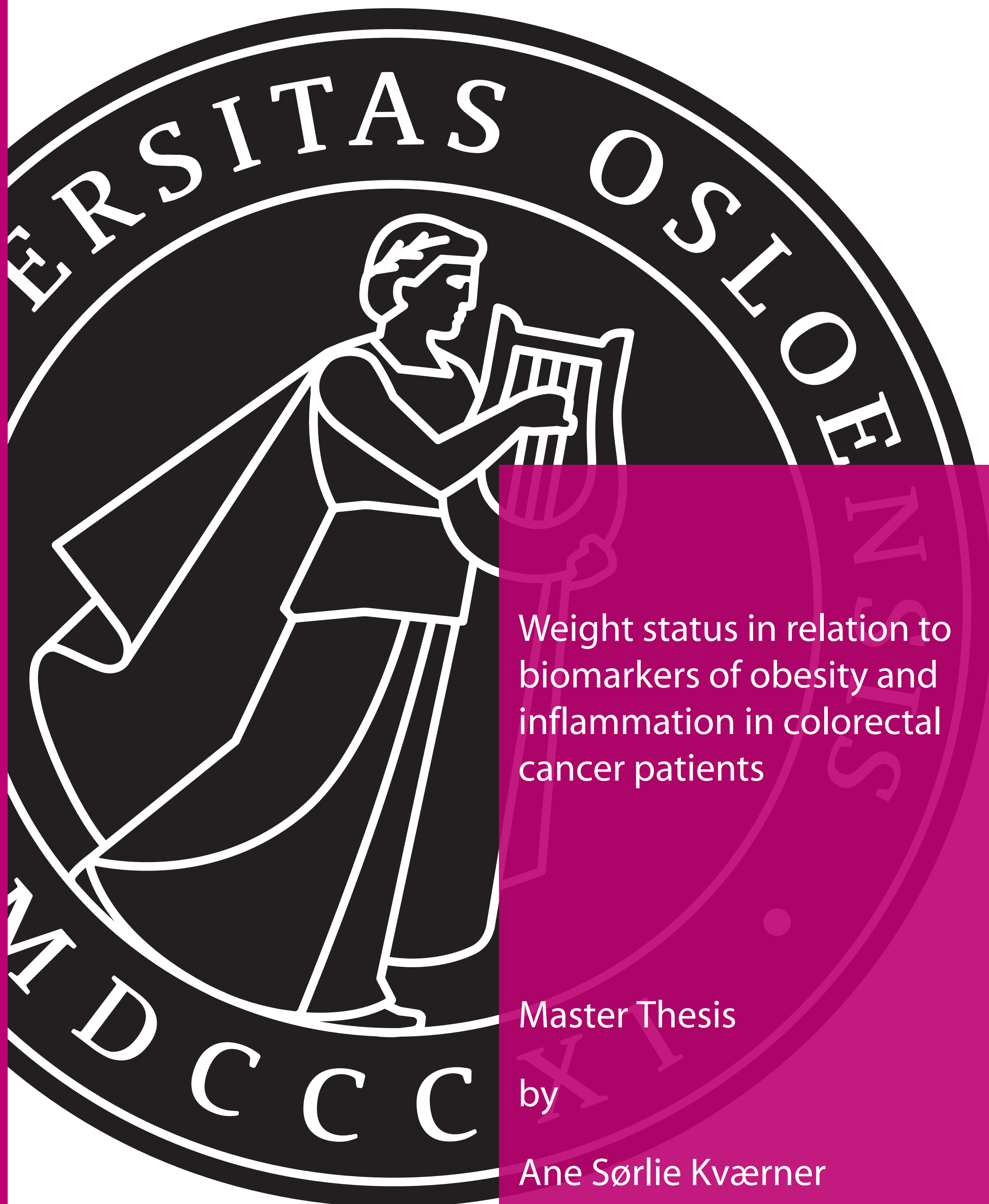

Weight status in relation to biomarkers of obesity and inflammation in colorectal cancer patients

Master Thesis

by

Ane Sørlie Kværner

Department of Nutrition
Faculty of Medicine
University of Oslo

June 2013

Weight status in relation to biomarkers of obesity and inflammation in colorectal cancer patients

Ane Sørlie Kværner

Supervisors:
Siv Kjølsrud Bøhn
Rune Blomhoff

Master thesis
Department of Nutrition
Faculty of Medicine

UNIVERSITY OF OSLO

June 2013

© Ane Sørlie Kværner

2013

Weight status in relation to biomarkers of obesity and inflammation in colorectal cancer patients

http://www.duo.uio.no/

Print: CopyCat, Forskningsparken, Oslo

Acknowledgements

The present work has been conducted at the Department of Nutrition, Faculty of Medicine, University of Oslo, from August 2012 to June 2013.

I am very grateful for the opportunity to conduct the work of this master thesis in the research group of Professor Rune Blomhoff. It has been an exciting and eventful year being part of a research team conducting a large human clinical intervention trial. I have expanded my knowledge in several areas within nutrition research. During this period I have appreciated all the enlightening discussions within the research group. I admire the group for their creative working environment, the versatility in the group and the high professional academic level.

I am deeply grateful to my main supervisor Post doc Siv Kjølsrud Bøhn for all valuable technical guidance, sound advices as well as the enthusiastic encouragement through this year. I will also like to express my sincere appreciation to my co-supervisor Professor Rune Blomhoff for his valuable and constructive suggestions during the planning and development of this thesis.

I acknowledge the members of the project group of the Norwegian Foods Study (NFS), consisting of Professor Rune Blomhoff, Post doc Siv Kjølsrud Bøhn, Post doc Ingvild Paur, PhD Hege Berg Henriksen, PhD Hanna Ræder, laboratory technician Siv Åshild Wiik and research assistant Kristine Lillebø Holm. It has been a privilege to work with all of you, and I really appreciate all guidance, assistance and support through this year.

I would like to thank my companion master students for their positive energy and enthusiasms, creating a positive working environment. I am particularly grateful for the close collaboration in the NSF with Anne Juul Skjetne and Mari Bøe Sebelien.

Last but not least, I would like to thank my life companion, my family and my friends for all their support and understanding during this year.

Abstract

Objective: Obesity is associated with increased risk of colorectal cancer (CRC), but the mechanisms linking the two conditions are not completely understood. The primary objective of this thesis was to investigate weight status in relation to comorbidities and biomarkers of obesity and inflammation in a subgroup of CRC patients from a multicenter randomized controlled intervention trial. In this trial, dried blood spot (DBS) will be used as the main blood collection method for measuring long-term effects of intervention, thus demanding the validation of new and relevant biomarkers. Therefore, we aimed at developing and validating a method to measure suPAR, a novel diagnostic biomarker, in DBS samples.

Subjects and methods: By May 2013, 41 newly diagnosed CRC patients aged 50-78 years were included in this study. Anthropometric measures and measures of physical function were recorded pre- and two months post-surgery. Pre-surgery, plasma adipokines and cytokines were analyzed by multiplex technology. suPAR was measured pre-surgery in DBS samples using a modified commercially available ELISA assay, and validated against plasma levels.

Results: We found that it was possible to measure suPAR in DBS and that DBS levels correlated significantly with that of plasma ($p<0.001$). A high prevalence of overweight and obesity was found in the study population with a mean BMI of 27.5 (26.0-29.0) kg/m^2. Both BMI and waist circumference were positively associated with several adipokines (resistin, adipsin, lipocalin-2, PAI-1 and leptin) and with insulin and suPAR. However, these anthropometric measures were not associated with established biomarkers of inflammation (CRP, MCP-1, TNFα, IL-1β, IL-6, and IL-8). Hypertension was significantly more common in obese compared to normal weight patients ($p<0.01$), and could be independently predicted by leptin, insulin and suPAR. During the two months period from pre- to post-surgery there was a significant reduction in body weight, BMI, waist circumference and waist-hip ratio.

Conclusions: We confirm the association between CRC and obesity and find an association between obesity, adipokines, insulin and suPAR. However, we do not find a link between obesity and inflammation with regards to the inflammatory markers measured. Although most CRC patients experienced weight loss during the postoperative period, these changes were not correlated with changes in physical function. Our work also adds suPAR to the growing panel of diagnostically valuable analytes validated in DBS samples, and report for the first time on an association between suPAR and diagnosed hypertension.

List of abbreviations

ADIPOR	Adiponectin receptor
AICR	American Institute for Cancer Research
AJCC	American Joint Committee on Cancer
APC	Adenomatous polyposis coli
BMI	Body mass index
BSA	Bovine serum albumin
CEA	Carcinoembryonic antigen
COPD	Chronic obstructive pulmonary disease
CRC	Colorectal cancer
CRP	C-reactive protein
CVD	Cardiovascular disease
DBS	Dried blood spot
DMT1	Diabetes mellitus type 1
DMT2	Diabetes mellitus type 2
EPIC	European Prospective Investigation into Cancer and Nutrition
FAP	Familial adenomatous polyposis
FFQ	Food frequency questionnaire
HbA1c	Glycated hemoglobin
HMW	High-molecular weight
HGF	Hepatocyte growth factor
HNPCC	Hereditary nonpolyposis colorectal cancer
ICD	International classification of diseases
IGF-1	Insulin-like growth factor 1
IGFBP-1	Insulin-like growth factor binding protein 1
IGFBP-2	Insulin-like growth factor binding protein 2
IL	Interleukin
IR	Insulin receptor
IGF1R	Insulin-like growth factor 1 receptor
MCP-1	Monocyte chemoattractant protein-1
NFS	Norwegian Foods Study
NGF	Nerve growth factor
PAI-1	Plasminogen activator inhibitor-1
PC	Principal Component
PCA	Principal Component analysis
RELMs	Restin-like molecules
sOB-R	Soluble leptin receptor
suPAR	Soluble urokinase plasminogen activator receptor
TNF-α	Tumor necrosis factor-α
TNM	Tumor node metastasis
uPA	Urokinase plasminogen activator
uPAR	Urokinase plasminogen activator receptor
UICC	International Union Against Cancer
WCRF	World Cancer Research Fund
WHO	World Health Organization

List of tables

Table 1 Staging of CRC according to the TNM system.

Table 2 Contributions directly related to this master project.

Table 3 Clinical and histopathological characteristics pre-surgery.

Table 4 Association between BMI and plasma biomarkers pre-surgery.

Table 5 Association between waist circumference and plasma biomarkers pre-surgery.

Table 6 Association between body weight and plasma biomarkers pre-surgery.

Table 7 Association between waist/hip-ratio and plasma biomarkers pre-surgery.

Table 8 Spearman's correlation coefficients between the different plasma biomarkers.

Table 9 Plasma biomarkers and tumor localization pre-surgery.

Table 10 Plasma biomarkers and TNM stage group pre-surgery.

Table 11 Hypertension and plasma biomarkers.

Table 12 Postoperative changes in weight status and physical function.

Table 13 Previously validated protein biomarkers related to obesity and inflammation.

List of figures

Figure 1 Anatomy of the large intestine.

Figure 2 Adipose tissue depots.

Figure 3 Suggested mechanisms linking obesity and CRC.

Figure 4 Structure of suPAR.

Figure 5 A flowchart of the recruitment and inclusion process including relevant samples and tests.

Figure 6 An overview of the study design with corresponding samples and tests.

Figure 7 Overview of the multiplex technology.

Figure 8 Illustration of PCA on three dimensions.

Figure 9 Protein concentration and amount in DBS samples.

Figure 10 Protein amount extracted in samples eluted in different types of buffers.

Figure 11 Association between suPAR measured in DBS and plasma samples.

Figure 12 Effect of storage in room temperature on suPAR concentration.

Figure 13 PCA on BMI, waist circumference and all plasma biomarkers pre-surgery.

Table of contents

1 Introduction 1
1.1 Colorectal cancer 1
1.2 Overweight, obesity and CRC 8
1.3 Mechanisms linking obesity and CRC 12
1.4 Biomarkers as indicators of health and disease 15
1.5 Biomarkers measured in dried blood spot (DBS) 19
2 Aims of the thesis 22
3 Materials 23
3.1 Equipment 23
3.2 Chemicals 23
3.3 Kits 24
3.4 Instruments 24
3.5 Software 24
4 Subjects and methods 25
4.1 Subjects 25
4.3 Study design 27
4.4 Assessment methods 29
4.6 Statistical analysis 37
4.7 My contribution to the research project 41
5 Results 43
5.1 Method development and validation 43
5.2 Subjects characteristics and biomarkers in CRC patients 50
6 Discussion 68
6.1 Method development and validation 68
6.2 Discussion of results 75
7 Conclusion 91
8 Future perspective 93
9 List of references 95

1 Introduction

This thesis is part of The Norwegian Foods Study (NFS), an ongoing large randomized controlled intervention trial on the effect of diet and physical activity after surgery for colorectal cancer (CRC). The main objective of the NFS is to investigate whether a diet and lifestyle intervention will reduce the risk of morbidity and mortality in a group of CRC patients.

The primary objective of this thesis was to investigate weight status in relation to comorbidities and biomarkers of obesity and inflammation. Data used in this thesis was mainly obtained during the master thesis period and comprises the first 41 participants included in the NSF. Furthermore, an aim of this thesis was to develop and validates the use of a self-administered blood collection method for biomarker measurements, which will be important for measuring long-term effects in the NFS.

1.1 Colorectal cancer

1.1.1 Epidemiology of colorectal cancer

Colorectal cancer (CRC), cancers of the colon and rectum constitute a significant proportion of the global burden of cancer morbidity and mortality. It is the third most commonly diagnosed cancer in men and woman combined, and the fourth most common cause of death (1). In 2008 there were estimated to be 1.2 million new cases of CRC and 608700 deaths (1). CRC is slightly more common among men than woman, and the disease risk increases with age until old age where it levels off (2).

CRC has generally been considered a disease of high income countries, but is now getting more common in middle- and low-income countries (2). The highest incidence rates are found in Australia, New Zealand, Europe and North America whereas the lowest incidence rates are found in Africa and South-Central Asia (1). The incidence rates of economically transitioning countries such as Japan, Singapore and eastern European countries are rapidly increasing (2).

1.1.2 Risk factors and causes of CRC

Both genetics and environmental factors contribute to the development of CRC. About five to ten percent of all CRC cases is a consequence of recognized hereditary causes, the most prevalent being familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC) (2). A further 20 % of CRC cases occur in people having a family history of CRC without fulfilling the strict criteria of hereditary CRC (2, 3). Having a background of inflammatory bowel disease such as ulcerative colitis or chrohn`s disease also increases the risk of developing CRC (2). In spite of the importance of familial predisposition, most CRC cases are sporadic, in which there is no family history of the disease (4). In these cases, cancer is assumed to develop as a complex interplay between multiple genes and environmental factors (5).

A wide geographic variation in incidence, together with observations from migrant studies, suggests that lifestyle factors including diet, physical activity, obesity and diabetes play a pivotal role in the etiology of CRC (6). In fact, it has been estimated that CRC is mostly preventable by appropriate diets and associated factors (2). World cancer Research Fund/American Institute of Cancer Research (WCRF/AIRC) has estimated that about 45 % of bowel cancer cases in the U.S., and about 43 % bowel cancer cases in the U.K. could be prevented through healthy patterns of diet, physical activity and weight maintenance (7). Furthermore, it has been estimated that overweight and obesity accounts for up to 23 % of the colon cancer incidence, physical activity up to 33 % of the colon cancer incidence, and fruit and/or vegetable intake up to 29 % of the colorectal cancer incidence (8). Clearly, eating healthy, being physical active and maintaining a normal body weight are of great importance for preventing CRC.

In the second expert report of 2007 (2), WCRF/AICR concludes that there is convincing evidence that physical activity reduces the risk of CRC, while there is probable evidence that food containing dietary fiber, garlic, milk and calcium reduces the risk of CRC. Furthermore, they conclude that there is convincing evidence that red meat, processed meat, alcoholic drinks (for men), body fatness, abdominal fatness and adult attained height increases the risk of CRC, while there is probable evidence that alcoholic drinks for woman increases the risk of CRC. The suggested mechanisms linking body fatness and abdominal obesity to development of CRC will be discussed in detail in later sections.

1.1.3 Anatomy and function of the large intestine

The large intestine extends from the terminal ileum to the anus, and consists of the caecum, the colon, the rectum and the anal canal (**Figure 1**) (9). The colon is the longest part of the large intestine (9), and includes the ascending colon, the transverse colon, the descending colon and the sigmoid colon (10). Partly digested food is moved into the colon where the remaining absorption of water and electrolytes take place (9). The chyme is mixed with mucus and bacteria and become feces (11). Feces is then transported into the rectum where it is temporary stored before leaving the body through the anal canal and anus (9).

The colon harbors a complex and abundant population of bacteria (12). In fact, it is estimated that about 10^{13}-10^{14} microorganisms live in the human colon (12). The microbiota provide several beneficial functions including enhancing intestinal immunity, promoting colonic motility as well as helping maintaining colonic mucosal integrity (13). Furthermore, the colonic microorganisms have important metabolic functions. For instance, the microorganisms synthesize a substantial amount of vitamins, in particular vitamin K and biotin. Furthermore, the microbiota generates short chain fatty acids, which acts as nutrients and growth signals for the colon epithelium (14). The microbiota also transform bioactive molecules and bind and facilitate the excretion of some toxins (14).

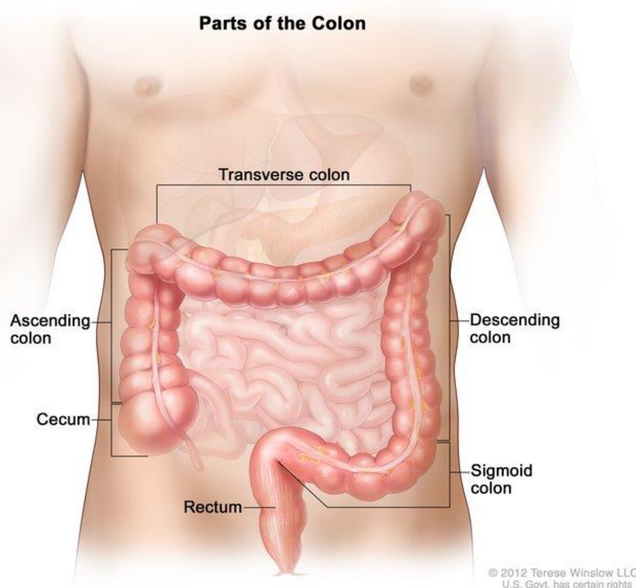

Figure 1 Anatomy of the large intestine. The large intestine can be divided in to four parts: the caecum, the colon, the rectum and the anal canal. The colon, the largest part of the large intestine can be further divided into the ascending, the transverse, the descending and the sigmoid colon. The picture is taken from the National Institute of cancer, Visuals Online with permission.

1.1.4 The pathogenesis of CRC

Several types of cancers can start in the colon or rectum, but the most common are adenocarcinomas, cancers originated from the glandular tissue. In fact, approximately 95 % of all CRC cases are adenocarcinomas (2). The majority of these cancers arise from adenomatous polyps, also called adenomas, masses that protrude into the lumen of the gut (15). These polyps develop as a result of disrupted regulation of cellular processes important for controlling the constant renewal of the colon epithelium, such as cell proliferation, differentiation and apoptosis (16). Disruption of these processes causes uncontrolled cell proliferation, loss of apoptotic regulation and uncontrolled tumor growth (16). Although the majority of CRC cases develops through adenomatous polyps, CRC can also arise from other types of polyps including serrated and hyperplastic polyps (17).

Most CRC cases develop slowly over several years. Specific genetic changes are thought to drive the transformation from normal colonic mucosa to invasive cancers (4). The genetic mutations can be both inherited and acquired. Germline mutations underlie common inherited syndromes, such as FAP and HNPCC, while sporadic CRC develops as a multistep process involving accumulation of multiple somatic mutations (4).

Different molecular mechanisms are involved in the pathogenetic process leading to CRC. Chromosomal instability is involved in 65-70 % of sporadic CRC (18) where the tumors are characterized by gross chromosomal abnormalities, including deletions, insertions and loss of heterozygosity (4). "Gain of function" mutations are often involved in the carcinogenetic process. Such mutations can result in either activation of growth promotion including oncogenes, or diminished activity of tumor suppressor genes or apoptotic pathways (4). Mutation of the tumor suppressor gene adenomatous polyposis coli (APC) gene is perhaps the most critical gene in the early development of CRC (4). It fact, somatic mutations in both alleles of this gene are present in 80 % of all sporadic CRC, and a single germline mutation of this gene is responsible for the inherited syndrome FAP (4). In 12-17 % of CRC cases mutations or epigenetic changes occur in genes that maintain genetic stability, e.g. mismatch repair genes (19). Silencing these genes can lead to accumulation of DNA errors throughout the genome, typically in simple repetitive DNA sequences called microsatellites (4). The tumors are therefore described as having the phenotype of microsatellite instability (4). A germline mutation in one of the mismatch repair genes give rise to the inherited syndrome HNPCC (4). Other molecular mechanisms leading to colorectal carcinogenesis involves

hypermethylation of CPG islands leading to lower or diminished expression of important genes involved in DNA stability and repair causing accumulation of DNA errors throughout the genome (4). Hypermethylation in such genes occurs in about 24-51 % of CRC cases (18).

1.1.5 Diagnosis of CRC

CRC can be symptomatic or asymptomatic at presentation. Common reported symptoms are abdominal pain, changes in bowel habits, hematochezia or melena, iron deficiency anemia and unexplained weight loss (20). As the symptoms are typically due to growth of the tumor into the lumen of the gut or into adjuvant structures, symptomatic presentation is often a manifestation of a relatively advanced tumor (20). CRC may be suspected by some of the symptoms mentioned above, or may be asymptomatic and discovered by routine screening of average and high risk patients (20). Colonoscopy is the gold standard method for diagnosing CRC since it is possible to both localize and biopsy lesions throughout the large intestine (19). Still abdominal ultrasound and chest radiography are routinely done as part of diagnosis process (19). A proper preoperative assessment is important to determine the extent of disease and the appropriate treatment approach.

1.1.6 Classification of CRC

CRC develops in the innermost layer of the colon or rectum, and can grow through some or all of the other layers. Staging of CRC depends to a great degree on how deeply the cancer has grown into these layers. The most commonly used staging system for CRC is that of the American Joint Committee on Cancer (AJCC/UICC) (10), known as the tumor node metastasis (TNM) system (**Table 1**). This system characterize how far the cancer has grown into the wall of the intestine and whether or not it has reached nearby structures (T), the extent of spread to nearby lymph nodes (N) and whether or not the cancer has metastasized (M). Cancer staging is one of the most important determinants of prognosis (21). In fact, the five-year survival of stage I CRC is estimated to be 80-95 % compared to 0-7 % for stage IV CRC (3).

Table 1 Staging of CRC according to the TNM system (19)

T	N	M	TNM stage
Tis	N0	M0	0
T1, T2	N0	M0	I
T3	N0	M0	IIA
T4	N0	M0	IIB
T1, T2	N1	M0	IIIA
T3, T4	N1	M0	IIIB
Any T	N2	M0	IIIC
Any T	Any N	M1	IV

T level indicate the size or the extent of the primary tumor. Tis: carcinoma in situ.
N level indicates the degree of spread to nearby lymph nodes. N0: No lymph nodes involved.
M level indicates whether or not the cancer has metastasized. M0: No distant metastasis.

1.1.7 Treatment of CRC

The principal first-line treatment for the majority of CRC patients is surgical resection (19). In fact, surgical resection of the primary tumor is performed in 80 % of patients diagnosed with CRC (22). Chemotherapy and radiotherapy are often used as an adjuvant to surgery depending on the type and grade of tumor (19). Several side effects are associated with CRC treatment. Commonly reported side effects are nausea, vomiting, abdominal pain, irregular bowel movements, gas and flatulence, malabsorption and diarrhoea (23, 24). These are all side effects that may negatively impact the nutritional status of the patient.

1.1.8 Comorbidity and CRC

Comorbidity is the presence of illnesses or disorders in addition to the primary disease of interest (25). Comorbid conditions are a common finding among CRC patients (25-27). Frequently reported comorbidities are cardiovascular disease (CVD), chronic obstructive pulmonary disease (COPD), diabetes mellitus type 2 (DMT2), hypertension and previous cancers (25-27). For most of these diseases, lifestyle factors such as diet, physical activity and body weight maintenance are assumed to be highly important (28). Presence of comorbid conditions is reported to be more common among patients who are older or of lower socioeconomic status (27). Furthermore, presence of comorbidity may vary according to tumor site. In a population-based study conducted in the Netherlands (26), number and types of associated comorbidities, in particularly diabetes mellitus, CVD and previous cancers varied according to gender and tumor location, being more prevalent in men with cancers in

proximal compared to distally located tumors. The authors suggested that the different prevalence rates could illustrate variation in the aetiology of CRC, with a marked influence of cardiovascular risk factors for cancers in the ascending colon.

1.2 Overweight, obesity and CRC

1.2.1 Obesity, abdominal obesity and risk of CRC

The worldwide prevalence of overweight and obesity is rising with epidemic proportions. In 2008, more than 1.4 billion adults were overweight, of which nearly 500 million were obese (29). It is estimated that by the year 2015, approximately 2.3 billion adults will be overweight, of which more than 700 million will be obese (30). Overweight and obesity are important contributors to the global burden of morbidity and mortality. In fact, body fatness, as measured by the body mass index (BMI) was ranked as the sixth most important risk factor of the global burden of disease in a newly published systemic analysis of the Global Burden of Disease Study (31).

Overweight and obesity is associated with increased risk of several chronic diseases including CVD, DMT2, musculoskeletal disorders and some caners (29). According to WCRF/AICR, there is convincing evidence that body fatness increases the risk of cancer in the esophagus, pancreas, breast (postmenopausal), endometrium, kidney and colorectum (2). For CRC, there is convincing evidence that both body fatness and abdominal obesity increases the risk (2). The relationship is consistent with a clear dose response relationship (2). It seems to be a stronger relationship between body fatness and CRC among men than woman (32), and a stronger relationship for cancers of the colon compared to cancers of the rectum (32).

In addition to being associated with increased risk of cancer development, excess visceral adipose tissue has been associated with increased tumor progression and reduced survival (33). Multiple molecular markers associated with CRC are found to be associated with both BMI and prognosis, suggesting that obesity may lead to a more aggressive CRC phenotype (34). In a large prospective study of more than 900 000 U.S. adults (35), increased BMI was associated with increased risk of mortality from all cancers at follow-up. Men and woman with BMI > 40 kg/m^2 had a 52 % and 62 % higher death rate from all cancers, respectively than men and woman with normal weight. The authors suggested that as much as 14 and 20 % of all cancer deaths in U.S. adult's aged 50 years or older in men and woman respectively, could be attributed to overweight and obesity.

1.2.2 Risk factors and causes of obesity

Obesity is a complex disease caused by several factors including genetic and environmental factors. The most important environmental factors are probably diet and lifestyle, in particular over-nutrition and a sedentary behavior, promoting the progression and the pathogenesis of this polygenic disease (36). An imbalance in energy intake and energy expenditure over time leads to accumulation of adipose tissue (37). This is accompanied by a moderate increase in adipocyte number, and a substantial enlargement of the individual adipocytes (38).

1.2.3 Anatomy and function of the adipose tissue

Adipose tissue is principally deposited in two compartments, subcutaneously and viscerally (**Figure 2**) (38). The subcutaneous adipose tissue is distributed over the body's surface in the hypodermal layer of the skin (38). The visceral adipose tissue surrounds inner organs in the abdominal cavity and mediastinum, and includes the omental and mesenteric fat depot, surrounding the intestines superficially and internally respectively, the retroperitoneal fat depot located near the kidneys, and fat localized in the mediastinum and around specific organs such as the heart, stomach and blood vessels (38). The distribution of fat is largely determined by genetic factors, with a typically different pattern in men and woman (2). Women tend to store more subcutaneous fat around their hips, buttocks and thighs, producing a "pear shaped" body profile. On the other hand, men tend to store more fat around their abdomen, producing a more "apple shaped" body profile.

White adipose tissue is primarily composed of large, tightly packed adipocytes, supported by a highly vascular loose connective tissue (38). The adipocytes have a considerable capacity for expansion, and varies greatly in size depending on the lipid content (39). Although the distinctive future of adipose tissue is the adipocytes, there are several cell types present including pericytes, preadipocytes, white and brown adipocytes, fibroblasts, endothelial cells, nerve cells and several types of immune cells (39).

The adipose tissue has several functions. It is crucial for storing energy as well as storage of cholesterol and lipid-soluble vitamins, in particular vitamin D and E (39). Moreover, it is a key regulator of energy metabolism, regulating the metabolism of lipids and carbohydrates (38). It also serves as an important thermic insulator and is important for mechanical protection (39). Furthermore, the adipose tissue has been widely recognized as an important

endocrine organ, secreting a multiplicity of protein and non-protein hormones in addition to other substances such as free fatty acids and lactate (39, 40). The protein hormones and cytokines secreted from the adipocytes are collectively referred to as adipokines.

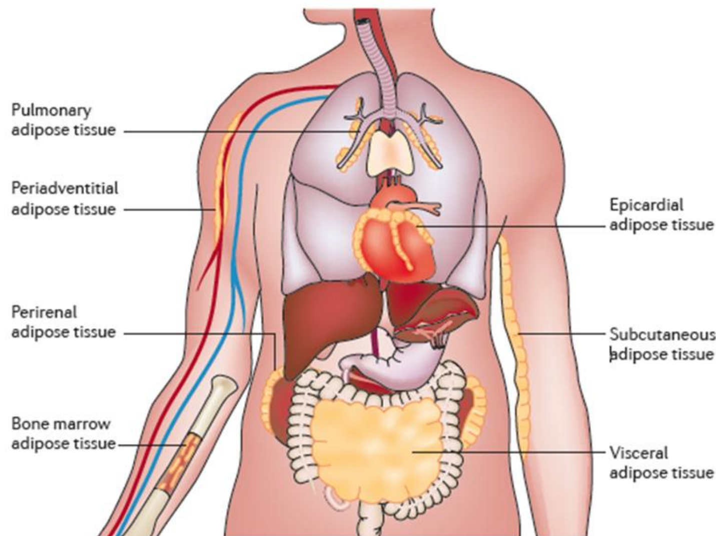

Figure 2 Adipose tissue depots. Adipose tissue is principally deposited in two compartments, subcutaneously and viscerally. Subcutaneous adipose tissue is distributed over the body's surface, while visceral adipose tissue surrounds the inner organs in the abdominal cavity and mediastinum. Different fat depots surrounding the heart, lungs, kidneys, bone marrow and blood vessels are shown in the figure. The secretion of protein hormones and cytokines from different fat depots may selectively affect organ function and systemic metabolism. The picture is taken from Ouchi et al (41) .

1.2.4 Visceral obesity and the metabolic syndrome

Excess visceral adipose tissue seems to be more strongly associated with pathological conditions than subcutaneous adipose tissue (39). This may be related to several factors including differences in metabolic activity, different production and secretion of adipokines and inflammatory cytokines as well as the different anatomic localization (38). Visceral adipose tissue is regarded as more metabolically active than subcutaneous adipose tissue (38). In particular, visceral adipose tissue is shown to have a higher lipogenic and lipolytic activity compared to subcutaneous adipose tissue (38). Furthermore, visceral adipose tissue have a higher infiltration rate of macrophages and also produce larger amounts of pro-inflammatory cytokines compared to subcutaneous adipose tissue (38). The anatomic location of visceral adipose tissue may also be important as blood from the omental and mesenteric adipose tissue is transported directly to the liver by the portal vein. In this way adipose tissue derived factors, such as hormones, cytokines and fatty acids are transported directly to the liver from the adipose tissue (42). This portal influx may affect liver metabolism, causing abnormal lipoprotein synthesis, hepatic insulin resistance and increased gluconeogenesis (42).

An excess amount of visceral adipose tissue is associated with several metabolic abnormalities including insulin resistance and lipid disturbances (42). A clustering of these metabolic abnormalities in combination with elevated blood pressure is collectively referred to as the metabolic syndrome. According to recent international criteria, the metabolic syndrome is defined as a constellation of metabolic risk factors associated with increased risk of CVD and DMT2 (43). The specific risk factors include elevated waist circumference (≥ 94 cm in men, ≥ 80 cm in women), elevated blood pressure (systolic ≥ 130 mmHg, diastolic ≥ 85 mmHg), elevated triglycerides (≥ 150 mg/dL), reduced HDL cholesterol (< 40 mg/dL in men, < 50 mg/dL in woman) and elevated fasting glucose (≥ 100 mg/dL) (43).

1.3 Mechanisms linking obesity and CRC

The mechanisms by which obesity and the associated metabolic syndrome are thought to promote tumorgenesis are manifold (**Figure 3**). Some suggested mechanisms involves insulin resistance and hyperinsulinemia, adipokine dysregulation as well as chronic inflammation (33).

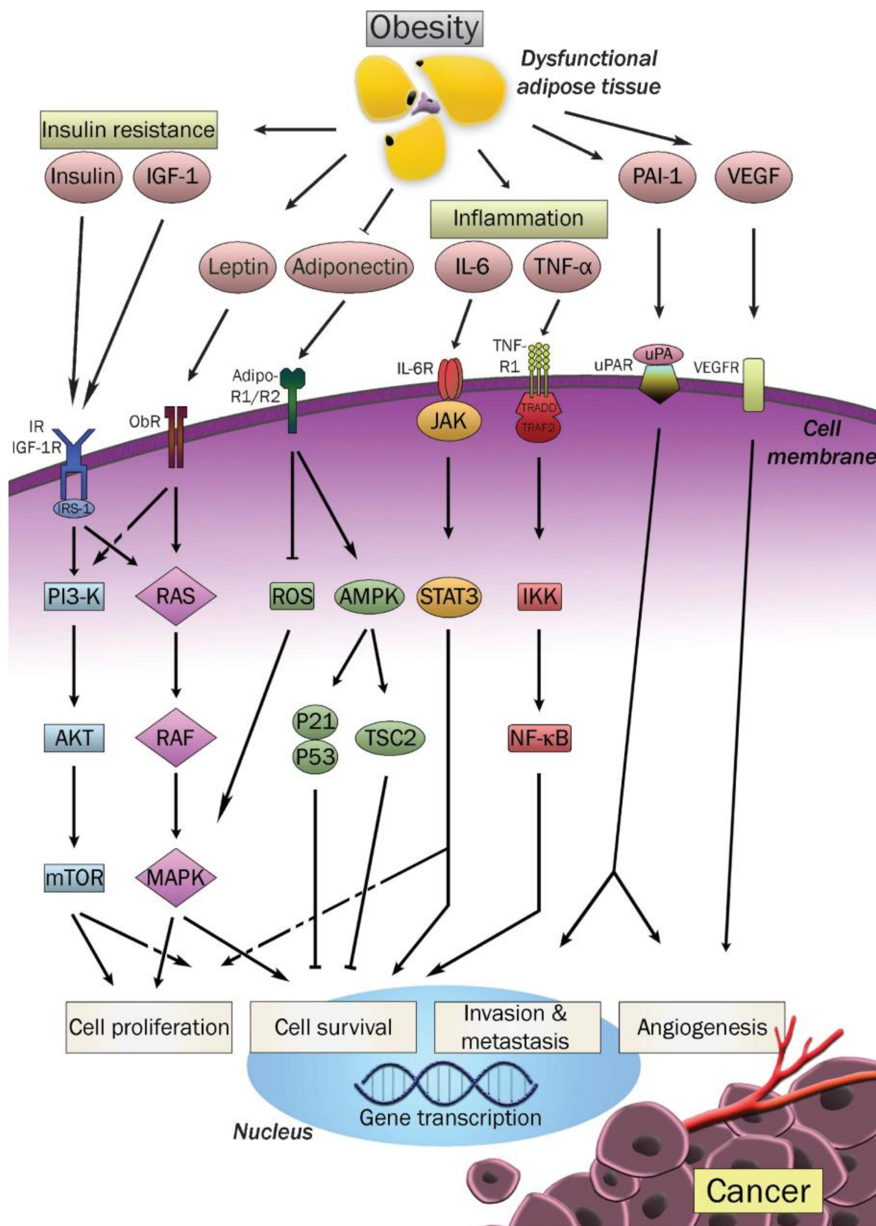

Figure 3 Suggested mechanisms linking obesity and CRC. Obesity is associated with insulin resistance, low-grade chronic inflammation and altered production and secretion of adipokines from the adipose tissue. As glucose levels rise, the body compensates by producing more insulin, causing hyperinsulinemia. Insulin is suggested to affect colorectal carcinogenesis both directly and indirectly through its effect on IGF-1. Binding of insulin and IGF-1 to their respective receptors activates intracellular signaling cascades important for regulation of cellular processes such as cell proliferation and survival. In the same way, adipokines and inflammatory cytokines secreted from both adipocytes and infiltrating immune cells within the adipose tissue may affect carcinogenesis by binding to their receptors, thereby activating intracellular signaling pathways important for regulation of cellular processes. The figure is taken from van Kruijsdijk et al (44).

1.3.1 Insulin resistance and hyperinsulinemia

Obesity and in particular abdominal obesity is associated with insulin resistance, a condition where insulin is less effective in reducing blood glucose levels (42). As a result blood glucose levels rise, and the body compensate by producing more insulin, causing hyperinsulinemia (42). Hyperinsulinemia has been suggested as an important mechanisms linking obesity to CRC (42). A plausible support for this hypothesis comes from observational studies showing an increased CRC risk among diabetic individuals compared to individuals without diabetes (42). In a recently published meta-analysis including 24 cohort and case-control studies (45), a significantly increased risk of CRC was seen among individuals with diabetes compared to individuals without diabetes. The risk increase was present for both sexes, as well as for both colon and rectum cancer. Use of insulin therapy was also found to be associated with CRC risk.

Insulin may influence colorectal carcinogenesis both directly and indirectly. In addition to being important in glucose homeostasis and energy supply to cells and tissues (46), insulin is an important regulator of cell growth, including differentiation and apoptosis (47). In particular, insulin is shown to be an important growth factor for colonic epithelial cells (47). Insulin also increases the bioactivity of insulin like growth factor 1 (IGF-1), both by up-regulating the hepatic production of IGF-1 and by reducing the hepatic secretion of the insulin like growth factor binding protein 1 (IGFBP-1) and 2 (IGFBP-2) (42). Insulin and IGF-1 mediates its effect through the insulin receptor (IR) and the IGF-1 receptor (IGF1R) (42). These receptors are widely expressed in normal tissue, as well as in epithelial colorectal cancer cells (42). Binding of insulin and IGF-1 to the respective receptors, leads to activation of intracellular signaling cascades, effecting gene transcription and cellular processes such as cell growth and differentiation (46).

1.3.2 The adipose tissue as an endocrine organ

The adipose tissue is an important endocrine organ secreting a substantial number of adipokines and inflammatory cytokines. Adipokines are biologically active proteins that may act in an autocrine, paracrine or endocrine fashion (40). At the paracrine level, adipokines are important for the communication between adipocytes and other cell types within the adipose tissue (40). At the systemic level, adipokines may influence a variety of biological processes including energy metabolisms, appetite, reproduction, immune function, angiogenesis and

extracellular matrix metabolism (39). So far, approximately hundred adipokines are discovered (40). The adipokines can be divided into groups according to physiological function (40). Some are classical cytokines, chemokines and growth factors, some are important for appetite and energy balance, some are important for vascular haemostasis while others are important for blood pressure control (40).

As adipose tissue expands, major changes in adipokine production can occur (40). Dysregulated production and secretion of adipokines have been suggested as a potential mediator of the increased risk of CRC associated with obesity.

1.3.3 Chronic inflammation

Inflammation is a physiological response to traumatic, infectious, post-ischaemic, toxic or autoimmune injury caused by a complex set of reactions involving soluble factors and cells of the immune system (48). When the inflammatory process persists it may contribute to tissue destruction and development of disease (48). The first indication of a possible link between inflammation and cancer was described as early as the 19th century (49). Nevertheless, clear evidence linking the two was first obtained last decade (49). Today, it is generally accepted that inflammation and cancer is closely connected. In fact, inflammation plays an important role in several aspects of the cancer process from initiation and tumor promotion, to metastatic progression (49).

Obesity is associated with a low-grade chronic inflammatory state (42). As adipose tissue expands it become infiltrated with macrophages and T-cells, leading to increased production and secretion of pro-inflammatory molecules, both by the adipocytes and the infiltrating immune cells (33). Several pro-inflammatory molecules have been shown to be elevated in obese individuals. For instance, obese individuals tend to have higher levels of C-reactive protein (CRP), tumor necrosis factor (TNF) alpha and interleukin (IL) 6 compared to lean individuals (2).

1.4 Biomarkers as indicators of health and disease

A biological marker, or biomarker can be defined as a characteristic that is objectively measured and evaluated as an indicator of normal physiological processes, pathogenetic processes or pharmacological responses to a therapeutic intervention (50). Biomarkers include a wide range of measures including physiological measurements, blood tests, imaging test as well as tested derived from tissue samples and bodily fluids (51). Biomarkers can be used as indicators of a variety of health and disease characteristics. They can be used as indicators of disease risk, to screen and detect subclinical and clinical disease, to diagnose diseases and specific abnormal conditions as well as to determine disease stage, treatment response and prognosis (52).

Biomarkers can also be used as a substitute for a clinical outcome, a characteristic or variable that reflects how the patients feels, functions, or survives (50). Biomarkers are then referred to as surrogate endpoints, *surrogate* meaning “substitute for”. A surrogate endpoint is expected to predict clinical benefit (or harm or lack of benefit or harm) based on epidemiologic, therapeutic, pathophysiologic, or other scientific evidence (50). Although the ultimate way of determining the effect of a therapeutic intervention is to study the effect on a well-defined clinical endpoint, this may be a highly expensive and time-consuming process requiring large sample sizes. Furthermore, surrogate endpoints may be easier to relate causally to the therapeutic intervention as they are closer in time compared to the clinical outcomes (52).

1.4.1 Obesity-associated biomarkers

Leptin, adiponectin and resistin are obesity-associated biomarkers that have been suggested as potential mediators of obesity associated CRC. The biological function of different adipokines as well as their relation to obesity and CRC risk will be discussed separately in the following section.

Leptin is expressed and secreted in proportion to adipocyte size and number (53). Accordingly, circulating levels of this adipokine is highly positively correlated with measures of obesity (54-56). Leptin was originally discovered as a long-term regulator of food intake and energy balance primary exerting its effect on the central nervous system (57). Today, leptin is widely recognized as an important endocrine molecule, acting both centrally and systemically. At the systemic level leptin is involved in regulation of several physiological

processes including energy homeostasis, metabolic function and inflammation (57). These are all mechanisms suggested as potential mediators of the effect of obesity on CRC risk (42). Leptin has been extensively studied as a potential mediator of obesity related colon cancer (16). In experimental studies, leptin has been shown to induce tumor angiogenesis, cell growth and migration as well as inhibit apoptosis (42). Results from observational studies is however inconclusive (58). Some have found an association between leptin concentration and increased risk of colon cancer and CRC (59, 60), while others have not (57). Leptin exerts its effect by binding to membrane bound receptors. These receptors also circulate in the blood and may affect the bioavailability of leptin (42). Soluble leptin receptors (sOB-R) have been inversely related to conditions associated with CRC risk such as insulin resistance, diabetes and obesity (42). Thus, sOB-R may be an important mediator of the obesity and CRC link (42). In a prospective, nested case-control study within the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort investigating the relationship between leptin, sOB-R and CRC risk (57), sOB-R, but not leptin was strongly inversely associated with CRC and colon cancer.

Adiponectin is an adipokine that is mainly secreted from adipocytes in the adipose tissue (33). In contrast to most other adipokines, circulating levels of this adipokine is shown to be inversely associated with both body fatness (61-63) and abdominal obesity (62). The adipokine is involved in several physiological processes including regulation of energy homeostasis, vascular reactivity, inflammation, cell proliferation and tissue remodeling (64). Adiponectin is suggested to provide a molecular link between obesity and CRC. In experimental studies, adiponectin is shown to inhibit cancer cell growth and to induce apoptosis (64). Furthermore, adiponectin is suggested to effect carcinogenesis indirectly by effecting pathways related to glucose metabolism, insulin resistance and inflammation (64). Despite of this, results from observational studies investigating the association between adiponectin and CRC risk are inconclusive (65, 66). Adiponectin circulates in plasma in three different isoforms including a trimer, a hexamer and a high-molecular weight (HMW) isoform (64). As these isoforms is suggested to have different biological activities, the isoforms may affect CRC risk in a different manner (64). This hypothesis was investigated in a prospective, nested case-control study within the EPIC cohort (64). Results from the study showed a significant inverse association between prediagnosis non-HMW adiponectin and CRC risk, but not for HMW adiponectin and CRC risk, supporting the hypothesis that different adiponectin isoforms may be of different important for the CRC pathogenesis.

Both leptin and adiponectin receptors (ADIPOR1 and ADIPOR2) have been found to be expressed by the colon epithelium (16). This provides support for the potential of adiponectin and leptin to influence regulation of cellular processes within the colon (16). Knowing that also insulin receptor is expressed by the colon epithelium indicates a potential cross talk between these signaling molecules and there target pathways (16).

Resistin is a member of the family of cysteine rich proteins called "resistin-like molecules" (RELMs) (67). It is mainly produced in monocytes and macrophages but is also detectable in adipocytes (67). It circulates in plasma in two forms, one abundant high-molecular weight hexamer and one less abundant but more bioactive trimer (67). The exact physiological function and regulation of resistin is yet to be established (67). Results from experimental studies have indicated a role of resistin in obesity-associated insulin résistance and inflammation (39). However, observational studies investigating the association between resistin, obesity and insulin resistance have shown controversial results (54, 55, 63, 68). In a study by Danese and coworkers including 40 CRC patients and 40 control subjects (67), a positive association was found between circulating resistin levels and CRC risk. The authors suggested that resistin may present a molecular link between inflammation and colorectal carcinogenesis (67).

1.4.2 Soluble urokinase plasminogen activator receptor (suPAR)

Soluble urkinase plasminogen activator receptor (suPAR) is assumed to play an important role in immune activation and in inflammatory processes (69). It is thought to reflect the level of immune activity of an individual, and hence to be a good marker of inflammation (69). suPAR has been suggested as a diagnostic biomarker of several inflammatory diseases and to be of prognostic value for age related diseases such as CVD, DMT2 and cancer (70, 71).

suPAR is the soluble form of the urokinase plasminogen activator receptor (uPAR), a membrane bound receptor connected to the cell surface by a glycolipid anchor (69) (**Figure 4**). Upon binding of urokinase plasminogen activator (uPA) to uPAR, the glycolipid anchor is cleaved, releasing suPAR into the circulation (72). Thus, the difference between uPAR and suPAR is the presence/absence of the glycolipid anchor (73). The uPA/uPAR system is involved in several physiological and pathological processes including regulation of pericellular proteolysis, cellular adhesion, migration and proliferation, as well as in the plasminogen activating pathway (72). The plasminogen activating pathway is thought to play

a key role in activation of cytokines and pro-metalloproteases, as well as in angiogenesis (71). suPAR is thought to regulate the uPA/uPAR system by acting as a uPA scavenger (69). Furthermore, the cleaved receptor is assumed to promote the immune response by acting as a chemotactic agent (69).

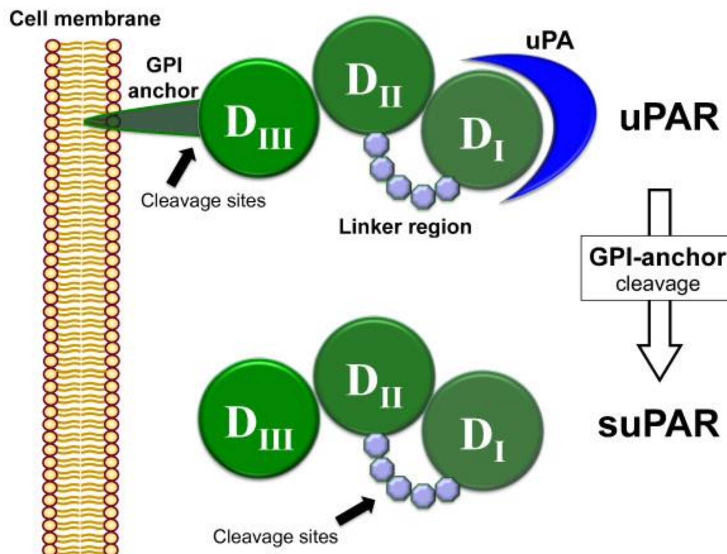

Figure 4 Structure of suPAR. Upon binding of urokinase plasminogen activator (uPA) to urokinase plasminogen activator receptor (uPAR), the glycolipid anchor is cleaved from the protein releasing a soluble form into the circulation. The soluble form is referred to as urokinase plasminogen activator receptor (suPAR). The picture is taken from Donadello et al (74).

Elevated plasma suPAR levels are associated with a number of inflammatory diseases, including bacterial, viral and parasitic infections, autoimmune diseases, as well as certain cancers (70). In a large prospective cohort study from Denmark, involving 2602 individuals aged 41 to 71 years (70), elevated suPAR levels were associated with development of cancer, CVD, DMT2 and mortality. The associations remained robust after adjustment for relevant risk factors of the common diseases and death. Elevated suPAR levels have also been found to predict outcome in CRC patients. In a retrospective study of 591 CRC patients (71), preoperative suPAR levels predicted survival independent of important prognostic markers such as stage, age and carcinoembryonic antigen (CEA), a glycoprotein associated with adenocarcinomas of the colon and rectum. It is therefore of particular interest to investigate the role of suPAR as a prognostic biomarker in CRC patients.

Although suPAR is assumed to play an important role in immune activation and inflammatory processes, its exact biological function is yet to be established (72).

1.5 Biomarkers measured in dried blood spot (DBS)

Regardless of its use, biomarkers should be reliable and reproducible, easy to interpret and well accepted by the patient. The latter is particularly important in clinical research as this may affect the patients' willingness and ability to participate in and complete the trial. Development and validation of minimally invasive methods to access physiological and pathological data is therefore of great importance as this may reduce the participant-burden associated with participation in large clinical trials. Dried blood spot (DBS) represents such a method and has gained much popularity because of its ease and feasibility. In the NSF, dried blood spot (DBS) cards will be the main blood collection method for measuring long-term effects of intervention. Although many biological molecules have been measured in DBS there is still a need to expand the use of DBS to include new and relevant biomarkers such as suPAR.

1.5.1 History and use

Dried blood spots (DBS), drops of whole blood collected on filter paper was first applied by Dr. Robert Guthrie in the early 1960s to measure phenylalanine in newborns (75). This novel application for collecting blood led to the nationwide population screening of newborns to detect treatable, inherited metabolic diseases (76). Today, DBS is used to detect several metabolic diseases, and in Norway screening for as much as 23 metabolic diseases is practised (77). More recently, DBS has been used in large population based studies and clinical trials. Numerous biological molecules have been measured including amino acids, proteins, DNA, lipids, carbohydrates, vitamins, trace elements and toxicological substances (76). Because of the feasibility of blood collection, blood sampling can be performed in a non-clinical environment, by trained technicians or by the participants themselves. Blood samples can then be transported by post to the laboratory where it is stored at appropriate conditions until later analysis. In this way, use of DBS can reduce the participant burden associated with participation in large clinical trials and possibly reduce dropout. Collecting whole blood on filter paper represents an easy and participant-friendly method of collecting blood with great potential in future research.

1.5.2 Sample collection, processing and storage

The procedure for sample collection and processing is relatively easy and straightforward. Whole blood is collected by a simple fingerprick or heelstick with a sterile, disposable lancet. The first drop of blood is wiped away and the subsequent blood drops are applied on filter paper into pre-printed circles. The blood spots are allowed to dry for a minimum of two hours and can then be stacked and stored in to air resistant bags with desiccant before storage. The filter paper matrix is assumed to stabilize most analytes giving some degree of flexibility when it comes to sample collection, processing and storage (75). Still, the rate of sample degradation may vary greatly by analyte, and analyte stability should always be evaluated before sample collection (75).

1.5.3 Analysis of blood spot samples

As the analytes are dried on filter paper, analytes must first be brought into solution before any analyses can be performed. This can be done by placing one or more discs of whole blood into an extraction buffer for a fixed amount of time while mixing the samples. In this way, the dried blood spot is reconstituted as hemolyzed liquid whole blood, and can then be used in multiple assay systems, much as for serum or plasma (75).

A perfect blood spot which fills the whole circle of sample area contains approximately 50 µL whole blood (75). Such a blood spots will yield seven 3.2 mm discs of blood (75). One 3.2 mm disc contains approximately 1.4 µL serum (75). The relatively small sample volume collected increase the demand for highly sensitive and specific detection methods (75). Although multiple discs can be used to increase the quantity of analyte introduced, assays requiring as much as 50-100 µL undiluted plasma or serum will be difficult to adapt to DBS samples (75).

1.5.4 Development and validation of assay protocols

The vast majority of laboratory protocols developed require serum or plasma (75). Analyzing biomarkers in DBS samples will therefore require the development and validation of assay protocols. Elution protocols need to be evaluated to establish the optimal combination of type and amount of elution buffer, eluting method, elution duration and temperature (75). An excellent way of validating DBS samples is to compare assay results from simultaneously collected DBS and plasma or serum samples (75). The relationship is typically analyzed by

linear regression or by inspecting residual plots for evidence of bias or inconsistent variability across the range of measurements (75). Furthermore, protocols should be validated for accuracy, precision, reliability and limits of detection (75). Analyses of analyte stability should also be conducted before starting sample collection as analyte degradation may vary greatly by analyte (75). This is particularly important in situations where blood sampling is performed outside the clinic/study center where samples will be exposed to different temperatures before appropriate storage in laboratory freezers.

2 Aims of the thesis

With a growing prevalence of obesity and CRC it is of great importance to understand the pathophysiological mechanisms linking the two conditions. The primary objective of this thesis was to investigate weight status in relation to comorbidities and biomarkers of obesity and inflammation in a group of CRC patients. In particular, we measured levels of adipokines and cytokines in plasma, and suPAR in DBS samples. As suPAR has not been measured in DBS samples previously, a part of this thesis was devoted to method development and validation of this biomarker.

Specifically, the aims of the master thesis were as follows:

- To develop and validate a method to measure suPAR in DBS with use of a commercially available ELISA assay, and to test for correlations between suPAR and biomarkers of obesity and inflammation in CRC patients pre-surgery.

- To investigate weight status in CRC patients and relate weigh status to biomarkers of obesity and inflammation pre-surgery.

- To compare the prevalence of comorbid conditions among normal weight, overweight and obese participants, and furthermore relate weight-associated comorbidities to biomarkers of obesity and inflammation measured pre-surgery.

- To investigate the relationship between clinicopathological characteristics, in particular tumor localization and TNM stage, and biomarkers of obesity and inflammation in CRC patients pre-surgery.

- To measure the change in weight status from pre- to two months post-surgery and its relation to hand grip strength as a measure of physical function.

3 Materials

This chapter provides a list of materials and equipment used for sample handling and analysis in the laboratory at the department of nutrition.

3.1 Equipment

Equipment	Manufacturer	Location
96 Well Microplates	Greiner Bio-One	Frickenhausen, Germany
Blood Collection Tubes, EDTA and Citrate	Becton, Dickinson and Company (BD)	Oxford, U.K.
Collections paper, 76x108, mm (Cat. no. WHAT10531018)	Whatman	VWR International AS, Norway
Foil-barrier Ziploc Bags (Cat. no. WHAT10534321)	Whatman	VWR International AS, Norway
Folded filters	Whatman	Dassel, Germany
Hole puncher	Whatman	Sanfold, USA
Microtubes and tubes, 2 and 50 ml	Sarstadt Ag & Co	Nümbrecht, Germany
Serological pipettes, 5, 10 and 15 ml	Becton, Dickinson and Company (BD)	Franklin Lakes, NJ USA
Thermowell Sealers	Corning Incorporated	Flintshire, U.K.

3.2 Chemicals

Chemical/compound	Manufacturer	Location
BioRad Protein Assay	Bio-Rad Laboratories, Inc	Hercules, USA
Ethanol	Arcus Kjemi AS	Drøbak, Norway
Milli-Q water	Millipore	Molsheim, France
Phosphate buffered saline	Sigma-Aldrich	Gillingham, U.K.
Protein Standard (BSA)	Bio-Rad Laboratories, Inc	Hercules, USA
Sheath Fluid	Bio-Rad Laboratories, Inc	Hercules, USA
Tween-20 Detergent	Millipore	Darmstadt, Germany

3.3 Kits

Kit name	Manufacturer	Location
Milliplex® MAP Human Adipokine Magnetic Bead Panel 1 (Cat. no. HADK1MAG-61K)	Millipore	Missouri, USA
Milliplex® MAP Human Adipokine Magnetic Bead Panel 2 (Cat. no. HADK2MAG-61K)	Millipore	Missouri, USA
suPARnostic® Standard kit (Cat. no. A001)	Virogates	Birkerød, Denmark

3.4 Instruments

Instrument	Manufacturer	Location
Bio-Rad Bioplex (100)	Bio-Rad Laboratories, Inc	Hercules, CA, USA
Branson 2510 Ultrasonic Cleaner	Cleanosonic	Richmond, USA
Multiscreen Vacum Manifold	Millipore	Missouri, USA
Polymax 1040 Microtiter shaker	Heidolph Instruments GmbH & Co	Schwabach, Germany
SL 40R Centrifuge	Thermo Fisher Scientific	Osterode, Germany
Thermo Scientific Multiskan® EX 2.3	Genesis Bio Solutions	Andhra Pradesh, India
Titertek Multiskan PLUS MK II	Rolla Biotech	Anaheim, USA
Vortex Genie-2 Mixer	Scientific Industries, Inc	New York, USA
Wellwash® 4 Mk 2	Genesis Bio Solutions	Andhra Pradesh, India

3.5 Software

Software package	Manufacturer	Location
Bio-Plex Manager software 6.0	Bio-Rad Laboratories, Inc	Hercules, CA, USA
Endnote X6	Thomson Reuters	California, USA
IBM SPSS Statistics 20	SPSS Inc	Chicago, USA
Labsystems Genesis 3.3	Genesis Bio Solutions	Andhra Pradesh, India
Microsoft Office XP	Microsoft Corporation	Redmont, WA, USA
Unscrambler® X 10.2	Camo Software AS	Olso, Norway

4 Subjects and methods

This thesis is part of the NFS, a large randomized controlled intervention trial on the effect of diet and physical activity after surgery for colorectal cancer. The study is a collaborative project between the department of nutrition at University of Oslo and hospitals in the Helse Sør-Øst region of Norway. The main objective of the NFS is to investigate whether a diet and lifestyle intervention will reduce the risk of morbidity and mortality in a group of CRC patients. Effect of intervention will also be studied on intermediate endpoints and other biomarkers.

4.1 Subjects

Patients were recruited from Oslo University Hospital in the time period spring 2012 to spring 2013. Men and woman aged 50-80 years with an assumed stage I-III CRC (pathological stage first confirmed after surgery) were invited to participate. Participants were invited in connection with their CRC surgery. Together with the invitation letter, participants received a letter of informed consent, which they signed the day before surgery.

By May 2013, 55 patients were invited to the study. Of these patients, 42 decided to participate. One participant withdrew consent after inclusion and patient material was thereby destroyed. The remaining study population consisted of 41 participants. Of these participants, 25 had started the intervention period. A flow chart of the recruitment and inclusion process including relevant samples and tests is shown in **Figure 5**.

For method development and validation, material from nine healthy volunteers from the research group was used. The samples were made anonymous according to the regulations from National Committee on pilot studies on method development, and used for the purpose of method development and validation only.

The Norwegian Foods study is registered in ClinicalTrial.gov (ID no. NCT01570010) and is approved by The National Committees for Research Ethics in Norway (REC no. 2011/836).

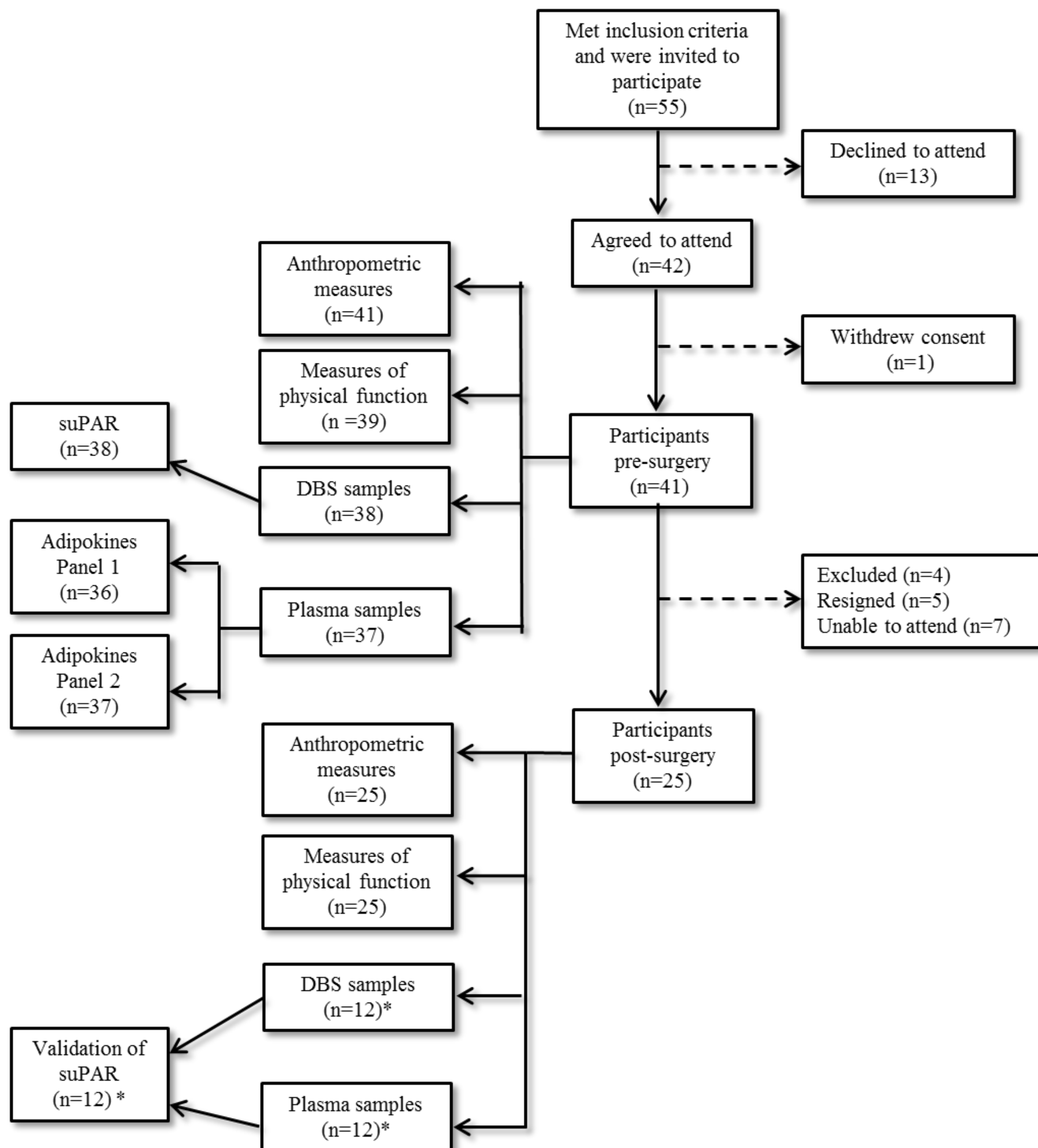

Figure 5 A flowchart of the recruitment and inclusion process including relevant samples and tests. By May 2013, 55 patients were invited to the study. Of these, 42 decided to participate. One patient withdrew consent after recruitment and patient material was thereby destroyed. The remaining study population consisted of 41 participants. Of these, 25 had completed baseline of intervention by May 2013. Measures of weight status and physical function were assessed pre-surgery and at baseline of intervention. Biomarkers of obesity and inflammation were measured in blood samples collected pre-surgery. Blood samples collected at baseline of intervention and at 6 months follow-up were used to validate suPAR. *Two of the twelve DBS and plasma samples were collected at 6 months follow-up.

4.3 Study design

The NFS is a multicenter randomized controlled, parallel two-arm one-year intervention trial. Participants were recruited one day prior to surgery (hereby referred to as pre-surgery) and later randomized to either one of two intervention groups, intervention group A (receiving a diet and physical activity intervention) or intervention group B (receiving a physical activity intervention). About two months after surgery, participants were invited to baseline of intervention. Participants are thereafter followed at 6 and 12 months, and at 1, 3, 5, 7, 10 and 15 years. A schematic overview of the study design with corresponding samples and tests is presented in **Figure 6**.

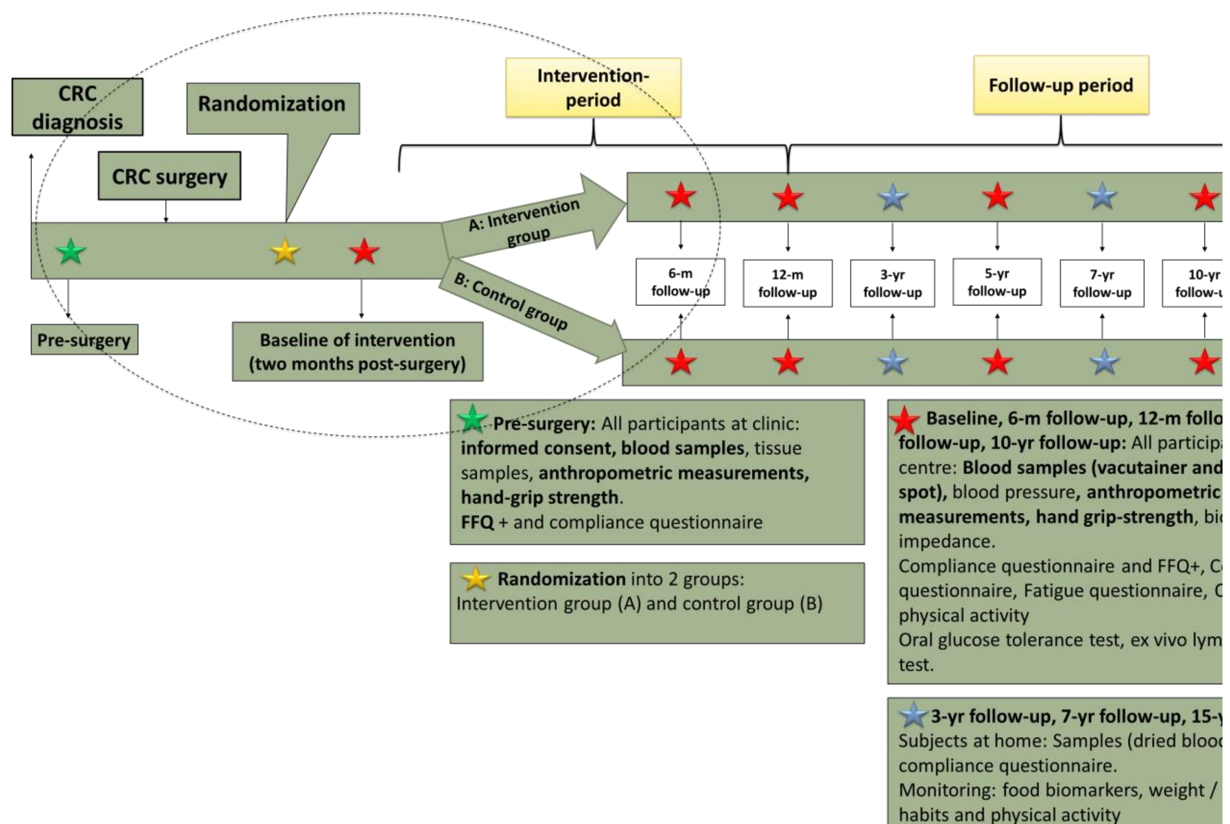

Figure 6 An overview of the study design with corresponding samples and tests. The NFS is a randomized controlled trial including a one-year intervention period. Patients are invited to the NFS in connection with CRC surgery (referred to as pre-surgery). They are then randomized to intervention group A (diet and physical activity) or intervention group B (physical activity). Two months post-surgery patients are invited to the study centre for baseline of intervention. Participants are then followed at 6 and 12 months, and at 1, 3 5, 7, 10 and 15 years. The assessment period relevant for the master thesis is highlighted in the figure. Furthermore, relevant assessment methods are marked in bold. The figure is taken from the project description of the NFS with permission.

The focus of the master thesis was on data collected before intervention start, more specifically on data collected pre-surgery and at baseline of intervention i.e. two months post-surgery.

To perform the measurements pre-surgery, patients were visited at the patient hotel at Oslo University Hospital one day prior to CRC surgery. The measures undertaken included weight, height (self-reported), waist and hip circumference, handgrip strength as measurement of physical function and blood samples (DBS and plasma samples). Patients were also asked to fill out a food frequency questionnaire (FFQ). All measurements were performed by trained nutritionists or scientists except the venipuncture which were performed by the nurses at the hospital. Baseline measurements and measurements at six months follow-up were carried out at the study center at Department of Nutrition, University of Oslo. Measures undertaken at baseline of intervention relevant to the master thesis included weight, height, waist and hip circumference, handgrip strength and blood samples (DBS and plasma samples). From the six months follow-up only blood samples (DBS and plasma samples) were used in the thesis. As for the measurements at the patient hotel pre-surgery, all measurements were performed by trained nutritionists or scientists except for the blood sampling which were performed by a medical laboratory technologist.

4.4 Assessment methods

4.4.1 Assessment of personal and clinicopathological characteristics

Information about personal and clinicopathological characteristics was collected from FFQs and medical records. Information about smoking status was available from FFQs (data processed by Mari Bøe Sebelien), while information about cancer localization, TNM stage and comorbidity was available from medical records. Cancer localization and comorbidity was assessed by use of the International classification of diseases (ICD) 10 coding system. Comorbid conditions registered without a ICD 10 code was also recorded. All comorbidities registered in connection with diagnosis and hospitalization was included. Comorbidities were grouped into the following categories: COPD, CVD (including coronary artery disease, aorta stenosis, pulmonary embolism, atrial fibrillation and atrial flutter, arrhythmia and conduction disorders), hypertension, DMT1, DMT2, hyperlipidemia, liver disease, kidney disease (including kidney stones and kidney failure), gastrointestinal diseases (including inflammatory bowel disease, gastroesophageal disease with esophagitis, esophageal hernia, umbilical hernia, celiac disease and irritable bowel disease), musculoskeletal and connective tissue diseases (including polyarthritis, spondylarthritis, Sjögren syndrome and osteoporosis), hematological diseases (included thrombocytopenia), neurological diseases (including polyneuritis) and other cancers (included breast cancer and prostate cancer). When possible, pathological staging (TNM stage assessed by the pathologist after surgery) was used. In the remaining cases, stage was determined based on a consultation with the surgeon. Results from routine blood samples taken pre-surgery (including CEA, CRP and glucose) were obtained from medical records.

4.4.2 Assessment of weight status and physical function

Weight, height and BMI

BMI is a simple index of weight-for-height used to measure adiposity. It is defined as the weight in kilograms divided by the square of the height in meters (kg/m^2) (30). According to World Health Organization (WHO), BMI can be categorized into the following categories: < 18.5, 18.5 to 24.9, 25.0 to 29.9, 30.0 to 34.9, 35.0 to 39.9 and 40.0 or more (78). These

categories correspond to “underweight”, “normal weight”, “overweight”, “obesity grade I”, “obesity grade II” and “obesity grade III”. BMI values are independent of age and can be used for both sexes (79). The same values are also recommended to be used worldwide, although BMI values may not respond to the same degree of fatness in different populations (79). This makes BMI an easy and practical measure to determine body fatness. For statistical comparison in the thesis, the categories normal weight, overweight and obesity (grade I, II and III combined) were used.

To measure the participant’s weight, a Marsden MS-4203 digital portable scale was used. Weight was measured with light clothing, empty pockets and with shoes taken off. Since weight was measured with cloths, a value of 0.5 kg was subtracted from all weight measurements. Both measurements pre-surgery and at baseline of intervention were performed in the morning. Measurements pre-surgery were performed in fasting and non-fasting participants. At baseline of intervention all participants were fasting. All measurements were recorded to the nearest 0.1 kg.

Height was self-reported pre-surgery and measured at baseline of intervention. The measurements were performed with the participant standing firmly against the wall with a straight back and head in a horizontal position. Height was recorded to the nearest 0.1 cm. Among patients that had completed baseline of intervention (n=25), measured height was used to calculate BMI. In patients where only self-reported height was available, a value of 1.35 cm (the mean difference between self-reported and measured height) was subtracted from the self-reported value.

Waist and hip circumference

Assessment of waist and hip circumference is an easy and inexpensive way to measure body fat distribution (42). According to WHO, a waist circumference above 94 and 80 cm for men and woman, respectively is associated with an increased risk of metabolic complications (78). A waist circumference above 102 cm for men and above 88 cm for woman is associated with a further increased risk of metabolic complications (78). For waist/hip-ratio, this corresponds to values above 0.9 and 0.85 cm for men and woman, respectively (78).

Waist and hip circumference were measured with a stretch resistant tape at a level parallel to the floor. Waist circumference was measured at the midpoint between the top of the iliac crest and the lower margin of the last palpable rib in the mid axillary line. Hip circumference was

measured at the largest circumference at the buttocks. All measurements were performed in the morning. As for weight, measurements pre-surgery were performed in fasting and non-fasting participants, while measures at baseline of intervention were performed in fasting participants. Measurements were recorded to the nearest 0.1 cm.

Physical performance

Measuring hand grip strength is a simple and non-invasive method to study muscle function of the upper extremities (80). It has been shown to correlate closely with whole body protein, body cell mass, anthropometrically measured muscle mass and BMI (80). Interestingly, it has been shown to respond earlier to nutritional deprivation than body composition parameters do, and hence become a popular marker of nutritional status (80). Impaired hand grip strength has proven to be a good marker of increased postoperative complications, increased length of hospitalization, higher rehospitalisation rate and decreased physical status (80).

Maximal voluntary handgrip strength was measured using a portable MAP handgrip dynamometer. The participants were asked to sit upright with the upper arm placed beside the trunk and with the elbow flexed to approximately 90’. The participants were then instructed to perform a maximal isometric contraction. Maximal handgrip strength was recorded to the nearest 0.1 kg. Test of maximal handgrip strength was performed on both arms. Different spring sets were used for men and woman; an 80 kg string set was used for men while a 40 kg string set was used for women.

4.4.3 Laboratory analysis

Blood collection, processing and storage

Arterial whole blood was collected on filter paper by a simple finger-prick performed by trained technicians. The participant’s finger was cleaned with alcohol, and then pricked with a sterile, disposable lancet. The first drop of blood was wiped away, and the subsequent blood drops were applied on filter paper. Samples were allowed to dry for at least two hours kept away from direct sunlight, and were then stacked and stored at -80’C with desiccant in aluminum bags.

Venous whole blood was collected by venipuncture in blood collection tubes added anticoagulants and cooled on ice. Citrate and EDTA samples were centrifuged for 15 minutes at 1500 and 3000 g, respectively. The supernatant liquid were removed and immediately frozen at -80´C. At day of analysis, samples were completely thawed and then properly mixed prior to use.

Extraction of protein from DBS

To optimize the protocol for protein extraction from DBS, protein was extracted under different conditions. Different number of discs (1-3 discs) were punched out from DBS card and placed in microtiter wells with different amount (50-200 uL) and type of extraction buffer. The extraction buffers PBS, PBS plus 0.01 % Tween-20 (a detergent used to solubilize membrane proteins) and PBS plus Brondiox (a preservative used to conserve proteins) was tested. Extractions were sonicated on an ultrasonic cleaner for ten minutes with or without the use of a multiplate shaker. The combinations were tested on extractions each made from 3.1 mm punches of filter paper containing dried whole blood from healthy volunteers.

Measuring protein concentration in DBS

To determine which extraction conditions that gave the highest amount of extracted protein, protein was measured using the BioRad protein assay. This is a simple and accurate method of quantifying protein based on the binding of the dye Coomassie brilliant blue G-250 to basic and aromatic amino acid residues. Binding of the acidic dye to the amino acid residues creates a shift in the absorbance maximum from 465 to 595 nm, which results in a color change in proportion to the amount of protein bound. Absorbance is measured using a microtiter plate reader at 595 nm. Protein concentration is then calculated based on the standard curve of a protein standard representative of the protein solution tested.

Protein concentration was measured according to the manufacturer’s instructions. In brief, the dye solution was prepared by diluting one part of the dye Coomassie brilliant blue G-250 with four parts distilled water, and then filtered through a Whatman filter. Protein standards were made from bovine serum albumin (BSA) in the concentrations 0.041, 0.061, 0.091, 0.150, 0.273 and 0.410 mg/ml. Protein samples were diluted with distilled water in the range 1:100-1:200, and then added to the microtiter plate together with standards and control in volumes of 10 µL. A volume of 200 µL of the diluted dye solution was then added to each microtiteter

plate well, and samples and reagent solution was mixed gently. Absorbance was measured at 595 nm. All protein solutions were assayed in duplicate or triplicate.

Measuring suPAR

suPAR was measured by the suPARnostic® ELISA assay, a commercially available immunoassay used for quantitative determination of suPAR in human plasma. The assay is a simplified double monoclonal antibody sandwich assay that utilizes monoclonal mouse and rat antibodies against human suPAR to determine suPAR concentration. Samples with unknown amount of suPAR are mixed with peroxidase-conjugated anti-suPAR, and then added to microwells precoated with anti-suPAR. During a one-hour incubation period, a sandwich consisting of a solid-phase antibody, suPAR and the peroxidase-conjugated antibody is formed. Unbound material is washed away, and a chromatographic substrate is added. A blue color develops in proportional to the amount of suPAR in the sample. The color development is stopped by the addition of sulphuric acid, which changes the color in the wells from blue to yellow. The absorbance is measured at 450 nm using a microtiter plate reader. suPAR concentration is then calculated on the basis of a calibration curve made from the suPAR standards included in the kit.

suPAR was measures with a slight modification of the protocol by the manufacturer: Four discs of whole blood were punched out from the filter paper blood spots using a paper punch and placed into 190 µL of dilution buffer (PBS + 0.05 % Brondiox). The extractions were sonicated for ten minutes, and 137.5 µL of the extracted solution were added to each well in the white microtiter plate. For plasma samples, standards, curve control and blank samples, 25 µL were added to each microtiter plate well followed by the addition of 112.5 µL dilution buffer. A volume of 112.5 µL peroxidase-conjugate solution (twice as concentrated as recommended) was added to each well, and contents were then mixed gently. A 100 uL aliquot of the mixed contents were transferred in duplicate into the clear coated plate and incubated for one hour at room temperature in the dark. Wells were washed five times with 250 µL of wash buffer, followed by 20 minutes incubation with 100 µL TMB substrate in the dark. The reaction was stopped by adding 100 µL stopping solution into each well. The absorbance was read at 450 nm.

Assay performance was evaluated by comparing results from paired, simultaneously collected DBS and plasma samples from 12 CRC patients. Intra-assay imprecision was calculated based on the coefficient of variation (CV; standard deviation divided by mean) of four different

blood spots from one individual run in replicates (n=4). Stability was evaluated by temporary storing filter paper cards from nine healthy volunteers in room temperature for different amount of time before storage at -80´C. The detection limit set by the manufacturer (0.1 ng/ml) was used as the detection limit for all assays run.

Measuring adipokines and cytokines

Adipokines and cytokines were measured using MILLIPLEX® MAP assays based on the Luminex® xMAP® technology, a technology that makes it possible to perform a variety of immunoassays at the same time on the surface of fluorescent-coded magnetic beads, called microspheres (**Figure 7**). These microspheres are internally colored with two fluorescent dyes giving each set of microspheres a unique color signature. The individual sets of microspheres are coated with different capture antibodies, making it possible to capture several analytes in one single assay. After the analytes of interest are captured by the different capture antibodies, a biotinylated detection antibody is introduced. The reaction mixture is incubated with strepdavidin-phycoerythrin conjugate, a reporter molecule that completes the reaction on the surface of each microsphere. The microspheres are then passed through two lasers, one excites the internal dye marking the microsphere set, and one excites the fluorescent dye on the reporter molecule. By using high-speed digital-signal processors it is then possible to both identify and quantify the different analytes based on the fluorescent dyes.

Adiponectin, adipsin, lipocalin-2, plasminogen activator inhibitor-1 (PAI-1) (total) and resistin were measured in citrate plasma by the Human Adipokine Magnetic Bead Panel 1 assay from Millipore. The measurements were performed according to the manufacturer's protocol. Before start of the protocol, citrate plasma was completely thawed, and then centrifuged to remove any particulates. A 96-well filter plate was prepared by prewetting the wells with 200 μL of assay buffer. A volume of 25 μL of assay buffer was added to each well, followed by the addition of 25 μL of standards, controls and plasma samples (diluted 1:400 in assay buffer) in duplicate. A volume of 25 μL antibody-immobilized beads was added to each well and the capture antibodies were allowed to react with their corresponding antigens during incubation on a plate shaker overnight at 4'C. Unbound material was removed from the beads by filtration on a Multiscreen Vacum Manifold from Millipore. The beads were then washed three times with 200 μL washing buffer per well. A mixture of 50 μL biotinylated detection antibodies was added to each well and allowed to incubate for one hour in room temperature. A 50 μL volume of strepdavidin-phycoerythrin was added to each well and incubated for an

additional 30 minutes. The beads were finally washed three times with 200 µL washing buffer per well. A volume of 100 µL sheath fluid was added to each well. After five minutes of shaking, samples were analyzed on the Luminex 200^{TM} according to the manufactures instructions. Concentration of analyte was calculated based on the calibration curves made from the standards included in the kit. All samples gave measurable analyte concentrations except for two samples where adiponectin concentration was above the upper limit of quantification. These samples were given the value of the upper limit of quantification.

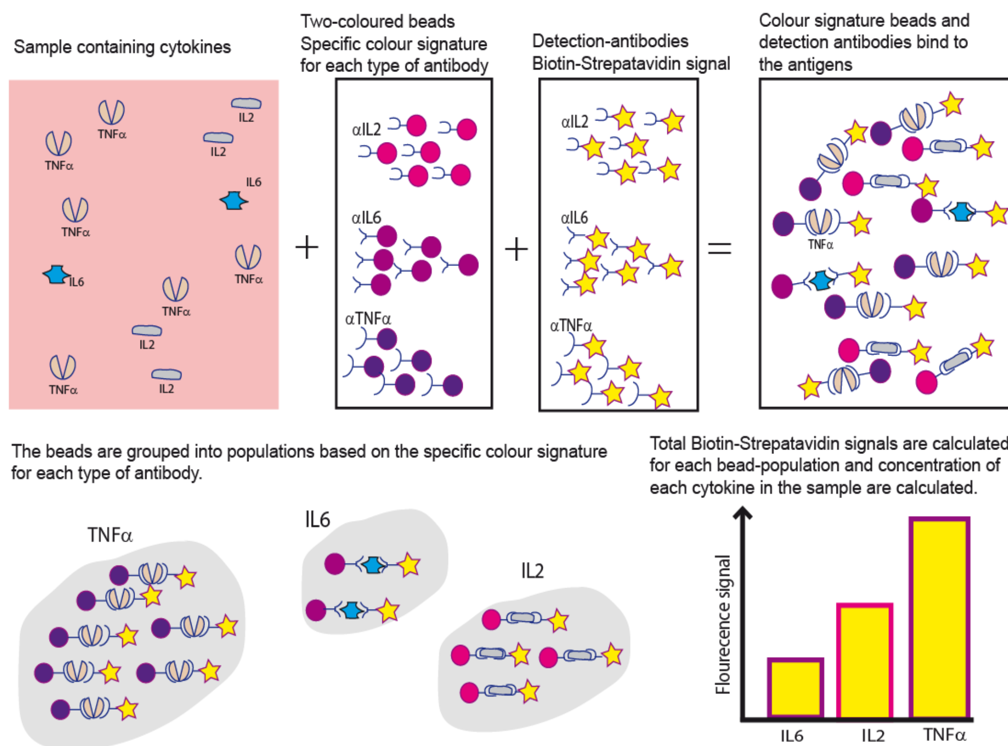

Figure 7 Overview of the multiplex technology. The figure shows how several biomarkers, here illustrated by TNFα, IL-2 and IL-6 can be measured at the same time with use of fluorescent-coded magnetic beads, called microspheres. These microspheres are internally colored with two fluorescent dyes giving each set of microspheres a unique color signature. The individual sets of microspheres are coated with different capture antibodies, making it possible to capture several analytes in one single assay. After the biomarkers of interest are captured, a biotinylated detection antibody is introduced. The reaction mixture is incubated with strepdavidin-phycoerythrin conjugate, a reporter molecule that completes the reaction on the surface of each microsphere. The microspheres are then passed through two lasers, one that excites the internal dyes marking the microsphere set, and one that excites the fluorescent dye on the reporter molecule. The different biomarkers are then identified and quantified based on the Biotin-Strepdavidin signal. The figure is taken from Siv Kjølsrud Bøhn, PhD thesis, with permission (81).

Leptin, insulin, nerve growth factor (NGF), hepatocyte growth factor (HGF), monocyte chemoattractant protein-1 (MCP-1), TNF-α, IL-1β, IL-6 and IL-8 were measured in citrate plasma by the Human Adipokine Magnetic Bead Panel 2 assay from Millipore. The measurements were performed according to the manufacturer's protocol. Before start of the protocol, citrate plasma was thawed, and then centrifuged to remove particulates. A 96-well

filter plate was prepared by prewetting the wells with 200 µL of assay buffer. A volume of 25 µL assay buffer and serum matrix was added to sample wells and standard and control wells, respectively. A 25 µL volume of standards, controls and plasma samples (neat) was added to the appropriate wells in duplicate. A mixture of 25 µL antibody-immobilized beads was added to each well and the contents were allowed to incubate on a plate shaker overnight at 4'C. Unbound material was removed from the beads by filtration. The beads were then washed three times with 200 µL washing buffer per wells. A mixture of 50 µL biotinylated detection antibodies was added to each well and the contents were allowed to incubate for one hour in room temperature. A 50 µL volume of strepdavidin-phycoerythrin was added to each well and incubated for an additional 30 minutes. The beads were finally washed three times with 200 µL washing buffer and a volume of 100 µL sheath fluid was added to each well. After five minutes of shaking, samples were analyzed on the Luminex 200™ according to the manufactures instructions. Concentration of analyte was calculated based on the calibration curves made from the standards included in the kit. For all analytes measured, analyte concentrations in samples were within the area of quantification, except for IL-1β where ten samples gave analyte concentrations below the lower limit of quantification. Values for IL-1β were therefore extrapolated based on a standard curve including the blank sample to get measurable data.

4.6 Statistical analysis

Normally distributed variable are expressed as mean with 95 % confidence intervals, while non-normally distributed variables are expressed as median with quartiles, calculated by the "Tukey's Hinges" method. Categorical variables are expressed by frequencies. *P*-values below 0.05 were considered significant. All statistical analyses were conducted with SPSS Statistics for Windows, Version 20.0.

The participants were divided into three BMI groups (normal weight, overweight and obesity) as described in the method section. The groups were compared with regards to personal and clinicopathological characteristics by the use of Fishers exact two-tail probability test for categorical variables. Post hoc analysis was conducted to detect which groups were significantly different. The same analysis was conducted according to age groups.

Several plasma biomarkers were not normally distributed and more skewed. Variables were thereby normalized by logarithmic transformation (strictly not necessary for suPAR, resistin, MCP-1 and TNF-α) before further statistical modeling. The variables CEA, CRP and glucose all included outliers disturbing the distribution of values in the study population. All analysis including these variables was therefore conducted with and without these outliers.

The associations between anthropometric measures (body weight, BMI, waist circumference and waist/hip-ratio) and plasma biomarkers (adipokines, cytokines/chemokines, insulin, CRP, suPAR and glucose) were analyzed by linear regression. Data were checked for normality, linearity and homoscedasticity of residuals to ensure that assumptions were not violated. Biomarker concentration was treated as the dependent variable while the anthropometric measures were treated as independent variables. The effect of anthropometric measures on biomarker concentration was expressed as ln (regression coefficient (b)) with ln (95 % CI) and p-value. Analyses adjusting for age and sex in the model were also conducted. Significant interaction terms between independent variables were included in the model. Both the unadjusted and adjusted regression coefficients with corresponding 95 % CI and p-values are presented in the table.

Levels of plasma biomarkers were also compared across tumor localization and prognostic groups based on TNM stage (I +II and III + IV) by linear regression. As for the previous analysis, data were checked for normality, linearity and homoscedasticity of residuals to ensure that assumptions were not violated. Biomarker concentration was treated as the

dependent variable while tumor localization and TNM-stage group were treated as independent variables. Analyses adjusting for age and waist circumference in the model were also conducted. Waist circumference was chosen as this anthropometric measure was shown to be most strongly associated with plasma biomarkers in the previous analysis. Sex was not adjusted for as sex distribution was approximately the same between groups compared. Results from the analysis are presented with unadjusted median with Q1-Q3. Both the unadjusted and adjusted *p*-values are presented.

Logistic regression was performed to determine the impact of plasma biomarkers on hypertension. Hypertension was treated as the dependent variables and plasma biomarkers as independent variables. Analysis was conducted without covariates in the model (model 1) and adjusting for age (model 2). Waist circumference was not adjusted for as this variable was highly correlated to several of the biomarkers analyzed. Results from the regression analysis are expressed as ln odds ratio (OR) with ln (95 % CI) and *p*-value for the two models.

No adjustments were made for multiple statistical comparisons in the regression models. Most of the multiple testing correction methods assume independency between tests (here biomarkers). Correction for multiple testing by these methods may therefore not be optimal in our analysis as several of the biomarkers tested were highly correlated. We therefore report nominal *p*-values for all regression analysis conducted.

Correlations between the different plasma biomarkers were investigating by Spearman´s rank correlation analysis. Correlation coefficients and level of significance are presented in the table. Correlations between the different biomarkers, BMI and waist circumference was also investigated by conducting principal component analysis (PCA) with use of Unscrambler, Version 10.2 from Camo Software AS. The main idea of PCA is to reduce the complexity or dimensions of large datasets (**Figure 8**). In a multidimensional space each individual can be defined by a large number of parameters. In the results section (**Figure 13**, score plot) the data including all measured biomarkers, BMI and waist circumference has been reduced into two dimensions: principal component (PC) 1 and PC2. The score plot visualizes how much the subjects are inter-related. Together they explain 48 % of the variation in the data. The *correlation loading* plot shows the importance of the different variables for the PCs. Variables that lie close to each other are correlated whereas if variables lie on opposite sides of the plot they would be inversely correlated. The variables lying in the outer circle have largest influence on the PCs. If a variable lie in the outer circle and is close to PC1 it is important for

PC1. If a variable lie in the outer circle and is close to PC2 it is important for PC2. Any placement in the outer circle between PC1 and PC2 means that the parameter influences both PCs.

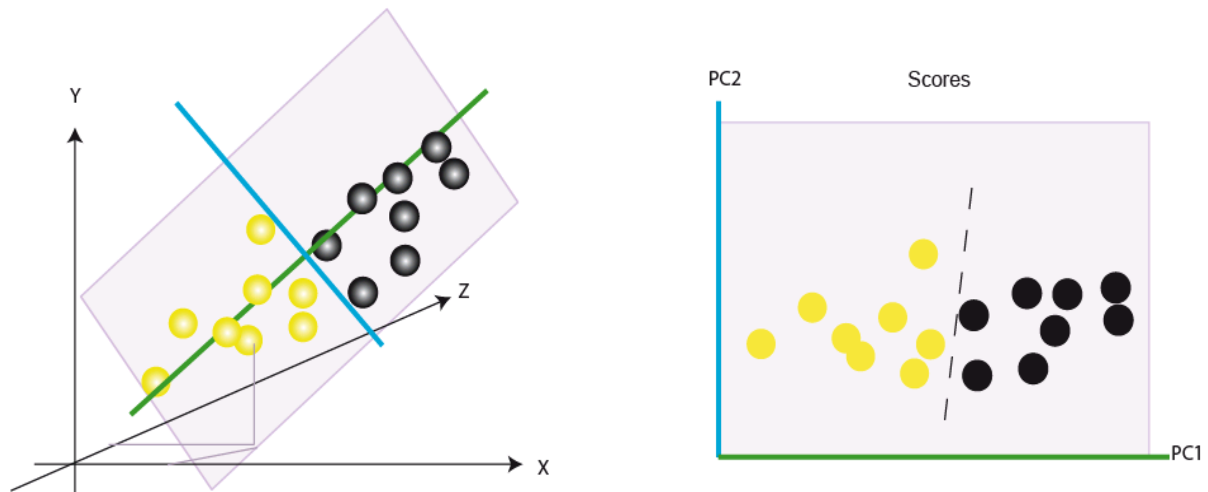

Figure 8 Illustration of PCA on three dimensions. Each individual is represented by a point in the three-dimensional space (left panel). The first principal component (PC1) is placed to account for as much of the variability in the data as possible (green line). Then PC2 accounts for as much as possible of the remaining variation (blue line). Each point is then projected into a two-dimensional space to obtain a scoring plot. The yellow and black spots represent individuals of two groups (e.g. different treatment) and the PC1/PC2 scoring plot separates the two groups. The figure and figure text is taken from Siv Kjølsrud Bøhn, PhD thesis, with permission (81).

Postoperative changes in anthropometric measures (weight, BMI, waist and hip circumference and waist/hip-ratio) and measures of physical function (handgrip strength measured in both arms) were analyzed by paired sample t-test or Signed rank test dependent on the distribution of the variables. Weight, BMI, waist and hip circumference and waist/hip-ratio were considered normally distributed and analyzed by paired sample t-test. The variables handgrip strength right and handgrip strength left were not normally distributed or symmetric and thereby analyzed by the non-parametric Signed rank test. Correlation analysis was conducted between anthropometric variables and variables of physical function, and between change in anthropometric variables and change in physical function.

The effect of experimental condition on protein amount extracted from DBS was analyzed by one-way-ANOVA and Mann Whitney U tests. Total protein amount in samples with different number of discs and buffer volumes were compared by one-way-ANOVA. Protein amount was treated as the dependent variable and number of discs and volume extraction buffer as the grouping variables. Post hoc analysis was performed with Bonferronis' correction. One-way ANOVA was also conducted to investigate the effect of buffer type on total amount of

protein. Protein amount was treated as the dependent variable and type of buffer as the grouping variables. Post hoc analysis was performed with Bonferronis' correction. Total protein amount in samples exposed to different mixing method was compared by Mann-Whitney U test as protein amount was not normally distributed across all groups of samples tested.

To determine the number of participants required to validate suPAR, a statistical formula for sample size calculations based on correlations was used. Expected correlation coefficient was included in the formula together with the desired power and significant level (set to 0.80 and 0.05, respectively). Comparable validation studies have obtained correlation coefficients in the range 0.88-0.99 between DBS and plasma/serum samples (82-91). To detect correlation coefficients in this range, about six to eight participants were needed. We anticipated that it could be difficult to obtain such correlation coefficients. Therefore a total of twelve participants were included in the validation analysis.

suPAR concentration in plasma and DBS samples was normally distributed in the CRC population (n=12). suPAR concentration measured in DBS and plasma samples were compared by Pearson correlation, and absolute levels were compared by linear regression and Bland-Altman analysis. Stability of suPAR was evaluated by performing a one-way ANOVA analysis across the different time-periods of sample storage (0, 1, 2 and 3 weeks).

4.7 My contribution to the research project

The NFS is a large and complex randomized controlled trial. The planning and preparation of the trial, recruitment and follow-up of patients, as well as the data sampling, processing and analysis require coordination of a large number of scientists and technicians. During this master period I have been part of this large organization and contributed to many aspects of the NFS. In addition to have the privilege to learn about running a randomized controlled trial, the study has also been the primary source of data for my master thesis. An overview of my contribution to the research project relevant to this master thesis is presented in **Table 2**.

At the time period the present thesis was conducted the project group of the NFS consisted of Professor Rune Blomhoff, post doc Siv Kjølsrud Bøhn, post doc Ingvild Paur, PhD Hege Berg Henriksen, PhD Hanna Ræder, laboratory technician Siv Åshild Wiik, research assistant Kristine Lillebø Holm and the master students Anne Juul Skjetne and Mari Bøe Sebelien and myself.

Table 2 Contributions directly related to this master project

Work assignment	Description	Responsible
Recruiting patients	Invite patients at the hospital for recruitment to the NFS	Project group, incl. Ane
	Undertake anthropometric measurements, hand grip strength and blood samples, deliver FFQ	
	Laboratory work including processing and storage of whole blood and plasma collected on filter paper cards and in vacutainer tubes, respectively.	
At Baseline of intervention and 6 month follow-ups	Undertake anthropometric measures, hand grip strength and blood samples	Project group, incl. Ane
	Laboratory work including processing and storage of whole blood and plasma collected on filter paper cards and in vacutainer tubes, respectively	
Screening of medical records	Record information about clinicopathological characteristics including tumor localization, TNM stage, comorbidity and routine blood samples from medical records	Hanna, Ane
Laboratory analysis	Develop and validate a method to measure suPAR in DBS by the use of a commercially available ELISA assay	Siv, Ane
	Measure suPAR in DBS samples collected pre-surgery with use of the established DBS ELISA assay method	
	Aliquotation of stored plasma samples collected pre-surgery to the multiplex analysis	
	Measure adipokines and cytokines in plasma samples collected pre-surgery by the use of multiplex technology	
Statistical analysis	Conduct PCA	Siv
	All other statistical analysis	Ane

5 Results

5.1 Method development and validation

Collecting blood on filter paper by the fingerprick method is an easy and participant-friendly method of collecting blood, associated with several advantages, including ease of sample collection, processing and storage. Although DBS have been used for public health purposes in almost 40 years, use of DBS in population-based and clinical research is still in the early phases. To be able to utilize DBS as an analytical platform for this purpose, assay protocols need to be adapted and validated prior to use. Thus, an aim of this thesis was to develop and validate a method of analyzing suPAR in DBS. suPAR was chosen as this is a new and promising prognostic biomarker associated with several inflammatory conditions.

5.1.1 Extraction of protein from DBS

Because the suPARnostic® ELISA assay is developed and validated for measuring suPAR in plasma, the assay procedure needed to be adapted for use on DBS samples. The first step in this process was to develop a procedure for protein extraction that would provide similar amount of protein from DBS to that of plasma samples. DBS samples from healthy volunteers were exposed to different experimental conditions (different number of discs and buffer volumes) for protein extraction. Total protein was then measured to evaluate which condition that gave the highest concentration of protein and highest total amount of eluted protein.

In the first experiment, one, two and three discs were eluted in 50, 100, 150 and 200 µL extraction buffer (PBS + Tween-20). The choice of extraction buffer was based on three papers reporting on biomarkers extracted from DBS (82, 84, 91). The protein concentration increased significantly with the number of discs for all buffer volumes (**Figure 9A**). Not unexpected, the concentration of protein was highest in samples with the lowest buffer volumes (50 µL). However in the 50 µL and 100 µL samples less protein were eluted as compared to the samples with larger volumes (150 uL and 200uL), as demonstrated by the lower amount of total protein in the samples (**Figure 9B**). In samples containing one disc, there was no significant difference in total protein amount between the samples eluted in 50, 100, 150 and 200 µL extraction buffer. In samples containing two and three discs there was a

significantly lower amount of protein extracted in both the 50 ($p<0.001$) and 100 µL ($p<0.001$) sample compared to the 200 µL sample. In samples containing two discs, there was also a significantly lower amount of protein extracted in the 150 µL sample compared to the 200 µL sample ($p<0.001$), but this was not apparent for the samples containing three discs. The results indicate that a volume above 150 µL is needed to extract the maximum obtainable protein from samples containing more than two discs.

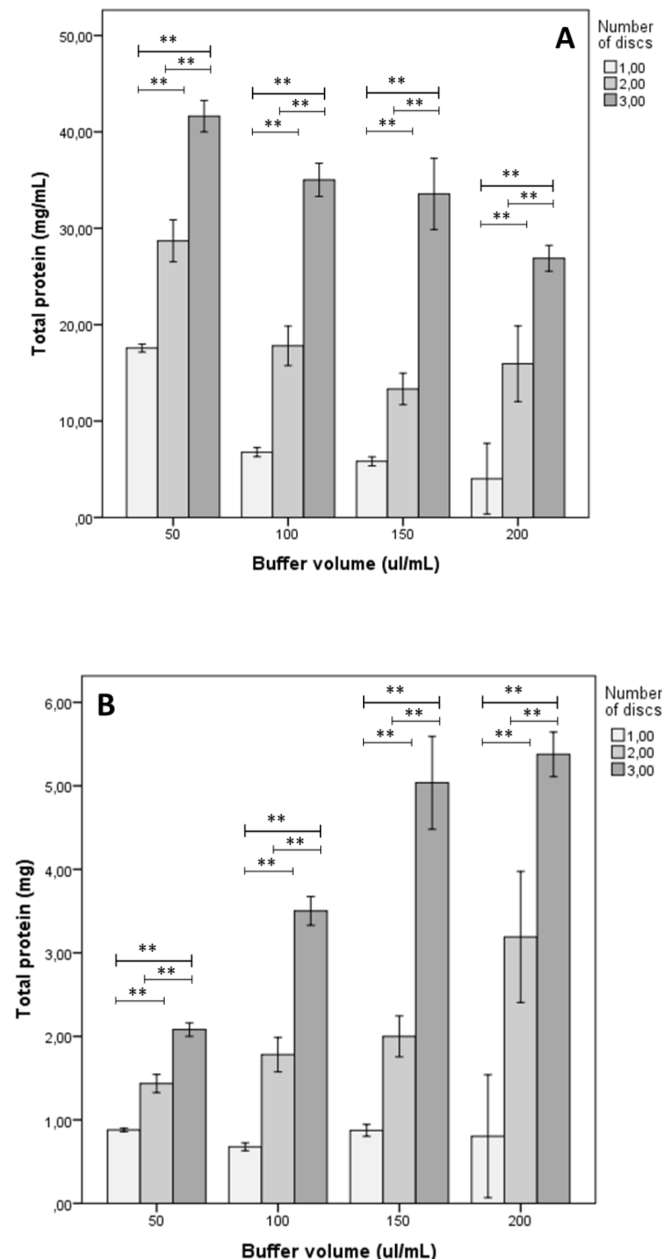

Figure 9 Protein concentration and amount in DBS samples. Protein concentration was analyzed in DBS samples containing one, two and three discs eluted in 50, 100, 150 and 200 µL extraction buffer. Difference in protein concentration and amount between samples containing one, two and three discs was compared by one-way ANOVA. **$p<0.001$. **Figure A** illustrates the mean (SD) protein concentration of the different samples while **Figure B** illustrates the mean (SD) protein amount.

In the second experiment, samples with different number of discs and volume extraction buffer were treated by two different mixing methods to determine which method that gave the highest amount of eluted protein. One plate of samples was sonicated for ten minutes while the other plate of samples was sonicating for ten minutes followed by 20 minutes of shaking on a multiplate shaker. When comparing the mixing methods including all samples in the analysis, no significant difference in protein amount was detected. When performing the analysis on the different samples separately, a significant difference was observed between the mixing methods in samples containing three discs. In these samples, sonication alone resulted in a significantly higher amount of protein extracted compared to sonication and shaking in combination (p=0.04). Otherwise, there were no significant differences in protein amount between the two methods. Overall, the results indicate that mixing the samples by sonication alone is sufficient to extract the protein from the DBS sample.

5.1.2 Optimization of the ELISA assay procedure

SuPAR was first measured in samples containing two to seven discs eluted in 150 µL extraction buffer according to the manufacturer's instructions. This resulted in suPAR concentrations just above the lower limit of detection.

To overcome this problem, we tested an alternative approach. As the ELISA assay required 25 µL undiluted sample, it was especially important to exploit as much as possible of the extracted protein from the sample. We therefore tested whether we could substitute the extraction buffer (PBS + Tween-20) previously used to extract protein with the dilution buffer (PBS + Brondiox) provided in the kit. In this way we could utilize a greater volume of extracted sample in the assay.

We eluted DBS samples, using three discs, in three different buffers (PBS, PBS + Tween and PBS + Brondiox) and measured the protein amount extracted. Results from the experiment are presented in **Figure 10**. Eluting the samples in PBS plus Brondiox resulted in a significantly higher amount of extracted protein compared to PBS ($p<0.001$) and PBS plus Tween-20 ($p<0.001$). The results indicated that we could substitute the previously used extraction buffer with the dilution buffer provided in the kit.

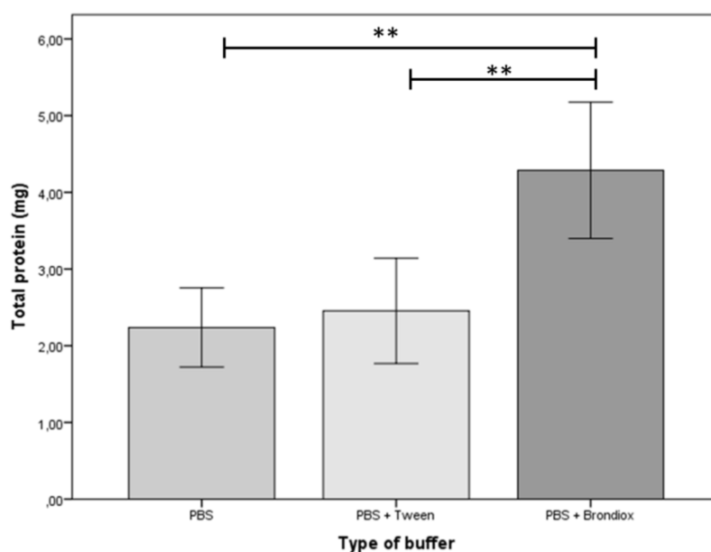

Figure 10 Protein amount extracted in samples eluted in different types of buffers. Protein concentration was analyzed in DBS samples eluted in three types of buffer: PBS, PBS + Tween-20 and PBS + Brondiox. Difference in protein amount between samples was analyzed by one-way ANOVA. $^{**}p<0.001$. The figure shows mean (SD) protein amount extracted.

SuPAR was then measured according to the protocol by the manufacturer with a slight modification. Because the DBS discs had been eluted directly in the dilution buffer we used a twice as concentrated peroxidase conjugate solution when mixing the samples with peroxidase-.conjugated anti-suPAR. Following the modified procedure, we obtained suPAR levels above the limit of detection in all samples tested (data not shown). From this point, all suPAR measurements were performed using this modified procedure.

5.1.3 Validation of suPAR, DBS versus plasma

Assay performance was evaluated by comparing results from paired, simultaneously collected DBS (using four discs per sample) and plasma samples (n=12). These comparisons showed a strong positive correlation between suPAR concentrations measured in DBS and plasma samples with a Pearson correlation coefficient of 0.84 ($p<0.001$). Normalization of samples to total protein did not influence the relationship, resulting in a correlation coefficient of 0.83 ($p<0.001$). The relationship between suPAR in DBS and plasma are illustrated by scatter plot and Bland Altman plot in **Figure 11A and 11B,** respectively. Based on these plots, the measurement imprecision seems higher among samples with low analyte concentration. The average DBS concentration from four discs was 0.35 (0.22-0.47) ng/ml, while the average plasma concentration using 25 uL as starting material was about six times higher i.e. 2.1 (1.6-2.5) ng/ml. The Bland Altman plot showed that the mean difference across the range of values was 1.7 (1.4-2.1) ng/mL with ± one SD of 1.1 and 2.3 ng/mL. Intra-assay coefficient of variation (CV) was calculated on the basis of four different blood spots from one individual run in replicates (n=4). This gave an intra-assay CV of 6.2 %.

Although we find that protein extraction from four DBS discs gives about six times less SuPAR than 25 uL plasma, results from the comparison analysis indicate that suPAR measured with our modified ELISA procedure correlates strongly with suPAR measured in plasma. However, measurement imprecision seems to be higher in sample with low analyte concentration.

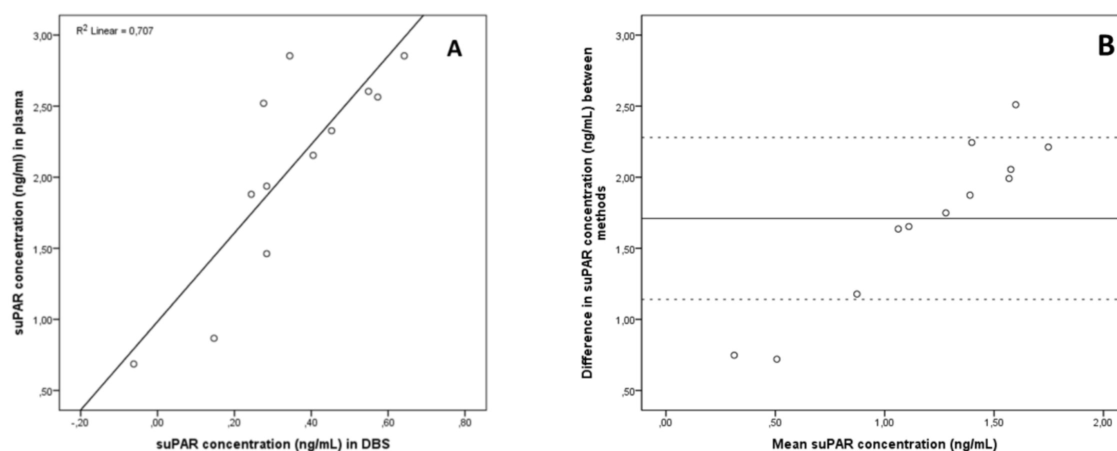

Figure 11 Association between suPAR measured in DBS and plasma samples. suPAR was measured in simultaneously collected DBS and plasma samples from 12 CRC patients. **A**) Association between suPAR concentration in DBS and plasma samples illustrated by a scatter plot, y=3.11x+0.99, r=0.84 ($p<0.001$). **B**) Bland Altman plots of difference between DBS and plasma supAR plotted against average suPAR concentration. The centerline indicates mean difference; the upper and lower lines indicate ± 1 SD.

5.1.4 Stability of suPAR

As collecting whole blood on filter paper is an easy and participant friendly method of collecting blood, blood sampling can be conducted at the participant's home and then be transported by post to the laboratory where it can be stored at appropriate conditions until later analysis. When transporting samples by post it is critical to know how the analyte stability responds to different storage conditions i.e. amount of time in room temperature. To analyze stability of suPAR in response to storage in room temperature, filter paper cards from nine healthy volunteers (four filter paper cards per person) were exposed to different amount of time in room temperature (0, 1, 2 and 3 weeks) before storage at -80'C. The results from the experiment are presented in **Figure 12**.

There was no significant difference in suPAR concentration between DBS samples kept in different amount of time in room temperature. This indicates that suPAR is stable in room temperature for at least three weeks.

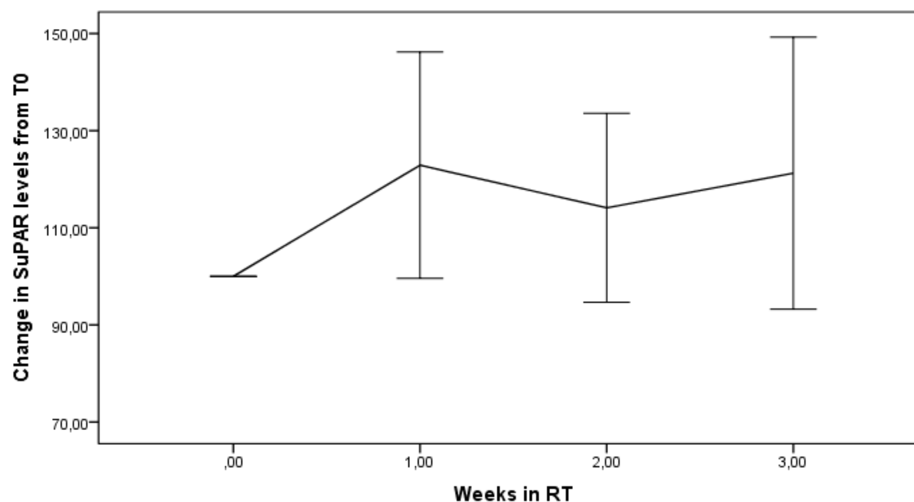

Figure 12 Effect of storage in room temperature on suPAR concentration. Stability of suPAR to room temperature (RT) was analyzed by exposing filter paper cards from healthy volunteers (n=9) to different amount of time in RT (0, 1, 2 and 3 weeks). Analyses was conducted by one-way ANOVA. Time point 0 is set to 1.

5.2 Subjects characteristics and biomarkers in CRC patients

5.2.1 Subject characteristics pre-surgery

During March 2012 to May 2013, 55 CRC patients were invited to the NFS. Of these patients, 42 decided to participate. One withdrew consent after recruitment, and the remaining study population consisted of 41 participants.

As the NFS is designed to include 500 participants, results presented in this thesis are interim, and should be interpreted accordingly.

Subject characteristics of the study population are presented in **Table 3**. The study population consisted of 19 men and 22 women with a mean age of 63 (95 % CI: 60.5-65.0) years. Information about cancer localization was available for 40 participants where 24 were diagnosed with colon cancer, five with cancers of the rectosigmoideum and eleven with rectum cancer. Combining the cancers of different localization, seven had stage I CRC, 15 stage II CRC, ten stage III CRC and three stage IV CRC. The most common comorbid conditions were hypertension (n=17), gastrointestinal diseases (n=8), CVD (n=7) DMT2 (n=6) and hyperlipidemia (n=6). Dividing the participants in to age groups by median age (62 years), we found a higher prevalence of hypertension, DMT2 and gastrointestinal diseases in the high compared to the low age group (data not shown). The difference between the age groups was however only significant for gastrointestinal diseases (p=0.02). We were not able to detect any significant differences between the age groups for the remaining comorbidities. The prevalence of comorbidity has been reported to be higher among participants with proximally compared to distally located tumors. We were not able to detect any significant differences in frequency of comorbid conditions in our data.

Mean BMI in the study population was 27.5 (26.0-29.0) kg/m^2. Out of the 41 participants, none of the participants were underweight (BMI < 18.5 kg/m^2), nine were normal weight (BMI 15.5-24.9 kg/m^2), 23 were overweight (BMI 25.0-19.9 kg/m^2) and nine were obese (BMI > 30.0). This corresponded to a total of 76 % overweight or obese participants in the study population as a whole. Among woman 68 % were overweight or obese. Among men 90 % were overweight or obese.

The BMI groups were significantly different with respect to prevalence of cancer localization (*p*=0.02) and hypertension (*p*=0.02). Post hoc analysis showed a significant difference in frequency of cancer localization between normal weight and overweight participants (*p*<0.01). Among the normal weight participants, 22 % were diagnosed with colon cancer, 11 % with rectosigmoid cancer and 67 % with cancer of the rectum. Among the overweight participants, 77 % were diagnosed with colon cancer, 9 % with rectosigmoid cancer and 14 % with cancers of the rectum. The highest frequency of hypertension was found in the obese participants (78 %), followed by the overweight (39 %) and normal weight (11 %) participants. Only the difference between normal weight and obese participants was significant (*p*=0.02) with regards to hypertension. For the other clinicopathological characteristics, there were no significant differences between the BMI groups.

Table 3 Clinical and histopathological characteristics pre-surgery

Characteristic	All patients	BMI category (kg/m^2) 18.5-24.9 (n=9)	25.0-29.9 (n=23)	≥30 (n=9)	*p**
Age, years, n (n=41)					
≤ 62	21	6	10	5	0.59
> 62	20	3	13	4	
Sex, n (n=41)					
Male	19	2	13	4	0.26
Female	22	7	10	5	
Smoking status, n (n=31)					
Smokes daily	8	2	4	2	0.68
Smokes occasionally	2	1	1	0	
Never smokes	21	3	13	5	
Tumor localization, n (n=40)					
Colon cancer	24	2	17	5	0.02*
Rectosigmoid cancer	5	1	2	2	
Rectum cancer	11	6	3	2	
TNM stage, n (n=35)					
Stage I	7	0	6	1	0.34
Stage II	15	3	6	6	
Stage III	10	3	6	1	
Stage IV	3	0	2	1	
Comorbidity, n (n=41)					
COPD	3	2	1	0	0.23
CVD	7	1	4	2	1.00
Hypertension	17	1	9	7	0.02*
DMT1	1	0	0	1	0.44
DMT2	6	1	4	1	1.00
Hyperlipidaemia	6	0	3	3	0.15
Liver disease	0	0	0	0	-
Kidney disease	2	1	0	1	0.19
GI diseases	8	2	5	1	0.88
MS/CT diseases	5	2	1	2	0.16
Hematol diseases	1	0	1	0	1.00
Neurological diseases	2	0	1	1	0.69
Other cancer	3	1	2	0	1.00

Fisher's exact two-tail probability test was used for all comparisons across BMI categories.
**p*-values <0.05
Post hoc analysis showed a significant difference in cancer localization (colon vs. rectum) between normal weight and overweight patients (p<0.01) and a significant difference in frequency of hypertension between normal weight and obese patients (p=0.02). *P*-values are nominal.
There were no underweight (BMI<18.5 kg/m^2) patients in the study population.
Abbreviations: BMI: Body mass index, COPD: Chronic obstructive pulmonary diseases, CVD: Cardiovascular diseases, DMT1: Diabetes Mellitus type 1, DMT2: Diabetes mellitus type 2, GI diseases: Gastrointestinal diseases, MS/CT diseases: Musculoskeletal and connective tissue diseases. Hematol diseases: Hematological diseases

5.2.2 Weight status and plasma biomarkers pre-surgery

Associations between weight status plasma biomarkers

Plasma biomarkers related to obesity and inflammation were measured pre-surgery to investigate the association between these variables and four different measures of weight status; body weight, BMI, waist circumference and waist/hip-ratio. The associations between biomarkers and anthropometric measures were analyzed by regression analysis. Results from the analysis are presented in **Table 4-7**.

There was a significant positive association between BMI and suPAR (p=0.03) lipocalin-2 (p=0.05), resistin (p=0.02), adipsin (p=0.02) and leptin (p<0.01) (**Table 4**). When adjusting for age and gender, associations remained significant for all analytes, except for lipocalin-2 that still showed a clear trend (p=0.05). After adjustments, the concentration of suPAR, resistin, adipsin and leptin increased by 2 %, 5 %, 6 % and 43 %, respectively for every kg/m^2 increase in BMI. Insulin tended to be associated with BMI with a p-value of 0.05 after adjustments for age and gender.

Waist circumference was significantly positively associated with suPAR (p=0.03), lipicalin-2 (p=0.04), resistin (p=0.02), adipsin (p=0.01), PAI-1 (p=0.03), insulin (p=0.04), and leptin (p<0.01) (**Table 5**). The associations remained significant after adjustment for age and gender for all analytes, except for lipocalin-2 that still showed a clear trend (p=0.05). After adjustments, the concentration of suPAR, resistin, adipsin, PAI-I, insulin and leptin increased by 1 %, 2 %, 2 %, 2 %, 22 % and 17 %, respectively for every cm increase in waist circumference.

There was no significant association between body weight and plasma biomarkers before adjustments for relevant covariates. However, after adjustment for age and gender in the model body weight was significantly positively associated with suPAR (p=0.03), adipsin (p=0.03) and leptin (p<0.001) (**Table 6**). For every kg increase in weight, concentration of suPAR, adipsin and leptin increased by 1 %, 2 % and 4 %, respectively.

There were no significant associations between waist/hip ratio and plasma biomarkers before adjustment for covariates, except for CRP, which showed a significant negative association with waist/hip-ratio (p=0.04). However, the association was no longer significant after adjustments (**Table 7**). Adjustment for age and gender resulted in a significant positive

association between waist/hip-ratio and adipsin (p=0.03), PAI-1 (p=0.02) and leptin (p=0.01). For every 0.01 increase in waist/hip-ratio adipsin, PAI-I and leptin increased by 3 %, 3 % and 4 %, respectively.

Overall, suPAR, lipocalin-2, resistin, adipsin, PAI-I, insulin and leptin seems to be positively associated with measures of obesity and abdominal obesity in CRC patients. In contrast, inflammatory cytokines and chemokines do not seem to be associated with these measures. Furthermore, waist circumference seems to be the most predictive measure of obesity-associated plasma biomarkers followed by BMI. Body weight and waist/hip-ratio seem to be less effective in predicting these biomarkers.

Table 4 Association between BMI and plasma biomarkers pre-surgery

		Unadjusted values		Adjusted values [b]	
Biomarkers	N	*b* (95 % CI)[a]	*P**	*b* (95 % CI)[a]	*P**
CRP, mg/L	9	-0.13 (-0.42-0.16)	0.31	-0.12 (-0.47-0.23)	0.42
suPAR, ng/mL	38	0.02 (0.00-0.04)	0.03*	0.02 (0.00-0.04)	0.02*
Adiponectin, pg/mL	36	-0.02 (-0.07-0.04)	0.58	-0.01 (-0.06-0.04)	0.60
Lipocal-2, pg/mL	36	0.04 (0.00-0.09)	0.05*	0.04 (0.00-0.08)	0.05
Resistin, pg/mL	36	0.05 (0.01-0.10)	0.02*	0.05 (0.01-0.10)	0.02*
Adipsin, pg/mL	36	0.06 (0.01-0.10)	0.02*	0.06 (0.01-0.10)	0.02*
PAI-1, pg/mL	36	0.04 (-0.01-0.08)	0.10	0.04 (-0.01-0.09)	0.09
Insulin, pg/mL	37	0.05 (-0.00-0.09)	0.06	0.05 (-0.00-0.09)	0.05
Leptin, pg/mL	37	0.10 (0.04-0.16)	<0.01*	0.36 (0.17-0.54)	<0.001**
NGF, pg/mL	37	0.019 (-0-01-0.05)	0.20	0.02 (-0.01-0.05)	0.22
HGF, pg/mL	37	-0.02 (-0.06-0.03)	0.49	-0.02 (-0.06-0.03)	0.42
MCP-1, pg/mL	37	-0.00 (-0.03-0.02)	0.79	-0.00 (-0.03-0.02)	0.87
TNF-α, pg/mL	37	0.01 (-0.02-0.04)	0.64	0.01 (-0.02-0.04)	0.64
IL-1β, pg/mL	37	0.04 (-0.07-0.14)	0.50	0.037 (-0.07-0.14)	0.46
IL-6, pg/mL	37	-0.03 (-0.10-0.04)	0.38	-0.03 (-0.10-0.04)	0.42
IL-8, pg/mL	37	-0.01 (-0.05-0.04)	0.78	-0.01 (-0.05-0.04)	0.83
Glucose, mmol/L	29	0.01 (-0.01-0.02)	0.41	0.01 (-0.01-0.02)	0.42

All variables are log-transformed and analyzed by linear regression. Variables are expressed as ln(*b*) with ln(95 % CI).
[a] A positive correlation coefficient *(b)* indicates an increase.
[b] Adjusted for age and gender. A significant interaction existed between BMI and sex for leptin and the interaction term was thereby included in the model.
**p*-values <0.05
***p*-values <0.001
Abbreviations: CRP: C-reactive protein, suPAR: Soluble urokinase plasminogen activator receptor, Lipocal-2: Lipocalin-2, PAI-1: Plasminogen activator inhibitor-1 (total), NGF: Nerve growth factor, HGF: Hepatocyte growth factor, MCP-1: Monocyte chemoattractant protein-1, TNF: Tumor necrosis factor, IL: Interleukin.
N indicates number of participants included in the analysis

Table 5 Association between waist circumference and plasma biomarkers pre-surgery

		Unadjusted values		Adjusted values [b]	
Biomarkers	N	*b* (95 % CI) [a]	*P**	*b* (95 % CI) [a]	*P**
CRP, mg/L	9	-0.06 (-0.15-0.02)	0.11	-0.06 (-0.16-0.05)	0.22
suPAR, ng/mL	38	0.01 (0.00-0.01)	0.03*	0.01 (0.00-0.01)	0.02*
Adiponectin, pg/mL	36	-0.01 (-0.02-0.01)	0.59	-0.00 (-0.02-0.01)	0.70
Lipocal-2, pg/mL	36	0.01 (0.00-0.027)	0.04*	0.01 (0.00-0.03)	0.05
Resistin, pg/mL	36	0.02 (0.00-0.03)	0.02*	0.02 (0.01-0.03)	<0.01*
Adipsin, pg/mL	36	0.02 (0.01-0.03)	0.01*	0.02 (0.01-0.04)	0.007*
PAI-1, pg/mL	36	0.02 (0.00-0.03)	0.03*	0.02 (0.00-0.03)	0.02*
Insulin, pg/mL	37	0.02 (0.00-0.03)	0.04*	0.20 (0.06-0.34)	<0.01*
Leptin, pg/mL	37	0.03 (0.01-0.05)	<0.01*	0.16 (0.11-0.20)	<0.001**
NGF, pg/mL	37	0.01 (-0.00-0.02)	0.10	0.01 (-0.00-0.02)	0.11
HGF, pg/mL	37	0.00 (-0.01-0.02)	0.70	0.00 (-0.01-0.02)	0.88
MCP-1, pg/mL	37	0.00 (-0.01-0.01)	0.83	0.00 (-0.01-0.01)	0.66
TNF-α, pg/mL	37	0.00 (-0.01-0.01)	0.49	0.00 (-0.01-0.01)	0.48
IL-1β, pg/mL	37	0.02 (-0.02-0.05)	0.30	0.02 (-0.01-0.05)	0.23
IL-6, pg/mL	37	-0.01 (-0.03-0.01)	0.43	-0.01 (-0.03-0.02)	0.49
IL-8, pg/mL	37	-0.00 (-0.02-0.01)	0.83	-0.00 (-0.02 (0.02)	0.93
Glucose, mmol/L	29	0.00 (-0.00-0.01)	0.64	0.00 (-0.00-0.01)	0.61

All variables are log-transformed and analyzed by linear regression. Variables are expressed as ln(*b)* with ln(95 % CI).

[a] A positive correlation coefficient (*b*) indicates an increase.

[b] Adjusted for age and gender. A significant interaction existed between waist and sex for leptin, waist and age for insulin and age and sex for resistin. These interaction terms were thereby included in the respective models.

**p*-values <0.05

***p*-values <0.001

Abbreviations: CRP: C-reactive protein, suPAR: Soluble urokinase plasminogen activator receptor, Lipocal-2: Lipocalin-2, PAI-1: Plasminogen activator inhibitor-1 (total), NGF: Nerve growth factor, HGF: Hepatocyte growth factor, MCP-1: Monocyte chemoattractant protein-1, TNF: Tumor necrosis factor, IL: Interleukin.

N indicates number of participants included in the analysis

Table 6 Association between body weight and plasma biomarkers pre-surgery

		Unadjusted values		Adjusted values[b]	
Biomarkers	N	*b* (95 % CI) [a]	*P**	*b* (95 % CI) [a]	*P**
CRP, mg/L	9	-0.05 (-0.13-0.03)	0.20	-0.05 (-0.16-0.07)	0.37
suPAR, ng/mL	38	0.01 (-0.00-0.01)	0.12	0.01 (0.00-0.01)	0.03*
Adiponectin, pg/mL	36	-0.01 (-0.02-0.01)	0.48	-0.00 (-0.02-0.02)	0.86
Lipocal-2, pg/mL	36	0.01 (-0.00-0.02)	0.15	0.01 (-0.00-0.03)	0.15
Resistin, pg/mL	36	0.01 (-0.00-0.02)	0.17	0.01 (-0.00-0.03)	0.08
Adipsin, pg/mL	36	0.01 (-0.00-0.03)	0.07	0.02 (0.00-0.033)	0.03*
PAI-1, pg/mL	36	0.01 (-0.01-0.02)	0.25	0.01 (-0.00-0.03)	0.13
Insulin, pg/mL	37	0.01 (-0.00-0.03)	0.08	0.02 (0.00-0.03)	0.05
Leptin, pg/mL	37	0.02 (-0.01-0.04)	0.13	0.04 (0.02-0.05)	<0.001**
NGF, pg/mL	37	0.01 (-0.00-0.02)	0.18	0.01 (-0.00-0.02)	0.17
HGF, pg/mL	37	0.00 (-0.01-0.01)	0.94	-0.00 (-0.02-0.01)	0.78
MCP-1, pg/mL	37	0.00 (-0.01-0.01)	0.96	0.00 (-0.01-0.01)	0.70
TNF-α, pg/mL	37	0.00 (-0.01-0.01)	0.60	0.00 (-0.01-0.01)	0.48
IL-1β, pg/mL	37	0.01 (-0.02-0.04)	0.47	0.01 (-0.02-0.05)	0.44
IL-6, pg/mL	37	-0.01 (-0.03-0.01)	0.38	-0.01 (-0.04-0.02)	0.45
IL-8, pg/mL	37	-0.00 (-0.02-0.01)	0.77	-0.00 (-0.02-0.02)	0.94
Glucose, mmol/L	29	0.00 (-0.00-0.01)	0.20	0.00 (-0.00-0.01)	0.16

All variables are log-transformed and analyzed by linear regression. Variables are expressed as ln(*b*) with ln(95 % CI).
[a] A positive correlation coefficient (*b*) indicates an increase.
[b] Adjusted for age and gender.
**p*-values <0.05
***p*-values <0.001
Abbreviations: CRP: C-reactive protein, suPAR: Soluble urokinase plasminogen activator receptor, Lipocal-2: Lipocalin-2, PAI-1: Plasminogen activator inhibitor-1 (total), NGF: Nerve growth factor, HGF: Hepatocyte growth factor, MCP-1: Monocyte chemoattractant protein-1, TNF: Tumor necrosis factor, IL: Interleukin.
N indicates number of participants included in the analysis

Table 7 Association between waist/hip-ratio and plasma biomarkers pre-surgery

		Unadjusted values		Adjusted values[b]	
Biomarkers	N	*b* (95 % CI)[a]	*P**	*b* (95 % CI)[a]	*P**
CRP, mg/L	9	-11.37 (-22.36—0.38)	0.04*	-11.44 (-27.14-4.26)	0.12
suPAR, ng/mL	38	0.31 (-0.65-1.26)	0.52	0.55 (-0.46-1.57)	0.28
Adiponectin, pg/mL	36	-0.86 (-3.39-1.68)	0.50	-0.24 (-2.87-2.39)	0.86
Lipocal-2, pg/mL	36	1.56 (-0.51-3.64)	0.14	1.66 (-0.64-3.95)	0.15
Resistin, pg/mL	36	1.76 (-0.50-4.01)	0.12	2.35 (-0.05-4.74)	0.05
Adipsin, pg/mL	36	2.05 (-0.22-4.33)	0.08	2.76 (0.30-5.22)	0.03*
PAI-1, pg/mL	36	1.99 (-0.17-4.14)	0.07	2.87 (0.45-5.29)	0.02*
Insulin, pg/mL	37	1.47 (-0.81-3.74)	0.20	1.90 (-0.69-4.49)	0.15
Leptin, pg/mL	37	0.90 (-2.30-4.11)	0.57	3.88 (0.90-6.87)	0.01*
NGF, pg/mL	37	0.80 (-0.59-2.19)	0.25	0.94 (-0.68-2.56)	0.25
HGF, pg/mL	37	1.21 (-0.83-3.24)	0.24	1.07 (-1.27-3.40)	0.36
MCP-1, pg/mL	37	-0.07 (-1.27-1.12)	0.90	0.16 (-1.22-1.53)	0.82
TNF-α, pg/mL	37	0.31 (-1.09-1.70)	0.66	0.45 (-1.11-2.01)	0.56
IL-1β, pg/mL	37	2.28 (-2.76-7.32)	0.36	2.87 (-2.55-8.29)	0.29
IL-6, pg/mL	37	-1.28 (-4.72-2.16)	0.46	-1.21 (-5.23-2.81)	0.54
IL-8, pg/mL	37	-0.54 (-2.78-1.71)	0.63	-0.35 (-2.97-2.27)	0.79
Glucose, mmol/L	29	-0.08 (-0.79-0.63)	0.81	-0.10 (-0.94-0.73)	0.80

All variables are log-transformed and analyzed by linear regression. Variables are expressed as ln(*b*) with ln(95 % CI).
[a] A positive correlation coefficient (b) indicates an increase.
[b] Adjusted for age and gender.
*p-values <0.05
Abbreviations: CRP: C-reactive protein, suPAR: Soluble urokinase plasminogen activator receptor, Lipocal-2: Lipocalin-2, PAI-1: Plasminogen activator inhibitor-1 (total), NGF: Nerve growth factor, HGF: Hepatocyte growth factor, MCP-1: Monocyte chemoattractant protein-1, TNF: Tumor necrosis factor, IL: Interleukin.
N indicates number of participants included in the analysis

Association between plasma biomarkers

To investigate the correlations between the different plasma biomarkers, Spearman correlation coefficients were calculated (**Table 8**). As shown in the table, several of the biomarkers were significantly correlated. Resistin, lipocalin-2, adipsin and PAI-1 was highly correlated with correlation coefficients in the range 0.56-0.90 (p<0.001). Resistin, lipocalin-2 and adipsin was also highly correlated with adiponectin (p<0.001). Furthermore, NGF, IL-6 and IL-8 were highly correlated with correlation coefficients in the range 0.60-0.70 (p<0.001). IL-8 was also significantly correlated with TNF-α (p<0.001). suPAR seemed to be more correlated to obesity-associated biomarkers than inflammatory biomarkers.

PCA was performed in order to identify possible patterns in the multidimensional data set, including BMI, waist circumference and all the plasma biomarkers (Figure 13). The score plot shows that 32 and 16 % of the total variation was explained by PC1 and PC2, respectively. Furthermore, the score plot, which was labeled according to gender, shows that there is no clear pattern in the data. This indicates that the genders were similar with regards to the parameters included in the analysis. The correlation loadings plot shows that the obesity relevant biomarkers (dotted black rectangle) are inter-correlated, since they lie close together, and are also correlated with waist circumference and BMI, thus confirming previous analysis using conventional statistics. The inflammatory biomarkers are also inter-correlated, but are not correlated with the anthropometric measures (green dotted rectangle).

Table 8 Spearman's correlation coefficients between the different plasma biomarkers pre-surgery

	Leptin	PAI-1	Resistin	Lipocal-2	Adipsin	Insulin	HGF	NGF	MCP-1	TNF-α	IL-6	IL-8	IL-1β	CRP	suPAR
Adiponectin	-0.01	0.24	0.58^{1}	0.60^{1}	0.64^{1}	0.06	0.16	0.33^{3}	0.13	0.42^{3}	0.26	0.35^{3}	0.05	0.55	0.46^{2}
Leptin		0.44^{2}	0.22	0.04	0.33^{3}	0.27	0.08	0.08	0.25	0.13	-0.28	-0.12	-0.16	0.51	0.09
PAI-1	0.44^{2}		0.59^{1}	0.56^{1}	0.73^{1}	0.40^{3}	0.13	0.44^{2}	0.29	0.27	0.25	0.37^{3}	0.11	0.75	0.14
Resistin	0.22	0.59^{1}		0.90^{1}	0.86^{1}	0.18	0.30	0.47^{2}	0.17	0.48^{2}	0.28	0.45^{2}	0.07	0.62	0.51^{2}
Lipocal-2	0.04	0.56^{1}	0.90^{1}		0.85^{1}	0.31	0.31	0.39^{3}	0.17	0.35^{3}	0.26	0.50^{2}	0.06	0.73	0.51^{2}
Adipsin	0.33^{3}	0.73^{1}	0.86^{1}	0.85^{1}		0.34^{3}	0.23	0.51^{2}	0.20	0.43^{3}	0.24	0.42^{3}	0.13	0.76^{3}	0.39^{3}
Insulin	0.27	0.40^{3}	0.18	0.31	0.34^{3}		-0.11	0.14	0.24	0.08	-0.02	0.24	-0.04	0.05	0.07
HGF	0.08	0.13	0.30	0.31	0.23	-0.11		0.21	0.28	0.30	0.14	0.32	-0.10	-0.02	0.25
NGF	0.08	0.44^{2}	0.47^{2}	0.39^{3}	0.51^{2}	0.14	0.21		0.27	0.49^{2}	0.61^{1}	0.60^{1}	0.51^{2}	0.21	0.32
MCP-1	0.25	0.29	0.17	0.17	0.20	0.24	0.28	0.27		0.23	0.24	0.48^{2}	0.08	0.36	-0.10
TNFα	0.13	0.27	0.48^{2}	0.35^{3}	0.43^{3}	0.08	0.30	0.49^{2}	0.23		0.47^{2}	0.60^{1}	0.21	0.22	0.38^{3}
IL-6	-0.28	0.25	0.28	0.26	0.24	-0.02	0.14	0.61^{1}	0.24	0.47^{2}		0.70^{1}	0.46^{2}	-0.12	0.15
IL-8	-0.12	0.37^{3}	0.45^{2}	0.50^{2}	0.42^{3}	0.24	0.32	0.60^{1}	0.48^{2}	0.60^{1}	0.70^{1}		0.44^{2}	0.40	0.37^{3}
IL-1β	-0.16	0.11	0.07	0.06	0.13	-0.04	-0.10	0.51^{2}	0.08	0.21	0.46^{2}	0.44^{2}		0.52	-0.05
CRP	0.51	0.75	0.62	0.73	0.76^{3}	0.05	-0.02	0.21	0.36	0.22	-0.12	0.40	0.52		0.32

Abbreviations: CRP: C-reactive protein, suPAR: soluble urokinase plasminogen activator receptor, PAI-1 (total): Plasminogen activator inhibitor-1 (total), Lipocal-2: Lipocalin-2, NGF: Nerve growth factor, HGF: Hepatocyte growth factor, MCP-1: Monocyte chemoattractant protein-1, TNF: Tumor necrosis factor, IL: Interleukin, ^{1} $P \leq 0.001$, ^{2} $P \leq 0.01$, ^{3} $P \leq 0.05$

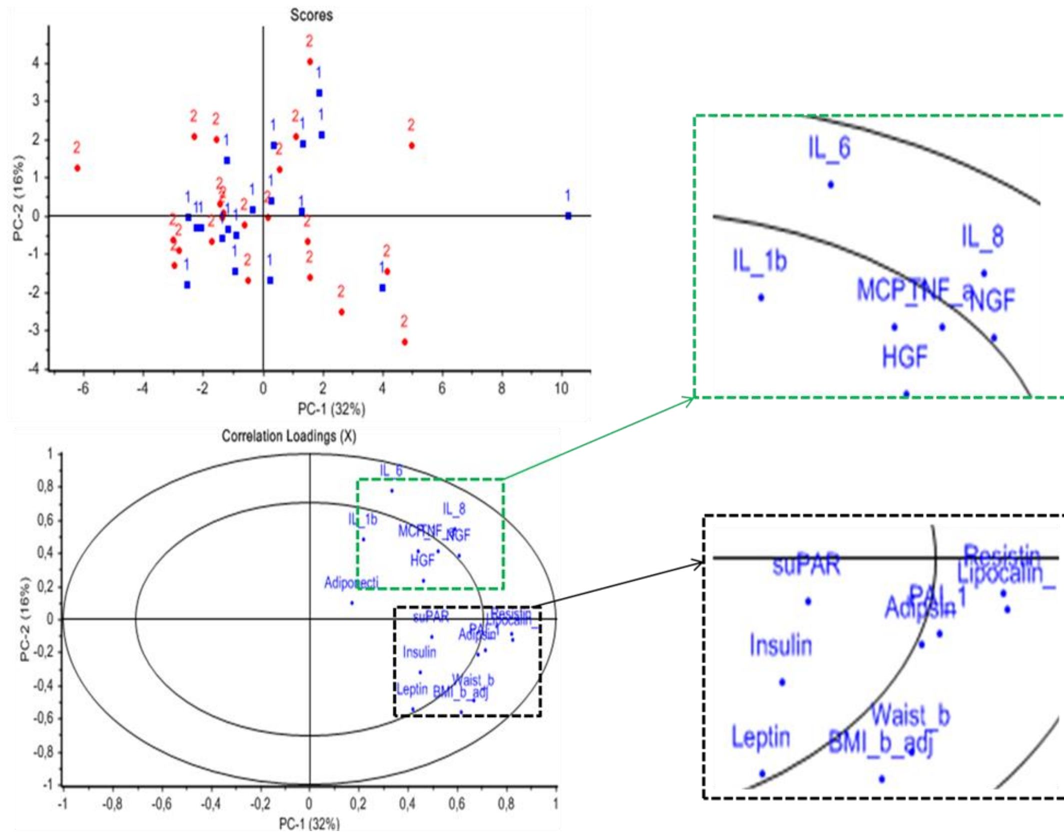

Figure 13 PCA on BMI, waist circumference and all biomarkers measured pre-surgery. The score plot, which was labeled according to gender shows that there is no grouping according to gender (1=Male, 2=Female). The *correlation loading* plot shows that the anthropometric measures and obesity relevant biomarkers (black rectangle) lie close to each other and are thus correlated in our population. The inflammatory biomarkers are also inter-correlated but are not correlated with the anthropometric measures (green dotted rectangle).

5.2.3 Clinicopathological characteristics and plasma biomarkers pre-surgery

Tumor characteristics and plasma biomarkers

Plasma biomarkers were measured pre-surgery to investigate the association between these biomarkers and tumor characteristics. Plasma biomarkers were compared across different tumor localization (colon and rectosigmoid + rectum cancer) and TNM stage (TNM stage I + II and TNM stage III + IV) groups by linear regression.

There was no significant difference in concentration of plasma biomarkers between participants with cancers of the colon compared to participants with cancers of the rectosigmoid and rectum (**Table 9**). However, there was a trend toward a difference for IL-1β (p=0.06), with a higher IL-1β level among patients with rectum or rectosigmoid cancer.

suPAR concentration was significantly higher in participants with TNM stage I and II compared to participants with stage III and IV CRC (p<0.01) with a median concentration of 3.61 and 2.71 ng/mL, respectively (**Table 10**). The difference in suPAR concentration between stage groups remained significant after adjustment for age and waist circumference (p=0.01). No significant associations were found between TNM stage groups and the other plasma biomarkers.

Table 9 Plasma biomarkers and tumor localization pre-surgery

Biomarkers	N	Tumor localization		p*	p*[a]
		Colon	Rectum[b]		
CEA, µg/L	11	3.20 (1.40-7.20)	2.00 (1.00-3.10)	0.21	0.26
CRP, mg/L	8	1.00 (0.80-1.45)	2.10 (1.00-3.90)	0.32	0.43
suPAR, ng/mL	34	3.27 (2.39-4.02)	3.00 (2.26-3.94)	0.42	0.84
Adiponectin, ng/mL	30	72.96 (48.51-106.32)	63.23 (44.28-71.27)	0.35	0.58
Lipocalin-2, pg/mL	30	487 (218-815)	270 (256-321)	0.17	0.35
Resistin, pg/mL	30	133 (62-206)	75 (61-99)	0.17	0.38
Adipsin, ng/mL	30	18.99 (8.86-30.73)	13.70 (10.14-25.30)	0.56	0.96
PAI-1, pg/mL	30	104 (68-197)	117 (75-187)	0.93	0.71
Insulin, pg/mL	31	363 (250-454)	308 (228-395)	0.29	0.24
Leptin, ng/mL	31	13.08 (8.37-29.36)	9.07 (6.02-18.04)	0.66	0.90
NGF, pg/mL	31	7.44 (5.32-8.71)	7.17 (5.35-9.08)	0.87	0.95
HGF, pg/mL	31	165 (126-282)	169 (150-209)	0.79	0.98
MCP-1, pg/mL	31	135 (107-151)	124 (110-194)	0.95	0.83
TNF-α, pg/mL	31	2.91 (2.28-3.96)	2.64 (1.77-2.91)	0.12	0.23
IL-1β, pg/mL	31	2.75 (0.30-4.30)	4.00 (0.60-14.30)	0.06	0.09
IL-6, pg/mL	31	6.02 (3.61-10.81)	6.02 (3.69-14.2)	0.81	0.74
IL-8, pg/mL	31	6.05 (3.47-13.02)	5.00 (4.23-7.15)	0.38	0.36
Glucose, mmol/L	23	5.40 (4.95-5.70)	5.30 (5.20-5.75)	0.79	0.73

All variables are log-transformed and analyzed by linear regression. Variables are expressed as median (Q1-Q3).
[a]Adjusted for waist circumference and age.
[b] Includes rectosigmoid and rectum cancer.
Abbreviations: CEA: Carcinoembrytotic antigen, CRP: C-reactive protein, suPAR: soluble urokinase plasminogen activator receptor, PAI-1: Plasminogen activator inhibitor-1 (total), NGF: Nerve growth factor, HGF: Hepatocyte growth factor, MCP-1: Monocyte chemoattractant protein-1, TNF: Tumor necrosis factor, IL: Interleukin.
N indicates number of participants included in the analysis.

Table 10 Plasma biomarkers and TNM stage group pre-surgery

Biomarkers	N	TNM stage I + II	TNM stage III + IV	*P**	*P**[a]
CEA, µg/L	11	2.00 (1.40-2.60)	3.60 (2.05-83.05)	0.21	0.28
CRP, mg/L	8	1.00 (1.00-1.90)	2.10 (1.50-3.00)	0.82	0.57
suPAR, ng/mL	34	3.61 (3.00-4.30)	2.71 (2.25-3.30)	<0.01*	0.01*
Adiponectin, ng/mL	30	67.15 (48.11-101.30)	70.96 (48.83-98.73)	0.68	0.56
Lipocalin-2, pg/mL	30	341 (243-824)	298 (192-523)	0.24	0.41
Resistin, pg/mL	30	112 (63-218)	90 (47-136)	0.38	0.65
Adipsin, ng/mL	30	16.88 (9.45-29.35)	14.68 (8.63-28.57)	0.74	0.82
PAI-1, pg/mL	30	131 (73-187)	90 (70-149)	0.43	0.73
Insulin, pg/mL	31	375 (250-438)	301 (218-411)	0.39	0.65
Leptin, ng/mL	31	13.81 (7.28-31.54)	9.07 (7.07-15.93)	0.42	0.79
NGF, pg/mL	31	6.68 (5.39-8.85)	7.17 (4.96 (8.95)	0.68	0.95
HGF, pg/mL	31	187 (148-271)	147 (132-171)	0.14	0.16
MCP-1, pg/mL	31	129 (107-154)	137 (104-194)	0.99	0.97
TNF-α, pg/mL	31	2.79 (2.28-3.73)	2.64 (1.78-3.78)	0.65	0.75
IL-1β, pg/mL	31	2.75 (0.15-5.00)	3.30 (1.65-15.55)	0.27	0.14
IL-6, pg/mL	31	5.72 (2.92-8.04)	12.61 (4.57-16.18)	0.15	0.21
IL-8, pg/mL	31	5.44 (4.00-10.38)	6.22 (3.43-7.91)	0.60	0.57
Glucose, mmol/L	23	5.35 (5.10-5.80)	5.40 (5.20-5.60)	0.49	0.56

All variables are log-transformed and analyzed by linear regression. Variables are expressed as median (Q1-Q3).
[a]Adjusted for waist circumference and age
* $p<0.05$.
Abbreviations: CEA: Carcinoembrytotic antigen, CRP: C-reactive protein, suPAR: soluble urokinase plasminogen activator receptor, PAI-1: Plasminogen activator inhibitor-1 (total), NGF: Nerve growth factor, HGF: Hepatocyte growth factor, MCP-1: Monocyte chemoattractant protein-1, TNF-α: Tumor necrosis factor-α, IL: Interleukin.
N indicates number of participants included in the analysis

Obesity-associated hypertension and plasma biomarkers

As adipokines are suggested as possible mediators of obesity-associated hypertension, logistic regression was performed to investigate the impact of the individual plasma biomarkers on hypertension (**Table 11**). Results from the logistic regression analysis showed that suPAR and insulin significantly predicted hypertension with OR of 16.23 (1.10-239.75) (*p*=0.04) and 4.65 (1.01-21.40) (*p*=0.05), respectively. When adjusting for age, suPAR, leptin and insulin significantly predicted hypertension with OR of 18.45 (1.03-332.07) (*p*=0.05), 2.36 (1.00-5.45) (*p*=0.05) and 5.08 (1.03-25.00) (*p*=0.05), respectively. For TNF-α, adipsin and lipocalin-2 there were trends in the data indicating an impact on hypertension. For the remaining plasma biomarkers, no associations were detected (data not shown).

Table 11 Hypertension and plasma biomarkers pre-surgery

		Unadjusted values		Adjusted values[b]	
Biomarker	N	OR (95 % CI)	*P**	OR (95 % CI)	*P**[a]
suPAR	38	16.23 (1.10-239.75)	0.04*	18.45 (1.03-332.07)	0.05*
Leptin	37	2.34 (1.00-5.51)	0.05	2.36 (1.00-5.45)	0.05*
Insulin	37	4.65 (1.01-21.40)	0.05*	5.08 (1.03-25.00)	0.05*
TNF-α	37	5.43 (0.78-37.84)	0.09	6.59 (0.85-51.29)	0.07
Adipsin	36	2.82 (0.90-8.91)	0.08	3.24 (0.97-10.80)	0.06
Lipocalin-2	36	3.04 (0.86-10.70)	0.06	3.58 (0.96-13.37)	0.06

Variables are log-transformed and analyzed by logistic regression. Variables are expressed as OR (95 % CI).
[a]Adjusted for age.
* p<0.05.
Abbreviations: suPAR: soluble urokinase plasminogen activator receptor, TNF-α: Tumor necrosis factor-α.
N indicates number of participants included in the analysis

5.2.4 Changes in weight status from pre to two-months post-surgery

Weight, height, waist and hip circumference and handgrip strength was measured pre- and two months post-surgery to investigate changes in weight status and physical function during the postoperative period. The results from the analysis are presented in **Table 12**. There was a significant reduction in weight ($p<0.001$), BMI ($p<0.001$), waist circumference ($p<0.01$) and waist/hip-ratio ($p=0.03$) from pre- to two months post-surgery. The mean difference in weight was -3.5 (-5.3—1.6) kg, corresponding to a weight loss of approximately 4 %. Of all participants, 19 lost weight during the postoperative period while six gained weight. The mean difference in BMI was -1.2 (-1.8—0.6) kg/m^2. Waist circumference and waist-hip/ratio was significantly reduced with a mean difference of -3.4 (-5.7—1.1) cm and -0.03 (-0.05—0.00), respectively. When conducting sub-analysis on waist and waist/hip-ratio excluding participants with stoma postoperative (n=8), the significant reduction in waist circumference and waist/hip-ratio disappeared. In a sub-analysis treating the genders separately, we found significant differences between pre- and postoperative weight, BMI and waist circumference only among women (n=16). Furthermore, a significant difference between pre and postoperative waist/hip-ratio was only found in men (n=9) (data not shown).

There was a trend towards a reduction in handgrip strength in both the right and left arm, but the differences were non-significant. Median change in handgrip strength was -1.6 kg in the right arm and -1.1 kg in the left arm.

There was a significant positive correlation between weight and waist/hip-ratio and handgrip strength in the right and left arm both pre- and two months post-surgery, except between waist/hip-ratio and handgrip strength in the right arm post-surgery. There was no significant correlation between change in anthropometric measures and change in handgrip strength.

Overall, the results indicate that CRC patients undergoing surgical resection experience weight loss, accompanied by a reduction in BMI, waist circumference and waist/hip-ratio during the postoperative period. However, changes in anthropometric measures do not correlate with changes in measures of physical function.

Table 12 Postoperative changes in weight status and physical function

Parameter	N	Pre-surgery	Post-surgery	Change	*P**
Weight, cm[a]	25	82.3 (75.4-89.3)	78.9 (72.3-85.5)	-3.5 (-5.3—1-6)	<0.001**
BMI, kg/m^{2}[a]	25	27.9 (25.7-30.1)	26.7 (24.7-28.7)	-1.2 (-1.8—0.6)	<0.001**
Waist, cm[a]	25	101.1 (94.6-107.5)	97.7 (91.6-103.7)	-3.4 (-5.7—1.1)	<0.01*
Hip, cm[a]	25	104.3 (100.1-108.6)	103.9 (99.5-108.3)	-0.4 (-2.3-1.5)	0.65
W/H-Ratio[a]	25	0.97 (0.93-1.01)	0.94 (0.90-0.97)	-0.03 (-0.05—0.00)	0.03*
Grip, R, kg[b]	24	26.7 (21.9-38.9)	27.4 (20.0-33.8)	-1.6 (-3.8-1.0)	0.15
Grip, L, kg[b]	24	24.6 (20.8-34.4)	22.7 (18.6-32.1)	-1.1 (-3.4-0.5)	0.06

**p*-values <0.05.
***p*-values <0.001.
[a] Data analyzed by paired samples t-test and expressed as mean (95 % CI).
[b] Data analyzed by Sign rank test and expressed as median (Q1-Q3).
Measured height from baseline of intervention is used for all BMI calculations.
Abbreviations: BMI: Body mass index, Waist: Waist circumference, Hip: Hip circumference, W/H-Ratio: Waist/hip-ratio, Grip: Handgrip strength, R: Right, L: Left.
N indicates number of participants included in the analysis.

6 Discussion

6.1 Method development and validation

suPAR is a biomarker of inflammation associated with disease risk and prognosis of several inflammatory diseases including cancer (70, 71, 73, 92). suPAR has previously been measured in serum, plasma, urine, saliva, ovarian cystic fluid and in the cerebrospinal fluid (93). To the best of our knowledge, there are no previous studies measuring suPAR in whole blood collected on filter paper. Therefore, we aimed at developing and validating a method of measuring suPAR in DBS samples collected by fingerprick with use of a commercially available ELISA assay.

Before initiating the method development, the literature was reviewed for relevant protein biomarkers measured in DBS. The papers were systematically screened on information about the extraction volumes, source and volume of sample, analytical method as well as elements used to evaluate assay performance (**Table 13**). On the basis of this literature review, we chose to start by eluting one to three discs in 50-200 μL extraction buffer.

6.1.1 Extraction of protein from DBS

Exploiting as much as possible of the sample material is extremely important in situations where sample volume is limited. We therefore tested protein extraction from DBS using different extraction volumes, different amount of starting material and different mixing conditions. As expected we found a significant increase in protein concentration with an increasing number of discs for all elution buffer volumes and found the concentration to be highest in samples with the smallest elution buffer volumes. However, the results indicate that extraction volumes less than 150 uL is insufficient to extract the maximum obtainable protein from the samples. We also experienced that the DBS discs absorbed fluid during sample processing which further complicated the use of small extraction buffer volumes.

In comparable validation studies, DBS samples have been mixed on a multiplate shaker in 30 minutes to two hours with or without overnight incubation prior to mixing the samples (84, 88, 91, 94). We tested whether we could mix the DBS samples by sonication alone as this is an easy and time saving method of extracting protein that has been published by Bastani et al in a method paper to measure isoprostanes from DBS (95). We showed that mixing the

samples by sonication alone was just as effective in extracting protein as sonication and 20 min shaking in combination. In DBS samples containing three discs, protein concentration was actually higher in the sonicated samples compared to samples that were sonicated and mixed for 20 minutes. A possible explanation for this difference may be that the samples mixed by both methods were exposed to room temperature for a longer time period than samples that were just sonicated. This may have made the samples more vulnerable to protein degradation, as these initial experiments were conducted without the use of protease inhibitors in the extraction buffer.

6.1.2 Optimization of the ELISA assay

In several published protocols, DBS discs have been eluted in an optimized volume of extraction buffer before a smaller volume of the extracted sample have been added to the assay (84, 88, 94). However, using this approach, sample material will not be completely exploited. When adapting the ELISA assay to DBS samples, this method for sample extraction resulted in suPAR concentrations barely above the lower limit of detection. To overcome this problem, we tested whether we could optimize the method by extracting the samples directly in the dilution buffer provided in the kit (PBS + Brondiox) to maximize the sample utilization. Others have tried similar approaches for measuring C-reactive protein, transferrin receptor and leptin from DBS cards (82, 89-91). Our results indicated that protein extraction efficiency from the DBS samples using the dilution buffer provided with the kit was better than the other extraction buffers. This finding might be explained by the presence of Brondiox in the extraction buffer, as it is a protease inhibitor in addition to being an antimicrobial agent (96). A protective effect of protease inhibitors has been reported previously in a validation study by Skogstrand and coworkers measuring 25 inflammatory markers and neurotrophins in DBS (97). When suPAR was measured after extracting the sample directly in the dilution buffer we were able to obtain suPAR concentrations above the lower limit of detection for all samples tested, although samples using two discs as starting material were only barely detectable. For the validation experiment we therefore chose to use four discs in the optimized assay protocol to avoid the likelihood of obtaining suPAR concentrations below the lower limit of detection.

In situations where highly concentrated samples are required it is a good option to elute DBS discs directly in the extraction buffer volumes in assay protocol

Table 13 Previously validated protein biomarkers related to obesity and inflammation

Biomarker	Volume/ Size	Source	Extraction buffer volume (µL)	Analytical method	Stability (freeze-thaw)	DBS vs. venous	Author (year)
Adiponectin	2 x 3,2 mm	C-WB	180	Luminex	Yes (no)	Yes	Klamer (2007)
	1 x 3,2 mm	C-WB	200	CB-ICE	Yes (no)	Yes	Mihalopou (2011)
Insulin	2 x 3,0 mm	C-WB	100	ELISA	Yes (yes)	Yes	Martin (2012)
	2 x 3,0 mm	V-WB	100	CLIA	Yes (yes)	Yes	Butter (2001)
	3 circles	C-WB	2000	RIA	No (no)	Yes	Dowlati (1998)
	2 x 6,0 mm	C-WB	350	ELISA	No (yes)	Yes	Kapur (2008)
	1 x 3,2 mm	C-WB	200	CB-ICE	Yes (no)	Yes	Mihalopou (2011)
HbA1c	2 x 3,2 mm	V-WB	500	TQAS/HPLC	Yes (no)	No	Jones (2010)
Apo A	1 x 3,0 mm	V-WB/C-WB	250	ELISA	Yes (no)	Yes	Wang (1989)
Apo B	1 x 3,0 mm	V-WB/C-WB	250	ELISA	Yes (no)	Yes	Wang (1989)
Lp (a)	1 x 3,0 mm	-	2500	ELISA	No (no)	Yes	Wang (1992)
Leptin	2 x 3,2 mm	C-WB	250	EIA	Yes (yes)	Yes	Miller (2006)
	1 x 3,2 mm	C-WB	200	CB-ICE	Yes (no)	Yes	Mihalopou (2011)
IGF-1	2 x 3,2 mm	V-WB	400	RIA	Yes (no)	Yes	Schutt (2003)
	1 x 8,0 mm	C-WB	200	ELISA	Yes (no)	Yes	Diamandi (1998)
IGFBP-2	2 x 3,2 mm	V-WB	250	RIA	Yes (no)	Yes	Schutt (2003)
IGFBP-3	1 x 3,2 mm	V-WB	1000	RIA	Yes (no)	Yes	Schutt (2003)
	1 x 8,0 mm	C-WB	500	ELISA	Yes (no)	Yes	Diamandi (1998)
CRP	1 x 3,2 mm	C-WB	250-5000	ELISA	Yes (no)	Yes	Brindle (2010)
	1 x 3,2 mm	C-WB	250	EIA	Yes (yes)	Yes	McDade (2004)
	1 x 6,0 mm	C-WB	200	ELISA	No (no)	Yes	Kapur (2008)
	1 x 6 mm	C-WB	100	ELISA	Yes (no)	Yes	Cordon (1991)
	2 x 3,2 mm	C-WB	50-200	Luminex	Yes (no)	No	Skogstrand (2005)

Analyte	Volume/Size	Source	Extraction buffer volume (µL)	Analytical method	Stability (freeze-thaw)	DBS vs. venous	Author (year)
IL-1β	2 x 3,2 mm	C-WB	50-200	Luminex	Yes (no)	No	Skogstrand (2005)
IL-2	2 x 3,2 mm	C-WB	50-200	Luminex	Yes (no)	No	Skogstrand (2005)
IL-4	2 x 3,2 mm	C-WB	50-200	Luminex	Yes (no)	No	Skogstrand (2005)
IL-5	2 x 3,2 mm	C-WB	50-200	Luminex	Yes (no)	No	Skogstrand (2005)
IL-6	2 x 3,2 mm	C-WB	50-200	Luminex	Yes (no)	No	Skogstrand (2005)
	4 x 3,2 mm	C-WB	100	ELISA	No (no)	Yes	Miller (2012)
IL-8	2 x 3,2 mm	C-WB	50-200	Luminex	Yes (no)	No	Skogstrand (2005)
IL-10	2 x 3,2 mm	C-WB	50-200	Luminex	Yes (no)	No	Skogstrand (2005)
IL-12	2 x 3,2 mm	C-WB	50-200	Luminex	Yes (no)	No	Skogstrand (2005)
IL-17	2 x 3,2 mm	C-WB	50-200	Luminex	Yes (no)	No	Skogstrand (2005)
IL-18	2 x 3,2 mm	C-WB	50-200	Luminex	Yes (no)	No	Skogstrand (2005)
TNF-α	2 x 3,2 mm	C-WB	50-200	Luminex	Yes (no)	No	Skogstrand (2005)
TNF-β	2 x 3,2 mm	C-WB	50-200	Luminex	Yes (no)	No	Skogstrand (2005)
IFN-γ	2 x 3,2 mm	C-WB	50-200	Luminex	Yes (no)	No	Skogstrand (2005)
RANTES	2 x 3,2 mm	C-WB	50-200	Luminex	Yes (no)	No	Skogstrand (2005)
MCP-1	2 x 3,2 mm	C-WB	50-200	Luminex	Yes (no)	No	Skogstrand (2005)
GM-CSF	2 x 3,2 mm	C-WB	50-200	Luminex	Yes (no)	No	Skogstrand (2005)
MIP-1α	2 x 3,2 mm	C-WB	50-200	Luminex	Yes (no)	No	Skogstrand (2005)
MIP-1β	2 x 3,2 mm	C-WB	50-200	Luminex	Yes (no)	No	Skogstrand (2005)
s-IL-6Ra	2 x 3,2 mm	C-WB	50-200	Luminex	Yes (no)	No	Skogstrand (2005)
TGF-β	2 x 3,2 mm	C-WB	50-200	Luminex	Yes (no)	No	Skogstrand (2005)
MMP-9	2 x 3,2 mm	C-WB	50-200	Luminex	Yes (no)	No	Skogstrand (2005)
TREM-1	2 x 3,2 mm	C-WB	50-200	Luminex	Yes (no)	No	Skogstrand (2005)
BDNF	2 x 3,2 mm	C-WB	50-200	Luminex	Yes (no)	No	Skogstrand (2005)
NT-4	2 x 3,2 mm	C-WB	50-200	Luminex	Yes (no)	No	Skogstrand (2005)

Abbreviations: HbA1c: Glycated hemoglobin, apo A: Apolipoprotein A, Apo B: Apolipoprotein B, Lp (a): Lipoprotein (a), IGF: Insulin like growth factor, IGFBP: Insulin like growth factor binding protein, CRP: C-reactive protein, IL: Interleukin, TNF: Tumor necrosis factor, IFN: Interferon, RANTES: Regulated upon activation normal T cell expressed and presumably secreted, MCP: Monocyte chemotactic protein, GM-CSF: Granulocyte-macrophage colony-stimulating factor, MIP: Macrophage inflammatory protein, TGF: Transforming growth factor, MMP: Matrix metalloproteinase, TREM: Triggering receptor expressed on myeloid cells, BDNF: Brain Derived Neurotrophic Factor, NT: Neurotrophin. WB: Whole blood, C: Capillary, V: Venous, CB-ICE: Chip-based immuneaffinity capillary electrophoresis, ELISA: Enzyme-linked immunosorbant assay, CLIA: Chemiluminescence immunoassay, RIA: Radioimmunoassay, TQAS: Tina-quant immunoturbidimetric method, HPLC: High performance liquid chromatography, EIA: Enzyme immunoassay.

6.1.3 Validation of suPAR

We developed and validated a method of quantifying suPAR in DBS samples based on a commercially available ELISA assay. suPAR measured using the DBS ELISA method agreed closely with those obtained in plasma samples, with a correlation coefficient of 0.084. Our findings is in agreement with those obtained in other validation studies, obtaining correlation coefficients in the range 0.88-0.99 (82-91) between DBS and plasma or serum samples. We found that suPAR levels measured from four DBS discs were about six times lower than suPAR levels measured in 25 uL plasma, which could be expected based on the different amount of starting material. One disc of DBS has been reported to contain approximately 1.4 uL serum (76). Theoretically, four DBS discs should therefore be equivalent to 5.6 uL serum/plasma.

In assay protocols requiring undiluted plasma samples, as in this ELISA assay, it can be particularly challenging to obtain protein concentrations within the detectable area. By using four DBS discs and the optimized assay for protein extraction as described in the previous section, we were able to obtain suPAR concentration above the lower limit of detection for eleven of the 12 samples analyzed. Four discs is a relatively large amount of discs compared to what have been used previously for other DBS biomarkers (table 13). However, results from our initial experiments showed that this amount of sample was necessary to obtain suPAR concentrations within the detectable range in the present assay. Furthermore, by inspecting the Bland-Altman plot it could seem like the measurement imprecision was higher among the samples with the lowest anlyte concentrations, thus supporting the use of four DBS discs. Similar observations were seen in a recently published validation study measuring IL-6 in DBS samples (83).

Collecting whole blood on filter paper has several advantages over venipuncture that makes it ideal for use in large clinical trials. First, sample collection is a relatively painless and noninvasive method of collecting blood. This makes it possible to collect blood from particularly vulnerable groups such as infants, children and critically ill patients. Second, sample collection can be performed in a non-clinical environment by individuals who are not medically trained, as the procedure is easy and straightforward, and entails a minimal risk of contamination. Third, DBS samples do not have to be centrifuged, separated or immediately frozen following collection. Sample storage and transportation are facilitated by the fact that

filter paper cards can be stacked and stored in airtight containers and kept at ambient temperature. This is of great benefit when conducting large population-based studies and clinical trials, particularly in projects involving international cooperation. As mentioned previously, DBS blood sampling can be conducted at the participant's home and transported by post to the laboratory. For the NFS, samples at 3, 7 and 15 years follow-up will be based on DBS samples only. Transportation by post can potentially take between one and five days. In clinical trials involving international cooperation, transportation of samples by post could take up to several weeks. It is therefore important to know how different analytes behave in DBS with regards to stability. The results of this thesis indicate that suPAR is stable in DBS kept at room temperature for up to three weeks. This gives some degree of flexibility when it comes to sample collection, processing and storage for future use of this DBS ELISA assay. However, as analyte stability is shown to vary greatly by analyte, analyte stability should be evaluated prior to sample collection and processing before measuring new biomarkers in DBS. For instance, glycated hemoglobin (HbA1c) and IGF-1 have been shown to be stable in room temperature for at least one month (94, 98), while levels of apolipoprotein A and adiponectin is shown to decline already after one and five days, respectively (99, 100).

Previous analysis have shown that investigators can attain the same degree of precision and reliability from DBS samples that can be expected from standard methods of sample collection (76). However, there are some challenges and sources of potential errors associated with sample collection and analysis that need to be discussed. First, obtaining perfect blood spots can be a practical challenge as the blood drop formation varies greatly among individuals. In some individuals one fingerprick is enough to fill out an entire filter paper card, while in others, multiple fingerpricks are required. Using several fingerpricks is not always an alternative as this may be uncomfortable for the patient and thereby compromise patient comfort. Furthermore, placing a second drop of blood on a previously collected drop of blood is not recommended as this may affect the absorbing properties of the filter paper (75). It is also important to obtain blood spots of comparable sizes, as blood drop size is shown to have an impact on the volume of serum contained within a single disc punched from the blood spot (76). Using filter paper cards with preprinted circles, as was done in this thesis, is a good way of overcoming, or at least minimizing the variation this may introduce. Second, there are important differences between DBS and plasma samples that may complicate the analyzing process. DBS samples are dried on filter paper and must therefore be brought into solution before any analyses can be performed. Drying of the sample may alter the

biochemical structure of the analyte (75). Furthermore, the efficiency of which analytes enter solution may vary (75). Another important difference between DBS and plasma samples is that DBS samples contain red and white blood cells in addition to the liquid fraction of blood. The presence of these cellular fragments may interfere with the assay system and affect assay sensitivity (75). In addition, due to the sample elution step, analyzing samples from DBS is more time consuming compared to the direct use of plasma samples.

One circle on a DBS card entirely filled with blood, as instructed in the protocol, will provide a maximum of 7 discs. However due to problems with the sampling process for many patients, the circles were often not filled out completely and sometimes it was only possible to obtain one-two discs per spot. Because we needed to use four discs, to obtain detectable suPAR concentrations we used a large quanta of the total DBS material for each patient. DBS validation studies of most other biomarkers have used less than four discs (Table 13).

Hematocrit level in blood is known to vary among individuals. Therefore, some laboratories correct DBS samples for hematocrit, as hematocrit level may influence the volume of blood collected on the filter paper. Our DBS results were not corrected for hematocrit, as this information was not available. Lack of correction may have affected the volume of plasma introduced into the assay, as DBS samples with low hematocrit values is shown to contain more serum than DBS samples with high hematocrit values (76). However, it has been reported that correcting for hematocrit is of minor importance for determining analyte concentration when values are within the normal physiological range (82, 94, 101).

Our results show that it is possible to measure suPAR in DBS material using four discs as starting material. However, because we needed more discs than reported for other biomarker assays a more sensitive assay would have been optimal.

6.2 Discussion of results

6.2.1 Subject characteristics

As obesity is associated with increased risk of CRC and possibly associated with prognosis (102), we aimed to investigate the weight status and its relation to common comorbidities in a group of CRC patients pre-surgery. We found a high prevalence of overweight (56 %) and obesity (22 %) among the CRC patients with a mean BMI of 27.5 kg/m^2, which is higher than expected in a healthy Norwegian population (Companion master thesis, Mari Bøe Sebelien). Approximately 76 % of the CRC patients were either overweight or obese, the prevalence being higher among men (90 %) than among woman (68 %). This has also been reported in other studies (103, 104). Our results agree closely with those reported in other studies. In a prospective cohort study from Australia, including 526 CRC patients, the prevalence of overweight and obesity was 45 and 24 %, respectively (104). In two other studies, investigating the nutritional status among Swedish and British CRC patients, the prevalence of overweight and obesity was slightly lower compared to what we found (22, 103). Karlsson and coworkers found a combined prevalence of overweight and obesity of 47 %, while Burden and coworkers found a combined prevalence of 61.5 % (22, 103). The results from these trials may not be directly comparable to our results as they used slightly different BMI cut-offs to define overweight and obesity, which may have resulted in a lower prevalence of overweight and obesity. Furthermore, the mean age as well as the distribution of cancer localization was slightly different compared to our population, with a higher mean age. The proportion of rectal tumors was higher in the study of Burden and coworkers compared to ours.

Comorbid conditions before cancer diagnosis may affect clinical appropriateness and effectiveness of treatment, the risk of developing another chronic disease after diagnosis as well as the survival after treatment (27, 105, 106). The most common comorbid conditions among our study participants were hypertension (41 %), gastrointestinal diseases (20 %), CVD (17 %), DMT2 (15 %) and hyperlipidemia (15 %). Similar findings have been reported in other studies. In a study by Shack and coworkers the most commonly reported comorbid conditions among CRC patients were hypertension (20 %), COPD (7.9 %), ischemic heart disease (7.1 %) and other cancers (6.0 %) (27). In a study by De Marco and coworkers, including a total of 3355 CRC patients, the most frequently reported comorbidities were

CVD, other cancers and hypertension (26). The same comorbid conditions were the most common among patients above and below 70 years of age, but the percentage were generally higher among those over 70. A higher prevalence of comorbid conditions among those over 70 years of age was also observed by Pedrazzani and coworkers (106). The most commonly reported comorbidities among patients below and above 70 years of age were CVD (30.7 and 51.1 %), COPD (3.6 and 7.9 %), diabetes mellitus (6.2 and 10.1 %), gastrointestinal diseases (12.0 and 11.2 %) and other cancers (4.0 and 8.4 %) (106). In our study, we found a higher prevalence of hypertension, DMT2 and gastrointestinal diseases in the high compared to the low age group when dividing the participants by median age (62 years). The difference was however only significant for gastrointestinal diseases. We were not able to detect any significant differences between the age group for the remaining comorbidities. The prevalence of comorbidity has been reported to be higher among participants with proximally located tumors compared to distally located tumors, and it has been suggested that subsite-specific occurrence of comorbid conditions may indicate that different factors are involved in development of cancer at different sites (106). We were not able to detect any significant differences in frequency of comorbid conditions in this interim analysis.

It is well established that obesity is a major risk factor of hypertension (107). Therefore, it is not surprising that we found a significant difference in frequency of hypertension between the normal weight and obese participants. In our study, approximately 78 % of the obese participants had hypertension compared to 39 and 11 % of the overweight and obese participants, respectively. Although it is well accepted that obesity is a contributing factor to hypertension, the exact mechanisms linking the two conditions are not completely understood. One suggested mechanism is the dysregulated production and secretion of adipokines associated with adiposity (107). The impact of adipokines on hypertension will be discussed later in the thesis.

Because the data on comorbid conditions were collected from medical records rather than being self-reported, this may have reduced the likelihood of under-reporting. For instance, cancer patients have been shown to under-report history of previous cancers (108). However, some diagnosis may have been missed as we only reviewed documents from the time period the patient was referred to the specialist health service. Although it is common that all comorbid conditions are registered at discharge, administrative discharge diagnoses may not be entirely accurate (109).

A stronger association has been found between obesity and cancer of the colon compared to cancer of the rectum (32). In our interim analysis, cancer localization was significantly differently distributed among the normal weight and overweight participants. Among the normal weight participants, 22 % were diagnosed with colon cancer, 11 % with rectosigmoid cancer and 67 % with cancer of the rectum. Among the overweight participants, 77 % were diagnosed with colon cancer, 9 % with rectosigmoid cancer and 14 % with cancers of the rectum. Similar findings have been reported by Karlsson and coworkers (103). In their study, 18 and 12 % of the colon cancer patients were overweight and obese compared to 10 and 7 % among the rectum cancer patients. The results are in contrast to what was observed by Hillenbrand and coworkers, where no significant difference in BMI was found between colon and rectum cancer patients (110).

6.2.2 Weight status and plasma biomarkers pre-surgery

Obesity is associated with insulin resistance, altered production and secretion of adipokines from the adipose tissue as well as low-grade chronic inflammation (41). These are all mechanisms suggested to be important for obesity-associated CRC (33). We aimed to investigate the relationship between weight status and obesity and inflammation-associated biomarkers.

Our results indicate that waist circumference, a measure of abdominal obesity is the most predictive measure of circulating levels of obesity associated biomarkers, followed by BMI, a measure of total body fatness. Body weight and waist/hip-ratio seemed to be less effective in predicting biomarkers of obesity and inflammation. The observation that waist circumference is a better predictor of adipokine dysregulation than BMI may be explained by the fact that waist circumference takes body fat distribution into account, as opposed to BMI which do not (42). Much research indicate that adipose tissue distribution and quality, not just the amount is important for cardiometabolic risk and risk of cancer (33, 111). Visceral adipose tissue, primarily located in the abdominal region, is assumed to better reflect the increased risk of metabolic abnormalities accompanying obesity compared to subcutaneous adipose tissue (111).

Our results indicate that leptin, adipsin, suPAR, lipocalin-2, resistin, PAI-1 and insulin are associated with measures of body fatness and abdominal obesity. The associations between obesity-related biomarkers and anthropometric data will be discussed separately below.

Leptin seemed to be most strongly associated with measures of obesity, in particularly BMI and waist circumference. Significant positive associations with leptin have been reported in other studies for BMI (54-56, 110), body weight (54), waist circumference (60) and waist/hip-ratio (54). Furthermore, leptin is shown to be significantly higher in obese individuals compared to normal weigh subjects (63, 110). Leptin is secreted at a higher rate from subcutaneous compared to visceral adipose tissue (111). Thus, circulating levels of this adipokines is reported to correlate more closely with total subcutaneous adipose tissue than total body fat (111). Leptin is an important regulator of appetite and energy balance. During starvation, leptin levels decline contributing to hyperpaghia, decreased metabolic rate as well as changes in hormone levels designed to restore energy balance (112). As circulating leptin

levels increase with adiposity, the concept of central leptin resistance has been introduced (112). The molecular basis for this concept is however yet to be determined (112).

Adipsin is an adipocyte secreted serine protease important for triglyceride synthesis and storage in adipose tissue (112). We found a significant association between adipsin and all measures of adiposity. A high correlation between adipsin and BMI have also been found in a study by Maslowska and coworkers (113). Furthermore, circulating levels of adipsin is reported to be low in individuals with anorexia nervosa and to increase with refeeding (114), further supporting a role of this adipokine in obesity.

We found a significant association between suPAR and BMI, waist circumference and body weight in our data. The literature regarding the role of suPAR in obesity is inconclusive (70, 115). Cancello and coworkers found significantly higher levels of suPAR in morbidity obese patients compared to lean controls, suggesting an association between this protein and obesity (115). suPAR levels were also significantly reduced in response to surgery-induced weight loss, although the significant reduction disappeared with time even though the metabolic and inflammatory parameters continued to improve (115). Andersen and coworkers investigated circulating suPAR levels in HIV-infected patients receiving highly active antiretroviral therapy (116). Interestingly, they observed that concentration of suPAR was significantly higher among patients with lipodystrophy, an adverse side effect of the antiretroviral treatment compared to patients without any manifestation of lipodystrophy. Furthermore, suPAR was positively associated with measures of insulin resistance and lipid disturbances, while it was negatively associated with percent limb fat. No association was observed between suPAR and BMI. Eugen-Olson and coworkers did not observe any associations between suPAR and BMI and waist circumference (70). Based on the lack of associations, they suggested that suPAR may be less related to anthropometric measures characterizing a dysmetabolic phenotype. The conflicting results regarding suPAR and obesity emphasize the need for more research to clarify the role of this biomarker in obesity and obesity associated abnormalities.

In our data resistin was significantly associated with BMI and waist circumference. In the literature, results regarding the role of resistin in obesity have been somewhat more inconclusive (54, 55, 60, 63, 68, 110). Pagano and coworkers observed a significant positive association between resistin and obesity, as measured by BMI in adults with Prader-Willi syndrome (63). A significant positive association between resistin and BMI was also found by

Degawa-Yamauchi and coworkers (117). In the latter study, resistin levels was also significantly higher in the obese subjects compared to the normal weight subjects. However, the results of this study have been questioned because of the different distribution of age and gender among the obese and normal weight subjects (118). Several studies have not been able to detect an association between resistin and obesity (54, 55). Furthermore, no difference in resistin concentration between obese and normal weight subjects has been reported in several studies (55, 63, 110).

There is growing evidence that overweight and obesity are associated with impaired fibrinolysis (119). PAI-1, the most important inhibitor of fibrinolysis, has been reported to be particularly evident among individuals with overweight and obesity and/or abdominal obesity (110, 119). In our analysis, PAI-1 was significantly associated with waist circumference and waist/hip-ratio, but not with BMI and body weight.

Obesity is associated with insulin resistance and hyperinsulinemia (42). Accordingly, we hypothesized that obesity would be associated with levels of insulin in our population. We found a significant positive association between insulin and waist circumference, and trends towards an association with BMI and body weight. Insulin secretion is reported to reflect the amount of visceral adipose tissue in the body (111). Furthermore, it is assumed that visceral adipose tissue may be more important for development of insulin resistance and hyperinsulinemia than subcutaneous adipose tissue (111). There are some limitations with our experimental design that needs to be addressed. First of all, we used both fasting and non-fasting blood samples which most certainly may have introduced an error in our data (will be discussed in further detail below). Second, we did not have any information about use of medications. This information would have been useful as seven of our patients were diabetic (including both DMT1 and DMT2) As we do not know which types of medications the patients used or whether or not they had taken these medications prior to blood sampling, we were not able to control for this in our analysis. Third, it is possible that measuring C-peptide, a by-product of insulin would be a better marker of glycemic status, as the peripheral concentration of this protein not is affected by large and varying hepatic extractions after secretion from the pancreas as insulin are (120). Furthermore, C-peptide reflects endogenous insulin production rather than total insulin and will therefore not be affected by insulin injections (121). Measurements of C-peptide will also not be influenced by the presence of insulin antibodies in the circulation (122). Despite some limitations with our experimental

design, we were still able to detect an association between waist circumference and insulin in our data. This may indicate that the association may be even stronger than what we observed from our data.

Adiponectin has been shown to be inversely associated with measures of obesity (60, 61, 63) and is reported to be significantly lower in morbidity obese patients compared to controls (110). However, we did not observe an inverse association between obesity and adiponectin in our data. Lack of association could be due to large interindividual variations in adiponectin levels in the study population. This was also reported in a study by Rokling-Andersen and coworkers (54).

CRP, a systemic marker of inflammation has been showed to be positively correlated to BMI (54, 123) and waist/hip-ratio (54, 123). Furthermore CRP levels has been shown to be higher among obese participants compared to participants with normal BMI (124). However, we did not observe any association between CRP and anthropometric measures in this interim analysis. The unexpected results may have occurred as a result of the small number of individuals included in this analysis (n=9).

As obesity is associated with low-grade chronic inflammation (42), we hypothesized that the inflammatory cytokines and chemokines would be associated with measures of obesity in our population. However we did not detect any associations between these biomarkers and obesity. It is possible that we not were able to capture the low-grade chronic inflammation associated with obesity, as other factors in this study population may have been more important for the inflammatory response than obesity. For instance, it is not unreasonable to assume that the CRC patients experience increased systemic inflammation as a result of the cancer disease per se. Furthermore, comorbidity may have been an important contributing factor to inflammation. Another possible explanation for the lack of association is that patients may have lost weight prior to surgery. This may have influenced the plasma level of the inflammatory biomarkers measured, as circulating level of some cytokines and chemokines are shown to change in response to weight loss (62, 125). Nevertheless, we cannot rule out that the lack of association is due to the small number of participants included in this interim analysis.

Results from the correlation analysis indicated that several of the obesity-associated biomarkers were strongly correlated including adipsin, lipocalin-2, resistin and PAI-1.

Interestingly, suPAR, was also significantly correlated with several of the obesity-associated biomarkers including adiponectin, resistin, lipocalin-2 and adipsin. In fact, suPAR seemed to be more correlated to obesity-associated biomarkers than the inflammatory biomarkers. The results support findings from previous research suggesting a role of suPAR in the pathogenesis of obesity (115). Several of the inflammatory biomarkers were inter-correlated. By inspecting the score plot from the PCA, IL-1b, IL-6, IL-8, TNF-a, MCP-1, NGF and HGF was inter-correlated. Similar inter-correlations between inflammatory biomarkers was observed by Rokling-Andersen and coworkers (54). Inter-correlated inflammation biomarkers suggest that mutually related pathways may be involved in their regulation (54).

There are some limitations with this thesis that needs to be discussed. As only a small number of the CRC patients met fasting on arrival at the patient hotel pre-surgery, analysis of plasma biomarker are based on both fasting and non-fasting samples. This may have introduced a source of error in our results as it well established that concentration of some of the plasma biomarkers may change in response to feeding. Based on the biological role of insulin as a regulator of glucose and lipid metabolism, we assume that the results obtained on insulin and glucose may be biased by the state of fasting. Hancox and coworkers showed that fasting and non-fasting insulin and glucose levels was only moderately correlated with correlation coefficients of 0.44 and 0.40, respectively (126). On the other hand, fasting and non-fasting measurements of leptin and adiponectin was highly correlated with correlation coefficients of 0.95 and 0.96, respectively, indicating that fasting is of minor importance for the levels of these adipokines (126). Furthermore, fasting is assumed to be of minor importance for plasma concentration of suPAR (74, 116) and resistin (55). In our population, there were no significant differences in biomarker concentration between samples from fasting and non-fasting participants. However it should be mentioned that the number of fasting participants was small, reducing the likelihood of detecting an actual difference between these groups.

Another limitation of this thesis is the lack of information about use of medications. In the NFS, medication use is registered at baseline of intervention. This information was therefore only available for a subgroup of the participants. Several commonly used medications is reported to have unintended effects on adipose tissue function. For instance, statins, aldosterone antagonists, thiazolidinedions and salicylates are assumed to influence the circulating levels of adipokines and cytokines (127). Lack of information about use of

medications may therefore have introduced a bias in our results, probably indicating that the associations observed are even stronger.

As no information about weight history prior to surgery existed, we do not know whether the patients experienced weight loss prior to surgery, As the level of some adipokines and cytokines are reported to changes in response to weight loss (54, 56, 62, 119, 125) and as weight loss is a common finding among newly diagnosed CRC patients (22, 103, 128), the concentration of adipokines and cytokines measured may have been influenced by a potential change in weight prior to surgery.

Despite some limitations, we demonstrate that several of the obesity-associated biomarkers were strongly associated with measures of body fatness and abdominal obesity in CRC patients.

6.2.3 Clinicopathological characteristics and plasma biomarkers pre-surgery

Tumor characteristics and plasma biomarkers

Much research has been conducted to investigate the impact of lifestyle factors on risk of CRC. Based on varying incidence rate of cancer at the various colorectal sub sites, as well as the variation in the aetiology of CRC, it has been suggested that lifestyle factors may impact CRC risk differently at the various anatomical sub sites (129). In our study population, tumor localization was differently distributed among patients with different BMI groups, colon cancer being most common among the overweight patients. Thus, we aimed to investigate potential differences between the anatomical sub sites further by comparing the concentration of obesity and inflammatory biomarkers among patients with different tumor localization. Our results showed no significant differences in biomarker concentration between patients with colon cancer compared to patients with rectosigmoid and rectum cancer. Several studies have investigating the impact on lifestyle factors on risk of cancer at the various sub sites (129-132). Some studies have reported a marked influence of cardiometabolic risk factors including abdominal obesity (130), physical inactivity (132) and dyslipidaemia (131) on cancers of the colon compared to cancers of the rectum (64). In a recently published meta-analysis including 30 cohort studies, a positive association between BMI and cancer risk was found for all anatomical sub sites, the most pronounced association being observed for the distal colon (129). In the same study, an inverse association was observed between physical activity and cancers of the proximal and distal colon, but not for cancers of the rectum (129).

Results from epidemiological and experimental studies have suggested a role of obesity and inflammatory biomarkers in tumor development and progression. We found a lower suPAR level in the most severe TNM stages, but no differences were observed for the other biomarkers. Although elevated suPAR levels has been associated with development of cancer and inflammatory conditions, its exact biological function is not yet established (72). This limits the interpretation potential of our results. In our population suPAR seemed to be more associated with an obesity phenotype based on the strong associations between suPAR, anthropometric measures and biomarkers of obesity.

Besides suPAR, there were no significant differences in biomarker concentration between the prognostic groups. However, recent literature has suggested an association between

adipokines and prognostic factors (58, 67, 68, 133). Special attention has been devoted to leptin, resistin and adiponectin

As leptin receptors are expressed in mucosa of the human colon, in adenomas as well as in colon cancer tissue, leptin signalling may be involved in regulation of cellular processes involved in the carcinogenesis (46). Several studies have found significantly lower leptin levels in CRC patients compared to controls (58, 110, 134). However, this has not been consistently reported (133). Furthermore, leptin concentration has been inversely associated with tumor stage (58) but results are inconclusive (110, 133).

Little is known about the role of resistin in CRC development and progression. Several studies have found significantly higher resistin levels among CRC patients compared to controls (58, 67, 68, 133, 134). However, this has not been reported in all studies (110). A possible explanation for an increased resistin concentration in CRC patients may be that the monocytes increases the production and secretion of resistin as part of the generalized inflammatory processes associated with cancer (68). Resistin has also been found to be correlated with tumor stage (67, 133). However, results are not consistent (58, 68).

Adiponectin levels are reported to be lower (68, 134) or non-significantly different (110, 133) in CRC patients compared to controls. Furthermore, studies have reported an inverse relationship between adiponectin and cancer stage (68), but result are inconsistent (133).

Although some studies have suggested an association between adipokines and prognostic factors, we did not detect any differences in biomarker concentrations between the prognostic groups. Thus, we do not confirm the proposed link between biomarkers of obesity and inflammation and prognostic factors in the present population.

There are some limitations that should be addressed. As the age distribution was slightly different among the groups compared we chose to adjust for age in both regression models. We also adjusted for waist circumference as this anthropometric measure was shown to be significantly associated with several of the plasma biomarkers investigated. However, there are some limitations associated with adjusting for covariates in small samples sizes. Tabachnick and Fidell recommend that the samples size should be greater than 50 plus eight times the number of independent variables when conducting multiple regression analysis (135). Still, we chose to include age and waist circumference in the regression model, as they were considered biologically relevant for the outcome of interest.

Because of the small sample size we chose to categorize CRC patients into two prognostic groups instead of four, patients with TNM stage I and II and patients with TNM stage III and IV. However, it is possible that categorizing the patients into four stage groups would be more optimal, as we would have been able to conduct stage-specific comparisons. Furthermore, we did not compare the level of plasma biomarkers between patients with and without lymph node involvement or metastasis due to the small number of affected cases. It would be interesting to investigate potential differences in the biomarker profile among these groups of patients in future analysis within the NFS.

Furthermore, a single measurement of the plasma biomarkers may not be representative of an individual's true exposure to these biomarkers as the measured levels may be influenced by both intra and inter-individual biological variability. However, previous research have showed that intraclass correlation coefficients is high for several of the plasma biomarkers measured (60), suggesting that a single measurement of circulating levels are reflective of the circulating levels of an individual. We tried to reduce inter-individual variability in our data to a minimum by adjusting for relevant covariates in all analysis.

As we measured plasma biomarkers in CRC patients only and cut-off values for most of the biomarkers measured do not exist (110), we cannot tell whether biomarker concentrations obtained were high or low compared to what is normal for a healthy population. Therefore, it would be interesting to include a control group in future biomarker analysis in the NFS to investigate whether levels of plasma biomarkers are significantly different in CRC patients compared to control subjects.

Multiple biomarkers are used to predict risk of CVD and DMT2. However, few plasma biomarkers exists predicting risk of cancer (70). Because the biomarkers measured in plasma in our study may have originated from multiple tissues we do not know if the biomarker profile is relevant for the tumor development or progression process. For adipokines and cytokines that are secreted from mononuclear phagocytes such as resistin, TNF-α and IL-6 it is possible that levels in tissue and the tumor microenvironment are more reflective of the tumor phenotype than levels in blood (60). However, since blood represents one of the most easy available, non-invasive source for biomarker measurement it is highly relevant to establish plasma biomarkers that can predict disease risk and make it possible to identify high-risk individuals and thereby initiate preventive strategies. Discovering plasma biomarkers related to cancer risk is therefore of particular importance to implement

preventive strategies in the early phases of disease development. Furthermore, there is a need to develop prognostic biomarkers as this can help to personalize the cancer treatment. Access to reliable prognostic biomarkers would for instance be valuable in determining which patients would benefit from adjuvant therapy and not. At the same time, it will spare a substantial number of low-risk patients from the adverse side effects associated with this treatment (71, 73). Although several biomarkers have been suggested as potential markers of prognosis in CRC, the number of markers that actually have proven clinically useful is currently limited (92). More research is needed to investigate the role of biomarkers in cancer development and progression.

Obesity associated hypertension and plasma biomarkers

It is well established that obesity increases the risk of hypertension. Elevated blood pressure is closely associated with increasing adiposity, and the vast majority of hypertensive patients are overweight or obese (107). Our results confirm the close association between obesity and hypertension. Although obesity and hypertension is clearly connected, the exact mechanisms linking the two conditions are not yet established. Obesity-associated hypertension is a multifactorial condition in which increased sympathetic nervous system activity, activation of the renin-angiotensin system and volume expansion are considered key components of the pathogenic process (136). The presence of adipokine receptors in vascular endothelium and smooth muscle cells supports a role of adipokines as a regulator of blood pressure (107). Some adipokines are also suggested to affect blood pressure via the central nervous system by regulating the activity of the sympathetic outflow (107). As adipokines are suggested as possible mediators of obesity-associated hypertension, we aimed at investigating the impact of the plasma biomarkers measured on hypertension. We found that suPAR, leptin and insulin significantly predicted hypertension in our population.

Few studies have investigated the relationship between suPAR and blood pressure regulation. In a study by Sehestedt and coworkers, suPAR concentration was significantly associated with blood pressure level (137). Furthermore, suPAR was significantly associated with the presence of plaque (137). In the SAfrEIC- study, investigating the relationship between suPAR and arterial stiffness in a bi-ethnic population, no independent association was found (138).

Several studies support a role of leptin in blood pressure regulation (136, 139, 140). In a recently published study, the Multi-Ethnic Study of Atherosclerosis (136), a significant association was found between leptin and systolic, diastolic, mean arterial and pulse pressure independent of relevant risk factors and anthropometric measures. Furthermore, patients having elevated leptin level had significantly higher odds for being hypertensive compared to patients with low leptin levels. A significant association between leptin and blood pressure was also found in a study of 457 untreated male employers independent of obesity measures (139). The association was also evident when conducting the analysis on normotensive subjects only. Evidence for an association between leptin and hypertension is further strengthening by the fact that obese patients with leptin deficiency have reduced sympathetic tone and normal blood pressure (140). Leptin is suggested to contribute to hypertension by stimulating the sympathetic nervous system, by effecting renal functions as well as by increasing the peripheral resistance, both by effect on the vascular endothelium, smooth muscle cells as well as macrophages (107).

Another mechanism that may contribute to obesity-associated hypertension is insulin resistance (141). DMT2 and hypertension are closely associated, and insulin resistance or hyperinsulinemia are present in most hypertensive patients (141). Possible mechanisms linking insulin resistance and obesity-associated hypertension are over-activation of the central nervous system, renal sodium retention, stimulation of smooth muscle cell growth and increased intracellular calcium and calcium sensitization, causing increased peripheral vascular resistance (141). Although, insulin resistance is suggested as a potential mediator linking obesity and hypertension, it is not clear whether hyperinsulinemia actually is causally related to hypertension (141). Insulin resistance may exerts its effect on hypertension via other mechanisms, such as inflammation and oxidative stress (141), or simply be a marker of a dysmetabolic state.

6.2.4 Changes in weight status from pre to two-months post-surgery

Unintentional weight loss can cause malnutrition in both the normal weight, overweight and obese patient (103). Patients that are normal weight, overweight or obese may appear well nourished although they actually may be loosing weight and have lost a significant amount of muscle mass (142). Monitoring weight changes is therefore of great importance to capture individuals at nutritional risk to be able to implement a nutritional intervention and thereby optimize nutritional status. Optimal nutritional status pre and post-surgery is important to aid recovery and to reduce the risk of postoperative complications and mortality (143). This thesis aimed to investigate the postoperative changes in weight status and physical function in CRC patients. We found a significant reduction in weight, BMI, waist circumference and waist/hip-ratio from pre- to two months post-surgery. Mean weight loss among CRC patients was -3.5 kg, corresponding to a percentage weight loss of 4 %. Mean reduction in BMI was -1.2 kg/m^2. Of the 25 participants with measurements before and after surgery 76 % lost weight. The results are in agreement with what was observed by Williams and coworkers, investigating the impact of surgery on skeletal muscle mass and protein turnover in 13 CRC patients (144). They observed a mean reduction in BMI of -1.5 kg/m^2 after surgery. In a study by Ulander and coworkers, as much as 89 % of the 75 CRC patients undergoing surgery lost weight during hospitalization (143). Mean percentage weight loss among the CRC patients were 6.1 and 3.3 % in men and woman, respectively (143).

Weight loss in the postoperative period may be explained by several factors. The nutritional intake may have been compromised, resulting in a negative energy balance. An insufficient energy intake during hospitalization was reported among most of the CRC patients in the study by Ulander and coworkers (143). Twenty-three percent of the CRC patients expended 40-60 % of estimated basal energy expenditure, 44 % expended 60-80 % of basal energy expenditure, 29 % expended 80-100 % of basal energy expenditure and 4 % expended 100-150 % of basal energy expenditure (143). Energy intake may also have been reduced in the time period after hospital discharge, as CRC surgery is associated with several side effects such as irregular bowel movements, gas and flatulence, obstipation, diarrhea, abdominal pain and stoma related complications. These side effects may have influenced the patients' energy intake postoperative and induced a state of negative energy balance.

Another contributing factor to weight loss may be surgical stress resulting in increased energy expenditure (143). Surgical stress is characterized by hypermetabolism causing accelerated

tissue breakdown and protein loss (143). In addition to causing weight loss, this stress response may result in impaired functional activity (143). Williams and coworkers observed a lower leg muscle mass in CRC patients compared to control subjects with and an additional decline after surgical resection (144). Postprandial muscle protein synthesis was significantly lower in CRC patients compared to controls, and a trend was observed towards a higher muscle protein breakdown (144). We did not find a significant reduction in handgrip strength during the pre- to post surgery period. Handgrip strength was significantly correlated with body weight both pre and postoperatively, but we did not detect any significant correlation between change in handgrip strength and change in body weight. This could indicate that the change in body weight observed in our study population not seemed to have an effect on physical function. Handgrip strength is only one out of several strategies to measure voluntary physical function. For instance muscle function can be measured by knee extension strength or hip flexion strength (80). However, handgrip strength is considered an attractive method to measure physical function, as it is validated and easy to use in clinical practice (80).

At the time period the present thesis was conducted, nutritional screening was not implanted as part of the preoperative assessment in the NFS. Thus, information about previous food intake and weight history was not available. Although weight loss and pronounced nutritional depletion often is a symptom of an advanced cancer disease, weight loss may occur early in the process, even before the tumor burden is significant (128). Weight loss prior to CRC surgery has been reported in various degree in the literature (22, 103, 128, 143). In the study by Karlsson and coworkers 30 % of the CRC patients were weight losing prior to surgery (103). In two other studies, the percentage of patients who lost weight was substantially higher at 52 and 77 % (22, 143).

Overall, the results from this interim analysis indicate that most of the CRC patients experience weight loss after surgery. However, weight loss does not seem to be important for physical function based on the lack of correlation between change in body weight and change in handgrip strength. Although most of the CRC patients experienced weight loss there were great interindividual differences in the response to surgery, highlighting the importance of individualized nutritional care in the postoperative period.

7 Conclusion

The rising occurrence of obesity worldwide is likely to have a significant impact on the prevalence of obesity-related cancers such as CRC in decades to come. Understanding the molecular mechanisms whereby obesity increases CRC risk is therefore of great importance to develop strategies to prevent a further increase in the prevalence of this disease. Plasma biomarkers reflecting endocrine and metabolic disturbances associated with obesity may not only aid in the understanding of colorectal carcinogenesis but also help define an “obesity phenotype” relevant to CRC development.

We have analyzed biomarkers of obesity and inflammation in relation to weight status in order to add knowledge as to how these processes may be connected. Both plasma and DBS have been used as sampling methods. Because DBS cards will be the main blood collection method for measuring long-term effects in the on-going clinical trial there is need to expand the panel of diagnostically valuable analytes validated in these samples. We therefore successfully developed and validated a method for measuring suPAR in DBS.

Our results indicate that CRC patients have a high prevalence of overweight and obesity pre-surgery. In addition we found that obesity and abdominal obesity was associated with levels of several adipokines in plasma, plasma insulin and suPAR measured in DBS. However we did not find an association between obesity and inflammation based on the inflammatory panel measured.

Out of all registered comorbid conditions among our study participants, hypertension was the only condition associated with obesity. Hypertension was more prevalent among the obese patients compared to patients with normal body weight, and could be independently predicted by leptin, insulin and suPAR.

Cancer localization was significantly differently distributed among the normal weight and overweight participants. There were no significant differences in biomarker concentration between participants with different tumor localization. Furthermore, we did not observe differences in biomarker concentrations between the TNM stage groups, except for suPAR, which was significantly lower among patients with the most severe TNM stages. The latter finding thus contradicts a role for suPAR as a prognostic marker in CRC patients.

Our results indicate that most CRC patients experience weight loss in the postoperative period. However, because there was no correlation between change in body weight and change in handgrip strength we suggest that weight loss does not seem to be important for physical function in our population.

In summary, our results confirm that CRC patients have a high prevalence of obesity and that adipokines, insulin and the novel biomarker suPAR are associated with obesity in this patient population. However, we do not confirm a link between obesity and low-grade inflammation with regards to the inflammatory biomarker panel measured. More research is needed to investigate the mechanisms linking obesity, inflammation and CRC.

8 Future perspective

The results of this thesis raise several interesting issues that should be followed. We find a high prevalence of overweigh and obesity in the present population, and thus confirm the association between CRC and obesity. As weight status may impact short and long-term outcome in cancer patients, it will be interesting to study the prognostic value of weight status in CRC patients pre- and post-surgery, as well the prognostic impact on weight changes during the postoperative period. Knowledge about how weight status effect prognosis is important to give optimized nutritional guidance in the follow-up of CRC patients.

Our results support a role of adipokines, as well as insulin and suPAR in the etiology of obesity. As studies indicate that several of these biomarkers are sensitive to changes in weight status, it will be interesting to investigate the impact of the diet and lifestyle intervention, with or without changes in weight, on levels of these biomarkers. Furthermore, because this thesis report for the first time an association between suPAR and diagnosed hypertension, it would be interesting to confirm the findings in the total NSF population and also examine whether changes in blood pressure during the intervention will be associated to changes in suPAR plasma levels. This could provide a potential mechanistic link to the reported association between suPAR and CVD (70).

The results from this interim analysis do not confirm an association between obesity and inflammation based on the inflammatory markers measured. Because of the small number of patients included in the analysis as well as the presence of possible confounders, caution should be made in the interpretation of these results. Whether the lack of association is caused by the limited number of participants included in the present thesis or due to an actual lack of association between obesity and inflammation will be investigated further in the NFS.

Results from correlation analysis can be used to generate hypothesis in terms of understanding which pathways that are mutually related. Our results indicate that several of the biomarkers measured are highly connected, and thus provides a basis for future analysis investigating the biological pathways involved in obesity-induced carcinogenesis. Understanding the pathophysiological mechanisms linking obesity and CRC will make it possible to implement preventive strategies in the primary and secondary prevention of this

disease. The biomarkers measured can also be used to investigate the possible prognostic impact on these biomarkers.

Our results indicate that most CRC patients experience weight loss in the postoperative period. As nutritional screening was not implemented at the time the present thesis was conducted, we do not know whether the changes in weight status are caused by an increased energy expenditure as a results of surgical stress or the cancer disease per se, or by a reduced energy intake, possibly due to treatment associated side effects. Some of these questions will be possible to answer when nutritional screening will be implemented as part of the NFS. Furthermore, nutritional screening will provide useful information about the patient's nutritional status together with bioelectrical impedance analysis, an assessment method already implemented as part of the measurements undertaken post-surgery.

We demonstrate in this thesis that it is possible to measure suPAR in DBS. The established DBS method can be used to investigate short and long-term effects of the NFS intervention on suPAR levels. The strategy used for method development used in this thesis will also be valuable to validate other relevant biomarkers. Because the long-term effects on biomarkers in the NSF study will depend solely on DBS samples, it will be necessary to validate the method for a wide range of disease related biomarkers.

9 List of references

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA: a cancer journal for clinicians 2011;61(2):69-90.

2. World Cancer Research Fund/American Institute for Cancer Research (WCRF/AICR). Food, Nutrition, Physical Activity, and the Prevention of Cancer: a Global Perspective. Washington DC: WCRF/AICR, 2007.

3. Weitz J, Koch M, Debus J, Höhler T, Galle PR, Büchler MW. Colorectal cancer. The Lancet 2005;365(9454):153-65.

4. UpToDate. Molecular genetics of colorectal cancer. Internet: http://www.uptodate.com/contents/molecular-genetics-of-colorectal-cancer (accessed 09.06.2013).

5. Drew JE, Farquharson AJ, Padidar S, et al. Insulin, leptin, and adiponectin receptors in colon: regulation relative to differing body adiposity independent of diet and in response to dimethylhydrazine. American journal of physiology Gastrointestinal and liver physiology 2007;293(4):G682-91.

6. Moghaddam AA, Woodward M, Huxley R. Obesity and risk of colorectal cancer: a meta-analysis of 31 studies with 70,000 events. Cancer Epidemiol Biomarkers Prev 2007;16(12):2533-47.

7. World Cancer Research Fund/American Institute for Cancer Research (WCRF/AICR). Landmark report: many cancers could be prevented across the globe. Internet: http://www.wcrf-uk.org/audience/media/press_release.php?recid=2 (accessed 02.01.2013).

8. World Cancer Research Fund (WCRF) International. Preventability of Cancer by Food, Nutrition, Physical Activity and Weight Management. York, UK: WCRF, 2008.

9. National Cancer Institute. NCI Dictionary of Cancer Terms. Large intestine. Internet: http://www.cancer.gov/dictionary?cdrid=45097 (accessed 09.06.2013).

10. American Joint Committee on Cancer. Colon and rectum. Internet: http://www.springer.com/cda/content/document/cda_downloaddocument/colon_rectum_chpt_14.pdf?SGWID=0-0-45-313390-0. (accessed 09.06.2013).

11. Wikipedia. Large intestine. Internet: http://en.wikipedia.org/wiki/Large_intestine (09.06.2013).

12. Zhu Q, Gao R, Wu W, Qin H. The role of gut microbiota in the pathogenesis of colorectal cancer. Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine 2013.

13. Sherwood L. Human Physiology: From cells to Systems. Sixth edition. Belmont, CA: Thomson Brooks/Cole, 2006.

14. Mai V. Dietary modification of the intestinal microbiota. Nutrition reviews 2004;62(6 Pt 1):235-42.

15. Kumar V. Abbas AK. Fausto N. Mitchell RN. Robins Basic Pathology. Eighth Edition. Philadelphia, PA: Saunders Elsevier, 2007.

16. Drew JE. Molecular mechanisms linking adipokines to obesity-related colon cancer: focus on leptin. Proc Nutr Soc 2012;71(1):175-80. doi: 10.1017/S0029665111003259. Epub 2011 Oct 21.

17. Jass JR. Pathogenesis of colorectal cancer. The Surgical clinics of North America 2002;82(5):891-904.

18. Grande-Puldio E. Riquelme-Oliveria A. Ballesteros-Bargues J. Guillen-Ponce C. Carrato A. The Challenge of Colorectal Cancer: A Review Book. Molecular biology of colorectal cancer. . Edtion ed. Kerala, India: Research Signpost, 2011.

19. Weitz J, Koch M, Debus J, Hohler T, Galle PR, Buchler MW. Colorectal cancer. Lancet 2005;365(9454):153-65.

20. UpToDate. Clinical manifestations, diagnosis, and staging of colorectal cancer. Internet: http://www.uptodate.com/contents/clinical-manifestations-diagnosis-and-staging-of-colorectal-cancer (accessed 09.06.2013).

21. American Cancer Society. Colorectal cancer. Internet: http://www.cancer.org/cancer/colonandrectumcancer/detailedguide/colorectal-cancer-what-is-cancer (accessed 09.06.2013).

22. Burden ST, Hill J, Shaffer JL, Todd C. Nutritional status of preoperative colorectal cancer patients. Journal of human nutrition and dietetics : the official journal of the British Dietetic Association 2010;23(4):402-7.

23. Nitenberg G, Raynard B. Nutritional support of the cancer patient: issues and dilemmas. Critical reviews in oncology/hematology 2000;34(3):137-68.

24. McGough C, Baldwin C, Frost G, Andreyev HJ. Role of nutritional intervention in patients treated with radiotherapy for pelvic malignancy. British journal of cancer 2004;90(12):2278-87.

25. Sarfati D, Hill S, Blakely T, et al. The effect of comorbidity on the use of adjuvant chemotherapy and survival from colon cancer: a retrospective cohort study. BMC cancer 2009;9:116.

26. De Marco MF, Janssen-Heijnen ML, van der Heijden LH, Coebergh JW. Comorbidity and colorectal cancer according to subsite and stage: a population-based study. European journal of cancer (Oxford, England : 1990) 2000;36(1):95-9.

27. Shack LG, Rachet B, Williams EM, Northover JM, Coleman MP. Does the timing of comorbidity affect colorectal cancer survival? A population based study. Postgraduate medical journal 2010;86(1012):73-8.

28. Eyre H, Kahn R, Robertson RM. Preventing cancer, cardiovascular disease, and diabetes: a common agenda for the American Cancer Society, the American Diabetes Association, and the American Heart Association. Diabetes care 2004;27(7):1812-24.

29. World Health Organization (WHO). Obesity and overweight. Internet: http://www.who.int/mediacentre/factsheets/fs311/en/index.html (accessed 09.06.2013).

30. World Health Organization (WHO). Obesity and overweight. Internet: http://www.mclveganway.org.uk/Publications/WHO_Obesity_and_overweight.pdf (accessed 09.06.2013).

31. Lim SS, Vos T, Flaxman AD, et al. A comparative risk assessment of burden of disease and injury attributable to 67 risk factors and risk factor clusters in 21 regions, 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet 2012;380(9859):2224-60. doi: 10.1016/S0140-6736(12)61766-8.

32. Ning Y, Wang L, Giovannucci EL. A quantitative analysis of body mass index and colorectal cancer: findings from 56 observational studies. Obes Rev 2010;11(1):19-30. doi: 10.1111/j.467-789X.2009.00613.x. Epub 2009 Jun 16.

33. Doyle SL, Donohoe CL, Lysaght J, Reynolds JV. Visceral obesity, metabolic syndrome, insulin resistance and cancer. Proc Nutr Soc 2012;71(1):181-9. doi: 10.1017/S002966511100320X. Epub 2011 Nov 3.

34. Jeon JY, Meyerhardt JA. Energy in and energy out: what matters for survivors of colorectal cancer? Journal of clinical oncology : official journal of the American Society of Clinical Oncology 2012;30(1):7-10.

35. Calle EE, Rodriguez C, Walker-Thurmond K, Thun MJ. Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. The New England journal of medicine 2003;348(17):1625-38.

36. Phillips CM. Nutrigenetics and metabolic disease: current status and implications for personalised nutrition. Nutrients 2013;5(1):32-57.

37. Prieto-Hontoria PL, Perez-Matute P, Fernandez-Galilea M, Bustos M, Martinez JA, Moreno-Aliaga MJ. Role of obesity-associated dysfunctional adipose tissue in cancer: a molecular nutrition approach. Biochim Biophys Acta 2011;1807(6):664-78. Epub 2010 Nov 24.

38. Wronska A, Kmiec Z. Structural and biochemical characteristics of various white adipose tissue depots. Acta physiologica (Oxford, England) 2012;205(2):194-208.

39. Haugen F, Drevon CA. The interplay between nutrients and the adipose tissue. The Proceedings of the Nutrition Society 2007;66(2):171-82.

40. Trayhurn P, Drevon CA, Eckel J. Secreted proteins from adipose tissue and skeletal muscle - adipokines, myokines and adipose/muscle cross-talk. Archives of physiology and biochemistry 2011;117(2):47-56.

41. Ouchi N, Parker JL, Lugus JJ, Walsh K. Adipokines in inflammation and metabolic disease. Nature reviews Immunology 2011;11(2):85-97.

42. Aleksandrova K, Nimptsch K, Pischon T. Influence of Obesity and Related Metabolic Alterations on Colorectal Cancer Risk. Curr Nutr Rep 2013;2(1):1-9. Epub 2012 Dec 16.

43. Alberti KG, Eckel RH, Grundy SM, et al. Harmonizing the metabolic syndrome: a joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. Circulation 2009;120(16):1640-5. doi: 10.161/CIRCULATIONAHA.109.192644. Epub 2009 Oct 5.

44. van Kruijsdijk RC, van der Wall E, Visseren FL. Obesity and cancer: the role of dysfunctional adipose tissue. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology 2009;18(10):2569-78.

45. Deng L, Gui Z, Zhao L, Wang J, Shen L. Diabetes mellitus and the incidence of colorectal cancer: an updated systematic review and meta-analysis. Digestive diseases and sciences 2012;57(6):1576-85.

46. Farquharson AJ, Steele RJ, Carey FA, Drew JE. Novel multiplex method to assess insulin, leptin and adiponectin regulation of inflammatory cytokines associated with colon cancer. Mol Biol Rep 2012;39(5):5727-36. doi: 10.1007/s11033-011-1382-1. Epub 2011 Dec 23.

47. Khaw KT, Wareham N, Bingham S, Luben R, Welch A, Day N. Preliminary communication: glycated hemoglobin, diabetes, and incident colorectal cancer in men and women: a prospective analysis from the European prospective investigation into cancer-Norfolk study. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology 2004;13(6):915-9.

48. Nathan C. Points of control in inflammation. Nature 2002;420(6917):846-52.

49. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. Cell 2010;140(6):883-99.

50. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. Clinical pharmacology and therapeutics 2001;69(3):89-95.

51. Fleming TR, Powers JH. Biomarkers and surrogate endpoints in clinical trials. Statistics in medicine 2012;31(25):2973-84.

52. Vasan RS. Biomarkers of cardiovascular disease: molecular basis and practical considerations. Circulation 2006;113(19):2335-62.

53. Drevon CA. Fatty acids and expression of adipokines. Biochimica et biophysica acta 2005;1740(2):287-92.

54. Rokling-Andersen MH, Reseland JE, Veierod MB, et al. Effects of long-term exercise and diet intervention on plasma adipokine concentrations. The American journal of clinical nutrition 2007;86(5):1293-301.

55. Lee JH, Chan JL, Yiannakouris N, et al. Circulating resistin levels are not associated with obesity or insulin resistance in humans and are not regulated by fasting or leptin administration: cross-sectional and interventional studies in normal, insulin-resistant, and diabetic subjects. The Journal of clinical endocrinology and metabolism 2003;88(10):4848-56.

56. Ostlund RE, Jr., Yang JW, Klein S, Gingerich R. Relation between plasma leptin concentration and body fat, gender, diet, age, and metabolic covariates. The Journal of clinical endocrinology and metabolism 1996;81(11):3909-13.

57. Aleksandrova K, Boeing H, Jenab M, et al. Leptin and soluble leptin receptor in risk of colorectal cancer in the European Prospective Investigation into Cancer and Nutrition cohort. Cancer research 2012;72(20):5328-37.

58. Salageanu A, Tucureanu C, Lerescu L, et al. Serum levels of adipokines resistin and leptin in patients with colon cancer. Journal of medicine and life 2010;3(4):416-20.

59. Stattin P, Lukanova A, Biessy C, et al. Obesity and colon cancer: does leptin provide a link? International journal of cancer Journal international du cancer 2004;109(1):149-52.

60. Ho GY, Wang T, Gunter MJ, et al. Adipokines linking obesity with colorectal cancer risk in postmenopausal women. Cancer research 2012;72(12):3029-37.

61. Arita Y, Kihara S, Ouchi N, et al. Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. Biochemical and biophysical research communications 1999;257(1):79-83.

62. Esposito K, Pontillo A, Di Palo C, et al. Effect of weight loss and lifestyle changes on vascular inflammatory markers in obese women: a randomized trial. JAMA : the journal of the American Medical Association 2003;289(14):1799-804.

63. Pagano C, Marin O, Calcagno A, et al. Increased serum resistin in adults with prader-willi syndrome is related to obesity and not to insulin resistance. The Journal of clinical endocrinology and metabolism 2005;90(7):4335-40.

64. Aleksandrova K, Boeing H, Jenab M, et al. Total and high-molecular weight adiponectin and risk of colorectal cancer: the European Prospective Investigation into Cancer and Nutrition Study. Carcinogenesis 2012;33(6):1211-8.

65. Wei EK, Giovannucci E, Fuchs CS, Willett WC, Mantzoros CS. Low plasma adiponectin levels and risk of colorectal cancer in men: a prospective study. Journal of the National Cancer Institute 2005;97(22):1688-94.

66. Lukanova A, Soderberg S, Kaaks R, Jellum E, Stattin P. Serum adiponectin is not associated with risk of colorectal cancer. Cancer epidemiology, biomarkers &

prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology 2006;15(2):401-2.

67. Danese E, Montagnana M, Minicozzi AM, et al. The role of resistin in colorectal cancer. Clinica chimica acta; international journal of clinical chemistry 2012;413(7-8):760-4.

68. Gonullu G, Kahraman H, Bedir A, Bektas A, Yucel I. Association between adiponectin, resistin, insulin resistance, and colorectal tumors. International journal of colorectal disease 2010;25(2):205-12.

69. Thuno M, Macho B, Eugen-Olsen J. suPAR: the molecular crystal ball. Disease markers 2009;27(3):157-72.

70. Eugen-Olsen J, Andersen O, Linneberg A, et al. Circulating soluble urokinase plasminogen activator receptor predicts cancer, cardiovascular disease, diabetes and mortality in the general population. J Intern Med 2010;268(3):296-308.

71. Stephens RW, Nielsen HJ, Christensen IJ, et al. Plasma urokinase receptor levels in patients with colorectal cancer: relationship to prognosis. J Natl Cancer Inst 1999;91(10):869-74.

72. ViroGates. What is suPAR? Internet: http://www.suparnostic.com/Key-Concepts/what-is-supar.html (accessed 09.06.2013).

73. Fernebro E, Madsen RR, Ferno M, et al. Prognostic importance of the soluble plasminogen activator receptor, suPAR, in plasma from rectal cancer patients. Eur J Cancer 2001;37(4):486-91.

74. Katia Donadello SS, Cecilia Covajes, Jean-Louis Vincen. suPAR as a prognostic biomarker in sepsis. BMC Medicine 2012.

75. McDade TW, Williams S, Snodgrass JJ. What a drop can do: dried blood spots as a minimally invasive method for integrating biomarkers into population-based research. Demography 2007;44(4):899-925.

76. Mei JV, Alexander JR, Adam BW, Hannon WH. Use of filter paper for the collection and analysis of human whole blood specimens. The Journal of nutrition 2001;131(5):1631S-6S.

77. Oslo Universitetssykehus HF. Nyfødtscreening. Internet: http://www.oslo-universitetssykehus.no/omoss/avdelinger/nyfodtscreening/Sider/enhet.aspx (accessed 09.06.2013).

78. World Health Organization (WHO). Waist Circumference and Waist-Hip Ratio: Report of a WHO Expert consultation Geneva: WHO, 2008.

79. Hjartaker A, Langseth H, Weiderpass E. Obesity and diabetes epidemics: cancer repercussions. Advances in experimental medicine and biology 2008;630:72-93.

80. Norman K, Stobaus N, Gonzalez MC, Schulzke JD, Pirlich M. Hand grip strength: outcome predictor and marker of nutritional status. Clinical nutrition (Edinburgh, Scotland) 2011;30(2):135-42.

81. Bøhn SK. Antioxidant-rich foods induce genes associated with stress defence. Implications for cancer and other oxidative stress related conditions. Department of Nutrition, Institute of Basic Medical Sciences, Faculty of Medicine. AIT Oslo, AS: University of Oslo, 2011.

82. McDade TW, Burhop J, Dohnal J. High-sensitivity enzyme immunoassay for C-reactive protein in dried blood spots. Clinical chemistry 2004;50(3):652-4.

83. Miller EM, McDade TW. A highly sensitive immunoassay for interleukin-6 in dried blood spots. American journal of human biology : the official journal of the Human Biology Council 2012;24(6):863-5.

84. Brindle E, Fujita M, Shofer J, O'Connor KA. Serum, plasma, and dried blood spot high-sensitivity C-reactive protein enzyme immunoassay for population research. Journal of immunological methods 2010;362(1-2):112-20.

85. Mihalopoulos NL, Phillips TM, Slater H, et al. Validity and reliability of perinatal biomarkers of adiposity after storage as dried blood spots on paper. American journal of human biology : the official journal of the Human Biology Council 2011;23(5):717-9.

86. Martin RM, Patel R, Zinovik A, et al. Filter paper blood spot enzyme linked immunoassay for insulin and application in the evaluation of determinants of child insulin resistance. PloS one 2012;7(10):e46752.

87. Dowlati B, Dunhardt PA, Smith MM, Shaheb S, Stuart CA. Quantification of insulin in dried blood spots. The Journal of laboratory and clinical medicine 1998;131(4):370-4.

88. Kapur S, Zava D. Cardiometabolic risk factors assessed by a finger stick dried blood spot method. Journal of diabetes science and technology 2008;2(2):236-41.

89. Miller AA, Sharrock KC, McDade TW. Measurement of leptin in dried blood spot samples. American journal of human biology : the official journal of the Human Biology Council 2006;18(6):857-60.

90. Cordon SM, Elborn JS, Hiller EJ, Shale DJ. C-reactive protein measured in dried blood spots from patients with cystic fibrosis. Journal of immunological methods 1991;143(1):69-72.

91. McDade TW, Shell-Duncan B. Whole blood collected on filter paper provides a minimally invasive method for assessing human transferrin receptor level. The Journal of nutrition 2002;132(12):3760-3.

92. Lomholt AF, Christensen IJ, Hoyer-Hansen G, Nielsen HJ. Prognostic value of intact and cleaved forms of the urokinase plasminogen activator receptor in a retrospective study of 518 colorectal cancer patients. Acta Oncol 2010;49(6):805-11

93. Gustafsson A, Ajeti V, Ljunggren L. Detection of suPAR in the Saliva of Healthy Young Adults: Comparison with Plasma Levels. Biomarker insights 2011;6:119-25.

94. Diamandi A, Khosravi MJ, Mistry J, Martinez V, Guevara-Aguirre J. Filter paper blood spot assay of human insulin-like growth factor I (IGF-I) and IGF-binding protein-3 and preliminary application in the evaluation of growth hormone status. The Journal of clinical endocrinology and metabolism 1998;83(7):2296-301.

95. Bastani NE, Gundersen TE, Blomhoff R. Dried blood spot (DBS) sample collection for determination of the oxidative stress biomarker 8-epi-PGF(2alpha) in humans using liquid chromatography/tandem mass spectrometry. Rapid communications in mass spectrometry : RCM 2012;26(6):645-52.

96. Ghannoum M, Thomson M, Bowman W, Al-Khalil S. Mode of action of the antimicrobial compound 5-bromo-5-nitro-1,3-dioxane (bronidox). Folia microbiologica 1986;31(1):19-31.

97. Skogstrand K, Thorsen P, Norgaard-Pedersen B, Schendel DE, Sorensen LC, Hougaard DM. Simultaneous measurement of 25 inflammatory markers and neurotrophins in neonatal dried blood spots by immunoassay with xMAP technology. Clinical chemistry 2005;51(10):1854-66.

98. Jones TG, Warber KD, Roberts BD. Analysis of hemoglobin A1c from dried blood spot samples with the Tina-quantR II immunoturbidimetric method. Journal of diabetes science and technology 2010;4(2):244-9.

99. Wang XL, Dudman NP, Blades BL, Wilcken DE. Changes in the immunoreactivity of Apo A-I during storage. Clinica chimica acta; international journal of clinical chemistry 1989;179(3):285-93.

100. Klamer A, Skogstrand K, Hougaard DM, Norgaard-Petersen B, Juul A, Greisen G. Adiponectin levels measured in dried blood spot samples from neonates born small and appropriate for gestational age. European journal of endocrinology / European Federation of Endocrine Societies 2007;157(2):189-94.

101. Butter NL, Hattersley AT, Clark PM. Development of a bloodspot assay for insulin. Clinica chimica acta; international journal of clinical chemistry 2001;310(2):141-50.

102. Kant P, Hull MA. Excess body weight and obesity--the link with gastrointestinal and hepatobiliary cancer. Nature reviews Gastroenterology & hepatology 2011;8(4):224-38.

103. Karlsson S, Andersson L, Berglund B. Early assessment of nutritional status in patients scheduled for colorectal cancer surgery. Gastroenterology nursing : the official journal of the Society of Gastroenterology Nurses and Associates 2009;32(4):265-70.

104. Haydon AM, Macinnis RJ, English DR, Giles GG. Effect of physical activity and body size on survival after diagnosis with colorectal cancer. Gut 2006;55(1):62-7.

105. Yancik R, Havlik RJ, Wesley MN, et al. Cancer and comorbidity in older patients: a descriptive profile. Annals of epidemiology 1996;6(5):399-412.

106. Pedrazzani C, Cerullo G, De Marco G, et al. Impact of age-related comorbidity on results of colorectal cancer surgery. World journal of gastroenterology : WJG 2009;15(45):5706-11.

107. Vlasova M, Purhonen AK, Jarvelin MR, Rodilla E, Pascual J, Herzig KH. Role of adipokines in obesity-associated hypertension. Acta physiologica (Oxford, England) 2010;200(2):107-27.

108. Yabroff KR, Lawrence WF, Clauser S, Davis WW, Brown ML. Burden of illness in cancer survivors: findings from a population-based national sample. Journal of the National Cancer Institute 2004;96(17):1322-30.

109. Iversen LH, Norgaard M, Jacobsen J, Laurberg S, Sorensen HT. The impact of comorbidity on survival of Danish colorectal cancer patients from 1995 to 2006--a population-based cohort study. Diseases of the colon and rectum 2009;52(1):71-8.

110. Hillenbrand A, Fassler J, Huber N, et al. Changed adipocytokine concentrations in colorectal tumor patients and morbidly obese patients compared to healthy controls. BMC cancer 2012;12:545.

111. Nedungadi TP, Clegg DJ. Sexual dimorphism in body fat distribution and risk for cardiovascular diseases. Journal of cardiovascular translational research 2009;2(3):321-7.

112. Ronti T, Lupattelli G, Mannarino E. The endocrine function of adipose tissue: an update. Clinical endocrinology 2006;64(4):355-65.

113. Maslowska M, Vu H, Phelis S, et al. Plasma acylation stimulating protein, adipsin and lipids in non-obese and obese populations. European journal of clinical investigation 1999;29(8):679-86.

114. Diamond F. The Endocrine Function of Adipose Tissue. Growth, Genetics & Hormones 2002;18(2).

115. Cancello R, Rouault C, Guilhem G, et al. Urokinase plasminogen activator receptor in adipose tissue macrophages of morbidly obese subjects. Obes Facts 2011;4(1):17-25.

116. Andersen O, Eugen-Olsen J, Kofoed K, Iversen J, Haugaard SB. Soluble urokinase plasminogen activator receptor is a marker of dysmetabolism in HIV-infected patients receiving highly active antiretroviral therapy. Journal of medical virology 2008;80(2):209-16.

117. Degawa-Yamauchi M, Bovenkerk JE, Juliar BE, et al. Serum resistin (FIZZ3) protein is increased in obese humans. The Journal of clinical endocrinology and metabolism 2003;88(11):5452-5.

118. Silha JV, Murphy LJ. Serum resistin (FIZZ3) protein is increased in obese humans. The Journal of clinical endocrinology and metabolism 2004;89(4):1977; author reply - 8.

119. Skurk T, Hauner H. Obesity and impaired fibrinolysis: role of adipose production of plasminogen activator inhibitor-1. International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity 2004;28(11):1357-64.

120. Autier P, Koechlin A, Boniol M, et al. Serum insulin and C-peptide concentration and breast cancer: a meta-analysis. Cancer causes & control : CCC 2013;24(5):873-83.

121. Hoekstra JB, van Rijn HJ, Erkelens DW, Thijssen JH. C-peptide. Diabetes care 1982;5(4):438-46.

122. Kuzuya H, Blix PM, Horwitz DL, Steiner DF, Rubenstein AH. Determination of free and total insulin and C-peptide in insulin-treated diabetics. Diabetes 1977;26(1):22-9.

123. Visser M, Bouter LM, McQuillan GM, Wener MH, Harris TB. Elevated C-reactive protein levels in overweight and obese adults. JAMA : the journal of the American Medical Association 1999;282(22):2131-5.

124. Hopkins MH, Flanders WD, Bostick RM. Associations of circulating inflammatory biomarkers with risk factors for colorectal cancer in colorectal adenoma patients. Biomarker insights 2012;7:143-50.

125. Pendyala S, Neff LM, Suarez-Farinas M, Holt PR. Diet-induced weight loss reduces colorectal inflammation: implications for colorectal carcinogenesis. The American journal of clinical nutrition 2011;93(2):234-42.

126. Hancox RJ, Landhuis CE. Correlation between measures of insulin resistance in fasting and non-fasting blood. Diabetology & metabolic syndrome 2011;3(1):23.

127. Westerink J, Visseren FL. Pharmacological and non-pharmacological interventions to influence adipose tissue function. Cardiovascular diabetology 2011;10(1):13.

128. Khalid U, Spiro A, Baldwin C, et al. Symptoms and weight loss in patients with gastrointestinal and lung cancer at presentation. Supportive care in cancer : official journal of the Multinational Association of Supportive Care in Cancer 2007;15(1):39-46.

129. Robsahm TE, Aagnes B, Hjartaker A, Langseth H, Bray FI, Larsen IK. Body mass index, physical activity, and colorectal cancer by anatomical subsites: a systematic review and meta-analysis of cohort studies. European journal of cancer prevention : the official journal of the European Cancer Prevention Organisation (ECP) 2013.

130. Pischon T, Lahmann PH, Boeing H, et al. Body size and risk of colon and rectal cancer in the European Prospective Investigation Into Cancer and Nutrition (EPIC). Journal of the National Cancer Institute 2006;98(13):920-31.

131. van Duijnhoven FJ, Bueno-De-Mesquita HB, Calligaro M, et al. Blood lipid and lipoprotein concentrations and colorectal cancer risk in the European Prospective Investigation into Cancer and Nutrition. Gut 2011;60(8):1094-102.

132. Giovannucci E. Metabolic syndrome, hyperinsulinemia, and colon cancer: a review. The American journal of clinical nutrition 2007;86(3):s836-42.

133. Nakajima TE, Yamada Y, Hamano T, et al. Adipocytokines as new promising markers of colorectal tumors: adiponectin for colorectal adenoma, and resistin and visfatin for colorectal cancer. Cancer science 2010;101(5):1286-91.

134. Kumor A, Daniel P, Pietruczuk M, Malecka-Panas E. Serum leptin, adiponectin, and resistin concentration in colorectal adenoma and carcinoma (CC) patients. International journal of colorectal disease 2009;24(3):275-81.

135. Tabachnick BG. Fidell LS. Using multivariate statistics. Fourth edition. New York: HarperCollins, 2001.

136. Allison MA, Ix JH, Morgan C, et al. Higher leptin is associated with hypertension: the Multi-Ethnic Study of Atherosclerosis. Journal of human hypertension 2013.

137. Sehestedt T, Lyngbaek S, Eugen-Olsen J, et al. Soluble urokinase plasminogen activator receptor is associated with subclinical organ damage and cardiovascular events. Atherosclerosis 2011;216(1):237-43.

138. Schutte AE, Myburgh A, Olsen MH, Eugen-Olsen J, Schutte R. Exploring soluble urokinase plasminogen activator receptor and its relationship with arterial stiffness in a bi-ethnic population: the SAfrEIC-study. Thrombosis research 2012;130(2):273-7.

139. Barba G, Russo O, Siani A, et al. Plasma leptin and blood pressure in men: graded association independent of body mass and fat pattern. Obesity research 2003;11(1):160-6.

140. Ozata M, Ozdemir IC, Licinio J. Human leptin deficiency caused by a missense mutation: multiple endocrine defects, decreased sympathetic tone, and immune system dysfunction indicate new targets for leptin action, greater central than peripheral resistance to the effects of leptin, and spontaneous correction of leptin-mediated defects. The Journal of clinical endocrinology and metabolism 1999;84(10):3686-95.

141. Kurukulasuriya LR, Stas S, Lastra G, Manrique C, Sowers JR. Hypertension in obesity. The Medical clinics of North America 2011;95(5):903-17.

142. Thoresen L, Frykholm G, Lydersen S, et al. Nutritional status, cachexia and survival in patients with advanced colorectal carcinoma. Different assessment criteria for nutritional status provide unequal results. Clinical nutrition (Edinburgh, Scotland) 2013;32(1):65-72.

143. Ulander K, Jeppsson B, Grahn G. Postoperative energy intake in patients after colorectal cancer surgery. Scandinavian journal of caring sciences 1998;12(3):131-8.

144. Williams JP, Phillips BE, Smith K, et al. Effect of tumor burden and subsequent surgical resection on skeletal muscle mass and protein turnover in colorectal cancer patients. The American journal of clinical nutrition 2012;96(5):1064-70.