid chromatography (UPLC) as described by Kylli *et al.* (2011) and the phenolic compounds were identified according to their UV spectra. Based on the results of these analyses, cloudberry extract that was added to the cell culture medium (5 µL/mL) provided final concentrations of 36.3 µg/ml ellagitannins, 19.1 µg/ml flavanols, 8.8 µg/ml hydroxycinnamic acids, 5.9 µg/ml hydroxybenzoic acids, and 5.3 ug/ml ellagic acid, but only 1.2 µg/ml flavonols and 0.02 µg/ml anthocyanins in cell culture medium.

7.2.2 Cell culture and migration studies

Both HGF-induced and intrinsic (*i.e.* without HGF-induction) cell migration in human HT29 and HCA7 colon adenocarcinoma cell lines were studied by cell scattering and wound healing experiments as described in detail in the original article of Study IV. Cells were treated either with 40 ng/mL HGF or HGF together with 5 µL/mL of cloudberry extract in growth medium.

Both scattering and wound healing experiments were monitored and recorded by time-lapse microscopy, during which cells were incubated at 37°C in a humidified chamber at 5% CO_2. Phase-contrast images of live cells were taken and archived every 5 min for 16 hours with Tempus software (Andor Technology, Belfast, UK), using a KPM1E/K-S10 CCD camera (Hitachi, Denshi, Japan) mounted on an Axiovert microscope using a 20X Plan/Neofluar objective (Zeiss).

Wound healing images were analysed quantitatively at various time points by determining the percentage of remaining wound area as compared to the area of the wound at the first time point (baseline). The wound area at each time point was measured with the geodesic active contours (Caselles *et al.* 1997) by setting a few initial circular contours in the middle of the wound, and making the contours iteratively propagate to fill the whole wound area. The scatter area evolution was measured as the wound area except that only the single initial contour that included whole scatter area was used. The contour was subsequently made to iteratively contract after which the area was defined as a combination of all isolated contours generated during the contractions of the initial contour. All contours were included in area analyses at all the time points as these merged into one larger area during the experiment. All

processing was carried out with BioImageXD software, an open access, high-throughput image-processing computer platform (Kankaanpää *et al*. 2012).

7.3 Methods

7.3.1 Immunohistochemical stainings

Immunohistochemical stainings are described in detail in the original publications of Studies II and IV. Briefly, serial 5 μm sections of samples were deparaffinized and subjected to heat-induced antigen retrieval in a microwave oven. Specific staining protocols and markers were used to analyze T and B lymphocytes, regulatory T cells, Met, and pMet; the marker CD3ε (clone SP7, RM-9107; NeoMarkers, Fremont, CA) was used for T lymphocytes, FoxP3 (clone FJK16s, 14-5773; eBioscience, San Diego, CA) for regulatory T cells, CD45R (clone RA3-6B2, RM2600; Invitrogen, Waltham, MA) for B lymphocytes, c-Met ab59884 for Met, and c-Met ab5662 (phospho Y1230 + Y1234 + Y1235) for pMet (both Met antibodies from Abcam, Cambridge, UK). The immunostained sections were photographed using a Leica DM4000 microscope (Wetzlar, Germany) and an Olympus DP70 camera (Tokyo, Japan). The densities of intraepithelial and mucosal lymphocytes in the digital photomicrographs were analysed using ImageJ software (Schneider *et al*. 2012). The intensity and localization of Met and pMet expression in adenoma tissue were analysed by visual inspection.

7.3.2 Analysis of bacterial diversity in mice caecum samples

Bacterial genomic DNA was extracted from cecum contents using the FastDNA Spin kit for Soil (QBiogene, USA) and the V6 and V8 regions of the bacterial 16S rDNA were amplified as previously described (Mättö *et al*. 2005). PCR products were separated in polyacrylamide gels with denaturating gradient as described in the original publication of Study II. The gels were stained with SYBR Green, visualized in UV light and photographed. The profiles were compared by calculating the similarity percentage using BioNumerics software version 5.1 (Applied Maths BVBA, Belgium). Clustering was performed using the Pearson correlation and the unweighted-pair group method (UPGMA). Amplicons with the total surface area of at least 1% were included in the similarity analysis. In addition, evolutionary modelling was performed using a maximum parsimony tree with a bootstrap value 100.

The amplification product characteristic for the bilberry-fed mice (“band g”, Fig. 3B in the original publication of Study II) was excised from the gel and the

DNA was obtained for re-amplification with the same primer pair as previously described. Amplification product was purified and sequenced as described in the original publication of Study II. Sequence was analysed with ABI PRISM 3100 capillary DNA cycle sequencer (Applied Biosystems) and checked and edited with the Chromas program (Technelysium, Australia). The sequence was identified through the GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and deposited there with the accession number KP257583.

7.3.3 RNA isolation and the Affymetrix microarray

Total RNA was extracted from the mucosa of each animal in Study I by using the RNeasy Mini kit (Qiagen). Equal amounts of total RNA from 10–12 mice of each diet group were used to prepare 3–4 pooled RNA samples (2–4 mice per pool) for each group for hybridization to Affymetrix microarray, which enabled the measurement of the expression of a large number of genes at the same time. The labelling, hybridization, and scanning of the arrays were performed at the Centre for Biotechnology, University of Turku, Finland.

7.3.4 Whole cell extracts, Western blot analyses and immunoprecipitation

Protein extracts from whole cells of the HT29 and HCA7 cell lines were prepared to study the effects of cloudberry extract on migration-related cell signalling. The preparation of cell extracts and Western blot analyses are described in detail in the original publication of Study IV. Briefly, cells were cultured as described above for scattering experiments and treated either with HGF or HGF and cloudberry extract for the stated times. Cells were lysed on ice in a lysis buffer pH 7.4 solution, the lysates were clarified by centrifugation and protein concentrations were determined using BioRad Bradford protein assay reagent. Lysates were denaturated in Laemmli sample buffer, heated and then stored in −70°C. The proteins were separated in SDS (sodium dodecyl sulphate) gels and transferred to polyvinylidene difluoride membranes. The membranes were blocked against non-specific binding of primary antibodies with bovine serum albumin (for phosphorylated AKT, pAKT, and phosphorylated ERK, pERK) or non-fat milk in Tris-buffered saline, and probed with primary antibodies as described in the original publication of Study IV. The proteins were detected using horseradish-peroxidase-conjugated secondary antibody, enhanced chemiluminescence kit (Amersham/GE Healthcare, UK), and the images were transferred to X-ray films. Band intensities were determined and quantified using a GS-800 densitometer and Quantity One software (BioRad, Hercules, CA). The quantities of phosphorylated and total protein of AKT and ERK were

determined using the same blots. After the determination of the phosphorylated form, the blots were stripped to remove the antibodies that had been used to detect the phosphorylated form to make the determination of total form possible. Small diameter (≤1 mm) and large diameter (≥1.1 mm) adenomas of the control and cloudberry-fed *Apc*^{Min} mice in Study I were analysed for pAKT, AKT, pERK, and ERK in the same way.

The effects of cloudberry extract on the activation of receptor Met were studied by immunoprecipitation and Western blotting for tyrosine phosphorylation. Cells were cultured, treated either with HGF or HGF and cloudberry extract and then lysed as described above and in the original publication of Study IV. Proteins from the lysates were incubated with anti-Met antibody conjugated to protein A/G PLUS-agarose and centrifuged to collect the pellets, which were re-suspended in denaturating sample buffer and analysed for tyrosine phosphorylation and total Met levels by Western blotting. The blots were developed and the bands quantified as described above.

7.3.5 Statistical analyses

The distributions between the groups of the adenoma data, immunohistochemical staining scores, and Western blot results from *in vivo* studies were tested for statistical significance by the Mann-Whitney U-test. The relations between adenoma and immunohistochemical staining data were tested by Spearman correlation, and the relations between adenoma and bacterial profile data by Pearson correlation. Male and female mice were pooled in the analyses since there were no differences in the number and size of adenomas between the sexes. Non-parametric tests were used due to the small number of samples (n=10–12 per group) and differences were considered significant for p-values below 0.05.

The Affymetrix data were normalized by using the GC-Robust Multichip Average algorithm and analysed by one-way ANOVA using the GeneSpring software (Agilent Technologies Inc., Palo Alto, CA, USA) with a cut-off value of $P<0.05$. In Study I, genes with a 2-fold or more increase or decrease in expression in a berry-group in comparison to the control group for all replicates in each group were tested using the Student's t test. In Study II, the genes that exceeded the 1.2-fold change limit between the control and either of the berry-fed groups were considered as candidates for being regulated genes. Statistics described above were used to detect the enriched GO functional categories (http://geneontology.org/) or KEGG pathways (http://www.genome.jp/kegg/) associated with the genes. This procedure diminishes the effect of false positives, as only a large body of genes with similar functions will show as significant enrichment. Differences in gene expression results were considered significant at $P<0.05$.

In Study IV, differences of standardized wound and scatter-data behaviour in treatment groups (HGF vs. HGF + cloudberry) over time were compared with parametric ANCOVA (group*time) interaction terms. The binary presence of HGF and type of cell (HCA7 and HT29) was also used as a stratifying variable, or as a group variable instead of the cloudberry treatment in the analyses.

Statistical analyses were carried out using StatView 5.0.1 software (SAS Institute Inc., Cary, NC) and PASW 18.0.1 (SPSS Inc., 2009, Chicago, IL).

8 Results

This section gives an overview on the effects of feeding whole cloudberry, its components, or bilberry on intestinal adenoma development. It also reports the effects of cloudberry and bilberry-feeding on intestinal immune response, gut microbiota and global gene expression in *Apc*^{Min} mice. The effects of cloudberry extract on cancer cell migration and cell signalling in human HT29 and HCA7 colon adenocarcinoma cells are also presented. More detailed results are given in the original publications enclosed in this thesis. The results of the effects of three berries (cloudberry, bilberry and lingonberry) on tumour formation and cell signalling in Study I have been thoroughly discussed in the original paper and in the PhD thesis of Marjo Misikangas (Misikangas 2007). This dissertation is focused on the different effects of cloudberry and bilberry.

8.1 Cloudberry, but not its components, reduced tumour development in *Apc*^{Min} mice (I, III)

In Study I, the diet containing 10% (w/w) freeze-dried cloudberries decreased the number (P<0.05) and size (P<0.01) of adenomas in the whole small intestine when compared to the control diet. Bilberries also decreased the number (P<0.05) of adenomas but increased the size (P<0.05) of them in the whole small intestine (Fig. 5). The inhibitory effect of cloudberry on the adenoma growth was associated with the decreased expression of nuclear cyclin D1 (P<0.01 when compared to the control diet) and a non-significant decrease in nuclear β-catenin (P=0.065) in the large diameter (≥1.1 mm) adenomas.

In Study III, the cloudberry was divided into seeds and pulp, which were fed to separate groups of *Apc*^{Min} mice. The concentration of seeds or pulp in the diet were equal to the whole-cloudberry diet in Study I, but the total concentrations of ellagitannins/ellagic acid in the diets were a bit less than half of their concentration in the previous whole-cloudberry diet. No differences between the cloudberry seed, pulp or pure ellagic acid-fed groups and the control group were found in the number of adenomas in the whole small intestine (Fig. 5). These diets had no effect on the mean adenoma size in the whole small intestine either (Fig. 3A in the original publication). However, the ellagic acid group had larger adenomas (mean diameter 1.50 ± 0.29 mm) in the duodenum than the control group (1.16 ± 0.31 mm; P=0.029; Fig. 3B in the original publication).

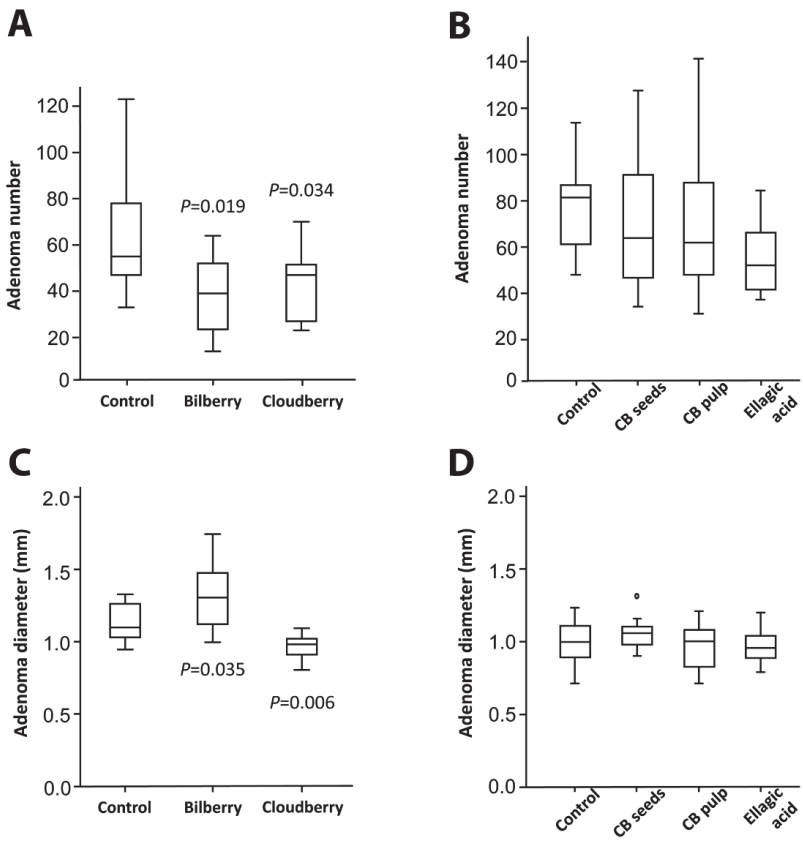

Figure 5. The number (A, B) and size (C, D) of adenomas in the whole small intestine of *Apc*^{Min} mice fed with a control diet without any added berries or diets containing 10% (w/w) freeze-dried cloudberries or bilberries in Study I (A, C), or 10% (w/w) freeze-dried cloudberry (CB) seeds, cloudberry pulp or 1564 mg/kg ellagic acid in Study III (B, D). The boxes represent the interquartile ranges (IQRs) and therefore contain 50% of the values. The median is indicated by the horizontal line across each box and the whiskers above and below the box extend to the maximum and minimum values.

8.2 Cloudberry-feeding attenuated intestinal cell-mediated immunity in *Apc*^{Min} mice (II)

The effects of cloudberry and bilberry-feeding on intestinal immunity in *Apc*^{Min} mice were compared with the control-fed group. The proportion of the intraepithelial to total mucosal CD3^{+} T lymphocytes was smaller in the cloudberry group than in the control group (Study II; Fig. 2A in the original publication; $P<0.05$), and there was a positive correlation ($P<0.05$) between

the total adenoma burden and the proportion of intraepithelial $CD3^+$ T lymphocytes in the small intestine (Study II; Fig. 2B in the original publication). The mean proportion of $FoxP3^+$ T lymphocytes to total $CD3^+$ T lymphocytes in the intestinal mucosa was also slightly but non-significantly smaller in the cloudberry group than in the control group (Study II; Fig. 2C in the original publication) and a significant correlation ($P<0.05$) was found between the $FoxP3^+/CD3^+$ ratio and the total adenoma burden in the small intestine (Study II; Fig. 2D in the original publication). Neither feeding whole cloudberry nor whole bilberry modulated the density of mucosal B lymphocytes, and interestingly, the bilberries had no effect on the density of any lymphocytes in the intestinal mucosa.

8.3 Cloudberry and bilberry-feeding affected gut microbiota differently in *Apc*^{Min} mice (II)

The predominant bacterial diversity (Study II, Fig. 3 in the original publication) was significantly smaller in the cloudberry group (35.5 ± 2.8) and in the control group (33.3 ± 2.7) as compared to the bilberry group (41.0 ± 5.8; $P<0.05$). Moreover, the caecal samples from the mice that had the smallest adenoma sizes clustered together and were obtained from the cloudberry-fed mice (Study II; Fig. 5 in the original publication). In addition, a particular band ('g'; Study II; Fig. 3 in the original publication) was weak or missing in the samples of cloudberry and control groups, but more prevalent and intense in the samples of the bilberry group. Sequencing followed by a BLAST search showed that the g-band was closest to the type strain of *Clostridium aldenese* RMA 9741 (identity 91%), whereas an uncultured bacterium clone abc22d07 (Sequence ID: gb|AY668073.2|) from mouse caecal sample was a complete match. The RDPII search found the closest type strain was *Blautia producta* ATCC 27340 (identity 90%).

8.4 Cloudberry and bilberry-feeding modulated global mucosal gene expression in *Apc*^{Min} mice (I, II)

An Affymetrix microarray assay was carried out to find potential differences in the intestinal mucosa at the gene expression level. One-way ANOVA of Study I data indicated diet-induced changes in the expression of 377 genes. The expression of 11 genes in the bilberry group and 10 genes in the cloudberry group differed from the control group at the cut-off point of 2-fold difference between the berry and control groups (Study I; Supplemental Table 2 in the original publication of Study I). The expression of adenosine deaminase and 5'-ectonucleoditase were consistently and similarly decreased by both the berries. The expression of many genes that encode immunoglobulins was also

changed. Therefore, the effects of berry-feeding on the intestinal immune response were studied further in Study II, and the gene expression data were analysed using gene enrichment analyses. For this purpose, the cut-off point was set at 1.2-fold change between the control and the berry groups, which revealed 16 up-regulated and 32 down-regulated genes for the cloudberry-added diet only. Moreover, 183 other genes were up-regulated and 56 down-regulated exclusively for the bilberry-added diet. Four genes were differently regulated by the two berries. Only one of these genes, the gene that encodes for phosphoenolpyruvate carboxykinase 1 *(Pck1),* was down-regulated by cloudberry and up-regulated by bilberry. Conversely, three other genes were up-regulated by cloudberry and down-regulated by bilberry, but the functions of these genes are not known yet (Fig. 6).

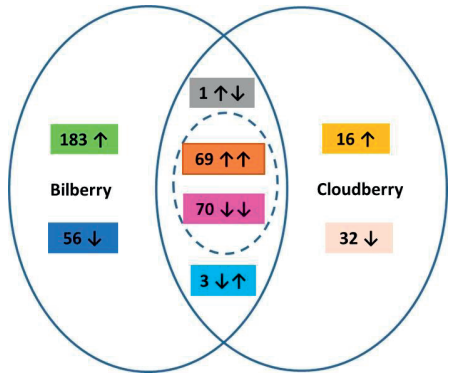

Figure 6. The expression of 183 genes were up-regulated (green) and 56 down-regulated (blue) by bilberry; the expression of 16 genes were up-regulated (orange) and 32 down-regulated (light pink) by cloudberry. The gene that encodes for phosphoenolpyruvate carboxykinase 1 *(Pck1;* grey*)* was down-regulated by cloudberry and up-regulated by bilberry; the expression of three genes (turquoise) were down-regulated by bilberry and up-regulated by cloudberry. The expression of 69 genes were up-regulated (brown) and the expression of 70 genes down-regulated (pink) by both berries. Analyses were made using 3–4 pooled RNA samples from 10–12 mice of each diet group. Comparisons were made to the control group and differences were considered significant at $P<0.05$ (ANOVA).

No KEGG pathways were enriched in the cloudberry group but eight KEGG pathways were enriched in the bilberry group. Bilberry increased the expression of many mitochondrial genes and consequently up-regulated four KEGG pathways related to oxidative phosphorylation. KEGG pathways for glycolysis/gluconeogenesis, glutathione metabolism and drug metabolism were also up-regulated by the bilberry enriched diet (Study II; Table 2 in the original publication).

Only three GO biological processes (GO-BP), all related to carbohydrate and energy metabolism, were enriched in the cloudberry group, whereas 36 GO-BPs were enriched in the bilberry group (Study II; Tables 3 and 4 in the original publication). The berries had opposite effects on the expression of energy metabolism-related genes and the differences were also seen in the enrichment of biological processes: cloudberry down-regulated and bilberry up-regulated GO-BPs related to energy metabolism.

In addition, as shown in Figure 6, the expression of 69 genes was up-regulated and the expression of 70 genes down-regulated by both berries. Consequently, one KEGG pathway (Huntington's disease) and 29 GO-BPs were shared between the berries; twenty of them were related to immunological processes and referred to attenuated antibody-dependent immunity (Study II; Table 6 in the original publication). Huntington's disease pathway included 13 genes, nine of which were associated with the electron transport chain and oxidative phosphorylation. This suggests that genes associated with oxidative phosphorylation were up-regulated by both berries, whereas those related to glycolysis and gluconeogenesis were up-regulated by bilberry only.

8.5 Cloudberry extract inhibited the migration of HT29 and HCA7 cells (IV)

The figures referred under the 8.5–8.8 inclusive are shown in the original publication of Study IV.

Cloudberry extract inhibited HGF-induced cell migration in both the HT29 and HCA7 cell lines. The scattering experiments showed that the increase in scatter area after 15 hours of the HGF treatment was 53 percentage points smaller in cloudberry treated HCA7 cells than in HCA7 control cells (Study IV, Fig. 1C; HGF vs. HGF+Cloudberry $P<0.001$). The scratch wound healing assay showed that cloudberry extract significantly inhibited HGF-induced wound healing in both cell lines (Study IV, Fig. 3C and 3D; in both $P<0.001$). Without HGF stimulation, cloudberry extract inhibited scratch wound healing in HT29 cells (Fig. 3E) but not in the HCA7 cells (Fig. 3F).

8.6 Cloudberry extract changed the cell signalling in HT29 and HCA7 cells (IV)

HGF stimulation of the HT29 and HCA7 cells led to the sustained activation of AKT and ERK, both of which increased rapidly after the addition of HGF, then reached a maximum level after 1–4 h, which subsequently gradually

decreased to nearly basal levels (Fig. 4A). The use of PI3K and MEK1 inhibitors confirmed that HGF-induced scratch wound closure and scattering in HT29 and HCA7 cells were dependent on the activation of PI3K and MAPK pathways (see Fig. 4B and 4C for HT29 cells). Cloudberry extract inhibited HGF-induced phosphorylation of AKT and ERK in HT29 cells (P=0.03 for pAKT/AKT; P=0.05 for pERK/ERK; Fig. 5B and 5C) and in HCA7 cells (P<0.001 for pAKT/AKT; P=0.018 for pERK/ERK; Fig. 5E and 5F), and the inhibitory effect of cloudberry extract on HGF-induced motility was therefore accompanied by the downregulation of PI3K and MAPK pathways.

Finally, cloudberry extract was shown to inhibit HGF-induced motility of HT29 and HCA7 by directly inhibiting the activation of their respective Met receptors. HGF treatment induced tyrosine phosphorylation of Met, but this was inhibited by cloudberry extract as assessed by the reduction in pMet/Met levels in HT29 (Fig. 6A and 6B; P=0.004) and HCA7 cells (Fig. 6C and 6D in Paper IV; P=0.036).

8.7 Cloudberry-feeding inhibited the AKT and ERK signalling in the adenomas of *Apc*^{Min} mice (IV)

Cloudberry was the most effective berry in reducing the size of adenomas in *Apc*^{Min} mice (Figure 1; P=0.006) in Study I. The activation of AKT and ERK in small diameter (≤1mm) and large diameter (≥1.1 mm) adenomas of cloudberry fed mice and control mice were analysed separately as a part of Study IV to evaluate further the anti-carcinogenic mechanisms of cloudberry. The ratio of pAKT/AKT in large adenomas of cloudberry-fed mice was significantly reduced (Study IV, Fig. 7C and 7D; P=0.015) and a similar but non-significant trend was seen in small adenomas (Fig. 7G). However, there was no change in pERK and ERK levels in either the large or the small adenomas.

8.8 Cloudberry-feeding changed the expression of Met and pMet in the adenoma tissue of *Apc*^{Min} mice (IV)

Total Met levels in large adenomas were analysed by Western blotting but no differences between the dietary groups were found. However, the variation in Met levels was notably smaller in the cloudberry-fed group (Study IV, Fig. 7C and 7F). Immunohistochemical analysis revealed a strong positive staining of pMet at the edge of the tumours in control-fed mice (Study IV, Fig. 8A and 8C) whereas the staining in cloudberry-fed mice predominantly localized in the crypt area of the mucosa (Figure 8D; as also observed in control mice in Fig. 8C; Study IV), and not in the adenoma (Figure 8B; Study IV).

9 Discussion

9.1 Whole-cloudberry feeding inhibited the formation of adenomas in *Apc*^{Min} mice whereas components of cloudberry did not (I, III)

The whole-cloudberry diet decreased the number and size of intestinal adenomas in *Apc*^{Min} mice, which indicates that it efficiently inhibited both the initiation and promotion steps of tumourigenesis. However, when cloudberries were fractionated into the pulp and the seeds, their chemopreventive effects were lost. Pure ellagic acid at a concentration similar to that in the whole-cloudberry diet had no chemopreventive effects either. Indeed, it even increased the size of adenomas in the most proximal part of the small intestine. The lack of any apparent chemoprevention by the diet containing pure ellagic acid supports the previous studies by suggesting that anticarcinogenic effects of berries and their polyphenols are at least partly produced due to the net synergism between the different compounds. For example, ellagic acid in combination with quercetin or resveratrol synergistically reduced proliferation and induced apoptosis in the MOLT-4 human leukemia cell line (Mertens-Talcott *et al.* 2003, 2005a, 2005b). Pomegranate juice also had a stronger antiproliferative effect than ellagic acid, punicalagin, or total pomegranate tannin extract in several cancer cell lines *in vitro* (Seeram *et al.* 2005). The chemopreventive effects of the whole-cloudberry diet may also be due to the synergistic effects of ellagitannins and other compounds of cloudberry. However, the lack of apparent chemopreventive effects of the diets that contained either cloudberry pulp or seeds may also be a result of the smaller concentrations of total polyphenols than those found in the whole-cloudberry diet in Study I. For example, the ellagitannin concentrations in the cloudberry pulp and seed diets may have been too small to produce any detectable chemopreventive effects. Therefore, it seems that both sufficient concentrations of polyphenols and synergistic or additional effects of different compounds of berries were needed for the effective prevention of adenoma formation. In addition, the differences in the total number of adenomas between the cloudberry pulp, seed or ellagic acid diets and the control diet in Study III may have at least partly been lost or hidden due to the exceptionally large variation in the numbers of adenomas found between the litters and between individual mice. Inbred mouse strains are genetically quite homogeneous and all the mice used in the studies for this thesis were of the same inbred *Apc*^{Min} mouse strain, thus the reason for the large variation of adenoma numbers observed in this study remains unknown. In summary, the results on the effects of whole-cloudberry are supported by the previous studies, which are listed and summarised in Table 2 on pages 27-

29: other ellagitannin-containing berries at corresponding concentrations than used here have also been found to reduce the number and/or size of intestinal tumours in rodent models. Contrary to the effects found for ellagic acid in Study III, two previous studies have reported chemopreventive effects of pure ellagic acid on carcinogen-induced intestinal tumourigenesis (Rao *et al.* 1991, Umesalma *et al.* 2010). However, the concentration of ellagic acid in the diet used by Rao *et al.* was more than five-fold the concentration used in Study III. Moreover, Umesalma *et al.* used aberrant crypt foci and not tumours as an endpoint, which makes the effects difficult to compare between studies.

Cloudberry effectively decreased the size of adenomas in *Apc*^{Min} mice in Study I, whereas bilberry did not. This difference in response to the two berries may be due to the changed Wnt and Met-signalling in the adenomas of cloudberry-fed mice. Even though immunohistochemical stainings revealed no changes in the expression of β-catenin and cyclin D1 in the normal-appearing mucosa, cloudberry decreased their levels in the nuclei of the large adenomas and also prevented the increase in their concentrations in the nuclei during the adenoma growth. Western blotting would perhaps have been a more sensitive method to show small differences in the expression levels but due to the lack of tissue samples these analyses were not possible in this study. A previous study showed that both ellagitannin-rich extracts, ellagic acid, and its metabolite urolithin A, inhibited Wnt-signalling in the human embryonic kidney 293T cell line (Sharma *et al.* 2010). These findings are in accordance with the decreased levels of β-catenin and cyclin D1 in the large adenomas of cloudberry-fed *Apc*^{Min} mice and suggest that the inhibition of the Wnt pathway in the tumours of *Apc*^{Min} mice may at least partly be produced by ellagic acid released from cloudberry ellagitannins and by urolithins produced from ellagitannins by the gut microbiota. In addition, the localization of pMet expression was also changed by whole-cloudberry feeding; pMet was strongly expressed at the edge of tumours in control-fed but not in cloudberry-fed mice. Even though no changes in the expression levels were found by Western blotting analyses, the localization of pMet at the invasive front of the tumour is important for the tumour growth (Bradley *et al.* 2016, Moon *et al.* 2005, Vermeulen *et al.* 2010).

9.2 Cloudberry-feeding attenuated cell-mediated intestinal immune response in *Apc*^{Min} mice (II)

Cloudberry feeding modulated the intestinal immunity in *Apc*^{Min} mice, whereas bilberry feeding did not. Cloudberry decreased the proportion of the intraepithelial to the total mucosal $CD3^+$ T lymphocytes, and non-significantly decreased the proportion of regulatory $FoxP3^+$ T lymphocytes to total $CD3^+$ T lymphocytes in the intestinal mucosa compared to the control group. The positive correlations between both the IEL/total $CD3^+$ and the $FoxP3^+$/total

$CD3^+$ ratios and the total adenoma burden in the small intestine suggest that the attenuation of intestinal immune response was one of the mechanisms that participated in slowing down the adenoma growth (Study II; Fig. 2 in the original publication). A clear but non-significant decrease in the mean adenoma burden was also seen in the bilberry-fed group. It is noteworthy therefore that the cellular immune response differed from the control only in the cloudberry group and no differences in the distribution of any of the analysed lymphocytes were found between the bilberry and control groups. This indicates that the decrease in the density of intraepithelial $CD3^+$ T lymphocytes or mucosal $FoxP3^+$ T lymphocytes in the cloudberry-fed group was a result of cloudberry feeding *per se*, and not of the decreased adenoma burden. The attenuated intestinal cell-mediated immunity may also be associated with the changes in the composition of gut microbiota in the cloudberry-fed group. No previous studies on the effects of berries or polyphenols on intestinal immunity and gut microbiota have been published as far as the author is aware. However, a recent study in which whole grains were substituted for refined grains for six weeks in human subjects, found modest changes both in the gut microbiota and in a few indicators of immune response. However, the effects on intestinal immunity were not studied (Vanegas *et al.* 2017).

9.3 Cloudberry and bilberry-feeding changed the composition of gut microbiota in *Apc*^{Min} mice (II)

Cloudberry-feeding changed the composition of gut microbiota and the changes it induced in cell signalling pathways may thus have been mediated by the native compounds of cloudberry or by their microbial metabolites produced in the colon and distal small intestine or a combination of both. The analyses that used the PCR-DGGE method showed that the composition of gut microbiota differed between the cloudberry, bilberry, and control-fed mice. In addition, the microbial profiles of cloudberry-fed mice clustered together and these mice also had the smallest adenomas of any group. Therefore, the changes induced in the gut microbiota by cloudberry may have participated in slowing down the growth of adenomas.

The composition of the microbiota was analysed from the contents of the caecum, whereas the effects of cloudberry on the development of adenomas were studied in the small intestine. The small intestine is the site where most of the adenomas in *Apc*^{Min} mice are formed. The distal part of the small intestine is also inhabited by gut microbes, and microbial metabolites can affect the epithelium of the site on which they are formed. It is also possible that the caecal and colonic metabolites ended up in the small intestine of the mice due to coprophagy of their own and/or their cagemates' faeces and thus the caecum contents were re-digested by the small intestine.

The microbial profiles were analysed using PCR-DGGE method, which reveals all the bacterial species that constitute more than 1% of the total bacterial population in the sample that will be analysed. Even though 16S rDNA based pyrosequencing would have allowed a more in-depth analysis (Kemperman *et al.* 2013), the simple and cost effective PCR-DGGE method made it possible to analyse the samples from each mouse separately. The PCR-DGGE also enabled the evaluation of the influence of the treatment on the general composition of gut microbiota, and also the interindividual variation of the response.

Berries are rich in polyphenols and also contain some fibre, of which at least 90% in cloudberries, and 85% in bilberries is insoluble. The fibre fractions of cloudberry have not been analysed in detail, but bilberry contains soluble pectin and insoluble hemicellulose, cellulose, cuticular waxes and cutin. The anthocyanins in bilberry may also be enclosed in the insoluble fibre matrix (Aura *et al.* 2015). The presence of fibre may affect the fermentability of the polyphenols and thus the yield of their microbial metabolites (Saura-Calixto *et al.* 2010). On the other hand, the dietary polyphenols may either enhance (Aprikian *et al.* 2003) or suppress the fermentation of dietary fibre and thus the production of short-chain fatty acids, particularly butyrate (Bazzocco *et al.* 2008). In addition, both ellagitannins and anthocyanins have been shown to inhibit starch degrading digestive enzymes (McDougall and Stewart 2005), and like tannins, they may also be able to bind enzymes in a non-specific manner (Santos-Buelga and Scalbert 2000), and thus affect the amounts of metabolites, such as butyrate that is produced at a site. Butyrate is an important source of energy for the colonic epithelial cells, and depending on the prevailing conditions, it may either enhance or suppress epithelial cell proliferation (O'Keefe 2016).

The changes in the gut microbiota found in this study may have been induced by both the polyphenols and dietary fibre contents of the berries. The fibre fractions of the berries and the berry components may also have affected the metabolism of berry polyphenols, whereas pure ellagic acid in the non-fibre diet was independent of these interactions. However, the total fibre content in the cloudberry diet was approximately 3.7% and in the bilberry diet 2.1%. Furthermore, the concentration of soluble, and thus fermentable, fibre was only 0.3% in both the cloudberry and bilberry diets. Because previous studies have shown that 5–15% (w/w) content of fermentable fibre (*e.g.* inulin and oligofructose) were needed to increase the caecal butyrate content (Pool-Zobel 2005), the soluble fibre contents in cloudberry and bilberry diets fed to the mice in the present study probably had no or minuscule effects on butyrate production, and thus they were not measured in this study.

9.4 Cloudberry and bilberry-feeding modified the expression of energy metabolism-related genes in *Apc*^{Min} mice (II)

Both cloudberry and bilberry-feeding modified the global gene expression in the intestinal mucosa of *Apc*^{Min} mice. The gene expression data from an Affymetrix microarray assay was first analysed in Study I using the cut-off point of a 2-fold difference in the gene expression between the berry and control groups. In Study II, more detailed analyses used the cut-off point of a 1.2-fold change difference in combination with gene enrichment analyses. Even though the analyses with the lower cut-off value were originally done due to the numerous changes in the expression of immunological genes in the previous analyses, the main findings from the pathway analyses were the changes found in the expression of energy metabolism-related genes. Among these, the most interesting finding was that the expression of the phosphoenolpyruvate carboxykinase 1 *(Pck1)* gene was decreased by cloudberries and increased by bilberries when compared with the control diet. The cytosolic enzyme Pck1 catalyses the formation of phosphoenolpyruvate from oxaloacetate, which is the main rate-controlling step of gluconeogenesis. The expression of glucose-6-phosphatase, which catalyses the last step of gluconeogenesis and glycogenolysis, was also decreased by cloudberry feeding. Thus, these changes in glucose metabolism in combination indicate attenuated glycolytic activity in the cloudberry-fed group. In contrast, the increased expression of *Pck1* in the bilberry-fed group was associated with increased expression of genes for other energy metabolism-related enzymes that catalyse gluconeogenesis and the citric acid cycle. Even though the changes in the expression of individual genes are small, when taken together they could modulate the glycolytic activity in the intestinal mucosa, attenuating glycolysis in the cloudberry-fed mice and enhancing it in the bilberry-fed mice.

The reprogramming of energy metabolism towards aerobic glycolysis, which is also known as Warburg effect (Potter *et al.* 2016), has been designated as a hallmark of cancer (Hanahan and Weinberg 2011). In a state of aerobic glycolysis, tumour cells start to use glycolysis in energy production, even in the presence of sufficient oxygen, in order to produce enough building blocks for proteins, lipids, and nucleotides needed for continuous cell division. Even though the Warburg effect has mainly been studied in fast-proliferating tumour cell models *in vitro*, the results of Study II suggest that the metabolic shift towards aerobic glycolysis may already happen in the normal-appearing mucosa of *Apc*^{Min} mice and may have been prevented by cloudberry and promoted by bilberry.

The results of this study suggest that the cloudberry diet suppressed the metabolic shift towards aerobic glycolysis and thus prevented the growth of

adenomas, whereas metabolic changes in the bilberry-fed group were the opposite and favoured the adenoma growth. The metabolic change in the bilberry-fed group is supported by the finding that lyophilized red grape pomace, which resembles the phenolic composition of bilberry also increased the activation of glycolysis and gluconeogenesis pathways in the colon mucosa of healthy C57BL/6J mice (Lizarraga *et al.* 2011). The upregulated expression of the genes that code for glycolytic enzymes may indicate enhanced glycolysis in the intestinal mucosa, which in turn may be a link between the metabolism of intestinal cells and the composition of gut microbiota. It is possible that increased glycolysis made the microenvironment in the intestinal mucosa more acid and thus facilitated the growth of tumour-promoting bacteria, which also participated in accelerating the growth of adenomas in the bilberry group.

9.5 Cloudberry extract inhibited the cell migration and underlying signalling pathways of HCA7 and HT29 cells (IV)

The investigations done for this thesis used the *Apc*^{Min} mouse strain to study the first two phases of carcinogenesis, initiation and promotion, whereas human colon adenocarcinoma cell lines were used to study the last phase, progression. Cell migration is a prerequisite for the progression and metastasis, and whole-cloudberry extract was found to inhibit cell migration in HT29 and HCA7 human colon adenocarcinoma cell lines. These cell lines differ in their status for the tumour suppressor adenomatous polyposis coli *(APC)* gene. The HT29 cells carry two mutated *APC* alleles, whereas HCA7 are wild type for *APC* (Ilyas *et al.* 1997). Both HT29 and HCA7 cells still have an epithelial morphology, similar levels of Met and responsiveness to HGF. Cell migration is stimulated by hepatocyte growth factor as determined by the scratch wound healing assay, which is a widely used model of cell migration in HGF-treated cell models (Nakamura *et al.* 2011). Cloudberry extract inhibited both HGF-induced wound healing, that is the migration, and activation of AKT and ERK in both cell lines. This demonstrates that HGF-induced cell migration in HT29 and HCA7 cells is dependent on the activation of the PI3K/AKT and Ras/ERK pathways, which contribute to HGF/Met-induced invasive growth program in cancer cells (Trusolino and Comoglio 2002).

Intrinsic migration without HGF treatment was inhibited by cloudberry extract in *APC*-mutated HT29 cells only. The protein coded by the *APC* gene also has a well-known role in regulating directional cell migration in the gut (Nelson *et al.* 2012), thus further studies must be conducted to find out whether this difference in intrinsic cell migration by cloudberry was due to *APC* allele status or due to differences in other signalling pathways between

the cell lines. However, the results demonstrate that the effect of cloudberry on the HCA7 cells was specific to HGF-induced migration. Furthermore, scratch wound healing in HGF-stimulated HT29 cells with cloudberry treatment resembled wound healing in these cells without HGF stimulation.

Cloudberry extract also inhibited HGF-induced tyrosine phosphorylation of Met in HT29 and HCA7 cells, which indicated that it reduced HGF-induced ERK and AKT activation and cell migration by directly inhibiting the activation of the Met receptor and thus preventing receptor signalling. It is possible that ellagitannins of cloudberry extract acted outside the cell and interfered with the binding of HGF to Met or specifically inhibited Met tyrosine kinase activity due to the complex molecular structure and high molecular weight of ellagitannins. However, previous findings of the inhibitory effect of ellagic acid on cell migration (Losso *et al.* 2004) and accumulation of ellagic acid in Caco-2 intestinal epithelial cells (Whitley *et al.* 2003) indicate that it is also possible that free ellagic acid of cloudberry extract entered the cells and acted inside them, even though the concentration of free ellagic acid was only one seventh (14%) that of the concentration of ellagitannins in the cloudberry extract. However, cloudberry extract directly inhibited the HGF/Met signalling pathway, which is one of the major therapeutic targets in cancer treatment (Sierra and Tsao 2011), thus the results suggest that ellagitannins may have some therapeutic value in reducing cancer progression.

9.6 Cloudberry-feeding inhibited the activation of AKT and altered the expression of pMet in the adenomas of *Apc*^{Min} mice (IV)

The cloudberry extract was found to inhibit the cell migration and Met signalling in colon adenocarcinoma cell lines. The aim, therefore, was to study whether the same effect could also be found in the preceding phase of carcinogenesis in the adenomas of *Apc*^{Min} mice. The reduced activation of AKT and altered localization of active pMet that were found in large adenomas of *Apc*^{Min} mice are consistent with the findings of the chemopreventive effects of cloudberry extract in HT29 and HCA7 cells. A strong positive staining of pMet was found at the edge of tumours in control-fed mice, whereas the staining in the cloudberry-fed mice predominantly localized in the crypt area of the mucosa, not in the adenoma (Figure 8 in the original publication of Study IV). This finding is in line with the effects of cloudberry extract on colon adenocarcinoma cells *in vitro* and suggests that cloudberry is likely to have a local effect on cellular responses relevant to cell invasion at the edge of tumours. This is probably more relevant for larger tumours that are beginning to invade the surrounding tissues, even though the trend for reduced AKT activation in the small adenomas indicates that these changes start in the early

stages of tumour development. Although Met is clearly expressed in human colorectal cancer (Wielenga *et al.* 2000), Boon *et al.* (2006) found no overexpression of Met in tumours of *Apc*^{Min} mice and the Met levels in general were near to the detection limit in the gut of the adult *Apc*^{Min} mouse. However, pMet and thus Met activity were not analysed by those authors (Boon *et al.* 2006). The results described in the present study suggest that the inhibition of the Met and PI3K/AKT pathways are involved in mediating the anti-carcinogenic effects of cloudberry both *in vitro* and *in vivo*.

9.7 Strengths and limitations of these studies

9.7.1 Animal studies

The C57BL/6 J *Apc*^{Min} mouse strain is well-documented and widely used for studying the effects of diet and drugs on intestinal tumourigenesis (Moser *et al.* 1990; Su *et al.* 1992, Fodde and Smits 2001, McCart *et al.* 2008, Taketo and Edelmann 2009). This model has been considered a suitable model for investigating the development of intestinal tumours due to the similarity between the murine *Apc* gene and the human *APC* gene; 90% of amino acids in the Apc/APC proteins are identical (Su *et al.* 1992). Intestinal tumours in the *Apc*^{Min} mouse and colon tumours in human develop in a similar adenoma-carcinoma sequence and are also morphologically similar (Preston *et al.* 2008). However, the tumours in the conventional *Apc*^{Min} mouse mainly develop in the small intestine, and colon tumours rarely exist. After the animal studies of this thesis had been completed, a novel A/J Min/+ mouse was established by transferring the Min/+ trait from C57BL/6J *Apc*^{Min} mouse to mice with an A/J genetic background was published (Sødring *et al.* 2016). The C57BL/6J *Apc*^{Min} mice develop a mean of 30 small intestinal tumours (Moser *et al.* 1990), and typically less than one tumour per animal in the colon (Bilger *et al.* 1996, Paulsen *et al.* 1997, 1999, 2001). The novel A/J Min/+ mice, on the other hand developed a mean of 21 small intestinal and eight colonic tumours between the ages of 4 and 60 weeks, even though the colonic tumours were not detectable before the age of 11 weeks. The conventional *Apc*^{Min} mouse model is, however, excellent for studying the effects of diet or drugs on the initiation and promotion phases of intestinal carcinogenesis since the effects are seen by the age of 15 weeks or even earlier (Pajari *et al.* 2003). The novel A/J Min/+ mouse, on the other hand, makes it possible to study also the progression phase of intestinal cancer because, unlike the adenomas in the C57BL/6J *Apc*^{Min} mice, both the small intestinal and colonic adenomas in the A/J Min/+ mice progress to carcinomas (Sødring *et al.* 2016). It would therefore be valuable to use this new rodent model to study the effects of cloudberry on the progression phase of intestinal carcinogenesis *in vivo*.

The berry content (10% w/w) in the whole-cloudberry and bilberry diets was relatively high and it would be nearly equivalent to the consumption of 90 g/day of freeze-dried berries by humans. An intake of 30–60 g/day of freeze-dried berry powder has been used in previous studies with human subjects (Chen *et al.* 2012, Kresty *et al.* 2016, Wang *et al.* 2011a, Wang *et al.* 2014a). Even though the corresponding berry content could not be reached in the human diet by consuming fresh berries, the concentration was chosen since no previous results on the effects of cloudberries and bilberries on the intestinal carcinogenesis *in vivo* had been published before. High concentrations are typically used to screen potential chemopreventive agents and to study the underlying mechanisms. Concentrations between 2.5–10% (w/w) of other berries have been used in studies with carcinogen-induced rodent models of oesophageal, oral, and colonic tumourigenesis (Carlton *et al.* 2001, Harris *et al.* 2001, Kresty *et al.* 2001, Casto *et al.* 2013) and the effects of lower cloudberry concentrations should be studied in *Apc*^{Min} mice and also in the novel A/J Min/+ mice.

A limitation of the study is that the microbial profiles were analysed in caecal contents only and not the epithelium directly, where the microbes are in close contact with epithelial cells (Dejea *et al.* 2014). Mucosal microbiota would also be a more sensitive indicator for changes in microbial composition related to intestinal tumourigenesis (Mira-Pascual *et al.* 2015). However, the strengths of these current studies include the wide mechanistic approach to the effects of cloudberry on intestinal tumourigenesis.

9.7.2 Cell culture studies

In this study, HT29 and HCA7 human colon adenocarcinoma cells were used to study the effects of cloudberry extract on HGF-induced migration and underlying cell signalling. These are well-characterized cell lines with different respective status of *APC* gene, which represent the two main forms of genetic defects in colorectal cancer aetiology of chromosomal instability and microsatellite instability, and were thus appropriate for this study (Ilyas *et al.* 1997). However, the results are limited only to these two cell lines used and studies using other cell lines would provide more information on the effects of cloudberry. It would also be important to study the effects of cloudberry extract on normal intestinal epithelial cells, but cell lines that represent normal adult gut mucosal cells were and are not available. ATCC offers cell lines derived from the intestine of human embryo which are claimed to be normal, but how well these cell lines represent mature adult gut mucosal cells is not clear.

The cells were treated with whole-cloudberry extract, which, unlike single, pure polyphenolic compounds, was a mixture of all methanol-extractable

compounds of cloudberries that contained polyphenols and carbohydrates. These compounds together may have additive, synergistic, or antagonistic effects. In comparison to the studies on single polyphenolic compounds, the studies using berry extracts may provide more relevant information on the effects of dietary components on gut cells, although it is not possible to study the mechanisms of single compounds when the whole extract is given as the treatment. The concentrations of the tested compounds in cell cultures often exceed the concentrations achievable *in vivo*. However, the concentration of cloudberry extract in the cell culture medium used in this study was moderate. For example, the concentrations of ellagitannins (36 µg/ mL) and total polyphenols (77 µg/mL) were in the same range as those used in some previous studies (Seeram *et al.* 2005). There is no clinical pharmacokinetic data on the metabolism of cloudberry ellagitannins in the human gut and it is therefore difficult to estimate the accurate concentrations that could be reached in the colon *in vivo*. In addition, ellagitannins are hydrolysed to release ellagic acid and then metabolized to urolithins in the colon *in vivo*. Thus, it would be important to study the effects of urolithins produced from the ellagitannins of cloudberry extract.

10 Conclusions and future perspectives

This thesis is the first study that investigated the effects of cloudberry, which is rich in ellagitannins, on the intestinal tumourigenesis *in vivo* and the possible molecular mechanisms that might mediate changes beyond those effects. This is also the first study that reports the effects of a source of ellagitannins on the cell migration and underlying cell signalling in human colon adenocarcinoma cells.

The results showed that whole cloudberry, but not its tested components, inhibited several steps of colon cancer development, from adenoma formation in the *Apc*^{Min} mouse to increased motility of human adenocarcinoma cells. The inhibitory effects of cloudberry on intestinal carcinogenesis in *Apc*^{Min} mice were associated with the inhibition of the Wnt and Met signalling pathways, and the modification of intestinal gut microbiota, immune response, and expression of energy metabolism-related genes. The changes were found to be simultaneous and therefore interactions between them are possible. The results in *Apc*^{Min} mice suggest that cloudberry consumption may reduce the risk of colorectal cancer and slow down the growth of colorectal adenomas, but long-term intervention studies in human subjects would be needed to confirm the results.

Cloudberry extract significantly inhibited cancer cell migration in HT29 and HCA7 human adenocarcinoma cell lines, and cell migration is a prerequisite for cancer progression and metastasis. Cloudberry also directly inhibited the HGF/Met signalling pathway, which is one of the major therapeutic targets in cancer treatment. The results therefore suggest that the use of cloudberry as an adjuvant therapy for *inter alia* the secondary prevention of colon cancer is a topic that is worth studying.

Reprogrammed energy metabolism of tumour cells was added to the second generation of hallmarks of cancer by Hanahan and Weinberg in 2011. They suggested that inhibitors of aerobic glycolysis should be one of the hot spots for the development of therapeutic drugs. Cloudberry was found to attenuate the expression of energy metabolism-related genes in normal appearing mucosa, therefore further studies should be conducted to address whether cloudberry inhibits aerobic glycolysis in those intestinal tumours that already have reprogrammed energy metabolism towards aerobic glycolysis. Constituents of cloudberry could also be studied as potential candidates for drug development in targeting both aerobic glycolysis and the HGF/Met signalling pathways.

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