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Brändstedt, Jenny

2014

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*Citation for published version (APA):*

Brändstedt, J. (2014). *Associations of sex, anthropometric and reproductive factors with clinicopathological and molecular characteristics of colorectal cancer*. [Doctoral Thesis (compilation), Tumor microenvironment]. Pathology, (Lund).

*Total number of authors:*

1

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# Associations of sex, anthropometric and reproductive factors with clinicopathological and molecular characteristics of colorectal cancer

Jenny Brändstedt



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## DOCTORAL DISSERTATION


by due permission of the Faculty of Medicine, Lund University, Sweden  
To be defended at the Main lecture hall, Department of Pathology  
Sölvegatan 25, Skåne University Hospital, Lund  
Friday 28<sup>th</sup> of March 2014 at 09:15 am

### *Faculty opponent*

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Organization LUND UNIVERSITY	Document name	
	Date of issue	
Author(s)	Sponsoring organization	
Title and subtitle: Associations of sex, anthropometric and reproductive factors with clinicopathological and molecular characteristics of colorectal cancer		
<p>Abstract: Colorectal cancer (CRC) is the third most common cancer globally, with approximately 1.2 million new cases every year. The highest incidence rates are seen in developed countries, thereby imposing dietary and lifestyle factors in the etiology of CRC. Accumulating epidemiological evidence suggests that obesity is a risk factor for CRC in particular in men, as weak or no associations are seen in women. The reason for this sex difference is not fully understood, but hormonal factors are suggested to play an important role. CRC is a largely heterogenous disease, and colorectal carcinogenesis can be regarded as a complex process involving multiple genetic and epigenetic alterations engendering tumours with different clinicopathological features.</p> <p>The aims of this thesis were to investigate the associations between obesity, measured as different anthropometric factors, and risk of CRC according to clinicopathological and tumour biological characteristics in men and women, respectively. In addition, the relationship between use of postmenopausal hormone therapy (HRT) and oral contraceptives (OC) with CRC risk was examined.</p> <p>All papers are based on tumours from incident CRC in the Malmö Diet and Cancer study, a large prospective population based cohort including 28098 individuals. Baseline examinations comprised anthropometric measurements, questionnaire on medications, socioeconomic- and lifestyle related factors. By the end of follow-up in 2008, 584 cases of incident CRC had been registered. Using the tissue microarray technique (TMA), immunohistochemical (IHC) expression of beta-catenin, cyclin D1, p53 and microsatellite instability (MSI) status of the tumours was investigated. KRAS/BRAF mutation status was assessed by pyrosequencing.</p> <p>Interestingly, we found that obesity was associated with an increased risk of more advanced CRC, i.e. tumour (T)-stage 3 and 4, lymph node positive and metastatic disease, predominantly in men. Further, obesity was associated with an overall increased risk of CRC in both sexes (Paper 1). Associations of anthropometric factors with the risk of various molecular subsets of CRC revealed that obesity was not related to risk of MSI tumours, indicating that obesity influences carcinogenesis through other pathways than the MSI pathway (Paper 2). Given the sex differences in the associations between obesity and CRC, and, that HRT has been shown to be a protective factor of CRC, we also examined the associations of HRT and OC use and CRC risk in the female cohort. The principle finding was that current use of HRT was not associated with a decreased overall CRC risk as expected, but with a decreased risk of T-stage 1 and 2 CRC. Further, HRT use was associated with a lower risk of lymph node negative-, non-metastatic disease and of p53 negative- and cyclin D1 negative tumours in the rectum, but not in the colon (Paper 3). Finally, we found that obesity was associated with an increased risk of both wild-type and KRAS-mutated colorectal tumours in men, and with an increased risk of BRAF wild-type tumours, but not with BRAF-mutated tumours, in both sexes (Paper 4).</p>		
Key words: colorectal cancer, carcinogenesis, MDCS, anthropometrics, risk factors, sex differences, hormonal therapy, beta-catenin, cyclin D1, p53, MSI, KRAS mutation, BRAF mutation		
Classification system and/or index terms (if any)		
Supplementary bibliographical information		Language: English
ISSN and key title 1652-8220		ISBN 978-91-87651-60-1
Recipient's notes	Number of pages	Price
	Security classification	

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# Associations of sex, anthropometric and reproductive factors with clinicopathological and molecular characteristics of colorectal cancer

Jenny Brändstedt



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The research presented in this thesis was supported by:

The Knut and Alice Wallenberg Foundation, the Swedish Cancer Society, the Gunnar Nilsson Cancer Foundation, the Crafoord Foundation, the Swedish Government Grant for Clinical Research, Lund University Faculty of Medicine and Skåne University Hospital Research Grants.

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Department of Clinical Sciences, Oncology and Pathology, Lund

Lund University, Faculty of Medicine Doctoral Dissertation Series 2014:35

ISBN 978-91-87651-60-1

ISSN 1652-8220

Cover illustration by Jesper Fermgård

Tryckt i Sverige av Media-Tryck, Lunds universitet

Lund 2014



L'essentiel est invisible pour les yeux, on ne voit bien qu'avec le cœur

Le petit prince, Saint-Exupéry

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# List of papers

## Papers included in the thesis

- I. **Brändstedt J**, Wangefjord S, Nodin B, Gaber A, Manjer J, Jirström K. Gender, anthropometric factors and risk of colorectal cancer with particular reference to tumour location and TNM stage: a cohort study. *Biology of Sex Differences* 3:23 (2012)
- II. **Brändstedt J**, Wangefjord S, Borgquist S, Nodin B, Eberhard J, Manjer J, Jirström K. Influence of anthropometric factors on tumour biological characteristics of colorectal cancer in men and women: a cohort study. *Journal of Translational Medicine* 11:293 (2013)
- III. **Brändstedt J**, Wangefjord S, Nodin B, Eberhard J, Jirström K, Manjer J. Associations of hormone replacement therapy and oral contraceptives with risk of colorectal cancer defined by clinicopathological factors, beta-catenin alterations, expression of cyclin D1, p53, and microsatellite-instability. *Submitted*
- IV. **Brändstedt J**, Wangefjord S, Nodin B, Eberhard J, Sundström M, Manjer J, Jirström K. Sex differences in the associations of anthropometric factors with KRAS and BRAF mutation status in colorectal cancer: a cohort study. *Submitted*

## Papers not included in the thesis

- Wangefjord S, **Brändstedt J**, Lindquist KE, Nodin B, Jirström K, Eberhard J. Associations of beta-catenin alterations and MSI screening status with expression of key cell cycle regulating proteins and survival from colorectal cancer. *Diagnostic Pathology* 8:10 (2013)
- Ehlén Å, Nodin B, Rexhepaj E, **Brändstedt J**, Uhlén M, Alvarado-Kristensson M, Pontén F, Brennan DJ, Jirström K. RBM3-regulated genes promote DNA integrity and affect clinical outcome in epithelial ovarian cancer. *Translational Oncology* 4: 212-21 (2011)

- **Brändstedt J**, Nodin B, Manjer J, Jirström K. Anthropometric factors and ovarian cancer risk in the Malmö Diet and Cancer Study. *Cancer Epidemiology* 35 432-7 (2011)
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- Nodin B, Zendeirokh N, **Brändstedt J**, Nilsson E, Manjer J, Brennan DJ, Jirström K. Increased androgen receptor expression in serous carcinoma of the ovary is associated with an improved survival. *Journal of Ovarian Research* 3:14 (2010)
- Brennan DJ, **Brändstedt J**, Rexhepaj E, Foley M, Pontén F, Uhlén M, Gallagher WM, O'Connor DP, O'Herlihy C, Jirstrom K. Tumour-specific HMG-CoAR is an independent predictor of recurrence free survival in epithelial ovarian cancer. *BMC Cancer* 10:125 (2010)

# Abbreviations

5-FU	5-flourouracil
ACF	abberant crypt foci
AJCC	American Joint Comittee on Cancer
APC	adenomatous polyposis coli
APR	abdomineoperineal rectal resection
BMI	body mass index
BRAF	v-Raf murine sarcoma viral oncogene homolog B1
BRAF <sub>wt</sub>	BRAF wild type
CDK	cyclin dependant kinase
CEA	carcinoembryonal antigen
CI	confidence interval
CIMP	CpG island methylator phenotype
CIN	chromosomal instability
CME	complete mesocolic resection
CIS	carcinoma in situ
CRC	colorectal cancer
CRT	chemoradiotherapy
CT	computed tomography
DCC	deleted in colorectal cancer
DNA	deoxyribonucleid acid
EGFR	epidermal growth factor receptor
ERK	extracellular-signal-regulated kinases
ER	estrogen receptor
FAP	familial adenomatous polyposis
GDP	guanosine diphosphate

GSK3 $\beta$	glycogen synthase kinase 3 $\beta$
GTP	guanosine triphosphate
Gy	Gray
HNPCC	hereditary non polyposis colorectal cancer
HP	hyperplastic polyp
HR	hazard ratio
HRT	hormone replacement therapy
IBD	inflammatory bowel disease
IGF	insulin like growth factor
IL-	interleukins
IHC	immunohistochemistry
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LAR	low anterior resection
LOH	loss of heterozygosity
MAPK	mitogen activated protein kinase
MDCS	Malmö Diet and Cancer Study
MEK	mitogen activated protein kinase kinase
MIN	microsatellite instability
MLH1	mutL homolog 1
MMR	mismatch repair
MRI	magnetic resonance imaging
MSH2	mutS protein homolog 2
MSH6	mutS homolog 6
MSI	microsatellite instability/unstable
MSS	microsatellite stable
NSAID	non steroidal anti-inflammatory drugs
OC	oral contraceptives
OS	overall survival
PCR	polymerase chain reaction
PMH	postmenopausal hormones

PMS2	postmeiotic segregation increased 2
RAF	rapidly accelerated fibrosarcoma
PPi	pyrophosphate
rRb	retinoblastoma protein
RT	radiotherapy
SA	serrated adenomas
SES	socioeconomic status
SSA	sessile serrated adenomas
TNF	tumour necrosis factor
TNM	tumour, node, metastasis
TMA	tissue microarray
TME	total mesorectal excision
TNM	tumour-node-metastasis classification
TME	total mesorectal excision
TMA	tissue microarray
UC	ulcerative colitis
UICC	International Union for Cancer Control
WHI	Women's Health Initiative
WHR	waist hip ratio
Wnt	Wingless-type MMTV integration site family members





# Introduction

Colorectal cancer (CRC) is one of the most common forms of human cancer worldwide with approximately 1.2 million new cases detected every year and represents a major health burden. CRC development is a multi-step process that spans 10-15 years, thereby providing an opportunity for early detection and even prevention [1]. The aetiology of CRC is debated, however life style factors have been shown to play an important role. Numerous epidemiological studies and meta-analyses have examined the relationship between body size and body mass index (BMI) and CRC, whereby most studies have shown a positive relationship between a high BMI and risk of colon cancer in men, whereas weak or no associations were reported in women [2-5]. The reasons for the apparently discrepant associations between BMI and CRC risk in men and women remain unclear, but are likely due to hormonal factors. To define obesity, different anthropometric factors have been used, but the most relevant predictor of CRC risk is not clear [2, 6-9].

Most former studies have focused on body measurements in relation to general CRC risk. CRC is, however, a largely heterogenous disease in terms of its biological properties. Colorectal carcinogenesis can be regarded as a complex process involving multiple genetic and epigenetic alterations [10, 11]. Three main pathways occur in CRC, including chromosomal instability (CIN), microsatellite instability (MSI) and epigenetic silencing through the CpG Island Methylator Phenotype (CIMP). These pathways have distinct clinical, pathological, and genetic characteristics, which can be used for molecular classification and tumour profiling for improved diagnostics, prognostication and treatment prediction in CRC. Accumulating evidence suggests that aetiological factors influence the carcinogenetic process differentially according to the tumour pathway. As traditional cancer epidemiology approaches have not generally taken tumour biological properties into consideration, the impact of body constitution on CRC risk may be further clarified by analyzing the molecular alterations involved in the different pathogenetic pathways [12]. Expression of beta-catenin, cyclin D1, p53, mutations in the KRAS and BRAF genes as well as microsatellite instability status of the tumours are known to play important roles in colorectal carcinogenesis [13-16].

Taken together, it can be hypothesized that risk of CRC differs according to life style related factors, gender and clinicopathological characteristics such as tumour location, TNM stage, and molecular subtypes of the tumours [2, 4, 8, 9, 17]. The aim of this thesis was therefore to examine some of these associations. By this molecular pathological epidemiology approach, we can refine risk estimates for specific subtypes of CRC and gain further insights into the potential influence of aetiological factors on different pathways of colorectal carcinogenesis.



# Colorectal cancer

## Epidemiology

Colorectal cancer (CRC) is a major cause of morbidity and mortality throughout the world. It accounts for 9.4% of all cancer incidence in men and 10.1% in women worldwide [18]. It is the third most common cancer with approximately 1.2 million new cases being detected every year, and the fourth most common cause of death worldwide [19]. There is a large geographic difference in the global distribution of CRC, as CRC is mainly a disease of developed countries [18]. The developed world accounts for over 63% of all cases [20]. The incidence rate varies up to 10-fold between countries with the highest rates and those with the lowest rates. Countries with the highest incidence rates include Australia, New Zealand, Canada, the United States, and parts of Europe. The countries with the lowest risk include China, India, and parts of Africa and South America [18]. However, these incidence rates may be biased due to underreporting in developing countries. Significant differences also exist within continents, with higher incidences in western and northern Europe than in central and southern Europe. Among immigrants and their descendants, incidence rates rapidly reach those of the adopted country, indicating that environmental and lifestyle related factors are important [20, 21].

In parts of Northern and Western Europe, the incidence rates of CRC have been stable during the last decades, but possibly declining gradually in the United States, due to implementation of screening programs [22]. Elsewhere, however, the incidence is rapidly increasing, particularly in countries with a high-income economy that have recently made a transition from a relative low-income economy, such as Japan, Singapore, and Eastern European countries [18, 20]. This trend is thought to be due to “westernization” with altered dietary habits and decreased physical activity. Incidence rates have at least doubled in many of these countries since the mid-1970s [18].

Incidence in men worldwide is approximately 1.4 times higher than in women [19]. In Sweden, approximately 6500 new cases of CRC are diagnosed every year and the trend is rather stable, although colon cancer in women has increased with 1.7 per cent per year during the last decade [23]. Mortality rates have steadily decreased in the past decades in Sweden and other developed countries, due to improved detection, and to advances in surgical and oncological management. In general, mortality rates are lower in women than in men [19]. Statistics of CRC in Sweden by 2011 is shown in Table 1[23].

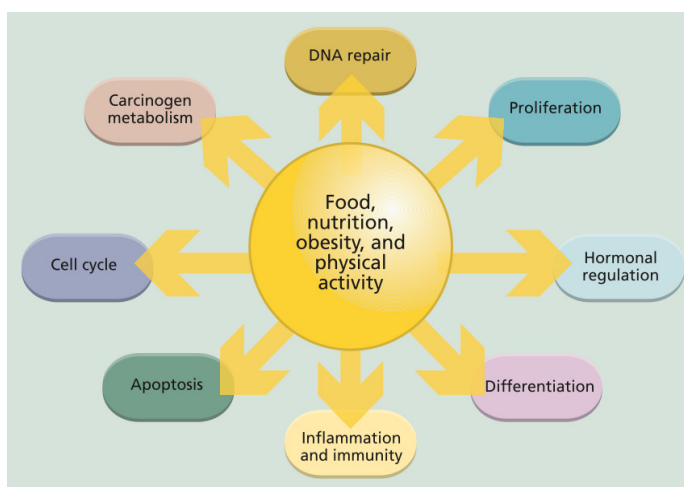
**Table 1.**

Statistics of colorectal cancer in Sweden 2011. Socialstyrelsen

	Colon cancer		Rectal cancer	
	men	women	men	women
Number of cases diagnosed	2081	2102	1158	821
Proportion of all cancers	6.9	7.6	3.9	3.0
Incidence per 100000	44.2	44.3	24.6	17.3
Prevalence total	13587	16556	8549	7297
Relative 5 year survival %	64.1	66.8	62.9	64.2
Relative 10 year survival %	57.9	62.3	53.9	58.0
Number of deaths	870	981	469	335

## Aetiology

The aetiology of sporadic CRC is debated, however a substantial amount of studies indicate that life style factors play an important role in the development of the disease. Aetiological factors implicated in colorectal carcinogenesis include red and processed meat, excess alcohol intake, obesity, physical inactivity, diabetes mellitus, smoking, family history of colorectal cancer, inflammatory bowel diseases, among others [24]. Risk factors are either non-modifiable, e.g. age, hereditary factors and inflammatory bowel disease, or modifiable, e.g. environmental and lifestyle factors.

**Figure 1.**

Life style and the cancer process. Food, Nutrition, Physical Activity, and the Prevention of Cancer: a Global Perspective. Used with permission by American Institute for Cancer Research. Washington DC: AICR, 2007 [25]

## **Risk factors**

### *Age*

CRC risk is strongly related to age. More than 75% of colorectal cancer cases occur in people aged 65 or older. Age specific incidence rates increase sharply from age 50, with the highest rates from age 85 and above [19].

### *Hereditary factors*

The majority of CRC cases occur in people without a family history of CRC. Nevertheless, up to 20-30% of people who develop CRC have other family members who have been affected by this disease [26]. People with a history of CRC or adenomatous polyps in one or more first-degree relatives are at increased risk. The risk is higher in people with a stronger family history, such as a history of CRC or adenomatous polyps in any first-degree relative younger than age 60; or a history of CRC or adenomatous polyps in two or more first-degree relatives at any age [27]. The reasons for the increased risk are not clear, but are likely due to inherited genes, shared environmental factors, or a combination of these.

By contrast, only 5% of colorectal cancers are a consequence of recognized hereditary conditions [28]. The most common inherited conditions are hereditary nonpolyposis colorectal cancer (HNPCC), also called Lynch syndrome, and familial adenomatous polyposis (FAP). The genes responsible for these forms of inherited colorectal cancer have been identified.

HNPCC is caused by germline mutations in genes involved in the DNA mismatch repair (MMR) system, namely the MLH1, MSH2, MHL6 or PMS2 genes, leading to microsatellite instability [28, 29]. Carriers of gene mutations in the MMR genes have a 50–80% lifetime risk of developing CRC. Inheritance is autosomal dominant and accounts for 2-5% of CRC [26, 30]. Clinical features of HNPCC are multiple generations affected with CRC at an early age (mean, approximately 45 years) with a predominance of right-sided tumours (approximately 70 percent proximal to the splenic flexure). There is an excess of synchronous and metachronous colorectal cancers, as well as an excess of extracolonic cancers, most frequently carcinoma of the endometrium. Further, HNPCC also displays specific histopathological features, such as poor differentiation, excess of mucinous or signet ring histology and lymphoid infiltration [31]. Personal and family cancer history, molecular testing of CRC tumour specimens for MSI and germline MMR gene mutation analysis should be performed to identify persons at risk.

FAP is the second most common inherited CRC syndrome and accounts for approximately 1% of all CRC cases. Unlike individuals with HNPCC, who develop only a few adenomas, FAP is easily identified, classically characterized by hundreds to thousands of adenomatous colorectal polyps that develop after the first decade of life, which confer a nearly 100% risk of CRC by age the age of 40 years in the absence of

any medical intervention [32]. Adenomatous polyps are usually discovered during endoscopic evaluation for symptoms such as gastrointestinal bleeding or during routine screening in individuals with a known family history of FAP. FAP is caused by a germline mutation in the adenomatous polyposis coli (APC) gene and is most often inherited in an autosomal dominant manner. However, up to 30% of cases can emerge as de novo gene mutations in the APC gene and consequently do not present with a family history of the disease. For known APC gene mutation carriers or individuals at risk, colorectal screening for polyps should begin with flexible sigmoidoscopy at the age of 10–12 years with annual colonoscopy once polyps are detected. Once the polyp burden is too numerous to be managed endoscopically, prophylactic colectomy is recommended [33].

### *Chronic inflammation*

Chronic inflammatory bowel disease (IBD): ulcerative colitis (UC) and Crohns disease are significant risk factors of CRC. The risk increases after 8-10 years and is highest in patients with early-onset and widespread manifestation [34, 35].

### *Obesity*

Obesity is one of the most serious public health problems worldwide, even in developing countries. Its prevalence has dramatically increased in the last few decades [36]. The proportion of men and women over 20 years of age in the U.S. who are obese has risen to 35% [37]. Epidemiological studies have shown that obesity is associated with an increased risk of several cancer types, including colon, breast, endometrium, liver, kidney, esophagus, gastric, pancreatic, gallbladder cancer, and leukemia [36]. Accumulating evidence suggests that insulin and the insulin-like growth factor (IGF) axis are putative mediators of the causal link between obesity and CRC. To date, several mechanisms have been proposed to explain the molecular associations between obesity and CRC, including insulin and insulin-like growth factors, leptin, adipose tissue-induced changes of estrogens and androgens and inflammatory molecules [38]. The high insulin and IGF levels observed in obese individuals may stimulate certain signaling pathways favoring pro-carcinogenic processes such as induction of proliferation and angiogenesis, and suppression of apoptosis [39, 40].

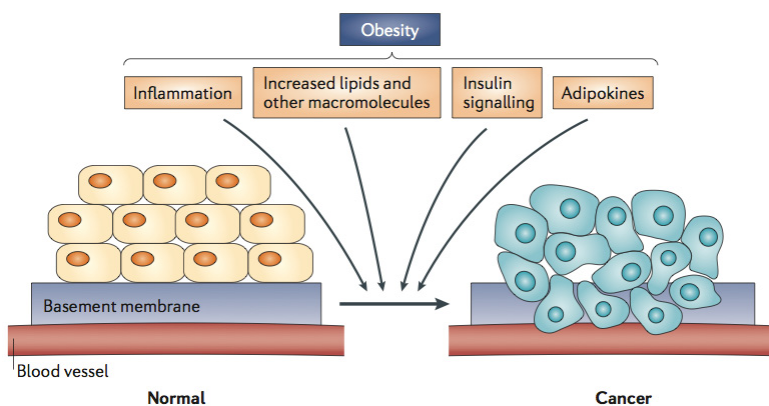
Leptin is a hormone and cytokine produced mainly in the adipose tissue, thus causing elevated levels in obese people. Leptin has been shown to both suppress apoptosis and stimulate proliferation of colonic epithelial cells in vivo [41].

Sex steroid hormones, including estrogens, androgens, and progesterone, are also likely to play a role in obesity and cancer. Adipose tissue is the main site of estrogen synthesis in men and postmenopausal women. Mechanistically, estrogen may prevent tumour growth by competitively preventing IGF from binding to its receptors. While estrogen exerts protective effects by binding IGF receptors, high levels of circulating insulin induced by excess adipose tissue may bind to the increased insulin receptors and increase CRC risk [42]. In contrast, in women with low levels of estrogen, its protective

effect is lost and small changes in circulating estrogen derived from excess adipose tissue has little effect on risk of CRC. Androgens in men may exert similar effects on the insulin pathways and thereby modify CRC risk [43, 44].

Obesity is further characterised by a low-grade chronic inflammatory state. The adipocyte produces pro-inflammatory factors, and obese individuals have elevated concentrations of circulating tumour necrosis factor (TNF)-alpha, interleukin (IL)-6, and C-reactive protein compared with lean people [45]. Such chronic inflammation can promote cancer development through production of reactive oxygen species and reactive nitrogen intermediates that can induce DNA damage and mutations by activated inflammatory cells [46].

Moreover, dietary energy restriction has been shown to reduce levels of circulating IGF-1 [38], which stimulate cell cycle progression [44]. Energy restriction has also been shown to decrease expression of cyclins and cyclin-dependent kinases (CDKs), and increases levels of CDK inhibitors, leading to inhibited cell cycle progression [47]. Energy restriction also decreases other inflammatory markers [38].



**Figure 2.** Pathways that may link obesity to cancer development. Used with permission, Nat Rev Cancer, 2011 [40]

### *Anthropometrics*

Body size is difficult to measure directly and accurately. Several weight-based measures are used as markers of body size. Anthropometric measurements cover a variety of parameters: height, weight, BMI, waist- and hip circumference, waist-hip-ratio and body fat percentage.

While height is regarded as a non-modifiable anthropometric factor, all other anthropometric factors are considered modifiable. Height is mainly determined by genetic factors, but is also suggested to be influenced by nutritional conditions in

childhood. Childhood and adolescence obesity poses a major public health problem through long-term adverse health outcomes such as insulin resistance, early-onset type 2 diabetes, hypertension, and hyperlipidemia [48]. However, the impact of early life obesity on cancer risk later in life is less well studied [36, 49-51]. Early life obesity is associated with alterations in basal insulin levels [52], which, in turn, lead to an increased activity of IGF-1 [53]. Both insulin and IGF-1 act as tissue growth factors and may thus enhance tumour development by stimulating cell proliferation and inhibiting apoptosis [54]. In several cohort studies, elevated height has been shown to be related to an increased risk of CRC [55-57], thus, the positive associations between height and CRC may be explained by the cancer promoting effects of IGF-1 [53].

The most common way to measure body size is body mass index (BMI), a measure of weight adjusted for height. BMI is calculated as weight in kilograms divided by height in metres squared ( $\text{kg/m}^2$ ). According to the WHO classification, overweight equals a BMI above 25, and obesity equals a BMI above 30. In most circumstances, BMI has been shown to be reliably linked to body fatness, but this method does not always provide an accurate measure. Numerous epidemiological studies and meta-analyses have examined the relationship between body weight or BMI and CRC [7, 58, 59], and most studies have shown a positive relationship between a high BMI and risk of colon cancer in men, but weak or no associations were reported in women [2, 3, 5, 7, 9]. When stratified according to cancer site, data suggest that the increased risk is more consistent for colon [5, 8, 17] than for rectal cancer [5, 60]. A clear dose- response relationship was apparent from cohort data for colorectal cancer [59, 60].

However, BMI may not be the ideal way to measure body fatness because of the changes in physiologic functions that to a certain extent depend on regional adipose tissue distribution. Available epidemiologic evidence suggests that abdominal obesity (high waist circumference and waist-hip-ratio) may be more predictive of CRC risk than overall obesity [2, 6, 9, 17, 61, 62].

### *Physical activity*

There is abundant evidence supporting that higher overall levels of physical activity are associated with a lower risk of CRC, including evidence of a dose-response effect, with frequency and intensity of physical activity inversely associated with risk [24, 63, 64]. The evidence is stronger for colon than for rectal cancer [65]. The biologic mechanisms potentially responsible for the association between reduced physical activity and CRC are not fully understood, but include a reduction in insulin resistance, the effects on endogenous steroid hormone metabolism, and reduced gut transit time [44, 64].

### *Diet*

Diet strongly influences the risk of CRC. Diets high in fat, especially animal fat, have been shown to be a risk factor for CRC in some studies. However, the associations between dietary fat (and types of fat) and the risk of CRC are somewhat inconsistent [59, 66, 67]. The implication of fat as a possible aetiologic factor is linked to the



concept of the typical Western diet, which is thought to favor the development of a bacterial flora that degrades bile salts to potentially carcinogenic nitrogen compounds [68].

A substantial amount of evidence shows that consumption of red and processed meat confers an increased risk of CRC. The finding that a high intake of red meat but not of chicken or fish might be associated with increased CRC risk was first reported in a prospective study by Willett et al. in 1990 from an analysis of 150 CRC patients in the Nurses' Health Study [69]. Results from a systematic review of observational and experimental studies and two meta-analyses also supported the initial finding [70, 71]. This is further confirmed in the EPIC study, concluding a consistent positive association between high intake of red and processed meat and CRC, and an inverse association between high intake of fish and CRC [72]. The positive association with meat consumption seems to be stronger for colon cancer than for rectal cancer [68].

A possible inverse association between dietary fiber intake and CRC was first proposed by Burkitt in 1971 [73]. Putative anti-carcinogenic mechanisms of dietary fiber within the bowel include the formation of short-chain fatty acids from fermentation by colonic bacteria, the reduction of secondary bile acid production, the reduction in intestinal transit time and increase of faecal bulk, and a reduction in insulin resistance. Most studies have demonstrated a decreased CRC risk with increased fiber intake. However, the evidence from prospective studies has been conflicting [74-76]. Intake of dietary fiber has been proposed to account for some of the differences in the incidence rates of colorectal cancer between Africa and Westernized countries [20].

### *Smoking*

The association between tobacco cigarette smoking and CRC risk is well established today. The association seems to be stronger for rectal than for colon cancer [77, 78]. Carcinogens from cigarette smoke cause irreversible genetic damage in the normal colorectal mucosa, but many years are required for completion of all carcinogenetic events after initiation [79]. Cigarette smoking is important for both formation and growth rate of adenomatous polyps, one of the precursor lesions of CRC [80].

### *Alcohol consumption*

A pooled analysis of eight cohort studies from North America and Europe found a modestly increased CRC risk with regular high alcohol intake ( $\geq 45$  g/day), compared with nondrinkers, in men and women. No increased risk was observed below intakes of 30 g/day [81]. However, the dose-risk relation of alcohol intake with CRC risk has not yet been investigated in detail. In particular, a more precise quantification of the association of light and/or moderate alcohol consumption with CRC risk, and the identification of a possible threshold of effect is warranted. Further, it is still unclear whether the effect of alcohol varies across colon and rectal subsites. Some studies have reported a stronger association of risk in the colon than in the rectum [82], whereas others have found a stronger [83] or similar [81] association for the rectum.

### *Educational level*

Education, an indicator of socioeconomic status (SES), has been shown to be inversely associated with the incidence of several cancers [84]. Many studies have shown a relationship between low SES and increased risk of CRC [85-87]. People with lower SES have been shown to be more likely to present with CRC in more advanced clinical stages compared to people with a higher SES [88, 89], most likely due to a delayed diagnosis.

### *Hormonal factors*

Data from prospective cohort studies suggest that circulating estrogen levels and life-time exposure to estrogen, increased by early menarche, late menopause, not bearing children, and late first pregnancy, are positively associated with CRC risk [90, 91]. The positive associations between endogenous estrogen level and the risk of colorectal cancer reported by these investigations are consistent with laboratory data demonstrating proliferative effects of exogenous estradiol in colorectal tissue and in colorectal cancer cell lines [92, 93]. By contrast, in a large meta-analysis conducted in 1999, Grodstein et al [94] found that hormone replacement therapy (HRT) use was associated with an approximately 35% decrease in colon cancer risk. This association was further confirmed by the Women's Health Initiative Clinical Trial [95, 96], a randomized, double blind placebo controlled clinical trial, where intervention with estrogen plus progestin yielded an even more striking 44% reduction in incident CRC, while estrogen alone did not appear to affect CRC risk. Subsequent epidemiological studies have observed similar, although not entirely consistent, inverse associations between HRT use and CRC risk, indicating that exogenous estrogen and/or progestin compounds may inhibit the development of CRC [97-100]. Taken together, data suggest that endogenous and exogenous sex hormones may play different roles in colorectal carcinogenesis [91].

The epidemiological evidence for an association between oral contraceptives (OC) and CRC risk is also inconsistent. Some studies have shown inverse associations [101-105], whereas others have found no associations [106-109]. A recent meta-analysis, summarising the results from seven cohort- and eleven case-control studies, reported a statistically significant 19% reduced risk among ever users of OC compared with never users, although there was no clear risk reduction with increasing duration of use [110].

### *NSAIDs*

Extensive evidence suggests that long-term, regular use of aspirin and other nonsteroidal anti-inflammatory drugs (NSAID) is associated with a lower risk of CRC [111, 112]. It has been shown that these drugs reduce CRC risk in a dose- and time-dependent manner. The use of specific COX2 inhibitors have been demonstrated to reduce CRC risk and slow progression of colorectal adenomatous polyps to carcinomas [113].

# Colorectal carcinogenesis

Colorectal cancer arises as the result of a multistep process by the accumulation of acquired genetic and epigenetic changes that transform normal glandular epithelial cells into invasive adenocarcinomas. Steps that transform normal epithelium to adenoma, followed by invasive carcinoma, and eventually metastatic cancer are described in the classic tumour progression model originally proposed by Fearon and Vogelstein in 1988 [114]. The adenoma-carcinoma sequence is characterized by a stepwise progression from normal epithelium to carcinoma due to a series of genetic changes. However, our understanding of the molecular pathogenesis has advanced considerably and led to several revisions of this model. The original adenoma-carcinoma sequence proposed that only tubular and tubulovillous adenomas had the potential to progress to invasive adenocarcinoma, and that the hyperplastic polyps were innocuous. It is now recognized that hyperplastic polyps and serrated adenomas also have the potential of malignant transformation by alternate pathways, and these polyps demonstrate characteristic molecular alterations not commonly seen in colorectal adenomas [115, 116] (Figure 3).

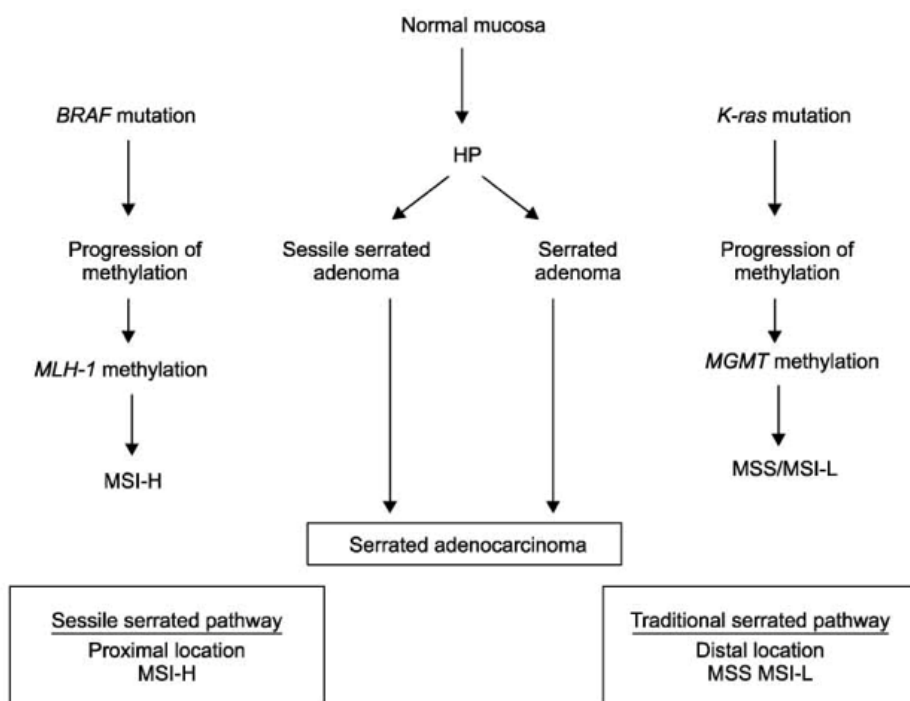
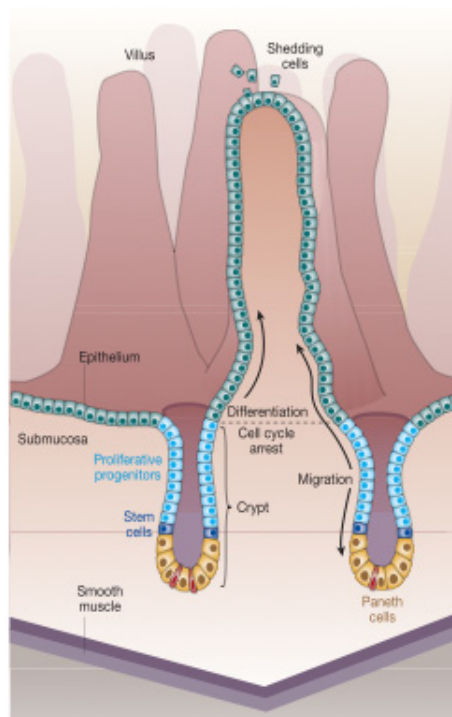


Figure 3.

Outline of the two different serrated pathways of CRC development.

## Aberrant crypt foci

The first step towards epithelial neoplasia is the development of early morphologic changes in clusters of epithelial crypts, so called aberrant crypt foci (ACF) [117, 118] (Figure 4). The digestive surface of the human large intestine is characterized by a monolayer of specialized epithelial cells that form invaginations called crypts. At the base of each crypt, 4-6 intestinal stem cells are located, from which the four cellular types that constitute the intestinal layer originate: columnar absorptive cells, the mucus secreting goblet cells, the neuroepithelial cells and the Paneth cells. By asymmetrical division, these stem cells are able to renew the complete layer in 3-8 days.



**Figure 4.**

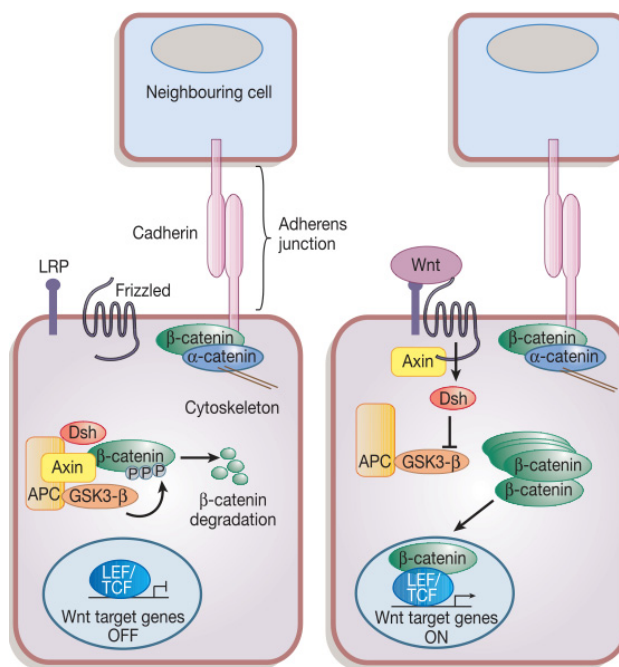
Aberrant crypt foci. Inactivation of the APC/beta- catenin (see below) pathway commonly initiates the process and results in extension of epithelial proliferation in dysplastic epithelium from the base of the crypts, where it normally occurs, toward or onto the luminal surface. Used with permission from Nature, 2005 [119].

ACFs are classified histologically as nondysplastic and dysplastic/hyperplastic [120]. Apart from their size, nondysplastic crypts are not remarkably abnormal, and their proliferative compartments are confined to the lower portion of the glands. However, they often display signs of hyperplasia and infolding of the epithelium into the crypt

lumen, a phenomenon referred to as serration. Dysplastic crypts, in contrast, present signs of cellular atypia (mucin depletion, nuclear enlargement, stratification, and are associated with mutation of the APC gene). They are found in the majority of FAP patients [121]. Around 60% of all healthy adults have a few ACFs in their colons, but these lesions are rarely dysplastic.

## Wnt signaling

The common denominator in the onset and progression of most precancerous lesions of the colorectum is aberrant activation of the Wnt signaling cascade. Beta-catenin is a membrane-associated protein with essential functions in the regulation of cellular adhesion and the major mediator of the Wnt-signaling pathway (Figure 5).



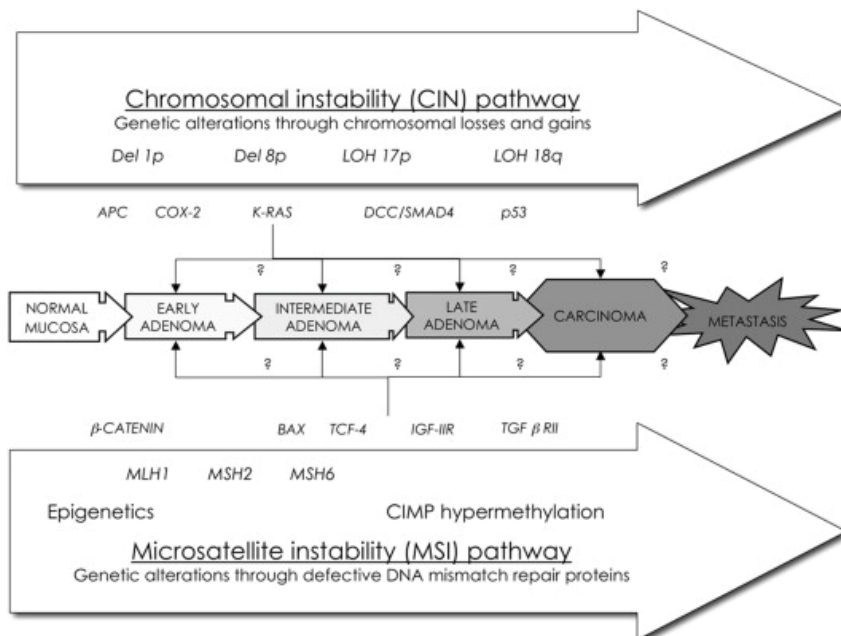
**Figure 5.**

The canonical Wnt signalling pathway. Used with permission, Nature, 2005 [119].

In the absence of Wnt, cytoplasmic beta-catenin will form a multiprotein complex with two other cellular proteins; axin and APC. Beta-catenin is then phosphorylated by GSK3 $\beta$  (glycogen synthase kinase 3 $\beta$ ), leading to destruction of beta-catenin by proteolysis, which explains the low steady state concentrations of beta-catenin normally present in the cytoplasm. When the Wnt signalling is activated by the Wnt ligand

binding to its Frz receptor (Frizzled family of transmembrane proteins), GSK3 $\beta$  is blocked and beta-catenin is saved from rapid destruction, leading to accumulation of unphosphorylated beta-catenin in the cytoplasm. This accumulation leads to translocation into the nucleus where beta-catenin binds to transcription factors, and activates transcription of target genes, including those involved in cell proliferation, for example cyclin D1, contributing to tumour progression. Constitutive Wnt signaling leads to an expansion of the proliferative compartment of the crypt by mutation of the tumour suppressor gene APC, hereby destroying the equilibrium between proliferation and differentiation, leading to the development of precancerous lesions [119, 122, 123].

Research conducted during the past decades has increased our understanding of the mechanisms involved in CRC initiation and development. The findings have demonstrated the existence of at least three major pathways of colorectal carcinogenesis: chromosomal instability (CIN), microsatellite instability (MSI) and the CpG island methylator phenotype (CIMP), all characterized by distinctive models of genetic instability and clinicopathological features [124, 125].



**Figure 6.** Genetic instability in colorectal cancer. The figure is derived from Søreide et al. Copyright British Journal of Surgery Society Ltd. Used with permission from British Journal of Surgery [124].

## **Chromosomal instability**

Chromosomal instability (CIN) is the most common and well-characterized carcinogenetic pathway. Approximately 70-80% of CRC develops through the CIN pathway and is clinically characterized by distal location, high differentiation grade and intermediate prognosis [124, 125].

The CIN pathway is associated with mutation in the adenomatous polyposis coli (APC) tumour suppressor gene, and/or loss of chromosome 5q, which harbours the APC gene, mutation of the KRAS oncogene, loss of chromosome 18q and deletion of chromosome 17p, harbouring the important tumour suppressor gene p53 [126, 127]. Only a very small minority of CRC characterized by CIN, however, possess a full complement of these molecular abnormalities [128].

The initial key event is the early mutation of the APC gene (Figure 6), involved in both sporadic CIN and, when germline mutated, in all FAP [129]. The APC suppressor gene is mutated in up to 80% of sporadic CRC.

The above-mentioned early mutations of the CIN pathway are then followed by subsequent events that promote new mutations and facilitate the progression to a malignant state. The adenoma to carcinoma transition is initially determined by the v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) gene, a proto-oncogene that is involved in the transduction and propagation of extracellular signals. KRAS mutations lead to a permanently active state that permits the cell to evade apoptosis and acquire a growth advantage.

Finally, loss of function of p53 by mutation is a key step in the later stages of colorectal carcinogenesis [130] (Figure 6). The p53 gene, also called “guardian of the genome”, is located on chromosome 17p and mutation leads to high proliferative activity through the loss of cell cycle control and apoptosis [131].

## **Microsatellite instability**

The MSI pathway represents a form of genomic instability involved in the development of approximately 15% of sporadic colorectal cancer and over 95 percent of HNPCC syndrome-associated tumours. CRC that develops through the MSI pathway presents distinct clinical features such as location in the proximal colon, poor differentiation and/or mucinous histology, and increased numbers of tumour infiltrating lymphocytes [132, 133].

In general, the prognosis and survival of patients affected by MSI-positive CRC is good, and MSI is relatively uncommon in metastatic CRC [134]. MSI is more frequent in women, especially older women, compared to men. MSI-high CRC has also been suggested to be less responsive to 5-fluorouracil-based chemotherapies [135].

Microsatellite instability refers to a change in the length of DNA microsatellites. Microsatellites are repetitive sequences distributed throughout the genome that consist of repeating units (usually 1–5 nucleotides long), which are frequently copied and inserted incorrectly in the new DNA by the DNA polymerases. It is estimated that each cell undergoes > 20 000 DNA damaging events and > 10 000 replication errors per cell per day [136]. One of the mechanisms to repair replication errors is the mismatch repair system (MMR). The MMR system, consisting of several proteins including MLH1, MSH2, MSH6 and PMS2, is responsible for the surveillance and immediate correction of these errors [124].

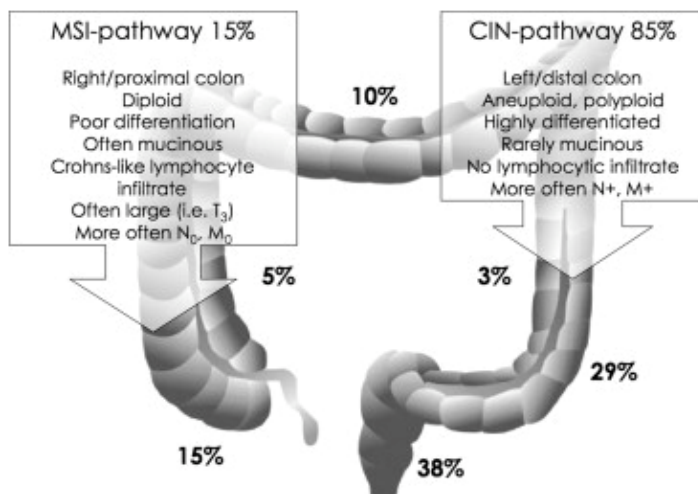
Whilst HNPCC causes the pure form of MSI, the majority of MSI-positive CRC occurs sporadically as a result of methylation of the MLH1 promoter and the consequent transcriptional silencing of MLH1 expression. Such cancers exhibit both CIMP and MSI, and therefore form part of the CIMP pathway. MSI positive tumours, whether sporadic or inherited, however, share similar clinicopathological characteristics [124, 137].

Determining the MSI status of CRC has a clinical use for identifying patients with HNPCC. In addition, MSI status, regardless of whether the causative defect is inherited or sporadic, may have a use in prognostic and therapeutic decision-making.

MSI is detected either indirectly by immunohistochemical (IHC) analysis of MMR proteins, or directly by polymerase chain reaction (PCR). MSI is tested through PCR amplification of a set of five specific microsatellite markers on tumour and normal DNA, followed by a comparison of the size of the amplified DNA by electrophoresis. The tumour is classified as MSI-high (MSI-H) if size alterations or shifts are observed in two or more of the five microsatellite markers. If only one marker shows instability, the tumour is classified as MSI-low (MSI-L), and finally, if none of the markers show instability the tumour exhibits a microsatellite stable (MSS) phenotype [138]. In clinical practice, MSI-L tumours do not differ from MSS, and is therefore generally sub-grouped together with MSS [138, 139].

Alternatively, IHC can confirm the presence or absence of MMR proteins. In general, MMR defects are the result of a germline mutation in one of the MMR genes, or due to changes in methylation of the promoter of a MMR gene (usually MLH-1) resulting in loss of protein expression. Tumours are determined as MSS or MSI when evaluated by IHC. Both IHC and PCR-based MSI testing show high sensitivity and specificity in detecting MSI [138].





**Figure 7.**

Clinicopathological distinctions between tumours exhibiting microsatellite instability (MSI) and chromosomal instability (CIN). Percentages indicate the anatomical distribution of colorectal cancers (TNM refers to the tumour node metastasis staging system). The figure is derived from Søreide et al. Copyright British Journal of Surgery Society Ltd, used with permission from British Journal of Surgery [124].

## CpG island methylator phenotype

Being relatively rare in conventional adenomas, the CIMP phenotype is found in 70–80% of all dysplastic serrated lesions of the right colon, and it is closely associated with older age, female sex, family history of CRC, smoking, mucinous histology, MSI and BRAF and KRAS mutations

Classically, cancer has been viewed as a set of diseases driven by progressive genetic abnormalities, including mutations in tumour-suppressor genes and oncogenes, and chromosomal abnormalities. It is however becoming increasingly apparent that cancer is also a disease that is driven by epigenetic changes, i.e. patterns of altered gene expression that are mediated by mechanisms that do not affect the primary DNA sequence [140]. CpG islands are regions of DNA that are often located proximally to the transcription start site of genes that contain a high frequency of CG dinucleotides [124, 141]. In cancer cells, CpG islands in various tumour-suppressor genes are frequently methylated, which results in repression of transcription. Thus, the expression of these tumour-suppressor genes in the cancer cell can be reduced or eliminated as an alternative mechanism to genetic mutation [141]. Subgroups of CRC exhibit widespread hypermethylation of the mismatch repair gene MLH1, referred to as the CpG island methylator phenotype (CIMP).

For detection of methylation, a panel of CpG markers is assessed by PCR. Tumours are categorized as CIMP- high or CIMP-low depending on the extent of methylation.

To summarize, these three pathways of colorectal carcinogenesis have distinct clinical, pathological, and genetic characteristics, all being of potential utility for a clinically relevant molecular classification of CRC for improved diagnostics, prognostication and treatment prediction [124]. However, no such classification has yet been implemented in clinical protocols. A molecular classification of CRC based predominantly on five features has been proposed by Jass in 2007 (Table 2) [142].

**Table 2.**

Summary of the Jass classification of CRC.

Type	Genetic instability	Morphologic correlate
1	CIMP high, MSI, BRAF mutation	Serrated pathway
2	CIMP high, MSS, BRAF mutation	Serrated pathway
3	CIMP low, MSS, KRAS mutation	Any polyp
4	CIMP neg, MSS	Adenoma-carcinoma sequence
5 (HNPCC)	CIMP neg, MSI	Adenoma-carcinoma sequence

## Clinical aspects

### Diagnosis

Symptoms of CRC are often diffuse and late presenting, also depending on the tumour site. Tumours in the right colon more seldom present with gastrointestinal symptoms, but sometimes with weight loss and iron deficiency/anemia. For tumours located in the left colon and rectum, bleeding, mucus in the stools and changed faecal habits are more common symptoms. Approximately 20% of CRC presents as an acute colonic obstruction [143].

Investigation to conclude diagnosis involves rectoscopy and colonoscopy with biopsy, and CT scan of the abdomen and thorax for assessment of potential liver and lung metastasis. For rectal cancers, a pelvic MR scan is added for assessment of local growth in relation to the mesorectal fascia and adjacent organs in the pelvis.

A great deal of effort has been spent in search of serological markers that would allow for early detection and diagnosis of CRC. The most widely studied marker is carcinoembryonic antigen (CEA). CEA has been proven to be of little use in detecting early colorectal cancer, although high preoperative concentrations of CEA correlate with poor prognosis [144, 145]. Serial CEA measurements can also detect recurrent colorectal cancer and liver metastasis [146, 147].

## Clinical staging

The extent of cancer at time of diagnosis is the key factor used to define treatment and is the strongest predictor of survival. Therefore, clinical staging is crucial for optimal patient management. In the past, several staging systems have been used, mostly known as Dukes and Astler-Coller classification systems [148, 149]. However, these systems are not considered elaborate enough, and today, the most widely used staging system is the TNM system, maintained by the American Joint Committee on Cancer (AJCC) [150]. This system codes the extent of the primary tumour (T), regional lymph nodes (N), and distant metastases (M) and provides a stage grouping based on T, N, and M (Table 3 and 4).

**Table 3.**

T-stage, N-stage, M-stage of CRC according to the American Joint Committee on Cancer (AJCC) [148].

T – Primary Tumour		N – Regional Lymph Nodes	
T0	No evidence of primary tumour	N0	No regional lymph node metastasis
Tis	Carcinoma in situ: intraepithelial or invasion of lamina propria	N1	Metastasis in 1–3 regional lymph nodes
T1	Tumour invades submucosa	N1a	Metastasis in one regional lymph node
T2	Tumour invades muscularis propria	N1b	Metastasis in 2–3 regional lymph nodes
T3	Tumour invades through muscularis propria into subserosa	N1c	Tumour deposit(s) in the subserosa, mesentery, or nonperitonealized pericolic or perirectal tissues without regional nodal metastasis
T4a	Tumour penetrates to the surface of the visceral peritoneum	N2	Metastasis in 4 or more regional lymph nodes
T4b	Tumour directly invades other organs or structures	N2a	Metastasis in 4–6 regional lymph nodes
		N2b	Metastasis in 7 or more regional lymph nodes
M – Distant Metastasis			
MX	Distant metastasis cannot be assessed		
M0	No distant metastasis		
M1a	Metastasis confined to one organ or site		
M1b	Metastases in more than one organ/site or the peritoneum		

**Table 4.**

Stage I-IV according to the American Joint Committee on Cancer (AJCC) [150].

Stage	T	N	M
0	Tis	N0	M0
I	T1, T2	N0	M0
IIa	T3	N0	M0
IIb	T4a	N0	M0
IIc	T4b	N0	M0
IIIa	T1-T2, T1	N1/N1c, N2a	M0
IIIb	T3-T4a, T2-T3, T1-T2	N1/N1c, N2a, N2b	M0
IIIc	T4a, T3-T4a, T4b	N2a, N2b, N1-N2	M0
IVa	Any T	Any N	M1a
IVB	Any T	Any N	M1b

## Tumour spread

Following transmural extension through the muscularis propria into pericolic or perirectal soft tissue, the tumour may involve contiguous structures. The consequences of direct extension depend on the anatomic site. An advanced rectal carcinoma may extend into pelvic structures such as the vagina and urinary bladder, but cannot gain direct access to the peritoneal cavity when it is located distal to the peritoneal reflection. By contrast, colonic tumours can extend directly to the serosal surface. Perforation can be associated with spread to the peritoneal cavity causing peritoneal carcinomatosis. Since the peritoneal surface infiltrated by tumour cells may become adherent to adjacent structures, direct extension into adjacent organs can also occur in colonic carcinomas that have invaded the peritoneal portion of the bowel wall. Spread via lymphatic or blood vessels lead to systemic disease, in which the most common sites of distant metastasis are the liver and the lungs.

## Prognosis

Despite the increasing knowledge on cancer biology, and vast research efforts, no prognostic biomarkers have yet been introduced into clinical practice. The TNM staging system continues to be the most powerful and reliable predictor of the clinical outcome of CRC patients. The prognosis of colon cancer is clearly related to the degree of penetration of the tumour through the bowel wall and the presence or absence of nodal involvement. The majority of patients presenting with stage I, II, or III disease (75%) can be treated with surgery alone or in combination with chemotherapy, and have a 5-year survival rate of 93.2%, 82.5%, and 59.5%, respectively, compared with only 8.1% survival rate of patients harboring stage IV disease [151]. Metastasis to numerous lymph nodes, those close to the mesenteric margin, or at great distance from the primary tumour, have been associated with poor prognosis while the prognostic

value of identification of micrometastasis in lymph nodes by immunohistochemical or molecular techniques is still controversial [152, 153].

Additional important parameters are the differentiation grade of the tumours, with the majority being moderately differentiated. The presence of an intense inflammatory infiltrate with leukocytes, lymphocytes, plasma cells, mast cells and histiocytes has been associated with an improved prognosis [154].

Further, the extent of surgical resection has considerable prognostic impact. The tumour status following treatment is described by the residual tumour (R) classification, as no residual tumour (R0), microscopic residual tumour (R1), or macroscopic residual tumour (R2). The R classification further influences treatment planning and is a strong predictor of prognosis [155].

Colorectal cancers manifesting MSI have been reported to have a lower frequency of metastasis and improved prognosis when compared to microsatellite-stable (MSS) tumours [156]. Moreover, bowel obstruction and perforation are clinical indicators of a poor prognosis [157]. Elevated pretreatment serum levels of CEA also have a negative prognostic significance [158].

## **Treatment**

### *Surgery*

Surgery is the primary treatment of CRC and curative resection is the most important factor for patient survival. The goal of surgery is a wide resection of the involved segment of bowel together with removal of its lymphatic drainage. The extent of the colonic resection is determined by the blood supply and distribution of regional lymph nodes, and the choice of surgical approach depends on preoperative TNM staging. Tumours located in the cecum and right colon should be removed by a right hemicolectomy, including ligation of the ileocolic, right colic and right branch of the middle colic arteries, followed by an ileocolic anastomosis. Tumours of the hepatic flexure, as well as tumors of the transverse colon, are treated with an extended right hemicolectomy, including ligation of the ileocolic, right colic, and middle colic arteries. Splenic flexure lesions require either previously described resections for transverse lesions or extended left hemicolectomy with ligation of the inferior mesenteric vessels after the blood supply has been ascertained. Descending or sigmoid colonic lesions are treated with left hemicolectomy with ligation of the inferior mesenteric vessels. Anatomic resection based on colonic blood supply assures both adequate margins as well as adequate anastomotic blood supply [159]. The resection should include a segment of colon of at least 5 cm on either side of the tumour, although wider margins are often included because of obligatory ligation of the arterial blood supply. Recently, the CME (complete mesocolic excision) technique has been introduced and is more frequently used [160]. The CME technique has been shown to improve overall survival

and lower the recurrence rate [160]. This is based on the same principle as the TME (total mesorectal excision) technique in rectal cancers, i.e. resection of tumour along embryologic tissue planes with the aim to separate the mesocolic from the parietal plane and true central ligation of the supplying arteries and draining veins right at their roots.

Local recurrence of rectal carcinoma is devastating, as a lateral spread of rectal cancer into the mesorectum is highly correlated with local recurrence rates. Total mesorectal excision (TME), as proposed by R.J. Heald more than 20 years ago [161], is nowadays the golden standard worldwide for optimal rectal cancer surgery. This technique is focused on a removal of the entire rectal mesentery as an intact package of the tumour and its main lymphatic drainage, requiring precise dissection in the embryologic tissue plane along the visceral fascia that envelopes the rectum and its mesentery. The main procedures performed are low anterior resection (LAR) for tumours in the upper, middle or distal third of the rectum, or an abdominoperineal resection (APR) applied for the most distal tumours [155].

### *Radiotherapy*

Radiotherapy (RT) is only administered for rectal cancers in the neoadjuvant setting for downstaging purposes, and is most often combined with chemotherapy, chemoradiotherapy (CRT) [155, 162]. In cases of locally advanced colon cancer without distant metastasis, neoadjuvant RT can be administered combined with capecitabin/5-FU. For rectal cancers in Sweden, neoadjuvant RT is given either as a short or longterm regimen [155]. The short-term regimen refers to treatment with 5 Gy per day for 5 days followed by surgery within a week. This regimen is administered to very low T2 and almost all T3 tumours. Long term RT applies to a setting with 1.8 Gy per day for 28 days, combined with chemotherapy, followed by surgery after 6-8 weeks. Long RT is given to T4 tumours. Palliative RT can be given to patients with bone metastasis and local recurrence for pain reduction [155].

### *Chemotherapy*

The aim of adjuvant chemotherapy is to reduce the risk of micrometastatic spread and local recurrence after surgery. Adjuvant chemotherapy is offered after complete surgical resection to patients with colon cancer in TNM stage III, a treatment which has been shown to reduce the relative risk of recurrence by 30-50% [163].

The potential value of adjuvant chemotherapy for patients with stage II colon cancer remains controversial and has been extensively investigated [164]. Although surgery alone is usually curative for stage II colon cancer, approximately 20% to 30% of these patients develop tumour recurrence. However, stage II patients are clearly a heterogeneous group and subgroups of patients with stage II colon cancer may be at a higher than average risk for recurrence, such as patients with inadequate lymph node sampling, T4 disease, involvement of the visceral peritoneum and a poorly differentiated histology [165]. Evidence for a beneficial effect on survival of adjuvant 5-FU-based chemotherapy compared with surgery alone is inconsistent [163]. In

Sweden, adjuvant chemotherapy is only recommended for stage III colon cancer. Adjuvant chemotherapy is generally not used for rectal cancers.

Historically, a few standard chemotherapies have been used in both adjuvant and palliative settings. 5-FU (5-fluorouracil) was the first drug widely used for treatment of colorectal cancer in the early 1990s. Today, four major chemotherapeutic agents are used in different combinations: 5-FU, which is often given with leucovorin (folinic acid), Capecitabine (Xeloda®), Irinotecan (Camptosar®) and Oxaliplatin (Eloxatin®).

Patients with MSI-high colon cancers have been shown to have longer overall survival (OS) and less tumour recurrence than stage-matched patients with MSS colon cancers [166]. MSI has been proposed to indicate resistance to 5-FU-based chemotherapy, however, findings are not conclusive [167, 168].

Novel therapeutic agents targeting the epidermal growth factor receptor (EGFR), such as Cetuximab® are currently used in combination with other therapies for treatment of metastatic CRC. The clinical effect of EGFR inhibitors has been thoroughly studied in recent years, with diverging results [169]. Moreover, KRAS mutation is associated with resistance to cetuximab in metastatic CRC [170]. Thus, KRAS mutation status might allow for the identification of patients who are likely to benefit from Cetuximab® and avoid a costly and potentially toxic administration of this treatment in nonresponders.

## Investigative markers

Even if CRC has been one of the most studied cancer forms at the molecular level during the last 30 years, the tumour staging system still remains the main predictor of survival and guide for therapy. A plethora of putative diagnostic, prognostic or treatment predictive biomarkers are under extensive investigation, but none has yet proven to be clinically useful.

### MSI

As described earlier, approximately 20 % of sporadic colorectal tumours display MSI, usually as a result of silencing of MMR genes by hypermethylation. MSI is associated with female sex, proximal location, low differentiation grade, mucinous histology, and, generally, good prognosis [171].

As regards the association of anthropometric factors, MSI and risk of CRC, previous studies present diverging results. One case control study found that MSI tumours were not associated with obesity [172], on the other hand, another presented data showing a positive relationship between a high BMI and microsatellite stable (MSS) tumours.

Only one prospective study has investigated the relationship between anthropometric factors and risk of CRC according to MSI status, demonstrating an association of high BMI with MSS tumours but not with MSI tumours [173].

## **p53**

The p53 tumor suppressor gene encodes for a transcription factor that regulates the expression of genes involved in the pathway of apoptosis, as well as angiogenesis, cell cycle progression, and genomic maintenance [131, 174]. p53 has an important regulatory role in various molecular pathways and it is altered in most cancers, whereby the mutated protein product cannot protect the genome, allowing mutations to accumulate [131, 175].

Inactivation of the p53 pathway by p53 mutations is the second key step in colorectal carcinogenesis, occurring late in the process, in the transition of large adenomas into invasive carcinomas [130]. Mutations occur in approximately 40-50% of CRC [125].

p53 also plays an important role in cellular energy metabolism and it has been shown that reduced nutrient or energy levels induce p53 [176-178]. However, how diet, lifestyle, environmental, or genetic factors interact with p53 mutations in CRC need to be further explored. There is a predominance for environmental factors affecting the type and/or location of p53 mutations in other tumours, for example in liver, lung and esophageal cancer, diseases all associated with tobacco usage [179].

Morikawa and colleagues further explored the role of p53 in energy balance and CRC risk, and described that among non-obese patients, p53 positivity was associated with reduced cancer-specific survival while an adverse effect of obesity on CRC patient mortality was observed in p53 negative subjects [180]. Associations between p53, and lifestyle factors and risk of CRC have only been shown in a few studies, with diverging results [181-183].

## **Beta-catenin**

Beta-catenin is a membrane-associated protein with essential functions in the regulation of cellular adhesion and the major mediator of the Wnt-signaling pathway [184], as previously described. Inactivation of kinases in the APC-complex leads to accumulation of cytoplasmic and nuclear beta-catenin, contributing to tumour progression [185]. Despite its crucial role in colorectal carcinogenesis, the clinical significance of altered beta-catenin expression in CRC is controversial, however, most previous results indicate an association of poor prognosis and more advanced clinical stages of CRC with beta-catenin overexpression [186]. However, in the here studied cohort, beta-catenin expression was found to be associated with a favourable prognosis [187]. Morikawa et al. have recently shown that BMI is associated with a higher risk of beta-



catenin negative-, but not of beta-catenin positive CRC [188]. Accumulating evidence supports a role for Wnt/beta-catenin signalling in adipogenesis, obesity and metabolic disorders [189], as well as in carcinogenesis.

## **Cyclin D1**

Cyclin D1 is activated by Wnt/beta-catenin signalling after mutation of the APC gene [123, 190]. Cyclin D1 is an important cell-cycle regulating protein that, together with its binding partners cyclin-dependent kinase CDK 4 and CDK 6, forms active complexes that promote cell cycle progression by phosphorylating and inactivating the retinoblastoma protein (rRb) [190]. Excessive cyclin D1 activation by APC mutation and beta-catenin activation in the Wnt signaling cascade contributes to the development of colorectal carcinogenesis by allowing the cell to escape apoptosis. Cyclin D1 overexpression is common in CRC [191, 192], but the findings regarding its prognostic value are conflicting, however, the largest study to date found an association between cyclin D1 overexpression and a prolonged survival from colon cancer [193]. In the MDCS, it has been shown that cyclin D1 overexpression is associated with prolonged survival in men, but not in women [194]. The association between obesity and anthropometric factors and cyclin D1 expression in CRC has, to our knowledge, not been studied previously.

## **KRAS**

KRAS is a proto-oncogene that encodes a GTPase protein with a central role in cellular signal transduction pathways that connect extracellular signals with nuclear transcription factors. When activated by binding of ligands (typically growth factors) to cell surface receptors, KRAS releases GDP and binds GTP, leading to activation of KRAS, which then activates RAF kinase. Activated RAF phosphorylates and activates MEK, which phosphorylates and activates MAPK (mitogen-activated protein kinase), which acts directly on proteins involved in gene regulation. Mutations in KRAS lead to a permanently active state that permits the cell to evade apoptosis, thereby acquiring a growth advantage [195, 196]. The MAPK/ERK cascade is a classical “survival” pathway, in that it promotes cell proliferation and prevents apoptosis and is frequently aberrantly activated in several cancers.

Target-based therapies are widely considered to be the future of cancer treatment and much attention has been focused on developing inhibitors of the MEK–ERK–MAPK signaling pathway [197]. Studies on the clinical effect of anti-EGFR treatment in metastatic CRC have presented conflicting results [169]. However, only a subgroup of patients with metastatic CRC has been shown to respond to anti-EGFR treatment, namely patients with mutations in the KRAS gene [170, 198]. Selecting the patients

with a positive effect from treatment is important and, consequently, KRAS testing has been introduced in routine clinical practice for patient selection.

In CRC, the predominant site of mutation in the KRAS gene is in codon 12, 13 and 61 [199]. Approximately 40% of CRCs have KRAS mutations in codon 12 or 13. Mutation of KRAS seems to be an early event in the process of colorectal transformation, and has been shown to be most prevalent in advanced CRC. The prognostic value of KRAS-mutated CRC has however been inconclusive [200, 201].

## **BRAF**

BRAF is a proto-oncogene that encodes for the serine/threonine protein kinase that is an immediate downstream effector of KRAS in the MAP kinase signaling pathway [202]. BRAF mutations are relatively rare in conventional adenomas, but closely associated with CIMP and MSI [203-205]. A mutation of BRAF is often present when the MLH1 gene is methylated, and do almost never occur in MSS CRC [206]. Evidence of MLH1 promoter hypermethylation or a BRAF mutation is highly predictive of a sporadic CRC with MSI, and consequently virtually absent in HNPCC associated tumours, thereby being a useful tool for distinguishing HNPCC from sporadic CRC with MSI [206]. KRAS and BRAF mutations are nearly always mutually exclusive. Further, BRAF mutated tumours are related to poor prognosis, in particular in combination with MSS [201, 207], which also have been shown in the here studied cohort [208]. Noteworthy, the anti-EGFR therapy in metastatic CRC is shown to be more effective in tumours that are BRAF wild type [209].

# The present investigation

## Aims of the thesis

The general aim of this thesis was to study the associations between obesity, measured as different anthropometric factors, and risk of CRC according to clinopathological and molecular features of CRC with the anticipation of refining risk estimates for specific subtypes of CRC and gain insights into how potential aetiological factors influence different carcinogenic pathways.

The specific aims of each paper are listed below:

- To study the associations between anthropometric factors and CRC risk by clinical stage and further according to sex and tumour location (Paper I)
- To study the associations between anthropometric factors and risk of CRC in men and women, respectively, according to the expression of beta-catenin, cyclin D1 and p53, as well as MSI status of the tumours (Paper II)
- To investigate the association between hormonal factors and risk of CRC according to clinopathological and molecular subsets of CRC in a female population (Paper III)
- To study the associations between anthropometric factors and risk of CRC in men and women according to KRAS and BRAF mutational status of the tumours (Paper IV)

## Subjects and methods

### **Study cohort**

The Malmö Diet and Cancer Study (MDCS) is a prospective population based cohort study. Participants were recruited from a background population of 74138 residents defined as all persons living in Malmö and born between 1926 and 1945. In 1994, the population was extended to include women born between 1923 and 1950 and men born between 1923 and 1945. The only exclusion criteria were mental incapacity and inadequate language skills in Swedish [210]. Recruitment was performed by public advertisement (posters and pamphlets) and personal invitations (letters and telephone

calls) [211]. Participation was voluntary and without economic compensation. Participation rate was 40%. At the end of baseline examinations, 28098 participants had completed all study parts. Of all participants, 17035 were women (60.6%) and 11063 (39.4%) were men [212]. The Malmö Diet and Cancer study is also forming part of the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort, where, in all, 34446 individuals performed at least some part of the baseline examination [213]. The 28098 individuals that have completed all study parts are in this thesis referred to as the MDCS study.

## **Baseline examinations**

Baseline examinations were initiated in March 1991 and conducted until September 1996. Participants filled in questionnaires concerning demographic, socioeconomic, reproductive and various life style factors, including dietary habits. Additionally, anthropometric measures and blood samples were taken.

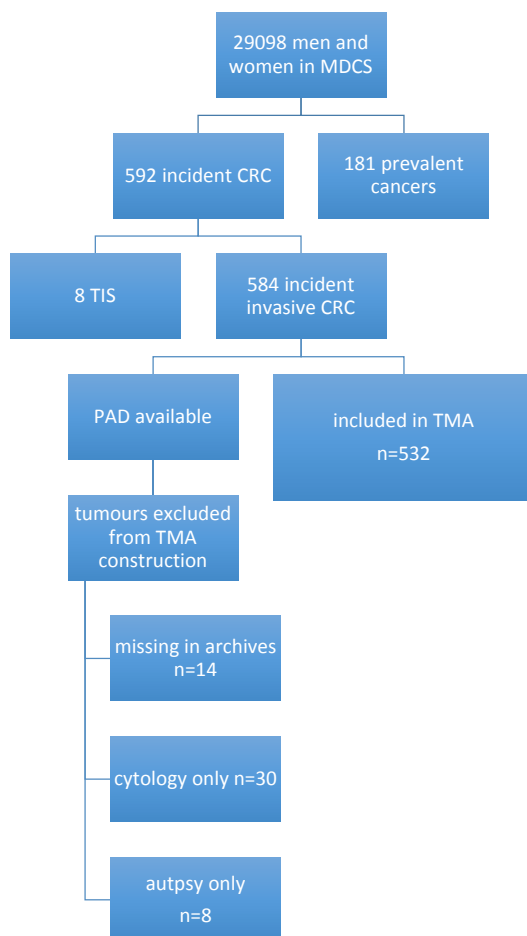
Anthropometrics were measured by a trained nurse; weight (multiples of 0.1 kg) and height (to the nearest 0.005 m) were measured and body mass index (BMI) was calculated as  $\text{kg/m}^2$ . Waist circumference was measured at the mid-point between the lower ribs and the iliac crest, and for hip circumference the level of greatest lateral extension was used. These measurements were estimated to the nearest 0.01 m. The waist and hip circumferences of each participant were used to calculate waist-hip ratio (WHR;  $\text{cm/cm}$ ) as an additional measure of fat distribution. Body composition was estimated using a single frequency bio-impedance methodology (BIA 103, RLJ-systems, Detroit, MI, USA) with tetrapolar electrode placement and subjects in a supine position. Lean body mass and fat mass were determined and served to calculate body fat percentage. The BIA method has previously been validated in Swedish middle-aged and elderly adults [214].

## **Follow-up**

Incident cases of invasive colorectal cancer in the MDCS were identified through the Swedish Cancer Registry and vital status was determined by record linkage with the Swedish Cause of Death Registry. End of follow-up was 31 December 2009. Information on vital status and cause of death was obtained from the Swedish Cause of Death Registry until 31 December 2009. Time on study was defined as time from baseline to diagnosis, death or end of follow-up on 31 December 2009. Median time from baseline until diagnosis was 8.6 (SD = 4.3) years and the median follow-up time in the entire cohort was 13.7 (SD = 3.2) years.

## Study population

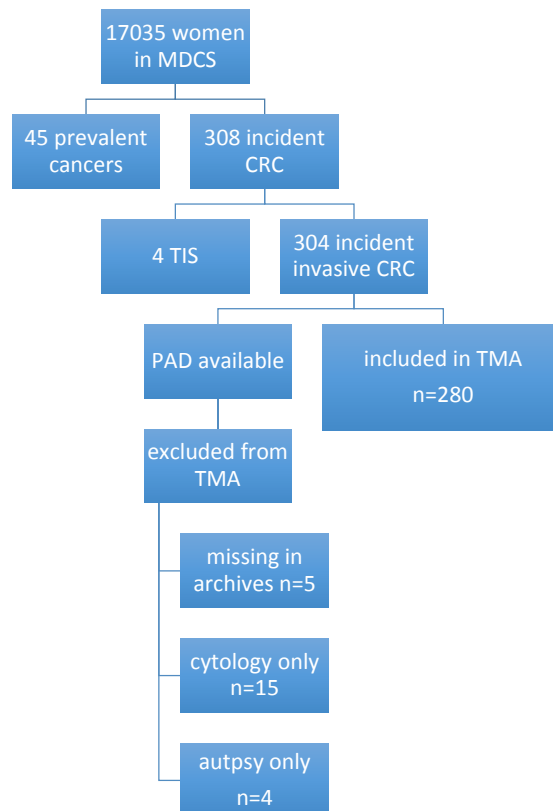
In Paper I, II and IV, 28098 men and women were included in the entire cohort. Among these, there were a total number of 584 cases of incident invasive colorectal cancer until 31 December 2009. Eight tumours were re-classified as intramucosal cancer upon histopathological re-evaluation, and these were not included as cases. A total number of 181 cases were diagnosed with CRC before baseline examination, i.e prevalent colorectal cancers, and therefore excluded from the study. Cases with other prevalent cancers were not excluded from the study.



**Figure 8.**  
Flow-chart of the MDCS and incident CRC up until Dec 31st 2008.

In Paper III, the female cohort consisted of 17035 women. A woman was considered postmenopausal if she had undergone (I) bilateral oophorectomy or (II) hysterectomy, but not bilateral oophorectomy, and if she was 55 years of age or (III) if the above criteria were absent and she affirmed that her menstruations had ceased at least during 2 years prior to baseline examinations. Use of HRT was assessed in two ways. All participants were asked to keep a diary of medications and moreover, medications were recorded in a questionnaire using an open-ended question on current use.

Use of HRT was divided into estrogen alone (ERT) and combined (estrogen+progesterin) HRT (CHRT), assessed as current use or not. The use of oral contraceptives was assessed as ever versus never use. A total of 12 583 (73.9%) women were classified as peri- or postmenopausal at baseline, consisting the study population in all HRT analyses. However, in the analysis related to OC, both pre-, peri- and postmenopausal women were included.



**Figure 9.**  
Flow-chart of the female MDCS cohort and incident CRC up until Dec 31st 2008.

## Tissue microarray

The tissue microarray technique is a high throughput approach for simultaneous analysis of multiple tissue specimens for a large number of markers, thereby decreasing the amount of tissue and reagent required for evaluation. The technique was first described by Kononen et al in 1998 [215]. By use of the TMA technique, selected tissue cores, generally 0.6 - 2 mm in diameter, are punched from selected archival tissue blocks and gathered into a novel paraffin block. The TMA block is then cut into sections and mounted on to glass slides, allowing for detection of proteins by immunohistochemistry (IHC) (Figure10).

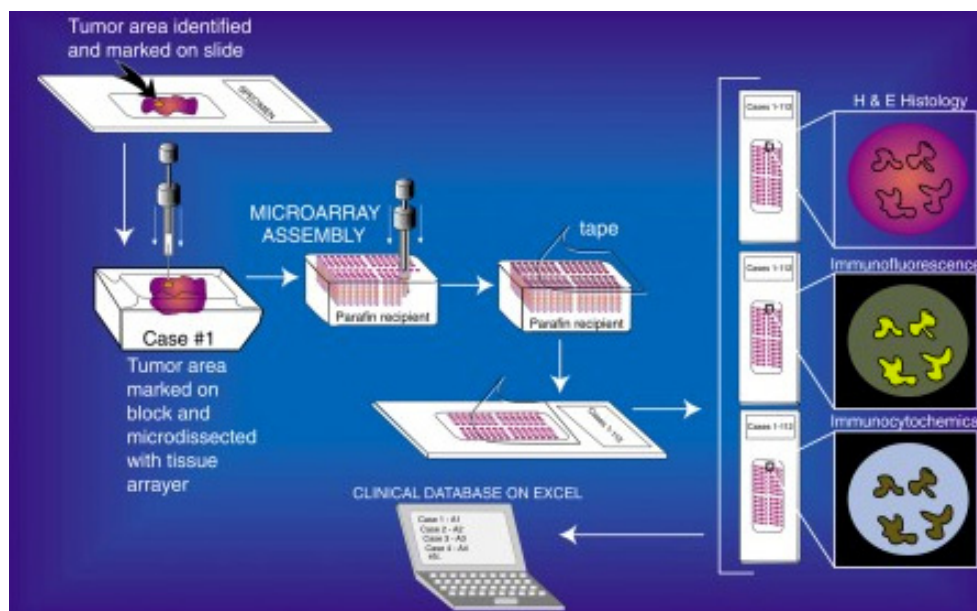


Figure 10.

Schematics of the tissue microarray technique. Used with permission from Johns Hopkins Pathology.

## Immunohistochemistry

Immunohistochemistry (IHC) is an antibody-based technique for detection of proteins in tissue. It is based on an antigen-antibody interaction that can be visualized by using antibodies labeled with an enzyme or fluorochrome that catalyzes a colour producing or fluorescent reaction. For IHC analysis in this study, 4  $\mu\text{m}$  sections were cut from the recipient block, dried and deparaffinised, rehydrated and treated in a citrate buffer (pH 6.0) for antigen retrieval [216]. The removal of paraffin allows for dipolar fluids to get into direct contact with the tissue, while the rehydration renders the cells permeable.

Formalin fixed paraffin embedded tissue needs to be pretreated before IHC staining due to formation of methylene bridges between proteins.

Immunohistochemical stainings of the MMR proteins MLH1, PMS2, MSH2 or MSH6 was denoted as negative when all tumour cells showed loss of nuclear staining. Surrounding stromal cells and tumour infiltrating lymphocytes served as internal controls for each biopsy core. A nuclear reaction of tumour cells was assessed as a positive staining. MSI screening status was defined as positive when tumour samples were lacking nuclear staining of MLH1, PMS2, MSH2 or MSH6, and negative (MSS) when tumour samples were positive for all four MMR proteins [187].

Immunohistochemical staining of beta-catenin was performed and evaluated as described by Jass et al [217] whereby membranous staining was denoted as 0 (present) or 1 (absent), cytoplasmic staining intensity as 0-2 and nuclear staining intensity as 0-2. In this study, the analyses were limited to nuclear expression of beta-catenin.

Cyclin D1 expression was recorded as intensity of nuclear expression (no, weak, moderate, strong) and the proportion of positive tumour cells (0=0-1%, 1=2-25%, 2=26-50%, 3, 51-75% and 4= > 75%) as described in Wangefjord et al [194]. For statistical analysis, cyclin D1 expression was dichotomized into negative versus positive staining.

p53 positivity was defined as  $\geq 50\%$  tumour cells with strong nuclear staining intensity in accordance with previous studies [194].

All immunohistochemical stainings were evaluated by two independent observers, who were blinded to clinical and outcome data. Scoring differences were discussed in order to reach consensus.

**Table 5.**

Antibodies used in Paper II and IV.

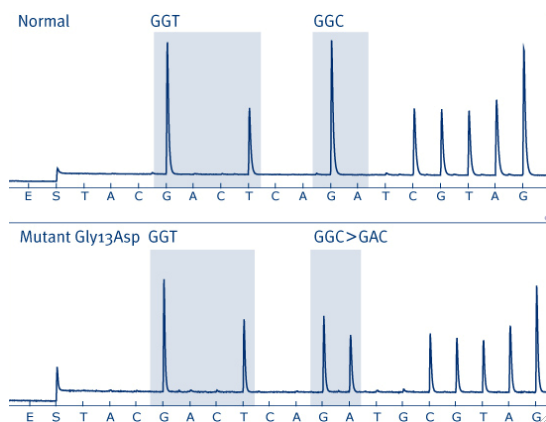
marker	manufacturer	clone	dilution	paper
Cyclin D1	Dako	DSC-6	1:50	II, III
Beta-catenin	BD Pharmingen	14/beta-catenin	1:5000	II, III
p53	Dako	DO-7	1:100	II,III
MLH1	Dako	ES05	1:100	II,III
PMS2	BD Pharmingen	A16-4	1:300	II,III
MSH2	Calbiochem	FE11	1:100	II,III
MSH6	Epitomics	EPR3945	1:100	II,III



## Pyrosequencing technology

Pyrosequencing is a method for determining the order of nucleotides in a gene segment based on the detection of released pyrophosphate (PPi) during DNA synthesis. Briefly, nucleotides are sequentially added to a DNA template in an order defined by the wild-type gene. If the added nucleotide is complementary to the single stranded DNA it binds to the DNA template and PPi is released proportionally to the amount of bound nucleotide. The released PPi is subsequently converted to ATP by ATP-sulfurylase, which provides the energy to luciferase to oxidize luciferin and hereby generates a visible light detected as a peak in the data output. The height of the peak correlates to the number of nucleotides incorporated. Because the added nucleotide is known, the sequence of the template can be determined, and the result of the sequencing is presented in a pyrogram [218].

In Paper IV, the PyroMark Q24 system (Qiagen GmbH, Hilden, Germany) was used for pyrosequencing analysis of KRAS and BRAF mutations. Genomic DNA was extracted from 1mm formalin fixed paraffin-embedded tumour tissue cores, punched from areas with >90% tumour cells using QIAamp MinElute spin columns (Qiagen). Amplification of DNA including codon 12 and 13 of the KRAS gene was performed for each patient by use of PCR and the resulting DNA product was analysed for mutation in the pyrosequencing assay. KRAS codons 12 and 13 were analysed using theascreen KRAS Pyro Kit (Qiagen). Analysis of BRAF mutation hotspots in codons 600 and 601 was performed using previously published PCR primers [219] and a novel BRAF sequencing primer (5'-TGATTTTGGTCTAGCTACA-3'), which was designed using the PyroMark Assay Design 2.0 software (Qiagen). The result of the sequencing is summarised and analysed in the pyrogram (Figure 11).



**Figure 11.**

A pyrogram demonstrating a KRAS wild type genotype (top) and a G13D (gly13-asn13) mutation in codon 13 (bottom).

## Statistical methods

All statistical analyses were conducted using SPSS version 16-20 (SPSS Inc. Chicago, IL, USA). All statistical tests were two sided and p-values less than 0.05 were considered significant.

Distribution of established and potential risk factors for CRC was compared between CRC cases and the rest of the study cohort in Paper I. In Paper II and IV, a Chi square test was applied for assessment of the distribution of investigative factors according to baseline characteristics. In paper III, distribution of hormonal treatment was compared between CRC cases and rest of cohort.

Anthropometric measurements were divided into quartiles. Quartile cut-offs were based on the distribution of each anthropometric factor in the whole cohort at baseline. Separate quartiles were calculated for men and women. A Cox proportional hazards analysis was used in order to compare risk of CRC between different categories of anthropometric factors in both sexes, and for women and men separately. This yielded relative risks (RR) with a 95% confidence interval. Time on study was used as the underlying time scale, defined as time from baseline to diagnosis, death or end of follow-up by 31 December 2009. The proportional hazards assumption was met as assessed by log-minus-log curves. In the multivariate Cox analysis potential confounders were included, which are described in detail in each paper. The confounders were chosen on the base of already established and potential risk factors of CRC. Trend was calculated as linear trend over quartiles. Missing categories were not included in the trend analysis. Using an unconditional logistic regression model in the case-to-case-analysis, the heterogeneity between relative risks was examined in paper II and IV.

## Methodological considerations

Molecular pathologic epidemiology has the same set of limitations as traditional epidemiology research, including those related to bias (selection bias, recall bias, measurement errors, and misclassification), confounding and representativity, as well as issues regarding tumour classification, antibody validity and problems imposed by multiple hypothesis testing.

## **Validity of tumour endpoints**

CRC endpoints in all studies were retrieved by record linkage to The Swedish Cancer Registry. This is a nationwide registry and all cancer cases in Sweden are reported to this registry by both clinicians and pathologists. The overall completeness of the Swedish Cancer Registry is considered high [220].

In studies II, III and IV, the investigative biomarkers were analysed using the TMA technique, which is a well-documented method for tumour tissue screening that enables high throughput simultaneous analysis of multiple tissue specimens. The major criticism of the TMA technique is that it uses only a small fraction of a tissue specimen, which may not be representative of the whole tissue section, especially for antigens with heterogeneous staining patterns in tumours. These concerns were extensively addressed in a series of early TMA studies. From these results, it could be concluded that all findings based on various methods on large sections could be fully reproduced in TMA-based studies [215, 221]. As another example, the immunohistochemical expression of proteins involved in the two main pathways of colorectal carcinogenesis; p53, as a marker of LOH, and MLH1 and MSH2, as markers of MSI, were evaluated in a study by Jourdan et al [222]. The results demonstrated that the analyse of three disks per case was comparable to the analysis of one whole section in 99.6% (p53), 98.8% (MLH1), and 99.2% (MSH2) of cases.

Analysis of different subgroups of CRC may potentially be affected by misclassification of the tumours. To minimize the risk of misclassification bias, all tumours were re-evaluated by a senior pathologist, thus eliminating inter-observer variation. Since the pathologist was blinded to all data on lifestyle factors, the pathology data is considered valid.

## **Misclassification**

Misclassification is a central issue in epidemiological studies using self-reported data since both under- and overreporting is common. Some previous studies on anthropometric factors and CRC risk have used self-reported anthropometric measures, whereas in this study, all anthropometric factors have been measured by a trained nurse. However, the validity of the anthropometric measurements may still be influenced by a potential inter-observer variation. Recommendations for the nurses performing baseline examinations described how participants should be dressed, in which position the participants should be examined, and location for the estimation of waist- and hip measurements. We therefore consider the risk of misclassification of anthropometric measurements to be low.

Another aspect is the validity of collected data. As anthropometric data were assessed only at baseline, it is possible that some individuals have gained and some have lost weight, which could influence the risk. History of weight gain in middle life may be

indicative of the formation of metabolic processes or diet-lifestyle characteristics that are not reflected adequately by a static measure of body fatness. Such a misclassification is likely to lead to an attenuation of risks and, if anything, observed risks may be underestimated.

In paper III, HRT use was divided into current users and non-users. The non-user cohort may include former users, which could imply a misclassification leading to a potential attenuation of risks and, hence, observed risks may be underestimated. Regarding exposure to OC, the risk of misclassification is probably lower, since we have used ever vs never use, and most women are peri- or postmenopausal at baseline and will most probably not have started treatment with oral contraceptives by that time. Information on duration of exposure of both HRT and OC is lacking in this study, which is a limitation as the effect may differ considerably according to the duration of treatment.

## **Confounding**

A true confounder is associated with both exposure and outcome variables without being caused by either of them. In paper I-IV, analyses were performed both crudely and adjusted for potential confounders, and the results did not differ considerably. Therefore, we consider it most relevant to present results from multivariate analysis since most previous important studies on this subject have used similar confounders as we do in our studies. In all papers in this study, confounders that are established risk factors of CRC such as age, educational level, smoking and alcohol consumption were included in the multivariate analyses [78, 81, 87].

A covariate which may have been appropriate to include due to its close relationship to overweight is physical activity. However, in the MDCS, the validity of physical activity has been questioned [223], hence we decided not to include physical activity in the adjusted analysis.

Another potential covariate of interest is heredity, as approximately 20-30% of CRC patients have some family history of CRC. In the MDCS, there is a substantial amount of missing information on CRC heredity, and consequently of poor interest to include in the multivariate analysis.

## **Representativity**

The MDCS cohort is population based, but with a participation rate of 40%. During recruitment, overall cancer incidence was higher in non-participants. Mortality was higher in non-participants both during, and following the recruitment period [212]. The incidence rates of CRC were similar in the MDCS and non- participants. The proportion reporting good health was higher in the MDCS than in the mailed health

survey (where 74.6% participated), which might indicate a greater health concern among MDCS participants as compared to non-participants. The socio-demographic structure was similar among participants and non-participants [212].

Noteworthy, the frequency of emergency surgery in the cohort was only 8.3%, which is lower than the commonly reported frequency of approximately 20% [51][194], which may reflect a higher awareness of CRC among study participants. On the other hand, the distribution of clinical stages at diagnosis is in line with the expected, both in women and in men [194].

It is also possible that participation in the MDCS was associated with body constitution, which may have lead to a potential selection bias. Different life style factors have been compared in the MDCS in relation to the background population, whereby an equal distribution of overweight and obesity was found [211]. Therefore, we do not believe that a potential bias in the distribution of anthropometric factors has affected our results.

However, within the cohort, there was a considerable difference in the distribution of examined exposures and tumour characteristics. We consider it possible to make internal comparisons comparing subjects with high versus low levels of the study measurements in order to obtain relative risks. However, it may be difficult to apply incidence rates, or information on prevalence rates for exposures, to the background population, even if the internal validity is good.

## **Selection bias**

In studies on tumour markers, one problem is using inappropriate sample sizes that are too small to conduct robust statistical analysis and draw meaningful conclusions.

Even when a study is large-scale, a molecular pathologic epidemiology study involves multiple exclusions based on availability of tumour tissue materials and valid assay results. The tumour tissue retrieval rate is almost inevitably less than 100 percent [224]. Selection bias is further possible due to treatment before surgical resection of the tumour. While this has not been a major issue in colon cancer, treatment prior to surgical resection of rectal cancer is now common. Treatment before surgery can eliminate most or all tumour cells in resection specimens in some patients, while treatment is ineffective in other patients. Further, it may also be possible that treatment itself may introduce molecular changes which may not naturally occur [12].

## **Chance findings and statistical power**

Multiple hypothesis testing is a common issue in epidemiology, and is even more problematic in molecular pathologic epidemiology. By definition, molecular pathologic epidemiology involves subset analyses of tumour subtypes, which raises the risk of false positive findings due to multiple hypothesis testing. If a wide range of lifestyle and other exposure variables are crossed with a variety of molecular changes, the likelihood for a significant chance finding is high.

In some of the papers in this thesis, a relatively large number of comparisons have been performed, which may be questioned. A type I error, often referred to as false positive, occurs when the null hypothesis is rejected when it is actually true, meaning that a difference is observed although there is none. A 95% confidence interval implies a 5% risk of observing false positive results due to coincidence of findings. False positive findings may potentially confuse the literature, scientific field, and clinical practice [225]. In order to reach a higher significance, large sample sizes are required.

Another statistical issue to be addressed is the rather small subgroups emerging in our studies and subsequently limited statistical power, ie a type II error, and a risk that true associations are not detected. Risk estimates in small groups often result in wide confidence intervals and, consequently, poor precision. Therefore, such risk estimates will also need careful interpretation.

# Paper I

## Aims

Body composition differs between men and women, as well as the association between obesity and risk of CRC. The reason for these sex differences in CRC risk remain unclear, as well as which anthropometric factor is the best predictor of CRC risk. The primary aim of this study was to examine the associations between anthropometric factors and risk of CRC in men and in women, respectively, and further to examine these associations according to tumour location and clinical stage of CRC.

## Summary of results

We compared baseline characteristics among individuals with incident CRC and the rest of cohort. Men was generally older than women in the rest of cohort, but among cases men were slightly younger than women. Mean values for all anthropometric factors were higher in cases as compared to the rest of cohort.

P-values for statistically significant associations are summarized in Table 6. The top quartiles of all anthropometric variables, except bodyfat percentage, were significantly associated with an overall increased risk of CRC, with the highest risk increase for waist (HR, 1.76; 95% CI: 1.37-2.27). When stratified for sex, the risk was more evident in men, where all anthropometric factors except height were associated with an increased risk of CRC. In women, an increased risk was seen with a high weight, hip measure and a high BMI.

While none of the anthropometric measures was significantly associated with risk of tumour (T)-stage I and II tumours, top quartiles of all anthropometric variables, except height, were significantly associated with an increased risk of tumours in T-stage III and IV in men, being most evident for weight (HR, 2.09; 95% CI: 1.37-3.19). In women, significant, although weaker, associations were seen with high weight and bodyfat percentage.

Further, in men, high quartiles of all anthropometric factors were significantly associated with an increased risk of lymph node positive (N1 and N2) disease, and there were no associations with N0 disease. Additionally, all factors except height were associated with an increased risk of non-metastatic (M0) disease and high weight, waist, and WHR were associated with metastatic (M1) disease. In women, there were no or

weak associations between obesity and risk of node-positive and metastatic disease, but statistically significant associations were seen between increased weight, bodyfat percentage, hip, BMI and M0 disease.

Further, we found an increased risk of colon but not rectal cancer in men, by increased measures of weight, hip-, waist circumference, bodyfat percentage, BMI and WHR. In contrast, in women, there were no associations with risk of colon cancer, but significant associations with increased risk of rectal cancer by high weight, bodyfat percentage, hip- and waist measures.

It should however be pointed out that sample sizes were rather small in the subgroup analyses by tumour location, and, thus, the results should be interpreted with caution.



**Table 6.**

Statistically significant p values (HR; p-trend over quartiles) in Paper I.

p-trend	CRC			T stage 1-2			T stage 3-4			N0			N1-N2		
	men	women	all	men	women	all	men	women	all	men	women	all	men	women	all
height	-	-	0.021	-	-	-	-	-	-	-	-	-	0.029	-	-
weight	0.001	0.007	<0.001	-	-	-	0.001	0.018	<0.001	-	0.034	-	<0.001	-	0.001
bodyfat	0.012	-	-	-	-	-	0.011	0.009	-	-	-	-	<0.001	0.021	-
hip	0.007	0.042	0.001	-	-	-	0.003	-	0.002	-	-	-	<0.001	-	0.002
waist	<0.001	-	<0.001	-	-	-	<0.001	-	<0.001	-	-	0.022	<0.001	-	0.001
BMI	0.001	0.027	<0.001	-	-	-	<0.001	0.017	<0.001	-	-	0.043	0.001	-	0.001
WHR	0.001	-	0.001	-	-	-	<0.001	-	<0.001	-	-	-	<0.001	-	0.003

p-trend	M0			M1			colon			rectum		
	men	women	all	men	women	all	men	women	all	men	women	all
height	-	-	0.037	-	-	-	-	-	0.041	-	-	-
weight	0.023	0.004	0.002	0.032	-	-	0.005	-	0.007	-	0.022	0.029
bodyfat	0.013	0.046	-	-	-	-	0.039	-	-	-	0.026	-
hip	0.025	0.012	0.001	-	-	-	0.019	-	0.049	-	0.009	0.003
waist	0.002	-	<0.001	0.009	-	0.049	<0.001	-	0.003	-	0.032	0.005
WHR	0.015	0.026	<0.001	-	-	-	0.004	-	0.001	-	-	0.044
BMI	0.021	-	0.013	0.028	-	0.017	<0.001	-	0.005	-	-	-

## Discussion

This study is the first to show a relationship between obesity, measured as several different anthropometric factors, and an increased risk of colorectal cancer of more advanced clinical stage, in both sexes, but particularly in men. These findings indicate that the association between obesity and risk of CRC varies by sex, cancer site and tumour aggressiveness.

A large number of epidemiological studies and meta-analyses have examined the relationship between body weight and body mass index (BMI) and risk of CRC [5, 7, 17, 55, 226]. There is undoubtedly a clear relationship between obesity and increased CRC risk in men, but weaker or no associations in women [2, 3, 7, 9, 57].

One potential reason for this discrepancy is that men and women have different body compositions. Fat makes up for a lower percentage of the body mass in men (approximately 20%) than in women (approximately 30%). Higher body weight is more closely related to abdominal obesity than lower body obesity in men and more closely related to gluteofemoral obesity than to abdominal obesity in women [6, 227]. Furthermore, upper-body fat has been shown to be more strongly associated with metabolic disorders than lower-body obesity [6]. Some studies have examined the association of body fat distribution, reflected by waist and hip circumference, and colon cancer risk, in which most have used self-reported rather than measured waist and hip circumference [2, 7, 8, 17, 55, 59].

A few of these studies suggest that abdominal obesity (high waist circumference and waist-hip-ratio) may be more predictive of CRC risk than overall obesity [7, 8, 17]. Two studies further investigated differences between men and women, demonstrating that waist circumference was an equally strong risk factor for colon cancer in men and women, and that it was a stronger risk factor than BMI in both sexes [2, 7]. Our results show that increased waist measurement was significantly associated with an increased risk of CRC in men, but not in women.

To our best knowledge, only two previous studies [17] have examined the associations between anthropometric factors and risk of CRC according to tumour aggressiveness, as reflected in the TNM-classification of the disease. These two studies examined the association between anthropometry and risk of colon cancer according to late (stage III and IV) vs early (stage I and II) stage colon cancer in men and women, respectively, and neither found any statistically significant associations. In contrast, our study demonstrates an increased risk of more advanced CRC (T stage III and IV, and lymph node positive disease) with top quartiles of all anthropometric factors in men. Moreover, our study provides full information on both tumour-, lymph node- and

distant metastasis staging according to the TNM classification, which is the accurate system of CRC staging used by clinicians today.

In agreement with most previous reports, we found a stronger association between body size and risk of colon compared to rectal cancer in general, in particular in men [2, 4]. Interestingly though, we found associations with an increased risk of rectal cancer for high weight, bodyfat percentage, hip- and waist measures in women, which is in contrast to most previous studies, including a large meta-analysis demonstrating a statistically significant association between BMI and an increased risk of rectal cancer in men, but not in women [4]. In line with these findings, a meta-analysis showed that physical activity, which is related to improved insulin sensitivity, was associated with a reduced risk of colon cancer, but not of rectal cancer [64]. This may suggest that insulin resistance, hyperinsulinemia and other factors related to obesity are stronger risk factors for colon than for rectal cancer.

The biologic mechanisms underlying the association between obesity and increased risk of CRC are unclear. Alterations in the metabolism of endogenous hormones, including insulin, insulin-like growth factors (IGFs), sex steroids, and possibly also adipocyte-derived factors such as leptin and adiponectin are suggested to play important roles. Studies have demonstrated that that high circulating concentrations of insulin and C-peptide [41, 228] as well as diabetes [229] are associated with a greater risk of CRC. Type 2 diabetes and hyperinsulinemia are also related to increased levels of insulin-like growth factor 1 (IGF-1) [230, 231]), that is known to have cancer promoting effects [53, 232, 233].

Exogenous estrogens in the form of hormone replacement therapy, HRT, have been associated with a decreased risk of colorectal cancer in several epidemiological studies and are considered a protective factor for CRC [94, 234]. The large EPIC study [2] demonstrated that abdominal adiposity was positively related to risk of colon cancer only in women who did not use postmenopausal hormones. Similarly, other prospective studies have found that BMI is positively related to risk of colorectal cancer in premenopausal women but not in postmenopausal women [5, 235], although discrepant findings have been reported [7].

In an additional analysis (not presented in Paper I), we have analyzed pre- and postmenopausal women separately, whereby the risk increase for CRC seen for weight, hip measure and BMI remained significant in postmenopausal women. However, the significance disappeared in the premenopausal group, most likely due to the very small number of premenopausal women available for analysis. Further analyses according to TNM stage and tumour location could not be performed since the number of cases in each strata was too small. Of note, inclusion of menopausal status in the multivariate analysis, did not alter any of the significant associations.

The protective role of HRT regarding CRC risk, has been shown to be even more efficient among lean women than among obese women [100, 236], which suggests that HRT might not offer additional benefit over that from the estrogen derived from

adipose tissue in postmenopausal obese women, which is the main source of endogenous oestrogen after the menopause.

Taken together, the potentially deleterious effects of obesity through increased insulin and IGF-1 levels in men, might, in postmenopausal women be set off by the effect of obesity on endogenous estrogen levels. Effect modification by menopausal status may therefore, at least in part, explain the inconsistent or weak findings in previous studies of women than the presumed lack of an association among women.

Moreover, and interestingly, geographic patterns for CRC and type 2 diabetes are strikingly similar in that both diseases were considered relatively rare before industrialization or Westernization and their incidence usually increases in regions undergoing economic development. The major environmental determinants of type 2 diabetes include high body mass index (BMI), increased central obesity, physical inactivity, excessive intake of energy and dietary patterns that stimulate secretion of insulin. These factors are remarkably similar to the constellation of risk factors emerging for CRC.

# Paper II

## Aims

The knowledge of the three main different pathways of colorectal carcinogenesis and their different clinicopathological characteristics imposed the question whether obesity may influence the risk of CRC differently according to certain molecular subsets of CRC, and, in analogy with our previous discussion, according to sex. The aim of the present study was to investigate the relationship between six anthropometric factors; height, weight, waist- and hip measurements, BMI and WHR, with risk of CRC according to expression of beta-catenin, cyclin D1, p53 and MSI screening status of the tumours in men and women, respectively.

## Summary of results

As in Paper I, the distribution of risk factors revealed that all anthropometric factors were higher in cases compared to rest of cohort, as well as age. Regarding the molecular features, cyclin D1 positive tumours were significantly associated with higher age in women, and p53 positive tumours were associated with height and were more frequent among never-smokers in women. MSS was associated with higher age in both men ( $p = 0.029$ ) and in women ( $p = 0.024$ ).

P-values for statistically significant associations are summarized in Table 7. In women, positive associations were seen between height, cyclin D1 positive and p53 negative tumours. The risk of p53 negative tumours in women was highest in the top quartile of height (HR, 2.17; 95% CI: 1.25-3.76, p-trend 0.004, p for heterogeneity = 0.013). In men, no significant associations were found between height and investigative factors.

Increased weight was associated with beta-catenin negative and p53 positive tumours in men, and with beta-catenin positive, cyclin D1 positive, p53 negative and MSS tumours in women. A high BMI was significantly associated beta-catenin overexpression, with the highest risk in the top quartile (HR, 2.25; 95% CI: 1.33-3.80, p-trend 0.004, p for heterogeneity = 0.048), and MSS tumours in women. In men, a high BMI was associated with beta-catenin, positive cyclin D1 positive and p53 positive CRC.

Increased WHR was significantly associated with beta-catenin overexpression, cyclin D1 positivity and p53 expression in men. The risk of beta-catenin positive, but not

beta-catenin negative CRC, was highest for the top quartile of WHR in men (HR, 2.14; 95% CI: 1.34-3.42, p-trend 0.004, p for heterogeneity = 0.015). High waist circumference was associated with beta-catenin positive, cyclin D1 expression, p53 positive and MSS tumours and a high hip circumference were associated with beta-catenin negative, cyclin D1 positive, p 53 positive and MSS CRC in men.

In women, an increased hip circumference was associated with beta-catenin positive, p53 negative and MSS tumours but waist circumference and WHR were not associated with risk of any of the molecular subsets of CRC.

**Table 7.**

Statistically significant p values (HR; p-trend over quartiles) in Paper II.

p-trend	height		weight		hip		BMI		WHR		waist	
	men	women	men	women	men	women	men	women	men	women	men	women
Beracatenin+	-	-	-	0.010	-	0.014	0.050	0.004	0.001	-	0.009	-
Beracatenin-	-	-	0.048	-	0.036	-	-	-	-	-	-	-
cyclinD1+	-	0.031	-	0.019	0.034	-	0.019	-	0.015	-	0.009	-
cyclinD1-	-	-	-	-	-	-	-	-	-	-	0.046	-
P53+	-	-	0.026	-	0.009	-	0.023	-	0.033	-	0.003	-
P53-	-	0.004	-	0.004	-	0.042	-	-	0.048	-	-	-
MSI	-	-	-	-	-	-	-	-	-	-	-	-
MSS	-	-	-	0.008	0.038	0.005	-	0.009	-	-	0.012	-

## Discussion

Findings from this large prospective cohort study demonstrate associations of several anthropometric factors with CRC risk according to the expression of beta-catenin, cyclin D1, p53 and MSI status, and that these associations differ in men and women. These findings further consolidate the theory that the influence of lifestyle factors on colorectal carcinogenesis may differ according to the pathogenetic pathway, and between sexes. Previous studies on this subject are quite sparse, and further investigations in large cohorts are needed.

We found no significant associations between any of the anthropometric measurements and risk of MSI tumours. However, significant associations were seen between high weight, BMI and hip circumference and MSS tumours in women. Among men, significant associations were found between increased waist and hip circumference and MSS tumours. These results are consistent with previous data from Hughes et al. [173], and also generally in agreement with the two previous case control studies from Slattery and Campbell [172, 237].

Knowing that MSI positive CRC is characterized by female sex, proximal location, old age and favourable prognosis, and that obesity is a well-established risk factor for CRC, these data suggest that obesity does not seem to constitute a risk factor for this subgroup of CRC.

As regards beta-catenin, accumulating evidence supports a role of Wnt/beta-catenin signalling in adipogenesis, obesity and metabolic disorders [189, 238] as well as in carcinogenesis [184]. Considering the dual roles of beta-catenin in both colorectal carcinogenesis and energy metabolism, we investigated potential links between obesity and beta-catenin alterations in CRC, and found somewhat inconsistent results in that obesity was associated with beta-catenin positive CRC in both sexes, but also with beta-catenin negative CRC in men. To our knowledge, only one former study by Morikawa et al. has addressed this question, however without stratifying for sex, whereby it was demonstrated that obesity is associated with a higher risk, and physical activity with a lower risk of beta-catenin negative but not of beta-catenin positive CRC [239]. These findings suggest that energy balance exposures (i.e. obesity and physical activity level) influence the risk of beta-catenin negative but not of beta-catenin positive CRC. Morikawa et al have also presented data showing that postdiagnosis progression of beta-catenin negative colorectal cancer is dependent on the patient's energy balance status, whereas beta-catenin positive cancer may progress regardless of the patient's energy balance status [188].

Notably, immunohistochemical staining of beta-catenin was initially denoted as membranous, cytoplasmic and nuclear staining, however the analyses in this paper were



limited to its nuclear expression. The reason for this is that the translocation of beta-catenin into the nucleus in the Wnt signaling cascade represents the active status of the Wnt signaling pathway, where beta-catenin coactivates transcriptional genes regulating cell proliferation and apoptosis. Additionally, most studies on the prognostic significance of beta-catenin have been performed on analysis of nuclear beta-catenin location [186], including the study on the relationship between beta-catenin, obesity and risk of CRC by Morikawa et al [239].

We further demonstrated that obesity is associated with an increased risk of cyclin D1 positive tumours, in both men and women. We are not aware of any previous studies on the influence of anthropometric factors on CRC risk according to cyclin D1 expression, although the prognostic role of cyclin D1 has been investigated in several studies, however with inconsistent results [191, 192, 240]. However, most recent studies indicate that cyclin D1 expression seems to be associated with good prognosis [192, 193, 241]. In the MDCS, it has been demonstrated that cyclin D1 expression is associated with a prolonged survival from CRC in men but not in women [194].

Of note, it would have been interesting to study these associations according to tumour location (colon vs rectum), however we decided not to do this due to the small subgroups available for analysis and, consequently, low statistical power.



# Paper III

## Aims

Epidemiologic evidence suggests that postmenopausal hormone replacement therapy (HRT) is a protective factor for CRC, even though the mechanisms behind this association remain uncertain. To address this question, we conducted a molecular epidemiologic pathology study hypothesizing that hormonal therapy influences molecular subsets of CRC differently. The main objective of this paper was therefore to evaluate the associations between HRT and oral contraceptives (OC) use and risk of particular molecular subgroups of CRC, overall and according to tumour site, in the female MDCS cohort.

## Summary of results

Among CRC cases, 35.2% were OC users and 15.1% were CHRT users compared to the entire cohort where 48.4% were OC users and 17.7% were CHRT users. Further, CHRT users were more often smokers among CRC cases than rest of cohort. Individuals using only estrogen treatment (ERT) were slightly older among both cases and non cases. BMI was slightly higher among cases than rest of cohort.

There were no statistically significant associations between HRT use, combined (CHRT) or estrogen only (ERT), and overall CRC risk. We found a significantly reduced risk of T stage 1 and 2 tumours among current users of CHRT (HR, 0.30; 95% CI: 0.09-0.96). There were no significant associations between neither CHRT, nor ERT use and risk of other particular subgroups of CRC.

When stratifying for cancer site, i.e.colon or rectum, we found significant associations of HRT use and overall risk (HR, 0.32; 95% CI: 0.14-0.71), T stage 1 and 2 tumours (HR, 0.03; 95% CI: 0.00-0.36), lymph node negativity (HR, 0.22; 95% CI: 0.06-0.77) and for non-metastatic disease (HR, 0.42; 95% CI: 0.18-0.98) in rectal, but not colon cancer. We also found significant associations between CHRT and cyclin D1 negative (HR, 0.07; 95% CI: 0.01-0.88) and p53 negative tumours (HR, 0.19; 95% CI: 0.04-0.96) in the rectum, but not in the colon. Additionally, in unadjusted analysis, CHRT use was associated with a reduced risk of MSS tumours in the rectum (HR, 0.46; 95% CI: 0.22-0.97). Of note, the analysis stratified by tumour location included very small subgroups and, hence, the results should be interpreted with caution.

Regarding use of oral contraceptives and CRC risk, we found no statistically significant results after adjustment for established CRC risk factors i.e. age, BMI, alcohol consumption, smoking habits and educational level. However, in unadjusted analysis, there was a statistically significant inverse association between ever-use of OC and overall CRC risk (HR, 0.56; 95% CI: 0.44-0.71). Similar statistically significant inverse associations were seen between OC use and the majority of clinicopathological and molecular subgroups, except for lymph node positive disease, negative nuclear beta-catenin expression and MSI tumours.

In the adjusted analysis stratified for cancer site, we found a significant increased risk of lymph node positive (HR, 1.81 95% CI: 1.00-3.28) and non-metastatic (HR, 1.55; 95% CI: 1.00-2.40) disease, as well as for cyclin D1 positive tumours (HR, 1.62 95% CI: 1.04-2.51) in the colon. No associations were found between OC use and specific subgroups of rectal cancer.

## Discussion

In this cohort, we could not see the expected overall risk reduction of CRC associated with HRT use among women, which is in contrast to the epidemiological evidence supporting the protective effect of HRT on CRC risk. On the other hand, our results indicate that HRT use reduces the risk of less aggressive tumours, i.e. T-stage 1 and 2 CRC.

In a metaanalysis, Grodstein et al. [94] reported a statistically significant inverse relative risk of 0.66 for current users of HRT compared with nonusers. Another two meta-analyses pooling at least 15 observational studies have concluded a  $\geq 15\%$  reduction in risk of CRC among ever users of HRT, with the risk reduction being more pronounced for more recent use and duration of use exceeding 5 years [234, 242].

These observational results were subsequently confirmed in the Women's Health Initiative (WHI) estrogen plus progestin randomized, placebo-controlled trial conducted among nearly 17000 post-menopausal women. In this WHI clinical trial, intervention with estrogen plus progestin yielded a striking 44% reduction in incident CRC. [95]. Of note, follow up time was only 5.2 years, thus lacking information on long term effects. Further analyses of the Women's Health Initiative data revealed that women assigned to the estrogen plus progestin arm were more likely to be diagnosed with advanced stage CRC. Taken together with our findings of a decreased risk of T-stage 1 and 2, this may indicate differential effects from HRT on heterogeneous pathways of colorectal carcinogenesis. Interestingly, in the WHI, unopposed estrogen did not appear to affect CRC risk, implying the question of the role of progestins in CRC. The biological mechanisms underlying an effect of progestins in the colorectum

are not well understood, although they may be synergistically amplifying the effects of estrogen.

It has been questioned whether HRT users simply represented a population that was healthier than women not using HRT and that the inverse association was, instead, due to the attributes of the users rather than the preparations themselves [243, 244].

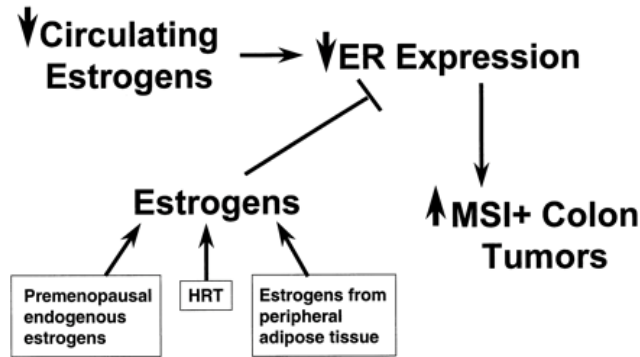
The associations between HRT use and tumour aggressiveness have been sparsely investigated previously. Grodstein et al reported a similar risk reduction with hormone therapy for higher and lower stages [245]. In the California Teachers study, the association between HRT and reduced CRC risk was stronger for more advanced stages [246], which is in contrast with our findings. Interestingly, we found a significant risk reduction of T-stage 1 and 2 tumours, as well as lymph node negative and non-metastatic tumour in the rectum, but not the colon.

Noteworthy, other prospective cohort studies suggest that circulating estrogen levels and life time exposure to endogenous estrogen are associated with an increased CRC risk [90, 91]. The positive associations between endogenous estrogen levels and the risk of CRC reported by these investigations are consistent with laboratory data demonstrating the proliferative effects of exogenous estradiol in colorectal tissue and in colorectal cancer cell lines [92, 93]. For example, estradiol has been shown to activate the MAPK cascade in colorectal cancer cell lines, hence inducing cell growth and proliferation [247].

However, the exact biological mechanisms underlying the effect of estrogen remain unclear, although endogenous and exogenous estrogens seem to play different roles in colorectal carcinogenesis [90, 248].

Interestingly, Issa et al. have shown that colon tumours almost universally arise from cells that have lost estrogen receptor (ER) expression [249]. Unlike the methylation silencing of MLH1, hypermethylation of ER is an age-related phenomenon, in the same way as the incidence of sporadic CRC is strongly age related [250]. Reduced circulating estrogen in ageing have been shown to be related to a decreased expression of ER in the colorectal mucosa, thus predisposing CpG hypermethylation and subsequent development of CRC.

Moreover, data on HRT use and risk of CRC by microsatellite instability (MSI) status are very limited [251, 252]. Newcomb et al presented a statistically significant association between estrogen plus progestin use and MSS, but not MSI, tumours, while Slattery et al [253] demonstrated in a case-control study, that recent users of HRT were at a reduced risk of MSI+ tumours and former HRT users were at an increased risk of MSI+ tumours. Further, obesity was associated with an elevated risk of both MSI+ and MSI- tumours in men, but only with MSI tumours in women.



**Figure 12.**  
Relationship between estrogen, ER and MSI suggested by Slattery et al [253]

Taken together, it is tempting to speculate that MSI status might be a key factor behind the sex-related differences in CRC risk. Slattery et al have proposed a model (Figure 12) in which estrogen protects against MSI and the lack of estrogen increases the risk of MSI, and that the increased risk can be modified by HRT use. The total lifetime estrogen exposure depends on reproductive factors, exogenously added estrogens (HRT and OC), as well as increased estrogen levels due to obesity and estrogen synthesis by the adipose tissue. It can therefore be hypothesized that obesity, and the consequent elevation of estrogens, may exert effects similar to HRT in postmenopausal women, thereby reducing the likelihood of ER methylation and loss of ER expression, thus lowering the risk of CRC [254].

One former study by Lin et al [255] reported on HRT use, CRC risk and MSI status, p53 expression and found no associations between HRT and CRC risk according to MSI or p53. In our study, we found a statistically significantly reduced risk for cyclin D1 negative and p53 negative rectal tumours with combined HRT use, and a similar association was seen for MSS tumours in unadjusted analysis.

Another previous prospective study has investigated the relationship between HRT use and risk of molecular subtypes of CRC. Limsui et al [251] demonstrated that HRT was associated with a statistically significantly lower risk for MSS tumours, as well as borderline statistically significant risk reductions for CIMP-negative and BRAF-wildtype tumours among women with prolonged exposure to HRT (>5 years duration). Conversely, HRT-related risk estimates for the MSI, CIMP-positive and BRAF-mutated CRC subtypes were not statistically significant [251]. These data suggest that HRT may have more pronounced inhibitory effects on the “traditional” pathway, as compared to the serrated or alternate pathways, of colorectal carcinogenesis.

The epidemiological evidence for an association between oral contraceptives (OC) and CRC risk is also somewhat inconsistent in that some studies have suggested inverse associations [101, 103, 256], whereas others have found no associations [106, 108,

109]. A recent meta-analysis, summarising the results from seven cohort- and eleven case-control studies, reported a statistically significant 19% reduced risk among ever users of OC compared with never users, although there was no clear risk reduction with increasing duration of use [18]. In the present study, we found significant associations of OC use and a reduced risk of all CRC subgroups, except lymph-node positive disease, negative nuclear beta-catenin expression and MSS tumours in the unadjusted analysis. In contrast, for tumours located in the colon, we found an increased risk of lymph node-positive and non-metastatic tumours, as well as for cyclin D1 positive tumours. However, in the adjusted analysis, there were no significant results.





# Paper IV

## Aims

Since KRAS and BRAF mutations in CRC are nearly always mutually exclusive, and signify tumours with different clinicopathological characteristics and prognosis, this paper addressed the question whether risk factors differ according to KRAS and BRAF mutational status of colorectal tumours.

## Summary of results

KRAS and BRAF mutations were successfully evaluated in 494 (84.6%) cases. A total number of 314 (63.7%) tumours were KRAS wild-type and 180 (36.4%) were KRAS-mutated. Among the KRAS-mutated, 149 (30.2%) were located in codon 12 and 31 (6.3%) were located in codon 13. Further, 423 (85.6%) of the tumours were BRAF wild type, and 71 (14.4%) were BRAF-mutated. KRAS and BRAF mutations were mutually exclusive. BRAF mutation was significantly associated with female sex, and KRAS mutation was significantly associated with height and BMI among men.

In women, there were no associations between any anthropometric factor and KRAS mutations, but in men, high weight, hip-, waist circumference, WHR and BMI were significantly associated with increased risk of KRAS codon 12 mutated CRC. Further high hip-, waist circumference and WHR were significantly associated with an increased risk of KRAS wild-type tumours, and elevated height was associated with KRAS codon 13 mutated CRC in men. In women, only high weight and bodyfat percentage were associated with an increased risk of BRAF wild-type tumours, whereas in men, all anthropometric factors, except height, were significantly associated with BRAF wild-type tumours. There were no significant associations between obesity and BRAF-mutated CRC in neither women nor in men. Statistically significant p-values are presented in Table 8.

**Table 8.**

Statistically significant p-values (HR; p-trend over quartiles) in Paper IV.

p-trend	BRAF wild-type		BRAF mutated		KRAS wild-type		KRAS codon 12		KRAS codon 13	
	men	women	men	women	men	women	men	women	men	women
height	-	-	-	-	-	-	-	-	0.002	-
weight	<0.001	0.042	-	-	-	-	0.002	-	-	-
BF	0.010	0.012	-	-	-	-	-	-	-	-
hip	<0.001	-	-	-	0.039	-	0.006	-	-	-
waist	<0.001	-	-	-	0.012	-	<0.001	-	-	-
whr	0.001	-	-	-	0.041	-	0.001	-	-	-
bmi	<0.001	-	-	-	-	-	<0.001	-	-	-

## Discussion

One well-defined subgroup of CRC arises through the (sessile) serrated pathway, via serrated epithelium or hyperplastic polyps [115], characterized by a number of genetic and epigenetic events such as BRAF mutation, MSI and CIMP, contributing to the resulting carcinoma.

We here demonstrate clear differences between the sexes in that no anthropometric factor was associated with KRAS mutation status in women, whereas in men, significant associations were found between several anthropometric factors and risk of both KRAS wild-type and codon 12 mutated tumours. However, since obesity is associated with both KRAS wild-type and mutated tumours in men, it is difficult to draw any firm conclusions from these results.

Only a handful studies have investigated the association between body size and characteristics of the (sessile) serrated pathway, and the majority of those only considered body mass index (BMI) as a risk factor [257-259], and central adiposity is thought to be a better predictor of CRC than BMI [2]. Two of these previous studies have investigated the associations between KRAS and/or BRAF status and CRC risk factors. Slattery et al [260] have demonstrated a reduced risk of KRAS-mutated rectal cancer with a high intake of vegetables, fiber and a high level of physical activity, factors being inversely associated with obesity, and it may thus be hypothesized that this indicate an increased risk by low levels of the above mentioned factors. Further, the same author showed that men with low levels of physical activity more frequently presented with KRAS-mutated tumours in the colon, and that women with high BMI were more likely to have KRAS-mutated tumours [261].

The prognostic role of KRAS mutations in CRC has however been more extensively investigated, whereby most studies have demonstrated an association of KRAS mutation with poor prognosis [200, 201, 207, 262]. Of note, in the here examined cohort, it has recently been shown that mutation in KRAS codon 13, but not codon 12, was associated with a significantly reduced cancer specific survival (CSS), which is

in line with some other publications [208, 263, 264]. Most previous studies have not taken into consideration how specific KRAS mutations affect clinical outcome in CRC patients and this study is the first to investigate how specific KRAS mutations, i.e. in codon 12 and 13, are associated with CRC risk.

As regards BRAF mutation status, several anthropometric factors were found to be associated with BRAF wild-type tumours in both sexes, but most evident in men, whereas BRAF mutation was not associated with any anthropometric factors, neither in men nor in women.

It is well established that BRAF mutation, in contrast to KRAS mutation, is associated with MSI and female sex [207, 265, 266]. MSI has generally been associated with good prognosis in most [156] but not all studies [267]. On the other hand, BRAF mutation is generally associated with a poor survival [207, 268, 269]. In the here studied cohort, BRAF mutation has previously been demonstrated to be an independent factor of poor prognosis in men, but not in women, in particular in MSS tumours [208]. These results are consistent with previous studies that demonstrate poor prognosis in patients with BRAF-mutated, MSS and CIMP high CRC [268-270]. Taken together, the prognostic role of BRAF mutations, as well as the role of risk estimates, seem to be related to MSI status, sex, and closely dependent on the different pathogenetic pathways of CRC.

In paper II, we presented data showing that obesity was not associated with MSI tumours, but that a high weight, hip circumference and BMI in women, and a high waist and hip circumference in men was significantly associated with microsatellite stable CRC. The findings from this paper demonstrate a significant association of obesity with BRAF wild-type tumours in both men and women, but that this association was particularly evident in men. These findings suggest that obesity is more related to MSS tumours, and to tumours lacking BRAF mutation.

Two previous studies have investigated the association between obesity and BRAF status in CRC tumours. Firstly, in a case-control study, Slattery et al reported that obesity was not associated with BRAF-mutated tumours, and that obese individuals were at 2-fold increased risk of CIMP-low colon cancer, but not of CIMP-high tumours [257]. As previously mentioned, BRAF mutations are closely related to CIMP-high CRC and almost never occur in CIMP negative CRC [271]. These findings indicate, in concordance with our results, that obesity does not seem to influence the development of the subgroup of colorectal tumours being CIMP high and lacking BRAF mutation within the serrated pathway.

Further, Hughes et al presented data showing that BMI and waist measurements were strong risk factors for BRAF wild-type tumours, as well as for MSS CRC, which is consistent with our findings [173].

In conclusion, these results provide further support to the accumulating evidence of the influence of lifestyle factors on different pathways of colorectal carcinogenesis. Obesity

was associated with KRAS mutation status is in men, but not in women, and with wild-type BRAF tumours in both sexes.

# Conclusions

A summary of the principle findings of this thesis:

- Obesity, measured as high height, weight, hip- and waist measurement, WHR and BMI, was associated with an increased risk of CRC in both men and women.
- Obesity was associated with an increased risk of more advanced CRC, i.e. T-stage 3 and 4, lymph node positive and metastatic disease, in men.
- No association was found between obesity and rectal cancer risk in men.
- Associations of anthropometric factors with the risk of various molecular subsets of CRC differed between sexes.
- Obesity was not related to risk of MSI tumours.
- Current use of postmenopausal hormone therapy was not associated with decreased overall risk, but with a decreased risk of T-stage 1 and 2 CRC.
- HRT use was also associated with a lower risk of T stage 1 and 2, lymph node negative-, non-metastatic disease and further of p53 negative- and cyclin D1 negative tumours in the rectum, but not in the colon.
- Obesity was associated with KRAS mutation status in men, but not in women.
- Obesity was associated with an increased risk of BRAF wild-type tumours in both sexes, but not with BRAF-mutated tumours.



# Implications and future perspectives

Molecular Pathologic Epidemiology is a multidisciplinary field that investigates the interrelationship between exposure factors with molecular signatures of the tumours [12]. CRC comprise a heterogeneous group of diseases with different sets of genetic and epigenetic alterations. In order to understand how a particular exposure influences the carcinogenic process, the exposure of interest has to be studied in relation to molecular alterations.

The increased understanding of the molecular biology in colorectal carcinogenesis has revealed several promising putative diagnostic, prognostic and predictive biomarkers. To date, KRAS is however the only biomarker implemented in clinical practice, serving as a predictor of EGFR resistance in metastatic CRC.

Screening for CRC will most probably increase rapidly in westernized countries, and it is thereby a great challenge to identify persons at risk of developing CRC. Detection and removal of adenomas are feasible by endoscopic techniques, but the majority of adenomas will probably never progress to cancer. Therefore, new markers indicative of an aggressive adenoma behaviour are needed. Thus, defining the “adenoma at risk”, and consequently, defining the “patient at risk”, by correlation of risk factors to specific molecular subgroups of CRC, is a major research challenge.

By elaborating the field of biomarkers, screening techniques such as faecal detection of mutated genes may become a reality. Moreover, by incorporating molecular features into clinical protocols, we could improve the staging system, prediction of chemotherapy response, elaborate individualized therapy, and develop novel targeted therapies.

CRC incidence is increasing in the developing countries, due to an adaptation to a westernized lifestyle. Given that the global prevalence of overweight and obesity continues to rise, it is of great importance to invest in primary prevention. Risk factors, such as diet, lack of physical activity and obesity, are de facto modifiable, and therefore there are good opportunities to develop accurate individualised prevention strategies.

In this thesis, we have chosen to explore the relationship between anthropometric factors and molecular and clinicopathological features of CRC. However, future studies should also consider several other lifestyle related factors, such as dietary aspects, the relation to diabetes and the metabolic syndrome.

Following up on paper III, where we have examined the role of hormone replacement therapy and risk of CRC, it may also be of interest to analyze other reproductive factors, e.g. numbers of pregnancies, age at menarche and menopause. According to the

discussion in Paper III, and the potential mechanisms underlying the relationship between obesity, estrogen and CRC risk, it would be relevant to examine the associations of estrogen receptor (ER) expression in CRC tissue, life style factors and CRC risk.

The findings in this thesis demonstrate that obesity is not associated neither with MSI nor with BRAF-mutated CRC, possibly indicating that obesity influences colorectal carcinogenesis mainly through the CIMP pathway. Therefore, it would be of interest to further investigate the influence of obesity on CRC risk by CIMP status of the tumours.

Given the potential biological effects of insulin, insulin-like growth factors, hyperinsulinemia and the metabolic syndrome on CRC risk, another research area of interest would be to investigate these factors in relation to obesity and CRC in the MDCS.

Moreover, as use of NSAID is considered to protect against CRC, and since obesity can be characterized as a chronic inflammatory state, it would also be of interest to relate the use of NSAID in the MDCS with CRC risk, according to anthropometry and tumour-specific COX-2 expression. COX-2 has been found to be overexpressed at the mRNA level in almost 80% of CRC [272], and high COX-2 expression has also been associated with a higher recurrence rate [273].

In conclusion, we need to continue the work of finding and elaborating new biomarkers in a molecular pathological epidemiological perspective, in order to develop improved strategies for prevention and treatment of CRC.



# Populärvetenskaplig sammanfattning på svenska

(Summary i Swedish)

Cancer i tjock- och ändtarmen, kolorektal cancer, är den tredje vanligaste cancersjukdomen hos både män och kvinnor. Globalt sett insjuknar ca 1.2 miljoner människor i kolorektal cancer årligen. I Sverige diagnosticerades 2011 ca 4000 fall av tjocktarmscancer, jämt fördelade mellan båda könen. Cancer i ändtarmen är vanligare hos män, med ca 1200 fall årligen i Sverige, mot 800 fall hos kvinnor. Majoriteten av dem som insjuknar i kolorektal cancer är över 65 år. Under de senaste årtiondena har prognosen förbättras, tack vare förbättrade diagnostiska möjligheter och bättre behandlingsmetoder. Sedan början av 1980-talet har den relativa 10-årsöverlevnaden ökat med cirka 20 procent för både män och kvinnor och 5-års överlevnaden är cirka 60% för båda könen.

I samband att diagnosen ställs delas cancer in i olika stadier (I-IV) beroende på hur djupt tumören växer i tarmväggen samt förekomst av spridning till närliggande lymfkörtlar och andra organ i kroppen. Denna stadieindelning är avgörande för val av behandling, samt är starkt förknippad med sjukdomens prognos. Behandlingen av kolorektalcancer består av kirurgi, samt vid avancerade stadier eventuellt tillägg av cellgifter och/eller strålning för att minska risken för återfall.

Kolorektal cancer anses vara en välfärdssjukdom, då förekomsten är låg i utvecklingsländer. Kostrelaterade riskfaktorer såsom fet och fiberfattig mat samt rött kött har visats öka risken för kolorektal cancer. Även andra livstilsfaktorer som rökning, alkohol, låg fysik aktivitet och övervikt ökar cancerrisken. Antiinflammatoriska läkemedel samt östrogenbehandling hos kvinnor har en skyddande effekt.

En stor mängd studier har visat att övervikt är en klar riskfaktor för kolorektal cancer bland män, men intressant nog ej hos kvinnor. Anledningen till detta är inte känd, men man har spekulerat i att det bland annat skulle kunna bero på hormonella faktorer. Hur man mäter övervikt och vilket mått som är bäst kopplat till risk för kolorektal cancer, är också ett debatterat ämne. Möjligen skulle detta kunna vara en tänkbar förklaring till könsskillnaderna. Man vet att det råder skillnad mellan kvinnors och mäns fettfördelning i kroppen, där män oftare har bukfetma sk abdominell fetma. Vidare skulle könsskillnaderna avseende övervikt kunna bero på att män och kvinnor helt enkelt utvecklar olika typer av kolorektal cancer. Man har länge studerat kolorektal cancer som en och samma sjukdom, men genom ökade kunskaper kring hur kolorektal cancer utvecklas från normal tarmslemhinna till cancer vet vi idag att det finns ett flertal

olika subgrupper av kolorektalcancer, baserade på olika molekyllära uttryck och genetiska förändringar i tumören. Man har kunnat identifiera ett antal proteiner som uttrycks i tumörerna, vilka är involverade i olika steg i bildandet av en cancercell, sk biomarkörer. Dessa olika varianter av cancer har olika kliniska kännetecken, såsom lokalisation, kön, aggressivitet och prognos. Målsättningen är att några av dessa biomarkörer skulle kunna implementeras i det kliniska arbetet i framtiden och leda till förbättrad diagnostik och i sin tur större möjligheter att förutse prognos samt bättre individualiserade behandlingsstrategier. Således bör kolorektal cancer betraktas som en heterogen sjukdom, där livstilsfaktorer har ett stort inflytande på uppkomst och utveckling av enskilda tumörer.

Eftersom 600000 människor runt om i världen dör i denna sjukdom varje år, och med tanke på att de flesta riskfaktorerna faktiskt är påverkbara genom vår livstil, är det av stor vikt att utveckla kunskaperna kring dessa samband för att i sin tur kunna utveckla individualiserade förebyggande metoder.

Syftet med denna avhandling har således varit att försöka identifiera sambanden mellan övervikt (här mätt som olika kroppsmått, antropometri) och olika molekyllära subgrupper av kolorektal cancer, samt olika kliniska stadier av sjukdomen. Vi har också studerat hur dessa samband skiljer sig mellan män och kvinnor.

I Malmö finns ett unikt material i den befolkningsbaserade studien Malmö Kost Cancer, där 28098 frivilliga individer deltog och lämnade uppgifter om matvanor, medicinering, sjukdomar, livstilsvanor, kroppsmått och mycket mer. Ur denna kohort har vi identifierat 584 fall av kolorektal cancer, inhämtat kliniska fakta från journaler och studerat olika proteinuttryck i de bortopererade tumörerna. Därefter har vi analyserat sambanden mellan övervikt och risken att insjukna i kolorektal cancer bland dessa patienter.

I det första delarbetet analyserades antropometriska mått och risk för kolorektal cancer av olika kliniska stadier. Vi använde sju kroppsmått; längd, vikt, kroppsfett i procent, höft- och midjemått, BMI samt en kvot på höft-och midjemått, WHR som ett mått på övervikt. Alla mått utom kroppsfett var signifikant kopplade till en generellt ökad risk för kolorektal cancer, och liksom förväntat var sambandet tydligare hos män än hos kvinnor, även om ökat BMI, höftmått och vikt hos kvinnor också ledde till en ökad risk. Intressant nog kunde vi visa att övervikt inte alls var förknippat med stadium I och II tumörer, dvs tumörer med mindre avancerad djupväxt i tarmen, utan istället starkt förknippat med ökad risk för de mer aggressiva stadium III och IV tumörerna hos båda könen, fast med starkast samband hos män. Utöver detta visade vi att det inte fanns några samband mellan övervikt och risk för cancer i ändtarmen hos män och inga samband mellan övervikt och cancer i tjocktarmen hos kvinnor.

I det andra delarbetet studerade vi sambanden mellan antropometri och risk för olika molekyllära subgrupper av kolorektal cancer. Ett antal proteiner som är involverade i uppkomsten av cancerutveckling på cellulär nivå analyserades med sk immunohistokemisk analys, där förekomst av de studerade proteinerna visualiseras i

mikroskop med hjälp av antikroppsbindning. Här valde vi att analysera beta-catenin och cyclin D1, båda cellcykelreglerande proteiner, samt p53 som är en tumorsuppressorgen med en central roll för att skydda kroppens celler mot cancer, en skyddseffekt som försvinner vid när genen stängs av genom mutation. Vidare analyserades något som kallas mikrosatellitinstabilitet (MSI) hos tumörerna. Detta innebär att tumörcellerna har nedsatt förmåga att korrigera de fel som uppstår vid DNA-replikation och celldelning. Mikrosatellitinstabil kolorektalcancer är starkt förknippat med ärftlig kolorektalcancer och är vanligare förekommande hos äldre kvinnor och har ofta en god prognos. I vår studie var det mest framträdande resultatet att övervikt inte var associerat med mikrosatellitinstabila tumörer, varken hos män eller kvinnor. Hos kvinnor var övervikt relaterat till en ökad risk för p 53 negativa-, cyclin D1 positiva- och betacatenin positiva tumörer. Hos män var sambanden inte lika entydiga, förutom att övervikt inte var förknippat med MSI tumörer.

Tredje arbetet utformades lite annorlunda i och med att vi bara studerade kvinnorna i kohorten. Vi analyserade risk för kolorektal cancer, inklusive olika kliniska stadier och molekylära subgrupper, i relation till östrogenexponering i form av behandling med p-pillar och klimakteriell hormonbehandling (HRT). Här fann vi, i motsats till majoriteten av tidigare studier, att HRT inte hade någon skyddande effekt mot kolorektal cancer. Däremot visade resultaten att HRT-behandling gav en minskad risk för mindre aggressiva tumörer, dvs stadium I och II, samt en minskad risk för stadium I och II-, lymfkörtelnegativa- och icke fjärrmetastaserade tumörer i ändtarmen, men inte i tjocktarmen.

Slutligen, i det fjärde arbetet, utvärderade vi sambanden mellan antropometri och risk för kolorektalcancer med mutationer i generna KRAS och BRAF. Dessa gener kodar för proteiner som fyller viktiga funktioner i cellen genom att vidarebefordra signaler från cellytan in till cellkärnan. Mutationer i dessa gener leder till en kontinuerlig aktivering med ohämmad tillväxt av tumörcellen som följd. Resultaten visade att övervikt var förknippat med tumörer med både icke-muterad, sk vildtyp, och muterad KRAS-gen hos män, medan övervikt inte alls hade några samband med KRAS-mutation hos kvinnor. Vidare var övervikt hos båda könen förknippat med en signifikant ökad risk för tumörer med BRAF av vildtyp, men inte för tumörer med BRAF-mutation.

Sammanfattningsvis har vi med detta avhandlingsarbete kunnat visa att övervikt utgör en risk för kolorektal cancer hos både män och kvinnor. Vi har också kunnat påvisa skillnader mellan könen avseende risken för olika molekylära och kliniska subgrupper av denna cancerform.



# Acknowledgements

Jag vill tacka alla som på ena eller andra sättet varit inblandade i detta arbete, men jag skulle vilja rikta ett särskilt stort tack till följande;

*Karin Jirström*, min huvudhandledare. Utan dig hade jag aldrig klarat av detta! Du är en fantastisk person, en verklig förebild! Tack för ditt oändliga engagemang och stöd, ditt positiva tänkande och för alla goa skratt! Jag hoppas att vi kommer att fortsätta att både jobba och ha kul ihop länge till...

*Jonas Manjer*, bihandledare för halva familjen Brändstedt! Tack för att du introducerade mig för Karin och den cancerforskning som jag letade efter! Din hjälp med statistiska och epidemiologiska frågor har varit ovärderlig.

Alla i forskargruppen: *Karolina Boman, Jacob Elebro, Richard Fristedt, Alexander Gaber, Charlotta Hedner, Björn Nodin, Liv Jonsson* och *Anna Larsson*. Ni är ett gött gäng!

*Elise Nilsson*, för alla fina TMA och immunofärgningar.

*Kristin Lindell*, för all administrativ hjälp.

*Nooreldin Zendehkrokh*, för hjälp med pyrosekvensering.

*Magnus Sundström*, för hjälp med BRAF och KRAS analyser.

Övriga medförfattare, *Signe Borgquist, Jakob Eberhard* och *Sakarias Wangefjord*.

*Peter Mangell*, för att jag får vara med på ett litet hörn på teamet, det betyder mycket!

*Alla kollegor på Kirurgen*, jag har världens bästa jobb!

*"Farmor" Solweig*, för all hjälp med barnen under alla år, du betyder mycket för oss!

*Mamma och Pappa*, för att ni alltid har trott på mig, stöttat mig och hjälpt mig på alla sätt och vis.

Mina syskon, *Johan* och *Jonas*, för att ni är de ni är, och för att ni alltid finns där när det behövs.

Mina älskade killar där hemma, *Johan*, för ditt lugn och för att allting aldrig är något problem! *Noel, Nils och Sixten*, för att ni får mig att inse vad som verkligen är viktigt och betydelsefullt i livet. Ni är mitt allt, Tack för att ni har stått ut med mig och peppat mig hela vägen! Vad mycket roligt vi ska göra nu!!



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