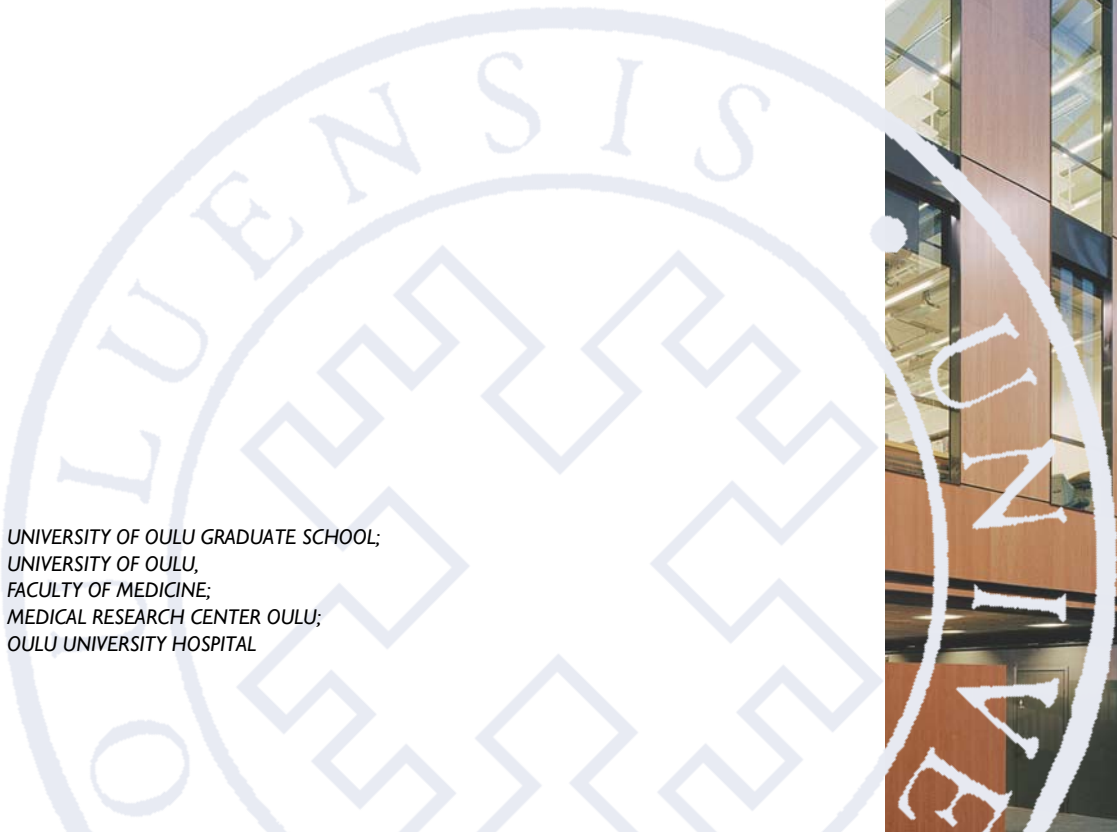


Päivi Sirniö

DETERMINANTS OF
SYSTEMIC INFLAMMATION
IN COLORECTAL CANCER

UNIVERSITY OF OULU GRADUATE SCHOOL;
UNIVERSITY OF OULU,
FACULTY OF MEDICINE;
MEDICAL RESEARCH CENTER OULU;
OULU UNIVERSITY HOSPITAL



ACTA UNIVERSITATIS OULUENSIS
D Medica 1539

PÄIVI SIRNIÖ

**DETERMINANTS OF SYSTEMIC
INFLAMMATION IN COLORECTAL
CANCER**

Academic dissertation to be presented with the assent of the Doctoral Training Committee of Health and Biosciences of the University of Oulu for public defence in Auditorium F101 of the Faculty of Biochemistry and Molecular Medicine (Aapistie 7), on 8 November 2019, at 12 noon

UNIVERSITY OF OULU, OULU 2019

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Acta Univ. Oul. D 1539, 2019

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ISBN 978-952-62-2401-5 (Paperback)
ISBN 978-952-62-2402-2 (PDF)

ISSN 0355-3221 (Printed)
ISSN 1796-2234 (Online)

Cover Design
Raimo Ahonen

JUVENES PRINT
TAMPERE 2019

Sirniö, Päivi, Determinants of systemic inflammation in colorectal cancer.

University of Oulu Graduate School; University of Oulu, Faculty of Medicine; Medical Research Center Oulu; Oulu University Hospital

Acta Univ. Oul. D 1539, 2019

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Abstract

Colorectal cancer (CRC) is the third most common cancer and the second leading cause of cancer-related deaths worldwide. In some CRC patients, the presence of the tumor elicits a systemic inflammatory response and metabolic derangements that lead to progressive tissue wasting. Systemic inflammation has been associated with decreased survival independent of tumor stage. However, the mechanisms and downstream effects of systemic inflammation in CRC are uncertain.

The aim of these studies was to examine the determinants of systemic inflammation in CRC. The study material consisted of tumor and serum samples collected from patients with stage I–IV CRC operated at the Oulu University Hospital (n=336). From preoperative serum samples, the levels of cell death marker keratin 18, matrix metalloproteinase 8 (MMP8), and ten metabolites (apolipoprotein A1 and nine amino acids) were measured.

CRC patients with systemic inflammation, assessed using a modified Glasgow Prognostic Score, had elevated serum levels of MMP8 and phenylalanine. On the contrary, the concentrations of apolipoprotein A1, glutamine, and histidine were lower compared to patients without systemic inflammation. Increased serum keratin 18 level associated with systemic inflammation in patients with metastatic disease. Elevated keratin 18 and MMP8 levels and decreased apolipoprotein A1 level were independent predictors of worse survival.

These studies describe biomarkers of systemic inflammation that provide insight into the mechanisms of systemic inflammation, have potential prognostic value in CRC, and are possible therapeutic targets. The results suggest that cell death and systemic inflammation are strongly connected in CRC, but the potential mechanistic link between them and tissues involved remain to be elucidated.

Keywords: colorectal cancer, metabolite, prognosis, serum, systemic inflammation

Sirniö, Päivi, Systemistä tulehdusta määrittävät tekijät paksusuolisyövässä.

Oulun yliopiston tutkijakoulu; Oulun yliopisto, Lääketieteellinen tiedekunta; Medical Research Center Oulu; Oulun yliopistollinen sairaala

Acta Univ. Oul. D 1539, 2019

Oulun yliopisto, PL 8000, 90014 Oulun yliopisto

Tiivistelmä

Paksusuolisyöpä on kolmanneksi yleisin syöpä ja toiseksi yleisin syöpäkuoleman aiheuttaja Suomessa. Osalla potilaista syöpään liittyy systeemisen tulehdusreaktion aktivoituminen ja aineenvaihduntahäiriö, joka johtaa yleiseen näivettymiseen. Systeemisen tulehduksen on havaittu olevan yhteydessä huonoon ennusteeseen riippumatta kasvaimen levinneisyydestä. Systeemisen tulehduksen aktivaatiomekanismit ja vaikutukset paksusuolisyövässä ovat kuitenkin huonosti tunnettuja.

Tutkimuksen tarkoituksena oli selvittää systeemistä tulehdusta määrittäviä tekijöitä paksusuolisyövässä. Aineisto koostui Oulun yliopistollisessa sairaalassa leikattujen paksusuolisyöpäpotilaiden (syövän levinneisyysaste I–IV) kasvain- ja seeruminäytteistä (n=336). Seeruminäytteistä mitattiin solukuolemaa osoittavan keratiini 18:n pitoisuudet, matriksin metalloproteiinaasi 8 (MMP8):n määrät sekä 10 metaboliitin (apolipoproteiini A1 ja 9 aminohappoa) tasot.

Paksusuolisyöpäpotilailla, joilla systeemistä tulehdusta osoittava mGPS-indeksi oli korkea, seerumin MMP8- ja fenyylialaniinitasot olivat koholla. Sen sijaan apolipoproteiini A1:n, glutamiinin ja histidiinin pitoisuudet olivat matalammat verrattuna potilaisiin, joilla ei ollut systeemistä tulehdusta. Kohonnut keratiini 18 -pitoisuus oli yhteydessä systeemiseen tulehdukseen etäpesäkkeistä tautia sairastavilla potilailla. Kohonnut keratiini 18- ja MMP8-taso sekä matala apolipoproteiini A1-pitoisuus liittyivät potilaiden huonoon ennusteeseen.

Tutkimuksessa löydettiin systeemisen tulehduksen merkkiaineita, jotka tuovat hyödyllistä tietoa systeemisen tulehduksen mekanismeista ja potilaiden ennusteesta paksusuolisyövässä, ja ovat mahdollisia terapeuttisia kohteita. Tulosten perusteella paksusuolisyövässä solukuolema liittyy vahvasti systeemiseen tulehdukseen, mutta lisätutkimuksia tarvitaan selvittämään näiden tapahtumien mahdolliset syy-seuraussuhteet sekä tapahtumaan liittyvät kudokset.

Asiasanat: paksusuolisyöpä, systeeminen tulehdus, ennuste, seerumi, metaboliitti

To all the patients involved in this study

Acknowledgements

This study was carried out at the Cancer and Translational Medicine Research Unit, Faculty of Medicine, University of Oulu in 2012–2019. I wish to thank all those who have participated this work.

I deeply thank my supervisors, Professor Markus Mäkinen and Docent Anne Tuomisto, for their support through this work. Markus is thanked for taking me as a member of his qualified and pleasant research group. I appreciate his encouraging attitude, vast knowledge, and kindness. Anne is thanked for the invaluable guidance throughout these years. She always made time for me, and her scientific knowledge, optimism and support made this thesis possible.

I wish to acknowledge the former Head of the Cancer and Translational Medicine Research Unit, Professor Emeritus Tuomo Karttunen, for the possibility to work at the Department of Pathology. I also want to thank Tuomo for all the wise and encouraging comments during these years.

I thank all the co-authors and collaborators: MSc Risto Bloigu, MD, PhD Kai Klintrup, Professor Jyrki Mäkelä, Professor Karl-Heinz Herzig, PhD Toni Karhu, MSc Shivaprakash Mutt, BM Ilkka Minkkinen, Professor Tuula Salo, Professor Timo Sorsa, and DDS, PhD Taina Tervahartiala. The former and current Chief Department Physicians, Docent Paavo Pääkkö and Docent Saila Kauppila, are thanked for providing facilities for my research at the Oulu University Hospital.

I wish to thank Docent Juha Hulmi and Docent Pia Österlund for careful review of the thesis manuscript. Their valuable comments significantly improved the manuscript and gave me new perspectives. Anna Vuolteenaho, MA, is acknowledged for the linguistic editing of this thesis.

I am very grateful for the members of my follow-up group, Docent Minna Ruddock, Docent Juha Saarnio, and Docent Pia Nyberg, for the useful comments and advice regarding my doctoral training plan, annual reports, and career plans.

I acknowledge the current and past members of our research group: Juha Väyrynen, Sara Väyrynen, Tiina Kantola, Anne-Mari Moilanen, and Karoliina Stefanius. Especially I wish to thank Juha for sharing his expertise and for always being helpful, and my former officemate Anne-Mari for all the good conversations during these years.

I thank Katja Porvari, Helena Kaija, and Madhura Patankar for the nice morning coffee breaks. Laboratory staff, especially Riitta Vuento, and other staff members at Pathology are also thanked for assistance and for creating a comfortable place to work.

I wish to thank my friends for all the special moments we have spent together. I warmly thank my parents, Pirjo and Vesa, who have always supported me and have taken excellent care of my children. I also deeply thank my sister Outi and her family for bringing happiness to my life.

Finally, I owe my deepest gratitude to my wonderful children Leevi and Senni and to my beloved partner Tuomas. I'm so lucky to have you in my life.

This study was financially supported by the Finnish Cancer Foundation, K. Albin Johansson Foundation, Orion Research Foundation, Emil Aaltonen Foundation, and Thelma Mäkikyrö Foundation.

Oulu, September 2019

Päivi Sirniö

Abbreviations

AA	amino acid
ABCA1	ATP binding cassette subfamily A member 1
ABCG1	ATP binding cassette subfamily G member 1
APC	Adenomatous Polyposis Coli
aKRT18	keratin 18 released during apoptosis
APOA1	apolipoprotein A1
APR	acute phase response
ATP	adenosine triphosphate
BMI	body mass index
BRAF	B-Raf proto-oncogene, serine/threonine kinase
CCL	chemokine (C-C motif) ligand
CEA	carcinoembryonic antigen
CETP	cholesteryl ester transfer protein
CI	confidence interval
CIMP	CpG island methylator phenotype
CIN	chromosomal instability
CRC	colorectal cancer
CRP	C-reactive protein
CRT	chemoradiotherapy
CSS	cancer-specific survival
CXCL	chemokine (C-X-C motif) ligand
DAMP	damage-associated molecular pattern
DFS	disease-free survival
ECM	extracellular matrix
e.g.	exempli gratia
ELISA	enzyme-linked immunosorbent assay
HDC	histidine decarboxylase
HDL	high-density lipoprotein
HR	hazard ratio
i.e.	id est
IFN	interferon
IL	interleukin
KRT18	keratin 18
KRAS	KRAS proto-oncogene, GTPase
LCAT	lecithin-cholesterol acyltransferase

LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor
MAPK/ERK	mitogen-activated protein kinase-extracellular signal-regulated kinase
mGPS	modified Glasgow Prognostic Score
MLH	MutL homolog
MMP	matrix metalloproteinase
MMR	mismatch repair
MSH	MutS homolog
MSI	microsatellite instability
MSI-H	microsatellite instability-high
mTOR	mechanistic target of rapamycin kinase
nKRT18	keratin 18 released during necrosis
NF- κ B	nuclear factor-kappa B
NLR	neutrophil to lymphocyte ratio
NRAS	NRAS proto-oncogene, GTPase
NSAID	non-steroidal anti-inflammatory drug
OS	overall survival
PAH	phenylalanine hydroxylase
PAMP	pathogen-associated molecular pattern
PDGF	platelet derived growth factor
PIK3CA	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PRR	pattern recognition receptor
P13K	phosphoinositide 3-kinase
ROC	receiver operating characteristics
ROS	reactive oxygen species
RT	radiotherapy
SAA1	serum amyloid A1
SD	standard deviation
SCARB1	scavenger receptor class B member 1
TGFB	transforming growth factor beta
TNF	tumor necrosis factor
tKRT18	total keratin 18
TP53	tumor protein 53
WHO	World Health Organization

List of original publications

This thesis is based on the following publications, which are referred throughout the text by their Roman numerals:

- I Sirmiö, P., Väyrynen, J. P., Mutt, S. J., Herzig, K.-H., Klintrup, K., Mäkelä, J., Karttunen, T. J., Mäkinen, M. J., & Tuomisto, A. Cell death related circulating keratin 18 fragments are associated with systemic inflammation and poor prognosis in colorectal cancer. *Manuscript*.
- II Sirmiö, P., Tuomisto, A., Tervahartiala, T., Sorsa, T., Klintrup, K., Karhu, T., Herzig, K.-H., Mäkelä, J., Karttunen, T. J., Salo, T., Mäkinen M. J., & Väyrynen, J. P. (2018). High serum MMP-8 levels are associated with decreased survival and systemic inflammation in colorectal cancer. *British Journal of Cancer*, *119*(2), 213–219.
- III Sirmiö, P., Väyrynen, J. P., Klintrup, K., Mäkelä, J., Mäkinen, M. J., Karttunen, T. J., & Tuomisto, A. (2017). Decreased serum apolipoprotein A1 levels are associated with poor survival and systemic inflammatory response in colorectal cancer. *Scientific Reports*, *7*(1), 5374.
- IV Sirmiö, P., Väyrynen, J. P., Klintrup, K., Mäkelä, J., Karhu, T., Herzig, K.-H., Minkkinen, I., Mäkinen, M. J., Karttunen, T. J., & Tuomisto, A. (2019). Alterations in serum amino-acid profile in the progression of colorectal cancer: associations with systemic inflammation, tumour stage and patient survival. *British Journal of Cancer*, *120*(2), 238–246.

In all studies, Päivi Sirmiö was involved in study design and data collection, performed statistical analyses, interpreted the results, and prepared the initial manuscripts.

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1 Introduction

Colorectal cancer (CRC) is the second most common cause of cancer death worldwide (Bray et al., 2018). Currently, the prognostic classification of CRC is largely based on TNM staging, which describes the extent of tumor growth and spread. The survival rate ranges from 90% for stage I to less than 10% for stage IV (Amin et al., 2017). However, CRC is a heterogeneous disease, and the survival outcome among patients within the same TNM category is variable (Nagtegaal et al., 2012). Thus, there is a need to identify new prognostic factors that can predict disease outcome and aid in selecting optimal therapy.

In addition to tumor-based characteristics, immune response has been shown to predict survival in CRC. Local inflammation is associated with favorable prognosis (Jass, 1986; Galon et al., 2006), whereas systemic inflammatory response predicts decreased survival (Park et al., 2016). During systemic inflammation in CRC, serum levels of several cytokines, including interleukin 6 (IL6) and C-X-C motif chemokine ligand 8 (CXCL8, also known as IL8), increase (Kantola et al., 2012). Pro-inflammatory cytokines induce the acute phase response and trigger metabolic responses. Systemic inflammation also contributes to the development of cancer-related malnutrition and sarcopenia, which deteriorate the well being and prognosis of cancer patients (Arends et al., 2017b). However, metabolic derangements associated with systemic inflammation in CRC are not well understood.

About one third of CRC patients have increased blood C-reactive protein (CRP) levels and decreased albumin levels, indicating systemic inflammation (Park et al., 2016). However, it is not clear what initiates systemic inflammation in these patients. According to our hypothesis, tumor necrosis could be the eliciting factor, as high amount of tumor necrosis has been shown to associate with increased blood IL6 levels (Richards et al., 2012a). Necrosis leads to loss of plasma membrane integrity and leakage of intracellular contents, including damage-associated molecular patterns (DAMPs). DAMPs can activate neutrophils and induce them to secrete various molecules, such as cytokines and matrix metalloproteinase 8 (MMP8) (Zhang et al., 2010). MMP8 degrades collagen and several other proteins, and has an important role in the regulation of immune responses (Van Lint & Libert, 2006), but its role in CRC-associated inflammation had not been studied.

The aim of this study was to evaluate the association of systemic inflammation, as evidenced by increased mGPS and cytokine levels, with circulating epithelial cell death marker keratin 18 (KRT18) (I), MMP8 (II), apolipoprotein A1 (APOA1)

(III), and nine amino acids (IV) in patients with stage I–IV CRC. We also analyzed the association between serum KRT18 level and the extent of tumor necrosis in the primary tumors (I), and the association between serum MMP8 level and local inflammatory cell infiltrate in the primary tumors (II). Moreover, we investigated the relationship between serum KRT18, MMP8, APOA1, amino acids, and survival (I–IV).

2 Review of the literature

2.1 Cell injury

Cells are constantly exposed to different types of stress. The adaptive responses allow the cell to survive and continue function. However, when cells suffer from severe stress so that they are no longer able to adapt, events leading to cell injury follow. Cell injury can be reversible up to a certain point, but when the stimulus is severe enough, the cell suffers irreversible injury and finally undergoes cell death (Kroemer et al., 2009).

The causes of cell injury are classified into exogenous and endogenous. Endogenous factors include immune reactions and genetic abnormalities, while exogenous factors include physical, chemical and infectious agents, nutritional imbalances and hypoxia (Fausto et al., 2014). Hypoxia is an important and common cause of cell injury. It is associated with depletion of adenosine triphosphate (ATP), anaerobic metabolism and cellular membrane disruption (Saikumar et al., 1998). ATP is the primary carrier of energy in cells and its depletion disturbs many critical cellular systems. Other biochemical mechanisms that contribute to cell injury include mitochondrial damage, increased cytosolic calcium, oxidative stress, and DNA damage (Fausto et al., 2014).

There are two principal types of cell death, apoptosis and necrosis (Kroemer et al., 2009). In response to irreversible injury, the cell undergoes necrosis. Necrosis was long considered merely uncontrolled cell death, but now it has become clear that it can also occur in a regulated manner (Galluzzi et al., 2012). Regulated necrosis mediated by death receptors is called necroptosis (Berghe et al., 2014). The morphological features of necrosis are diverse, but it can be defined by cellular swelling (oncosis), swelling of cell organelles, and loss of plasma membrane integrity (Zong & Thompson, 2006). In necrosis, DNA is cleaved in a non-specific way and the nucleus disintegrates late (Ziegler & Groscurth, 2004). On the contrary, apoptosis is programmed cell death that can occur as a part of physiological events. In apoptosis, cells shrink and at least initially, maintain plasma membrane integrity (Elmore, 2007).

Necrotic cell death triggers a host inflammatory response due to early leakage of intracellular contents. When cells undergo necrosis, the tissue is rapidly infiltrated with leukocytes, which is seen, for example, in burns and in the experimental implantation of necrotic cells into mice (Chen et al., 2007; Kono &

Rock, 2008). In contrast to necrosis, apoptosis is regulated by caspases and produces apoptotic bodies that are usually phagocytosed without any ensuing inflammatory reaction (Elmore, 2007). However, if the apoptotic cells are not cleared rapidly enough, they can also stimulate a host response (Kono & Rock, 2008).

Different techniques can be used to identify apoptotic and necrotic cell death. These techniques rely on cell morphology, cell surface markers, intracellular markers and release of extracellular markers (Krysko et al., 2008). Markers of apoptosis that can be measured in blood include FAS ligand, cytochrome C, and selected caspases, particularly caspase-3 and its specific cleavage products such as caspase-cleaved keratin 18 (KRT18) (Ward et al., 2008). Blood biomarkers of necrosis include lactate dehydrogenase, full-length KRT18, and high-mobility group box 1 (Chan et al., 2013; Yang et al., 2014).

Circulating keratin levels can be used to detect specifically epithelial cell death in the body (Ueno et al., 2005). Keratins are the major subgroup of intermediate filament proteins in epithelial cells that provide structural support to the cell. KRT18 is expressed by a variety of single layered epithelial cells, such as hepatocytes, intestinal epithelial cells, exocrine pancreas, and tumor cells arising from simple epithelia (Omary et al., 2009). During necrosis and apoptosis, different forms of KRT18 are released into the blood where they remain relatively stable (de Haas et al., 2008). If the cell undergoes apoptosis, caspase-cleaved KRT18 fragments (aKRT18) are released, whereas in necrosis, uncleaved KRT18 (nKRT18) is released (Fig. 1) (Caulín et al., 1997; Schutte et al., 2004). The level of aKRT18 in serum or plasma can be measured by the M30 apoptosense enzyme-linked immunosorbent assay (ELISA), whereas the M65 ELISA measures total KRT18 (tKRT18) (Kramer et al., 2004). Previously, serum aKRT18 and tKRT18 levels have been used as diagnostic or prognostic biomarkers in patients with different carcinomas such as colorectal and breast cancer (Ausch et al., 2009; Greystoke et al., 2012; Oven Ustaalioglu et al., 2012; Oyama et al., 2013; Yang et al., 2018), and in patients with liver diseases (Bechmann et al., 2010; Papatheodoridis et al., 2010).

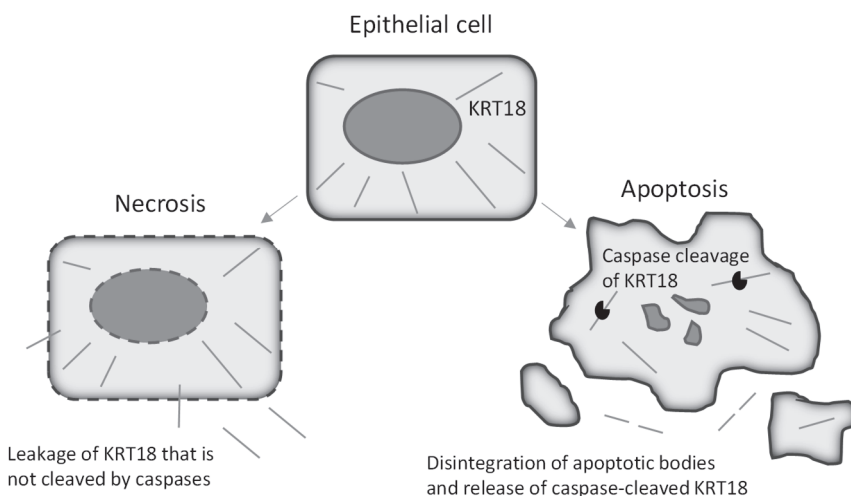


Fig. 1. Epithelial cell death due to apoptosis leads to release of caspase-cleaved keratin 18 (KRT18) fragments, whereas during necrosis KRT18 is not cleaved by caspases.

2.2 Host defense mechanisms and inflammation

Host defense mechanisms are used by the body to protect itself against infection or sterile tissue injury (Rock & Kono, 2008). These mechanisms include physical barriers, immunologically active cells and a variety of chemicals. Nonspecific host defenses, termed innate immunity, consist of natural mechanisms that are not directed specifically toward a particular pathogen. On the contrary, specific host defense, termed adaptive or acquired immunity, must be developed uniquely for each pathogen through the activity of specialized white blood cells. However, innate and adaptive immunity are highly linked and act together to provide optimal defense (Hoebe et al., 2004).

Inflammation is responsible for activating both innate and adaptive immune systems to resolve the damage and restore homeostasis. Inflammation occurs in vascularized tissues and comprises the recognition of injury or pathogens, recruitment of leukocytes and other immune system components, elimination of the cause of injury and repair of tissues (D'Elia et al., 2013).

Following infection and injury, the body's first response is acute inflammation. Acute inflammation starts rapidly and leads to resolution often in a few days. It is characterized by pain, redness, heat and swelling at the area of injury due to

changes in local blood vessels (Freire & Van Dyke, 2013). The blood vessels dilate and become more permeable, leading to local swelling and an accumulation of blood proteins that aid in defense (Poher & Sessa, 2014). At the same time, the endothelial cells at the blood vessel walls are activated to express cell adhesion proteins that facilitate the attachment and migration of leukocytes. The production of proinflammatory mediators from a local inflammatory response can later lead to a prominent systemic response known as acute phase response (APR) (Gruys et al., 2005).

When the agent causing the acute inflammation cannot be eliminated unresolved chronic inflammation occurs (Sansbury & Spite, 2016). Chronic inflammation lasts for several weeks or longer. It is mediated mainly by lymphocytes, plasma cells and macrophages, whereas in acute inflammation neutrophils are important contributors. In chronic inflammation, the production of reactive oxygen species (ROS), proteases and growth factors by neutrophils and macrophages leads to tissue destruction and abnormal collagen accumulation (Chen & Nuñez, 2010).

The inflammatory response can be local or systemic. Injury always instigates a local inflammatory response, but major host insults may also evoke systemic inflammation, which is mediated by hormonal, metabolic, and immunological factors (Brøchner & Toft, 2009). During systemic inflammation, secretion of several stress hormones, such as cortisol and adrenalin, is increased. In chronic inflammatory systemic diseases, signs and symptoms commonly include increased body temperature, fatigue, anorexia, muscle wasting, insulin resistance, increased IL6 serum levels, and dyslipidemia (Straub & Schradin, 2016).

2.2.1 Innate immunity

The innate immune responses occur within minutes or hours of injury (Netea et al., 2015). They are non-specific and evolutionarily the oldest component of the human immune system (Delves & Roitt, 2000). Innate immunity consists of barriers to infection (skin and other epithelial surfaces), blood proteins (i.e. complement system, cytokines) and cellular components (i.e. neutrophilic granulocytes, macrophages, dendritic cells, and natural killer cells). If a pathogen crosses an epithelial barrier, it is immediately faced by macrophages that reside in tissue (Alberts et al., 2002a). Tissue macrophages are soon reinforced by the recruitment of large numbers of neutrophils. Macrophages and neutrophils recognize pathogens,

leading to phagocytosis of the pathogen and its death (Silva & Correia-Neves, 2012).

Several soluble factors are involved in the innate immune response (Riera Romo et al., 2016). At first, these mediators are secreted by local cells at the site of injury, and later they originate from recruited immune cells and plasma proteins. Immune cells produce a wide variety of mediators, including vasoactive amines and peptides, eicosanoids, ROS, cytokines and chemokines (Abdulkhaleq et al., 2018). Plasma mediators are typically derived from molecules that are normally present in the plasma as inactive precursors and are activated by a proteolytic cascade. Plasma derived mediators include the complement system, kinin system and clotting system. The complement system consists of a number of proteins that are made mainly by the liver and circulate in the blood and extracellular fluid (Lubbers et al., 2017). Once activated, they react with one another to enhance chemotaxis and to target pathogens for phagocytosis and lysis (Dunkelberger & Song, 2010).

2.2.2 Recognition of microbes and cell damage

The main targets of the innate immune recognition are certain evolutionarily conserved structures on pathogens that are absent in the host (Medzhitov & Janeway, 1997). These pathogen-associated molecular patterns (PAMPs), such as microbial nucleic acids, lipoproteins and surface glycoproteins, are detected by the immune cells through pattern recognition receptors (PRRs). Activated PRRs stimulate many intracellular signaling pathways, leading to translocation of nuclear factor- κ B (NF- κ B) into the nucleus and release of inflammatory mediators, such as cytokines (Fig. 2) (Liu et al., 2017).

In the case of sterile inflammation caused by tissue damage, PRRs recognize certain endogenous DAMPs that are only released from injured and necrotic cells (Matzinger, 1994). DAMPs is a heterogeneous group that can be broadly divided into intracellular molecules, such as nuclear proteins, mitochondrial DNA, uric acid and ATP, and molecules that originate from the extracellular matrix (ECM), like hyaluronan and heparin sulfate (Kaczmarek et al., 2013). ECM-derived DAMPs are generated as a result of ECM degradation during tissue injury (Chen & Nuñez, 2010).

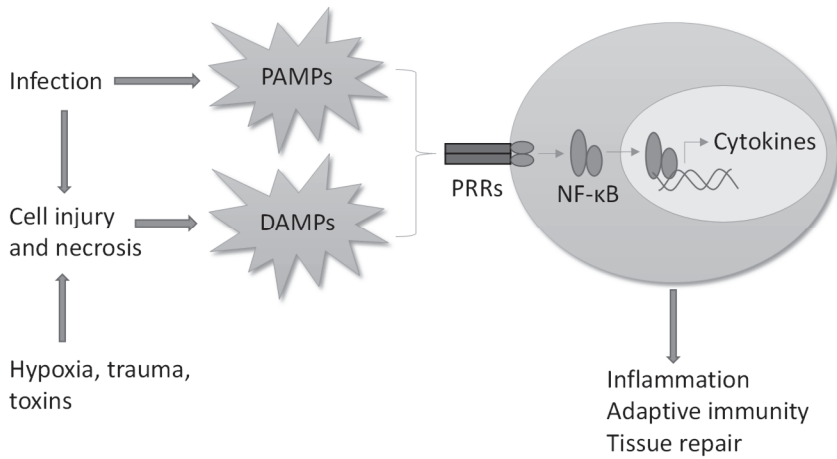


Fig. 2. Infection and cell injury result in the release of PAMPs and DAMPs that promote the inflammatory response via PRRs. Abbreviations: PAMPs: pathogen-associated molecular patterns, DAMPs: damage associated molecular patterns, PRRs: pattern recognition receptors, NF-κB: nuclear factor-κB. Modified from Brennan et al. 2010.

2.2.3 Adaptive immunity

Adaptive immune responses occur after a delay of several days following the initial exposure. They are activated when the innate immune response is insufficient to control the threat. Compared to innate immunity, adaptive immunity is more specific and can provide long-lasting memory (Medzhitov & Janeway, 1997). There are two types of adaptive immune responses: the cell-mediated response, which is carried out by T lymphocytes, and the humoral response, which is characterized by the secretion of antibodies by B lymphocytes.

T and B cells are activated when the surface receptors of these cells bind to an antigen. B cells recognize antigens in their native form, whereas activation of naïve T cells requires recognition of antigens presented by antigen-presenting cells (Delves & Roitt, 2000). Antigen-presenting cells such as dendritic cells, B cells, and macrophages can engulf pathogens and display processed antigens on their surface by coupling them to major histocompatibility complex molecules (Paul, 2011). Naïve T cells recognize these antigens and mature into functional immune cells (Brenchley et al., 2002). Activated T and B cells that are specific to the particular antigen proliferate and attack the infecting pathogen. T cells can kill

pathogens directly, and B cells secrete antibodies that enhance the phagocytosis of pathogens and disrupt the infection (Delves & Roitt, 2000).

2.2.4 Immune cells

Neutrophils

Neutrophils are short-lived cells and the most abundant leukocytes in blood (Kumar et al., 2018). They are major players during acute inflammation, and usually the first cells to be recruited to the site of infection. Neutrophils destroy pathogens through phagocytosis and intracellular degradation, release of granules, and forming the neutrophil extracellular traps (Rosales, 2018). Once neutrophils have accomplished a round of phagocytosis, they undergo spontaneous apoptosis and are cleared by macrophages, subsequently resulting in resolution of the inflammatory response (Bagaitkar, 2014).

Neutrophils are densely packed with granules containing an assortment of molecules, such as lysozyme, myeloperoxidase, defensins and cathepsins, that are released upon stimulation (Lacy, 2006). One of the granule-derived molecules is MMP8, also known as neutrophil collagenase and collagenase 2 (Van Lint & Libert, 2006). MMPs are zinc-dependent enzymes that can cleave proteins of ECM, as well as other substrates, and are involved in many physiological processes and pathological conditions (Rodríguez et al., 2010). MMP8 is synthesized in neutrophils during their maturation, and stored in a latent form in specific granules (Murphy et al., 1977). At sites of inflammation, MMP8 is secreted as an inactive pro-enzyme that is activated by ROS produced by neutrophils or by a variety of proteases like cathepsin G and MMPs (Okamoto et al., 1997; Van Lint & Libert, 2006).

Once activated, MMP8 degrades the ECM components and allows the neutrophil to migrate through the tissues (Lin et al., 2008). However, in addition to collagens it can cleave a wide range of other substrates, including cell adhesion proteins, growth factors, and cytokines (Van Lint & Libert, 2006). MMP8 has been shown to cleave and activate chemokines CXCL5 and CXCL8 to promote neutrophil recruitment during acute inflammation (Tester et al., 2007), and to modulate the activity of cytokines, such as tumor necrosis factor (TNF) (Lee et al., 2014). It also appears to contribute to the resolution of inflammation, as MMP8-deficiency in mice leads to sustained accumulation of neutrophil infiltrates during

wound healing, skin tumorigenesis, and autoimmune arthritis (Balbin et al., 2003; Gutiérrez-Fernández et al., 2007; Cox et al., 2010).

Macrophages

Macrophages are long-lived cells that differentiate from circulating monocytes (Murray & Wynn, 2011). They reside in tissues throughout the body and are among the first cells to encounter invading microbes. Like neutrophils, macrophages recognize and engulf pathogens and destroy them by producing degradative enzymes, antimicrobial peptides and ROS (Silva & Correia-Neves, 2012). Macrophages also remove cellular debris released during cell injury and necrosis (Brouckaert et al., 2004). When macrophages are activated, they can present antigens and secrete inflammatory mediators (Murray & Wynn, 2011). Macrophages have different functional phenotypes that arise in response to environmental signals (Martinez & Gordon, 2014).

Lymphocytes

Lymphocytes originate from the bone marrow-derived progenitors (Blom & Spits, 2006). They are found in large numbers in lymph, blood and lymphoid tissues, such as thymus, spleen and lymph nodes (Alberts et al., 2002b). Circulating lymphocytes consist of B cells, T cells, and natural killer cells.

T lymphocytes mature in the thymus and express T cell receptors on their surface (Kruisbeek, 1999). Exposure of naive T cells to antigens leads to their clonal expansion and differentiation. The two major T lymphocyte subsets are CD4 and CD8 cells, which can be identified by their surface expression of either CD4 or CD8 molecules (Ellmeier et al., 1999). CD4 cells act as helper cells. They provide signals that enhance the activation of CD8 T cells and macrophages, and maturation of B cells into plasma cells (Zhu & Paul, 2010). CD8 T cells directly kill infected cells by producing cytotoxic proteins (Barry & Bleackley, 2002).

B cells develop in the bone marrow and their activation occurs in peripheral lymphoid organs (Pieper et al., 2013). B cell activation begins when a specific antigen binds to the B cell receptor. Activated B cells differentiate into both memory B cells and plasma cells that synthesize antibodies which are able to bind to the antigen (Pieper et al., 2013).

Natural killer cells can develop at multiple sites (Blom & Spits, 2006). They are lymphocytes of the innate immune system that do not require specific antigen

activation to detect and destroy target cells (Caligiuri, 2008). Natural killer cells mediate killing of virally infected cells and transformed cells by lysis and secretion of interferon gamma (IFNG) (Caligiuri, 2008).

Eosinophils

Eosinophils are important mediators in allergic reactions and in host protection against parasites (Rothenberg & Hogan, 2006). They have granules containing highly cytotoxic substances that are capable of causing tissue damage (Acharya & Ackerman, 2014). In addition, eosinophils secrete chemoattractants and other proinflammatory mediators (Rothenberg & Hogan, 2006).

Basophils and mast cells

Basophils and mast cells contain cytoplasmic granules with heparin, histamine, proteolytic enzymes and other inflammatory mediators (Min, 2008; Wernersson & Pejler, 2014). Immediate hypersensitivity and inflammatory reactions cause their degranulation and mediator release (Wernersson & Pejler, 2014). Both mast cells and basophils develop from a common bone marrow-derived hematopoietic precursor cell (Huang & Li, 2014). Basophils mature in the bone marrow and then enter the circulation, whereas mast cells circulate as immature precursors and mature after entering the tissue (Min, 2008). Mast cells are distributed in connective tissues, generally clustered at epithelial surfaces and around blood vessels (Abraham & St. John, 2010).

Dendritic cells

Dendritic cells are innate immune cells that provide a link between the innate and adaptive immunity. They migrate from blood to peripheral tissues, where they sample the environment through PRRs for self- and non-self antigens (Castell-Rodríguez et al., 2017). On contact with antigen, immature dendritic cells undergo a process of maturation and migrate to secondary lymphoid organs (Dalod et al., 2014). In the secondary lymphoid organs, dendritic cells activate T lymphocytes by presenting them with antigens (Medzhitov & Janeway, 1997). Dendritic cells also produce a number of cytokines, such as IL12 (Heufler et al., 1996).

2.2.5 Cytokines

The release of cytokines from immune cells is a key modulator of the immune response. Cytokines are small secreted polypeptides that mediate both activating and inhibiting signals between cells and regulate many biological functions (Zhang & An, 2007). The two major producers are helper T cells and macrophages, but many different cell types, including immune cells, endothelial cells and fibroblasts, can secrete them (Turner et al., 2014). Cytokines act mainly in a paracrine and autocrine manner on nearby cells and themselves, but they can also act in an endocrine manner on distant cells. They are often released in a cascade, as one cytokine stimulates its target cells to make additional cytokines (Zhang & An, 2007).

Cytokines can be broadly classified into proinflammatory and anti-inflammatory, based on whether they facilitate or inhibit inflammation. However, cytokine networks are complex and each cytokine performs specific roles depending on the type and location of the target cell (Cavaillon, 2001). The main pro-inflammatory cytokines include IL1, IL6, CXCL8, and TNF (Dinarello, 2000), while the major anti-inflammatory cytokines comprise IL1 receptor antagonist (IL1RN), IL4, and IL10 (Opal & DePalo, 2000).

TNF and IL1 are produced early during the response to trauma or bacterial infections (Foëx & Shelly, 1996). They are short-lived cytokines released mainly from macrophages and monocytes. Both can stimulate immunological cells, induce the production of other cytokines such as IL6 and CXCL8, and trigger systemic changes, including fever and metabolic alterations (Dinarello, 1988; Tosato & Jones, 1990; Kaplanski et al., 1994; Matsuki et al., 2003; Popa et al., 2007; Lee et al., 2013). TNF activates intracellular signaling pathways through two receptors, TNF receptor superfamily member 1A and 1B, to regulate various cellular responses such as proliferation, survival, differentiation, and apoptosis (Bradley, 2008). IL1 binds to receptor IL1R1 and has an important role in the activation of T and B cells and in mediating the inflammatory response to cell death (Chen et al., 2007). IL1RN is another cytokine that binds to the IL1R1, but it does not activate downstream signaling and thereby blocks IL1-mediated cellular changes (Zhang & An, 2007).

The levels of IL6 also increase rapidly during acute inflammation (Gebhard et al., 2000). IL6 is a multifunctional cytokine produced by different cell types. Its functions include stimulation of the hepatic acute phase response, activating neutrophils and natural killer cells, and regulating lymphocyte differentiation

(Hunter & Jones, 2015). IL6 also coordinates anti-inflammatory activities that are crucial for the resolution of inflammation (Maggio et al., 2006).

CXCL8 is secreted by a variety of tissue and blood cells, including leukocytes, endothelial cells, fibroblasts, and tumor cells exposed to inflammatory stimuli or environmental stress (Xie, 2001). The main role of CXCL8 in inflammation is the chemoattraction and activation of neutrophils (Hammond et al., 1995).

Proinflammatory cytokines can induce the expression of chitinase 3 like 1, also known as YKL-40 (Bhardwaj et al., 2015). YKL-40 is a glycoprotein produced by a vast array of cells and associated with inflammation and tissue remodeling (Lee et al., 2011). Elevated serum levels of YKL-40 have been found in several cancers and chronic inflammatory diseases (Kazakova et al., 2017; Fuksiewicz et al., 2018), but the biological function of YKL-40 is not completely understood.

2.2.6 Acute phase response

The liver responds to cytokines, especially IL6, by initiating the APR (Castell et al., 1989). APR is a complex systemic defense system that constitutes an essential component of the innate immune response. During APR, the synthesis of positive acute phase proteins, such as CRP, serum amyloid A1 (SAA1) and fibrinogen, increases in hepatocytes. At the same time, there is a decrease in the production of normal blood proteins, like albumin and transferrin, which represent the negative acute phase proteins (Gabay & Kushner, 1999).

CRP is the most prevalent measure used to evaluate the magnitude of systemic inflammatory response in several disorders (Gabay & Kushner, 1999). After stimulus, CRP level increases rapidly and peaks 2 to 3 days at concentrations that reflect the extent of tissue injury. After the disappearance or removal of the stimulus, the CRP level drops rapidly with a constant half-life of roughly 19 hours (Vigushin et al., 1993). In healthy individuals, CRP concentration is usually less than 3 mg/L (Imhof et al., 2003). Mildly elevated levels (3–10 mg/L) can be seen in a range of conditions, such as obesity and hypertension, but during acute inflammation values may increase to more than 500 mg/L (Pepys & Hirschfield, 2003; Kushner et al., 2006). A major function of CRP is binding to phosphocoline found on the surface of bacteria and damaged tissue to activate the complement and phagocytosis (Volanakis & Wirtz, 1979; Gershov et al., 2000).

SAA1 is another acute phase protein that is sensitive in reflecting inflammatory activity. Its plasma levels can rise up to 1,000-fold 24 hours after APR onset (Sack, 2018). SAA1 is found in blood associated with high-density lipoprotein (HDL)

(Benditt & Eriksen, 1977) and has the potential to influence cholesterol metabolism during inflammation. Its biological function is not well understood, but various effects in the inflammatory process have been reported, including opsonization, chemotaxis of leukocytes and induction of cytokine release (Badolato et al., 1994; Patel et al., 1998; Shah et al., 2006).

2.2.7 Metabolic alterations

Metabolism can be separated into two major pathways: catabolism and anabolism. Catabolism generates energy by the breakdown of molecules, while anabolic pathways consume energy to synthesize molecules. During significant injury or illness, a catabolic state is induced to furnish substrates for the healing process. It is mediated by catabolic hormones (glucagon, catecholamines and corticoids), insulin resistance as well as by cytokines, eicosanoids, oxygen radicals, and other local mediators (Sobotka & Soeters, 2009). Glucose is a crucial source of energy in hypoxic and inflammatory tissues, and increased hepatic glucose production along with a reduced glucose clearance result in hyperglycemia (Sobotka & Soeters, 2009). Triglycerides in the adipose tissue are hydrolysed to release free fatty acids and glycerol into the circulation, and muscular proteins are degraded to amino acids.

Protein metabolism

Proteins are continuously broken down into amino acids and resynthesized through the process of protein turnover. During infections and injuries, the whole body protein turnover is elevated and amino acids are redistributed from physiological processes towards processes important in the inflammatory response and tissue repair (Macallan et al., 1995; Biolo et al., 1997). Increased protein degradation predominates over increased protein synthesis, leading to a net protein catabolism. The protein catabolic response after injury was first described by Cuthbertson, who suggested that nitrogen loss in patients with bone injuries indicated whole-body protein loss (Cuthbertson, 1932).

Protein catabolism is stimulated by altered levels of cytokines and hormones (Le Floch et al., 2004). Glucocorticoids are known to produce an anabolic effect on the liver but a catabolic effect on skeletal muscle, and they participate in mediating the onset of systemic proteolysis after trauma (Şimşek et al., 2014). Insulin resistance also contributes, as the normal anabolic effect of insulin is reduced in response to injury (Sobotka & Soeters, 2009). Proinflammatory

cytokines, such as TNF and IL6, can increase muscle protein breakdown by activating NF- κ B signaling and the ubiquitin-proteasome system (Lorite et al., 2001; Wyke & Tisdale, 2005).

Glutamine and alanine comprise more than 50% of the amino acids released by catabolic muscle (Weissman, 1990). Glutamine is the most abundant amino acid in blood and is normally considered nonessential. In healthy humans, circulating glutamine is mostly consumed in the gut and kidney (Hensley et al., 2013). However, glutamine levels decrease rapidly after trauma and sepsis, and it can become conditionally essential (Fürst et al., 1989; Engel et al., 2003). Glutamine acts as a major fuel for rapidly proliferating cells, such as fibroblasts, enterocytes, leukocytes, and other cells involved in tissue repair. T cells increase glutamine uptake 5- to 10-fold upon activation, and the depletion of glutamine blocks their proliferation and cytokine production (Carr et al., 2010). To meet enhanced demand, glutamine is synthesized mainly in the muscle from glutamate and ammonia by glutamine synthetase, an enzyme that is induced by glucocorticoids (Gaunitz et al., 2002).

HDL and APOA1

Infections and inflammation are associated with a variety of alterations in lipid metabolism. A common change is the increase in serum triglyceride and very low-density lipoprotein levels as a result of adipose tissue lipolysis, increased *de novo* hepatic fatty acid synthesis, and suppression of fatty acid oxidation (Feingold & Grunfeld, 2000; Khovichunkit et al., 2004). On the contrary, the levels of HDL cholesterol and apolipoprotein A1 (APOA1) are often decreased (Choi & Seeger, 2005; Apostolou et al., 2010). Low-density lipoprotein (LDL) cholesterol levels are more variable, but are often decreased as well (Feingold & Grunfeld, 2000).

The main function of HDL is reverse cholesterol transport, shuttling excess cholesterol from peripheral tissues to the liver (Fig. 3). In addition, HDL has antioxidant, anti-inflammatory and anti-apoptotic properties and it exerts beneficial cardiovascular influences (Jacobs et al., 1990; Zamanian-Daryoush & DiDonato, 2015). HDL particles contain a lipid core composed of cholesteryl esters and triglycerides surrounded by a membrane of phospholipids, free cholesterol, and apolipoproteins (Kontush et al., 2015). APOA1 is the predominant protein in HDL and plays an essential role in facilitating the numerous biological functions of HDL (Mangaraj et al., 2016). APOA1 initiates HDL synthesis by promoting ATP binding cassette subfamily A member 1 (ABCA1)-mediated cholesterol and phospholipid

efflux, and acts as a cofactor for lecithin-cholesterol acyltransferase (LCAT), an enzyme that converts cholesterol to cholesteryl esters (Sorci-Thomas et al., 2009).

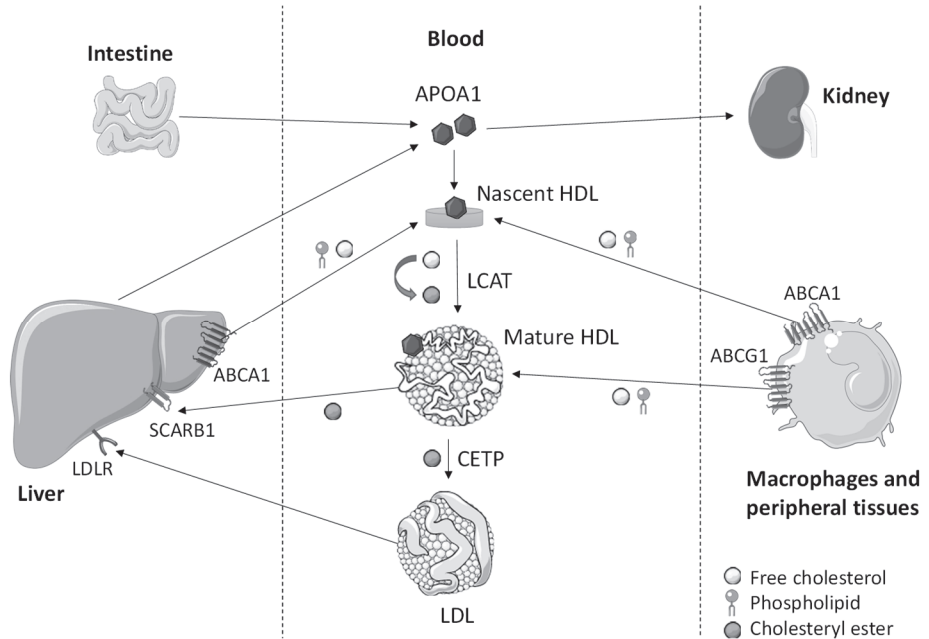


Fig. 3. Reverse cholesterol transport. APOA1 is synthesized mainly in the liver and the small intestine. It acquires free cholesterol and phospholipids via ABCA1-mediated efflux from the liver, macrophages and peripheral tissues to form nascent HDL particle. Esterification of free cholesterol to cholesterol ester by LCAT generates a mature HDL particle. This larger HDL particle can acquire additional lipids from macrophages via efflux mediated by ABCG1. Cholesteryl esters are off-loaded from HDL into hepatocytes via SCARB1. Alternatively, cholesteryl esters are transferred to LDL under mediation by CETP and then delivered to the liver via LDLR. The final step of reverse cholesterol transport is the catabolism of free APOA1 by the kidney with excretion in the urine. Modified from Joy & Hegele 2008 and Khera & Rader 2010. Abbreviations: ABCA1/ABCG1: ATP binding cassette subfamily A/G member 1, CETP: cholesteryl ester transfer protein, HDL: high-density lipoprotein, LDL: low-density lipoprotein, LCAT: lecithin-cholesterol acyltransferase, LDLR: low-density lipoprotein receptor, SCARB1: scavenger receptor class B member 1. The figure was created using Servier Medical Art templates, <https://smart.servier.com>.

HDL levels decrease in acute infections and chronic inflammatory diseases (Apostolou et al., 2010; Choi & Seeger, 2005), but the exact mechanism behind this is uncertain. However, the APR is known to induce marked alterations in HDL composition and function (Feingold & Grunfeld, 2016). These changes lead to the formation of acute-phase HDL with different characteristics from normal HDL, such as impaired cholesterol efflux capacity (Jahangiri et al., 2009). Normally SAA1 circulates in low levels bound to HDL, but during inflammation the synthesis of SAA1 in the liver is increased in response to cytokines (Castell et al., 1989). SAA1 can contribute up to 80% of acute-phase HDL apolipoprotein composition, and it has been shown to displace APOA1 in HDL *in vitro* (Coetzee et al., 1986; Cabana et al., 1989). This may promote APOA1 catabolism in the liver and kidney and impair the anti-inflammatory properties of HDL during inflammation (Catapano et al., 2014; Han et al., 2015).

2.3 Colorectal cancer

2.3.1 Incidence and risk factors

In both sexes combined, colorectal cancer is the third most commonly diagnosed cancer worldwide (Bray et al., 2018). There is a significant variation in prevalence across the world, with the highest incidence rates seen in the developed countries with westernized lifestyle, and the lowest incidence in less developed regions (Bray et al., 2018). In Finland, 3,359 new cases were diagnosed in 2017, and CRC was the third most common cancer diagnosis after breast cancer and prostate cancer (Finnish Cancer Registry 2017). The incidence of CRC progressively increases with age, being low up to 40 years and rising sharply after age 50 (Siegel et al., 2017).

The most important hereditary CRC syndromes are Lynch syndrome and familial adenomatous polyposis. They account for 2–6 % of all CRC cases, but affected individuals have a lifetime risk for CRC 39–100 % (Jasperson et al., 2010; Valle, 2014). In addition, 20–30 % of CRCs have a familial component and potentially identifiable genetic cause (Lichtenstein et al., 2000).

Most CRC cases are thus sporadic (Arvelo et al., 2015). Risk factors include advanced age, family history of cancer, male sex, previous CRC and its precursor lesions, inflammatory bowel diseases, and environmental factors such as high consumption of red meat and low intake of dietary fiber (Eaden et al., 2001;

Cunningham et al., 2010). Heavy long-term continuing smoking (Botteri et al., 2008), excessive alcohol consumption (Su & Arab, 2004), obesity (Gathirua-Mwangi et al., 2017) and physical inactivity (Slattery, 2004) also increase the risk.

Patients with inflammatory bowel disease are at significantly increased risk for developing CRC, and the risk increases with duration of illness and extent of inflammation (Eaden et al., 2001). Chronic inflammation induces DNA damage and produces inflammatory mediators, creating a microenvironment that promotes the development of cancer (Terzic et al., 2010).

The association between diet and CRC risk has been evaluated in numerous studies, focusing either on the intake of single food items or on dietary patterns (“Western diet” vs. “healthy diet”). Western dietary pattern, and especially the intake of red and processed meat, has been linked to increased CRC risk (Mehta et al., 2017). Red meat contains potential carcinogenic compounds, but the exact mechanisms underlying the association between red meat and CRC remain unclear (Aykan, 2015). High cholesterol intake may also increase CRC risk (Järvinen et al., 2001). On the contrary, high fruit and vegetable consumption as well as high intake of dietary fiber are associated with reduced risk of CRC (van Duijnhoven et al., 2009; Aune et al., 2011). Potential mechanisms for the protective effect of high fiber consumption include increased stool bulk and dilution of fecal carcinogens, shorter gut transit time, and bacterial fermentation of fiber to short chain fatty acids (Lipkin et al., 1999; Baena & Salinas, 2015).

Type 2 diabetes and metabolic syndrome are associated with increased risk of CRC (Jarvandi et al., 2013; Jinjuvadia et al., 2013). It is unclear whether this risk is because of the shared risk factors, like obesity and poor diet, or whether diabetes- and metabolic syndrome-related factors, such as hyperinsulinemia, hyperglycemia, diabetes medications and chronic inflammation, increase the risk of CRC (Jarvandi et al., 2013; González et al., 2017). However, a link between hyperglycemia and cancer has been shown, as sustained hyperglycemia can promote tumor growth by destabilizing the tumor suppressor TET2 (Wu et al., 2018).

The use of aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) has been associated with reduced CRC risk (Friis et al., 2015). A large population-based case-control study reported that the protective effect of aspirin was achieved with a low dose (75 mg per day) after 5 years use in the general population (Din et al., 2010). However, the precise mechanism by which NSAIDs reduce CRC risk is not clear, and in patients with inflammatory bowel disease, they do not appear to reduce the risk (Burr et al., 2016).

2.3.2 Pathogenesis

Hanahan and Weinberg originally proposed that virtually all human cancers must acquire the same six hallmark capabilities: sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis, and resisting cell death (Hanahan & Weinberg, 2000). In 2011, two emerging hallmarks were added: reprogramming of energy metabolism and evading immune destruction (Hanahan & Weinberg, 2011). Additionally, two characteristics that enable the acquisition of hallmark functions, genomic instability and inflammation, were added.

Molecular pathways

A classic model for CRC progression, called adenoma-carcinoma sequence, was first proposed in the 1980s (Fearon & Vogelstein, 1990; Vogelstein et al., 1988). It describes the transformation of normal epithelium to an adenoma and finally to invasive and metastatic carcinoma with the accumulation of mutational activation of oncogenes and the inactivation of tumor suppressor genes. Since this model was initially introduced, it has become evident that CRC can develop via several distinct molecular pathways.

Normal mutation rates are usually insufficient to account for the multiple mutations detected in many cancers, and thus cancers must acquire some form of genomic instability that increases their mutation rate (Loeb et al., 2003). In CRC pathogenesis, there are at least three different pathways of genomic instability: the chromosomal instability (CIN), microsatellite instability (MSI), and CpG island methylator phenotype (CIMP) pathways.

CIN pathway is based on the adenoma-carcinoma sequence and occurs in approximately 70–85% of CRCs. CIN tumors are characterized by the accumulation of numerical or structural chromosomal abnormalities, resulting in imbalances in chromosome number (aneuploidy) and loss-of-heterozygosity. Chromosomal abnormalities in CIN tumors are associated with a characteristic set of mutations. The earliest genetic events are mutations in the *APC* (*Adenomatous Polyposis Coli*) gene, which activate the Wnt signaling pathway by increasing β -catenin levels (Pino & Chung, 2010). *APC* mutation is followed by activating mutations of *KRAS*, leading to activation of the mitogen-activated protein kinase-extracellular signal-regulated kinase (MAPK-ERK) signaling pathway, and

additional mutations in the TGF β , PIK3CA, and TP53 pathways drive subsequent malignant transformation.

The second pathway, MSI-high (MSI-H) (15% of all CRCs) is defined by inactivating mutations in the DNA mismatch repair (MMR) genes that are responsible for correcting DNA replication errors. MMR deficiency can be sporadic (12%) or hereditary (3%, Lynch syndrome-associated CRC) (Boland & Goel, 2010). Sporadic cases are mainly caused by silencing of the MMR genes, mostly *MLH1*, by promoter hypermethylation (Herman et al., 1998) and Lynch syndrome by germline mutations in the mismatch repair genes *MLH1*, *PMS2*, *MSH6*, or *MSH2* (Lynch et al., 2009).

CIMP is a pathway of epigenetic instability. It is characterized by a widespread hypermethylation of promoter CpG island sites, resulting in transcriptional inactivation of several tumor suppressor genes or other tumor-related genes (Toyota et al., 1999). CIMP cancers often harbor *BRAF* mutation (Weisenberger et al., 2006).

Invasion and metastasis

The multistep process of metastatic cascade begins when tumor cells from the primary site lose cell-cell adhesion and detach from the ECM. Local invasion of cancer cells is followed by intravasation into nearby blood and lymphatic vessels, migration through the circulation and lymphatic system, movement into tissue (extravasation), the formation of micrometastases, and finally, the growth of a secondary tumor (Hanahan & Weinberg, 2011). Transformed epithelial cells can acquire the ability to invade by a dynamic program of dedifferentiation known as epithelial-mesenchymal transition. In CRC, epithelial-mesenchymal transition occurs at the invasive front where tumor-host interactions are important determinants of tumor dissemination. The balance between pro-tumor and anti-tumor factors at the invasive front may be decisive in determining tumor progression (Zlobec & Lugli, 2009).

Tumor microenvironment

The development and progression of human CRC is determined not only by genetically abnormal cells, but also by intricate interactions between malignant cells and the surrounding microenvironment. The tumor microenvironment is composed of the ECM and several non-malignant cell types such as endothelial cells, adipocytes, fibroblasts and immune cells. These cells can act as both

suppressors and promoters of tumor initiation, tumor growth, invasion, and metastasis.

Immune system is a critical component of the tumor microenvironment and is thought to represent a host immune response against tumor (Markman & Shiao, 2015). Cells from both innate immune system and adaptive immune system interact with the tumor by direct contact or through chemokine and cytokine signaling. T cells can recognize tumor antigens (Blankenstein et al., 2012), and nascent transformed cells can be destroyed by the immune system. However, elimination eventually leads to selection of tumor cells that can escape immune recognition and inhibit immune cells by different mechanisms (Kim et al., 2007).

Intestinal microbiota

The normal intestinal microbiota has a specific function in maintenance of a gut mucosal barrier against the external environment, host nutrient metabolism, immunomodulation, and protection against pathogens. Microbial structures differ between CRC patients and healthy individuals (Chen et al., 2012), and increased evidence has demonstrated that disruption of the gut microbiota is related to the development of CRC. Mechanisms by which the bacterial microbiome modulates carcinogenesis include disruption of epithelial barrier leading to increased inflammation, release of toxins that induce DNA damage, and metabolic actions that activate or inactivate carcinogens (Schwabe & Jobin, 2013).

2.3.3 Prognosis and prognostic markers

Survival trends for CRC have been generally flat or increasing in recent years (Allemani et al., 2018). The worldwide analysis of cancer survival (CONCORD-3) reported that for patients diagnosed with colon or rectal cancer during 2010–14, age-standardized 5-year net survival was 60–69% in many countries, although the variation was wide (Allemani et al., 2018). In Finland, CRC is the second most common cause of cancer death when men and women are combined, and the 5-year survival is about 65% (Finnish Cancer Registry 2017).

However, CRC is a heterogeneous disease and outcomes for patients are variable. Prognostic markers provide information about an individual patient's risk of disease recurrence and progression (Riley et al., 2009). They are useful to stratify patients into different risk groups and thus to guide the selection of treatment strategies (Erstad et al., 2015). In prognostic studies, several clinical endpoints can

be used to measure survival time. In overall survival (OS) the endpoint is death irrespective of cause, in cancer-specific survival (CSS) death caused by the same cancer, and in disease-free survival (DFS), any sign or symptom of the disease after curative treatment (Punt et al., 2007). Progression-free survival is the length of time that a patient lives with the disease but the disease does not get worse. Identified prognostic markers in CRC are presented in Table 1.

TNM Stage

CRC survival is greatly dependent upon stage of disease at diagnosis. Typically, 5-year survival rate ranges from 90% for cancers detected at the localized stage to 10% for metastatic cancer (Amin et al., 2017). Several staging systems for CRC exist, but the most commonly used is the tumor/node/metastasis (TNM) classification system produced by the American Joint Committee on Cancer and the Union for International Cancer Control. In this system, T describes the spread of the primary tumor to nearby tissue, N the spread of the tumor to nearby lymph nodes and M metastasis. The TNM system is continuously updated. Table 2 shows the current (8th edition) TNM classification, and Table 3 previous classifications (6th and 7th edition) that were also used in the studies included in this thesis. Stage classifications are presented in Table 4. Stage I tumor is localized and does not penetrate the muscle wall of the colon or rectum. Stage II tumor has grown through the muscle wall of the colon or rectum but has not spread to the nearby lymph nodes. Stage III tumor has invaded any of the local lymph nodes, but not to other distal parts of the body. Stage IV tumor has metastasized to distant organs.

Table 1. Some prognostic factors in CRC.

Prognostic factor	Markers of favorable prognosis	Markers of poor prognosis	References
Routinely used			
Resection margin	Negative	Positive	Amin et al. 2017
Examined lymph nodes	At least 12	Less than 12	Chang et al. 2007
TNM stage	N0, M0	Any N, any M	Amin et al. 2017
Tumor grade	Low grade	High grade	Halvorsen & Seim 1988, Newland et al. 1994
Histologic subtype	Medullary morphology	Signet ring-cell, micropapillary, undifferentiated	Cohen et al. 1991, Fleming et al. 2012, Lee et al. 2013b, Yun et al. 2017
Lymphatic invasion	Absent	Present	van Wyk et al. 2013
Blood vessel invasion	Absent	Present	Liang et al. 2007, van Wyk et al. 2013
Perineural invasion	Absent	Present	Yang et al. 2015
MSI in stage I–II tumors	MSI-H	MSS	Klingbiel et al. 2015
Serum CEA level	High	Low	Huang et al. 2019, Ahmed et al. 2018
Emerging markers			
Configuration of tumor border	Non-infiltrative	Infiltrative border, tumor budding	Graham et al. 2015, Lugli et al. 2017
Tumor necrosis	Low	Moderate or extensive	Pollheimer et al. 2010, Väyrynen et al. 2016
Microvessel density	Low	Increased	Des Guetz et al. 2006
Local infiltration of T cells	High	Low	Jass 1986, Galon et al. 2006
BRAF mutation in MSS tumors	No	Yes	Gavin et al. 2012, Bläker et al. 2019
Systemic inflammation markers	Low mGPS, low NLR	Elevated mGPS, elevated NLR	Walsh et al. 2005, Park et al. 2016

Abbreviations: CEA: carcinoembryonic antigen, mGPS: modified Glasgow Prognostic Score, NLR: neutrophil to lymphocyte ratio. MSS: microsatellite stable. MSI: microsatellite instability. MSI-H: microsatellite instability-high

Table 2. TNM8 Classification.

Classification	TNM8
Primary tumor (T)	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
T1	Tumor invades submucosa
T2	Tumor invades muscularis propria
T3	Tumor invades through the muscularis propria into pericorectal tissues
T4	Tumor invades through the visceral peritoneum and/or directly invades other organs or structures
T4a	Tumor invades through the visceral peritoneum (including gross perforation of the bowel through tumor and continuous invasion of tumor through areas of inflammation to the surface of the visceral peritoneum)
T4b	Tumor directly invades or adheres to other adjacent organs or structures
Regional lymph nodes (N)	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in 1–3 regional lymph nodes, or any number of tumor deposits present and all identifiable lymph nodes negative
N1a	Metastasis in 1 regional lymph node
N1b	Metastasis in 2–3 regional lymph nodes
N1c*	Tumor deposits in the subserosa, mesentery or non-peritonealised pericolic or perirectal/mesorectal tissue without regional lymph node metastasis
N2	Metastasis in 4 or more regional lymph nodes
N2a	Metastasis in 4–6 regional lymph nodes
N2b	Metastasis in 7 or more regional lymph nodes
Distant metastasis (M)	
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Metastasis to 1 or more distant sites or organs or peritoneal metastasis
M1a	Metastasis confined to 1 organ or site
M1b	Metastasis to 2 or more sites or organs
M1c	Metastasis to 1 or more sites or organs or the peritoneal surface alone or with other site or organ metastases

* In cases with lymph node metastasis, the number of tumor deposits is not added to the number of positive lymph nodes. The presence of tumor deposits does not change the primary tumor T category, but does change the node status (N) to N1c if all regional lymph nodes are pathologically negative. Adapted from Brierley et al. 2017.

Table 3. TNM6 and TNM7 Classification.

Classification	TNM6	TNM7
Primary tumor (T)		
TX	Primary tumor cannot be assessed	Primary tumor cannot be assessed
T0	No evidence of primary tumor	No evidence of primary tumor
T1	Tumor invades submucosa	Tumor invades submucosa
T2	Tumor invades muscularis propria	Tumor invades muscularis propria
T3	Tumor invades through the muscularis propria into pericorectal tissues	Tumor invades through the muscularis propria into pericorectal tissues
T4	Tumor invades through the visceral peritoneum and/or directly invades other organs or structures	Tumor invades through the visceral peritoneum and/or directly invades other organs or structures
T4a	-	Tumor invades through the visceral peritoneum
T4b	-	Tumor directly invades or adheres to other adjacent organs or structures
Regional lymph nodes (N)		
NX	Regional lymph nodes cannot be assessed	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis	No regional lymph node metastasis
N1	Metastasis in 1–3 regional lymph nodes	Metastasis in 1–3 regional lymph nodes
N1a	-	Metastasis in 1 regional lymph node
N1b	-	Metastasis in 2–3 regional lymph nodes
N1c*	-	Tumor deposits in the subserosa, mesentery or non-peritonealised pericolic or perirectal soft tissue without regional lymph node metastasis
N2	Metastasis in 4 or more regional lymph nodes	Metastasis in 4 or more regional lymph nodes
N2a	-	Metastasis in 4–6 regional lymph nodes
N2b	-	Metastasis in 7 or more regional lymph nodes
Distant metastasis (M)		
MX	Distant metastasis cannot be assessed	Distant metastasis cannot be assessed
M0	No distant metastasis	No distant metastasis
M1	Distant metastasis	Distant metastasis
M1a	-	Metastasis confined to 1 organ or site
M1b	-	Metastasis to 2 or more sites or organs

*If tumor deposits are observed with lesions that would otherwise be classified as T1 or T2, then the T classification is not changed, but the nodule(s) is recorded as N1c. If a nodule is considered by the pathologist to be a totally replaced lymph node (generally having a smooth contour), it should be recorded as a positive lymph node and not as a satellite, and each nodule should be counted separately as a lymph node in the final pN determination. Adapted from Sobin & Wittekind 2002 and Sobin et al. 2009.

Table 4. Stage Classification.

Stage	TNM6	TNM7	TNM8
I	T1–2, N0, M0	T1–2, N0, M0	T1–2, N0, M0
II	T3–4, N0, M0	T3–4, N0, M0	T3–4, N0, M0
IIA	T3, N0, M0	T3, N0, M0	T3, N0, M0
IIB	T4, N0, M0	T4a, N0, M0	T4a, N0, M0
IIC	-	T4b, N0, M0	T4b, N0, M0
III	T1–4, N1–2, M0	T1–4, N1–2, M0	T1–4, N1–2, M0
IIIA	T1–2, N1, M0	T1–2, N1, M0	T1–2, N1, M0
	-	T1, N2a, M0	T1, N2a, M0
IIIB	T3–4a, N1, M0	T3–4a, N1, M0	T3–4a, N1, M0
	-	T2–3, N2a, M0	T2–3, N2a, M0
	-	T1–2, N2b, M0	T1–2, N2b, M0
IIIC	T1–4, N2, M0	T4a, N2a, M0	T4a, N2a, M0
	-	T3–4a, N2b, M0	T3–4a, N2b, M0
	-	T4b, N1–N2, M0	T4b, N1–N2, M0
IV	T1–4, N0–2, M1	T1–4, N0–2, M1a–1b	T1–4, N0–2, M1a–1c
IVA	-	T1–4, N0–2, M1a	T1–4, N0–2, M1a
IVB	-	T1–4, N0–2, M1b	T1–4, N0–2, M1b
IVC	-	-	T1–4, N0–2, M1c

Adapted from Sobin & Wittekind 2002, Sobin et al. 2009 and Brierley et al. 2017.

Grade of differentiation

The prognosis of patients with poorly differentiated CRCs is typically reported to be unfavorable (Halvorsen & Seim, 1988; Newland et al., 1994). The conventional histological grading system in CRC is the WHO classification, which currently categorizes adenocarcinomas into well, moderately and poorly differentiated adenocarcinomas, and undifferentiated carcinomas based on the percentage of glandular formation (Hamilton et al., 2010). However, in a forthcoming WHO2019 classification, adenocarcinomas will only be divided into two classes, low-grade and high-grade adenocarcinomas (Nagtegaal et al., 2019).

Genetic markers in clinical use

MSI-H CRCs have a more favorable prognosis at early local stages of disease (Klingbiel et al., 2015). Tumors arising through the MSI pathway have a high mutational load that creates many tumor neoantigens, and these tumors are associated with increased local immune response (Llosa et al., 2015). *BRAF* mutations are associated with worse survival in MSS tumors, but the presence of MSI may attenuate their adverse impact (Amin et al., 2017; Bläker et al., 2019). In stage IV CRC, the prevalence of *BRAF* mutations and MSI is low, but both markers confer an inferior prognosis, and MSI testing has a predictive value for the use of immune checkpoint inhibitors (Van Cutsem et al., 2016; Schrock et al., 2019). *KRAS* and *NRAS* mutations predict poor response to epidermal growth factor receptor-specific antibody therapy in stage IV CRC (Allegra et al., 2009; De Roock et al., 2010).

Necrosis

Abundant tumor necrosis has been reported to associate with poor prognosis in CRC (Pollheimer et al., 2010; Komori et al., 2013; Väyrynen et al., 2016). Necrosis is often present in solid tumors and is thought to reflect hypoxia and nutrient depletion due to rapid tumor growth (Bredholt et al., 2015; Lee et al., 2018).

Local inflammation

Evaluation of the local inflammatory reaction also provides information on prognosis. The most frequent tumor infiltrating immune cells in CRC include T cells and monocyte-macrophage lineage cells, and the immune cell densities generally decline as tumor stage advances (Ge et al., 2019; Guo et al., 2019; Väyrynen et al., 2013). Various studies have shown that a strong local inflammatory response, especially a high number of tumor infiltrating lymphocytes, is associated with better outcome in CRC regardless of stage (Jass, 1986; Galon et al., 2006; Ogino et al., 2009; Roxburgh & McMillan, 2012). The study by Jass (1986) was the first classification of lymphocytic infiltration in CRC. Since then, several scoring systems for the local inflammation have been reported. Graham and Appelman (1990) found that evaluating the intensity of Crohn's-like lymphoid reaction around tumors may have prognostic value. In Klintrup-Mäkinen score, generalized inflammatory cell infiltrate is evaluated from haematoxylin and eosin

slides (Klintrup et al., 2005), and ImmunoscoreTM is based on the numeration of CD3 and CD8 T lymphocyte subsets (Galon et al., 2014).

Systemic inflammation

Systemic inflammation is activated in about one third of CRC patients at the time of operation, and it has been linked with muscle loss, impaired nutritional status, poor performance status, increased comorbidity, increased pro-inflammatory and angiogenic cytokines, complications on treatment, and decreased survival (Kantola et al., 2012, 2013; McMillan, 2013; Park et al., 2016). The initial studies used CRP as a measure of an ongoing systemic inflammation, and reported independent prognostic value in curatively operable CRC (McMillan et al., 1995). Later, it was shown that a cumulative prognostic score based on CRP and albumin (subsequently termed the Glasgow Prognostic Score, GPS) had similar prognostic value to that of stage and performance status (Forrest et al., 2003). Based on further investigation, the GPS was modified, as it was found that hypoalbuminemia on its own was not associated with decreased survival (McMillan et al., 2007). Table 5 presents the comparison of GPS and modified GPS (mGPS).

Table 5. Comparison of Glasgow Prognostic Score (GPS) and modified Glasgow Prognostic Score (mGPS).

Description	GPS	mGPS
CRP ≤10 mg/L and albumin ≥35 g/L	0	0
CRP ≤10 mg/L and albumin <35 g/L	1	0
CRP >10 mg/L and albumin ≥35 g/L	1	1
CRP >10 mg/L and albumin <35 g/L	2	2

Abbreviations: CRP: C-reactive protein

Since the initial work, the use of GPS or mGPS has been examined and validated in numerous studies with a variety of operable and inoperable cancers (McMillan, 2013). Park et al (Park et al., 2016) showed that the combination of TNM and mGPS effectively stratifies outcome in stage I–III CRC patients amenable for curative resection, with CSS at 5 years varying from 100% in patients with stage I and mGPS0 to 32% in patients with stage III and mGPS2. Also in stage IV CRC, elevated mGPS has been shown to predict worse CSS and OS (Køstner et al., 2016; Lu et al., 2019).

In addition to mGPS, several other inflammation-based markers have been related to unfavorable survival in CRC. These include a high neutrophil to lymphocyte ratio (NLR, blood neutrophil count divided by blood lymphocyte count) (Walsh et al., 2005), high lymphocyte to monocyte ratio (Stotz et al., 2014), elevated platelet count (Wan et al., 2013) and a systemic immune-inflammation index based on neutrophil, lymphocyte, and platelet counts (Xie et al., 2018). GlycA is a novel nuclear magnetic resonance inflammatory marker that identifies N-acetyl glycan groups mostly attached to acute phase glycoproteins (Otvos et al., 2015). Elevated baseline levels of GlycA have been shown to associate with increased risk of incident CRC and mortality (Chandler et al., 2016). Moreover, increased serum level of YKL-40 is a predictor of poor OS in patients with rectal cancer (Fuksiewicz et al., 2018) and in patients with metastatic CRC treated with liver resection (Holsey Gramkow et al., 2017).

2.4 Cancer-related malnutrition

Patients with cancer often experience some degree of malnutrition because both the disease and its treatments threaten their nutritional status (Arends et al., 2017b). Unlike simple malnutrition, the negative energy balance and skeletal muscle loss seen in cancer patients are driven by a combination of reduced food intake and metabolic derangements. Systemic inflammation and catabolic factors provoke elevated resting metabolic rate, insulin resistance, lipolysis, and proteolysis, and thus promote wasting (Arends et al., 2017a). Cancer-related malnutrition can impair response to treatments, lower quality of life, worsen muscle function, and increase the risk of post-operative complications (Pressoir et al., 2010; Aaldriks et al., 2013; Fukuda et al., 2015; Gellrich et al., 2015). Moreover, it can lead to reduced survival (Pressoir et al., 2010; Martin et al., 2015). Malnutrition is most often seen among patients with gastrointestinal tract cancer, head and neck cancer, and lung and liver cancers (Arends et al., 2017b).

2.4.1 Definitions of malnutrition, sarcopenia, and cachexia

Malnutrition, sarcopenia, and cachexia are partially overlapping conditions, and there is also some overlap in their definitions (Arends et al., 2017b). Disease-related malnutrition with inflammation has been defined as a catabolic condition that results from the activation of systemic inflammation by an underlying disease such as cancer (Cederholm et al., 2017). Sarcopenia is a condition characterized by loss

of muscle mass and muscle strength (Muscaritoli et al., 2010). Cachexia is a multifactorial syndrome defined by an ongoing loss of skeletal muscle mass (with or without loss of fat mass) that cannot be fully reversed by conventional nutritional support and leads to progressive functional impairment (Fearon et al., 2011). Cachexia can be further classified into three stages of clinical relevance: pre-cachexia, cachexia and refractory cachexia (Fearon et al., 2011). Besides weight loss, additional criteria for cancer cachexia have been suggested, including decrease in muscle strength, fatigue, anorexia, lean tissue depletion, high CRP, high IL6, low albumin, and low hemoglobin (Evans et al., 2008; Argilés et al., 2011).

2.4.2 Mechanisms

The pathophysiology of cancer-related malnutrition is presented in Figure 4. The mechanisms are multifactorial and have not been fully elucidated, but it is known that systemic inflammation plays a central role (Arends et al., 2017a). Cytokines elicit the production of hepatic acute phase proteins and contribute to induction of muscle proteolysis, adipose tissue lipolysis, and neuroendocrine control of appetite in brain. The liver is a key metabolic organ that contributes to cancer-related malnutrition by increasing gluconeogenesis and worsening inflammation by secreting acute phase proteins (Porporato, 2016).

The role of cytokines in wasting was first suggested in 1985, when Cerami et al showed that the culture medium from lipopolysaccharide-activated macrophages caused body weight loss when injected into mice (Cerami et al., 1985). The molecule causing weight loss was first termed cachectin, but subsequent analyses revealed that it was actually TNF (Beutler et al., 1985). In addition to TNF, the levels of several other proinflammatory cytokines, including IL1, IL6, and CXCL8, are elevated in malnourished cancer patients (Shenouda et al., 1993; Pfitzenmaier et al., 2003; Daniele et al., 2017). Especially IL6 has a well-documented role in wasting (Oldenburg et al., 1993). It has the potency to induce anorexia, metabolic alterations, muscle wasting, and gut barrier dysfunction (Iwase et al., 2004; Flint et al., 2016; White, 2017; Bindels et al., 2018). In animals, chronic administration of cytokines mimics cachexia, and the administration of specific antagonists of cytokines can relieve cachexia (Sherry et al., 1989; Strassmann et al., 1992; Zaki et al., 2004). However, treatments with antibodies targeting a single cytokine have failed to significantly ameliorate the wasting syndrome, and it has become evident that it is a set of cytokines and other mediators that is responsible for wasting in cancer patients (Penna et al., 2010).

The mechanisms underlying muscle loss have been intensively studied in recent years, but discrepancies have been reported between different experimental models as well as in patients with different cancer types (Mueller et al., 2016; Aversa et al., 2017). However, it is apparent that specific pro-cachectic factors are upregulated (e.g., members of the ubiquitin-proteasome system, activin A, angiotensin II and apoptosis inducing factors) whereas anti-cachectic molecules (e.g., insulin-like growth factor 1, muscle growth and regeneration factors) are downregulated in tissue wasting (Suzuki et al., 2013; Mueller et al., 2016). Tumor derived proteolysis-inducing factor and cytokines such as TNF can activate NF- κ B signaling and the ubiquitin-proteasome system leading to muscle proteolysis (Lorite et al., 2001; Wyke & Tisdale, 2005). Insulin and insulin-like growth factor 1 activate the anabolic P13K/Akt/mTOR pathway, and insulin resistance has been shown to accelerate muscle protein degradation (Wang et al., 2006). Impaired muscle protein synthesis may also contribute to muscle loss (Horstman et al., 2016).

Lipolysis can be induced by several serum factors secreted by tumor or host cells, including cytokines such as IL6, IL1, and TNF, hormones like glucocorticoids and catecholamines, and zinc- α_2 glycoprotein (Tisdale, 2010; Petruzzelli & Wagner, 2016). The progressive switch from white to brown adipose tissue, termed browning, may take place before muscle wasting and could contribute to increased energy expenditure in cancer patients (Shellock et al., 1986; Porporato, 2016).

Decreased appetite and anorexia are characteristic for end-stage cancer patients (Argilés et al., 2014). Regulation of food intake involves hormonal networks, primarily in the hypothalamus, that are altered in cancer-associated malnutrition (Porporato, 2016). Starvation or loss of body fat normally lead to decrease in adipokine leptin, which in turn leads to increased production of gastric hormone ghrelin and other appetite-stimulating neuropeptides, and decreased activity of anorexigenic neuropeptides (Suzuki et al., 2013). In cancer-associated anorexia, cytokines may modulate central nervous system neurotransmitter pathways and mimic the negative feedback action of leptin (Patra & Arora, 2012).

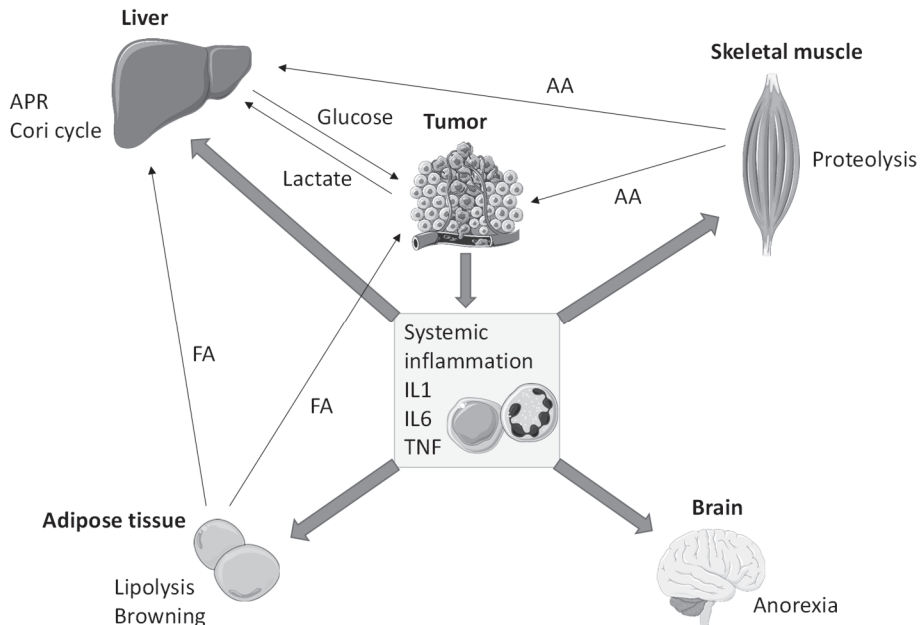


Fig. 4. Mechanisms involved in cancer-related malnutrition. The tumor releases proinflammatory cytokines and other catabolic factors that act on target tissues to elicit excess catabolism. Amino acids (AA) are released from the muscle and can be used by the liver to sustain gluconeogenesis and acute phase response (APR), or by the tumor for synthesis of protein and DNA. Fatty acids (FA) released in lipolysis can be used by the tumor or by the liver. The tumor generates excess lactate that can enter the Cori cycle in the liver and be recycled to glucose, which can return to the tumor. Modified from Argiles et al. 2017. The figure was created using Servier Medical Art templates, <https://smart.servier.com>.

2.4.3 Treatment

Cachexia is an irreversible condition that cannot be reversed by conventional nutritional care, but processes preceding it may be reversible. Thus, interventions initiated as early as possible give the most clinical benefit to the patient (Cederholm et al., 2017). It has been suggested that malnutrition intervention in cancer patients should include multimodal treatment consisting of combinations of nutrition, exercise, and medication to counteract catabolism and the underlying inflammatory process (Arends et al., 2017b). Drug therapy can be used in severely malnourished patients with advanced disease to stimulate appetite and/or gut motility, to attenuate

systemic inflammation and/or hypercatabolism, or to increase muscle mass and/or improve anabolism (Arends et al., 2017a).

The only two drugs presently widely prescribed for cachectic patients near the end of life are progestins and corticosteroids. Both have been shown to improve appetite, but they do not improve survival, and their adverse effects make them unsuitable for long-term use (Loprinzi et al., 1999; Gullett et al., 2011; Ruiz Garcia et al., 2013). Clinical trials have reported some improvements in weight gain and inflammatory markers in NSAID-treated patients with cancer cachexia (Falconer et al., 1995; Mantovani et al., 2010), but there is no reliable support to recommend the widespread use of NSAIDs (Solheim et al., 2013). In addition, monoclonal antibodies that target inflammatory cytokines, androgens that increase muscle mass, and cannabinoids that could improve appetite have been investigated as potential agents to manage cachexia (Jatoi et al., 2010, Bayliss et al., 2011, Crawford et al., 2016, Turcott et al., 2018). However, the available evidence is too limited and inconsistent to recommend their use in clinical practice (Arends et al., 2017a). The peptide hormone ghrelin has a key role in increasing appetite and also possesses anti-inflammatory actions (Li et al., 2004; Wei et al., 2015). It has been shown to increase energy intake in cancer patients (Neary et al., 2004) and several clinical trials with ghrelin and ghrelin agonists are currently on going (ClinicalTrials.gov, 2019).

It is unclear whether enteral or parenteral supplements can reverse lean body mass depletion in pre-cachectic patients. A meta-analysis of oral nutritional interventions in malnourished cancer patients found that nutritional intervention had a beneficial effect on some aspects of quality of life, but had inconsistent effect on body weight and no effect on mortality (Baldwin et al., 2012). Eicosapentaenoic acid, an omega-3-fatty acid found abundantly in fish oil, can downregulate proinflammatory cytokines and suppress ubiquitin-proteasome-induced muscle proteolysis (Giacosa & Rondanelli, 2008). Some clinical trials have reported improvements in body weight or physical activity in cachectic patients using eicosapentaenoic acid rich nutritional supplements (Moses et al., 2004), but others have found no clear benefit (Fearon et al., 2006). Dietary protein supplementations could mitigate muscle wasting, and the recommended protein supply for cancer patients with systemic inflammation is up to 1.5 g/kg/day (Arends et al., 2017a).

3 Aims of the study

Systemic inflammation is associated with metabolic derangements, malnutrition, and decreased survival in CRC, but the underlying factors and downstream effects of CRC-related systemic inflammation are not well understood. The aim of this study was to provide new information on the determinants of systemic inflammation in CRC patients. The specific objectives were:

1. To analyze the association of systemic inflammation markers (mGPS, CRP, albumin, cytokines, and immune cells) with serum levels of KRT18 fragments, MMP8, APOA1, and nine amino acids (I–IV).
2. To assess the relationships between circulating KRT18 levels and the extent of tumor necrosis in the primary tumor (I).
3. To evaluate serum MMP8 levels in relation to local immune cell infiltrate in the primary tumor (II).
4. To assess the prognostic value of serum KRT18, MMP8, APOA1, and amino acid levels (I–IV).

4 Materials and methods

4.1 Patients (I–IV)

These studies were based on a cohort of electively operated CRC patients in Oulu University Hospital between April 2006 and April 2014. Other than primary tumor operations were excluded from the study. The studies were introduced to all newly diagnosed CRC patients, and patients who signed an informed consent to participate and were also eligible for the study were included. The patients with other earlier or simultaneously diagnosed malignant diseases were excluded. Preoperative blood samples and surgical specimens were collected from a total of 357 patients, and of those 328 were included in study I, 271 in study II, 144 in study III, and 336 in study IV. The characteristics and outcome of patients are presented in Table 6. In study II, those who received preoperative radiotherapy/chemoradiotherapy (RT/CRT) (n=70) were excluded to avoid confounding, as RT/CRT might affect the inflammatory reaction around the tumor (Nagtegaal et al., 2002). In study III, only patients operated between April 2006 and January 2010 were included (n=149). Cases with inadequate serum sample material or insufficient serum quality for analyses were excluded (study I: n=23, study II: n=16, study III: n=5, study IV: n=15). In addition, six cases were excluded in studies I and IV due to other earlier or simultaneous malignancy that was noticed during review of the pathological slides. The study design was approved by the Ethical Committee of Oulu University Hospital (58/2005, 184/2009). All the experiments were conducted in accordance with the Declaration of Helsinki. The details of age, gender, height, weight, medication, and previous illnesses of the patients were collected from clinical records and by a questionnaire. The 120-month survival data was acquired from the clinical records and from Statistics Finland.

Table 6. Characteristics and outcome of patients operated for primary colorectal cancer.

Characteristic	Study I (n=328)	Study II (n=271)	Study III (n=144)	Study IV (n=336)
Time of operation	2006–2014	2006–2014	2006–2010	2006–2014
Age, mean (SD)	68.2 (11.3)	69.5 (11.6)	66.6 (11.0)	68.2 (11.5)
Gender				
Male	177 (54.0%)	138 (50.9%)	77 (53.5%)	180 (53.6%)
Female	151 (46.0%)	133 (49.1%)	67 (46.5%)	156 (46.4%)
BMI				
<25	112 (34.1%)	94 (35.7%)	58 (40.8%)	118 (36.1%)
25–30	134 (41.9%)	106 (40.3%)	58 (40.8%)	136 (41.6%)
>30	74 (23.1%)	63 (24.0%)	26 (18.3%)	73 (22.3%)
Preoperative RT/CRT				
No	260 (79.3%)	271 (100%)	112 (77.8%)	267 (79.5%)
Yes	68 (20.7%)	0 (0%)	32 (22.2%)	69 (20.5%)
Tumor location				
Proximal colon	107 (32.6%)	115 (42.4%)	49 (34.0%)	117 (34.8%)
Distal colon	70 (21.3%)	72 (26.6%)	27 (18.8%)	71 (51.1%)
Rectum	151 (46.0%)	84 (31.0%)	68 (47.2%)	148 (44.0%)
WHO grade				
Well differentiated	69 (21.1%)	67 (24.8%)	19 (12.2%)	73 (21.9%)
Moderately differentiated	218 (66.7%)	171 (63.3%)	105 (73.4%)	217 (65.0%)
Poorly differentiated	40 (12.2%)	32 (11.9%)	19 (13.2%)	44 (13.2%)
TNM stage				
I	68 (20.7%)	59 (21.9%)	25 (17.4%)	71 (21.2%)
II	110 (33.5%)	89 (33.0%)	54 (37.5%)	110 (32.8%)
III	109 (33.2%)	81 (30.0%)	44 (30.6%)	110 (32.8%)
IV	41 (12.5%)	41 (15.2%)	19 (13.2%)	44 (13.1%)
mGPS				
0	258 (78.9%)	209 (77.4%)	113 (78.5%)	262 (78.4%)
1	66 (19.3%)	54 (20.0%)	26 (18.1%)	64 (19.2%)
2	6 (1.8%)	7 (2.6%)	5 (3.5%)	8 (2.4%)
Lymphatic invasion				
No	178 (54.3%)	144 (53.7%)	80 (57.1%)	179 (53.3%)
Yes	147 (44.8%)	124 (46.3%)	60 (42.9%)	153 (45.5%)
MMR enzyme screening status				
Deficient	35 (10.7%)	38 (14.1%)	11 (7.7%)	38 (11.3%)
Proficient	293 (89.3%)	232 (85.9%)	132 (92.3%)	297 (88.7%)
Immunohistochemistry with BRAF V600E- mutation specific antibody (VE1)				
Negative	297 (90.8%)	242 (89.3%)	129 (90.2%)	304 (90.7%)
Positive	30 (9.1%)	29 (10.7%)	14 (9.8%)	31 (9.3%)

Characteristic	Study I (n=328)	Study II (n=271)	Study III (n=144)	Study IV (n=336)
Median follow-up time for patients alive in months (range)	77.5 (11.3–120.0)	75.5 (11.3–120.0)	60.0 (36.0–60.0)	75.7 (11.3–120.0)
Number of patients who died during follow-up	108 (32.9%)	93 (34.3%)	44 (30.5%)	110 (32.7%)
Recurrence after operation				
Recurrence during follow-up time	59 (18.0%)	44 (16.2%)	23 (16.0%)	59 (17.6%)
Recurrence within 3 months after resection	5 (1.5%)	2 (0.7%)	4 (2.8%)	5 (1.5%)
Recurrence after 3 months and within 3 years after resection	42 (12.8%)	38 (14.0%)	16 (11.1%)	42 (12.5%)
Recurrence after 3 years	12 (3.7%)	4 (1.5%)	3 (2.1%)	12 (3.6%)
Disease-free survival				
Alive, no recurrence	189 (57.6%)	154 (56.8%)	86 (59.7%)	194 (57.7%)
Alive after/with recurrence	24 (7.3%)	17 (6.3%)	9 (6.3%)	25 (7.4%)
Died of colorectal cancer	71 (21.6%)	63 (23.2%)	32 (22.2%)	74 (22.0%)
Died of non-colorectal causes	37 (11.3%)	30 (11.1%)	12 (8.3%)	36 (10.7%)

Abbreviations: BMI: body mass index. MMR: Mismatch repair. mGPS: modified Glasgow Prognostic Score. WHO grade: WHO histological grade of differentiation. RT/CRT: radiotherapy/chemoradiotherapy.

4.2 Histopathological analysis (I–IV)

4.2.1 Stage and grade (I–IV)

In studies II and III, the tumors were staged according to TNM6 classification (patients operated between April 2006 and January 2010) or TNM7 (patients operated between February 2010 to April 2014). In studies I and IV, all cases were restaged according to TNM8 classification. Original pathology reports and tissue slides were reviewed and TNM stage was corrected accordingly in five cases. The WHO2010 criteria were used in grading (Hamilton et al., 2010).

4.2.2 Tumor necrosis (I)

The percentage of tumor tissue showing coagulative necrosis (characteristic necrotic appearance with increased eosinophilia and nuclear shrinkage, fragmentation, and disappearance in the hematoxylin and eosin stained sections) (Fig. 5) was evaluated from haematoxylin and eosin stained sections by inspecting manually all available slides (Väyrynen et al., 2016). To determine the accuracy of

the visual assessment, one haematoxylin and eosin stained section per case of 50 randomly selected cases was scanned using the Aperio AT2 image-capturing device (Leica Biosystems, Wetzlar, Germany). Two researchers independently performed visual necrosis percentage estimations by marking the tumor necrosis area by hand and calculating its proportion with ImageJ (US National Institutes of Health, Bethesda, MD, USA) (Väyrynen et al., 2016). As necrosis percentage does not indicate the extent of necrosis, tumor necrosis percentage was multiplied by the maximum tumor diameter mentioned in the pathology report. To estimate the total area of necrotic tumor tissue, this tumor necrosis index was used in the analyses.

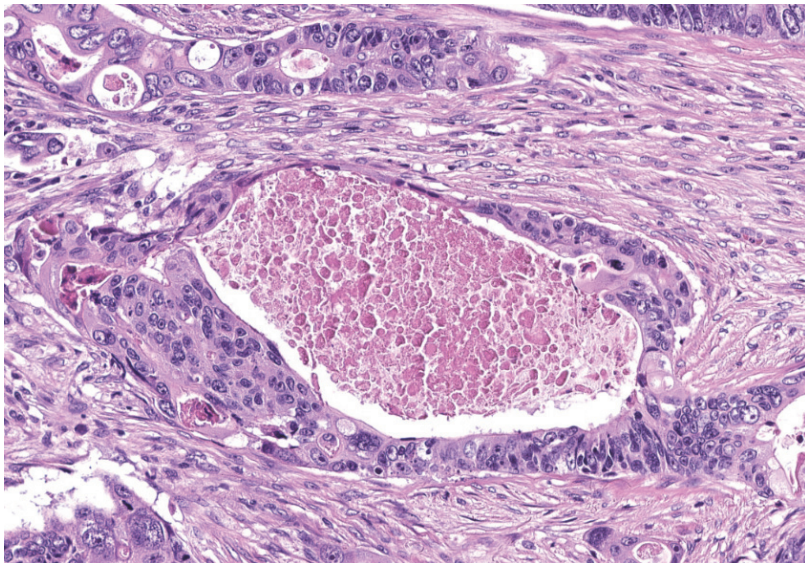


Fig. 5. Haematoxylin and eosin stained section showing necrotic area in colorectal cancer.

4.2.3 Tumor-infiltrating immune cells (II)

Tissue microarrays were constructed to facilitate the analysis of local inflammatory reaction. These arrays included 1–4 cores of 3.0 mm diameter, depending on the size of the tumor, including both the invasive margin and the center of the tumor (Väyrynen et al., 2013, 2014). Immunohistochemistry was conducted on 3.5- μ m sections cut from the TMA paraffin blocks for five immune cell markers (CD3 for T cells, CD8 for cytotoxic T cells, FoxP3 for regulatory T cells, mast cell tryptase

for mast cells, and neutrophil elastase for neutrophilic granulocytes). For immune cell counting, images were captured from the center of the tumor and the invasive margin, and the cell densities were counted using a computer-assisted cell counting method that utilizes ImageJ (Väyrynen et al., 2012a) .

4.3 Serum analyses (I–IV)

Preoperative serum samples were collected into BD Vacutainer® tubes. The samples were centrifuged at 2,500 x g for 10 min at room temperature after which serum was frozen within 4 h of collection at -20°C and transferred into -70°C during the same day.

4.3.1 Inflammatory markers (I–IV)

A leukocyte differential count, serum CRP levels and serum albumin levels were measured in the laboratory of Oulu University Hospital. mGPS was calculated from CRP and albumin values (McMillan et al., 2007). Serum levels of 27 cytokines were measured by Bio-Plex Pro Human pre-manufactured 27-Plex Cytokine Panel (Bio-Rad, Hercules, CA, USA) from patients operated between April 2006 and January 2010 (Kantola et al., 2012). Of the measured cytokines, 14 had several values below or above the assay detection limits. Cytokines with less than four values outside the assay working range (n=13) were included in these studies: IL1RN, IL4, IL6, IL7, CXCL8, IL9, IL12, IFNG, CXCL10, chemokine (C-C motif) ligand 2 (CCL2), CCL4, CCL11, and platelet derived growth factor subtype BB (PDGF-BB).

4.3.2 KRT18 (I)

Concentration of caspase-cleaved KRT18 released during apoptosis (aKRT18) was measured by M30 Apoptosense® sandwich ELISA, and total soluble KRT18 (tKRT18) by M65® sandwich ELISA (Peviva AB, Bromma, Sweden) (Fig. 6). All determinations were done in duplicate according to manufacturer's instructions. Units were defined against a synthetic peptide standard (1 U/L=1.24 pM). As the M65 ELISA measures total KRT18 released from dying cells (due to both apoptosis and necrosis), the level of KRT18 released during necrosis (nKRT18) was calculated as M65-M30.

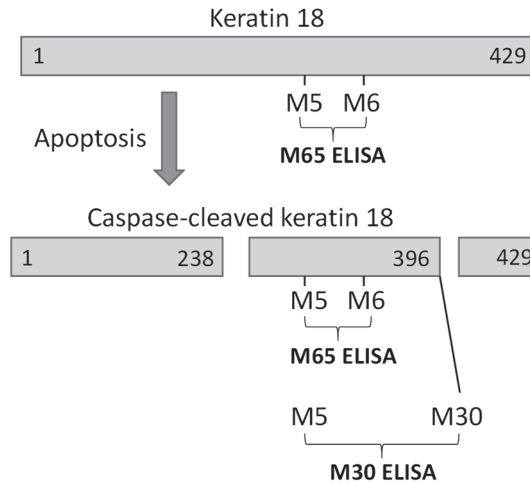


Fig. 6. Schematic presentation of the keratin 18 (KRT18) epitope map targeted by the antibodies used in the M30 and M65 ELISAs. During apoptosis, caspases cleave KRT18 at two sites, after Asp238 and Asp396, and M30 antibody recognizes a new epitope formed after cleavage at Asp396. Therefore, the M30 ELISA, using M5 antibody as a catcher and M30 antibody for detection, detects the soluble apoptosis-released KRT18 fragment. The M65 ELISA, using antibodies M6 and M5, recognizes a common epitope present in both full-length protein and in the caspase-cleaved fragment and thus measures total soluble KRT18. Modified from Kramer et al. 2004.

4.3.3 MMP8 (II)

Serum levels of MMP8 were measured by a time-resolved immunofluorometric assay (Medix Biochemica, Kauniainen, Finland) in accordance with the manufacturer's instructions with 1:5 dilutions of serum (Tuomainen et al., 2007).

4.3.4 Metabolites (III–IV)

A high-throughput serum nuclear magnetic resonance metabolomics platform equipped with Bruker AVANCE III 500 MHz and Bruker AVANCE III 600 MHz spectrometers (Bruker, Billerica, MA, USA) (Soininen et al., 2009), was used to quantify altogether 233 metabolic measures. From these variables, the levels APOA1 and nine amino acids (alanine, glutamine, glycine, histidine, isoleucine, leucine, phenylalanine, tyrosine, and valine) were included in this study.

4.4 Statistical analyses (I–IV)

The statistical analyses were conducted using IBM SPSS Statistics for Windows version 22 and 25 (IBM Corporation, Armonk, NY, USA). Normally distributed continuous variables were presented as mean (standard deviation, SD), whereas other continuous variables were presented as median (interquartile range, IQR). Statistical significances of the associations between categorical and continuous variables were analyzed by independent samples t-test or Mann-Whitney U test (comparing two classes), and by one-way analysis of variance or Kruskal-Wallis test (comparing three or more classes). Pearson correlation coefficients (r) were used in examining the bivariate correlations between two continuous variables. A logarithmic transformation was applied to variables with positive skewness (aKRT18, tKRT18, nKRT18, MMP8, CRP, blood cell counts, and cytokines). Multiple linear regression analyses were performed to adjust the correlations for additional parameters by the enter method, and to analyze the most important predictors of variables by the stepwise method. The factors included in the multivariable linear regression models were those considered theoretically important (stage variables T1–T2 vs. T3–T4, N0 vs. N1–2, M0 vs. M1) and those that had significance in the univariate analysis. The 2D visualization of the bivariate correlations between variables was created with Cytoscape software platform (Shannon et al., 2003), utilizing the Prefuse force directed algorithm weighted by the statistical significances of the correlations between individual variables. Multiple comparisons are accompanied by an increased possibility of type I error (false positive), and it is necessary to adjust p value accordingly. However, there is no universally accepted single method for that. As these analyses were mostly exploratory rather than confirmatory, a strict adjustment for multiple comparisons is less critical (Althouse, 2016). We adjusted the level of statistical significance to $p=0.01$ and interpreted the results with caution. All p values are two-tailed.

4.4.1 Survival (I–IV)

In the survival analyses, CSS was defined as time from the operation to death from the same cancer, OS as time from the operation to death, regardless of cause, and DFS as time from radical tumor operation to recurrence. Follow-up time was 60 months in study III and 120 months in studies I, II and IV. Receiver operating characteristics (ROC) analysis was used to determine an optimal cut-off point for continuous variables in discriminating survivors from non-survivors. Cut-off points

with the shortest distance to the coordinate (0,1), representing 100% sensitivity (no false negatives) and 100% specificity (no false positives), were chosen. Kaplan-Meier curves were conducted to visualize the estimated survival probability at any time. The Cox's proportional hazards regression analysis was used to investigate the difference between survival times of different groups. In the Cox regression, hazard ratio (HR) provides an estimate of the ratio of the death rates between two independent comparison groups over the entire study duration. Multivariate Cox regression analyses were conducted with the enter method to assess the independent prognostic value of variables.

5 Results

Table 7 shows the observed serum levels of KRT18, MMP8, APOA1, and nine amino acids in CRC patients. The blood reference values (Contois et al., 1996; Thraikill et al., 2005; Laposata, 2014; M30Apoptosense® and M65® ELISA kit instructions, Peviva AB) and how the levels in CRC patients fit in these ranges are also described in Table 7.

Table 7. Concentrations of the studied serum analytes in CRC patients.

Serum analyte	Median (IQR) or mean (SD)	Reference value	Number of patients with level outside the reference range
tKRT18, U/L	483.4 (366.5–697.1)	≤450	187 over (57.0%)
aKRT18, U/L	185.9 (142.3–254.0)	≤150	231 over (70.4%)
nKRT18, U/L	298.9 (191.4–471.0)	-	-
MMP8, ng/mL	64.3 (27.9–118.4)	1.39–79.02	1 under (0.4%), 110 over (40.6%)
APOA1, g/L	1.30 (1.23–1.43)	>1.20	28 under (19.4%)
Alanine, μmol/L	452.4 (89.1)	210–661	0 under, 9 over (2.7%)
Glutamine, μmol/L	488.6 (87.0)	420–700	66 under, 0 over (19.8%)
Glycine, μmol/L	282.1 (60.5)	120–560	0 under, 0 over
Histidine, μmol/L	57.6 (10.6)	32–110	4 under, 0 over (1.2%)
Isoleucine, μmol/L	55.6 (18.7)	40–100	67 under (20.0%), 12 over (3.6%)
Leucine, μmol/L	77.8 (24.3)	75–175	172 under (51.3%), 0 over
Valine, μmol/L	173.2 (49.0)	145–315	101 under (30.1%), 5 over (1.5%)
Phenylalanine, μmol/L	84.6 (18.4)	35–90	0 under, 96 over (28.6%)
Tyrosine, μmol/L	57.4 (16.6)	20–90	1 under (0.3%), 15 over (4.5%)

Abbreviations: tKRT18: total keratin 18. aKRT18: keratin 18 released during apoptosis. nKRT18: keratin 18 released during necrosis. MMP8: matrix metalloproteinase 8. APOA1: apolipoprotein A1. IQR: interquartile range. SD: standard deviation.

5.1 Serum markers associated with systemic inflammation (I–IV)

5.1.1 tKRT18, aKRT18, and nKRT18 (I)

Serum tKRT18 and nKRT18 levels were significantly increased in CRC patients with elevated mGPS (1–2) compared to patients with low mGPS (0) preoperatively (median 680.4 U/L vs. 464.8 U/L and 441.7 U/L vs. 284.9 U/L, respectively; $p < 0.001$ for both), and aKRT level showed a non-significant trend of association with mGPS ($p = 0.021$). The analysis was also performed separately for patients with local disease (stage I–III) and patients with distant metastasis (stage IV). In patients

with stage IV CRC, mGPS 1–2 significantly associated with high tKRT, high aKRT18, and high nKRT18 ($p < 0.001$ for all). In stage I–III CRC, none of the KRT18 forms showed statistically significant association with mGPS.

In univariate analysis, all KTR18 forms had a significant positive correlation with CRP, blood neutrophil count, and the levels of several cytokines. In multivariable linear regression analysis using the enter method (correlations adjusted for tumor stage variables, BMI, and preoperative RT/CRT), tKRT18 had a significant positive correlation with CRP ($\beta = 0.190$, $p = 0.001$), and aKRT18 and nKRT18 showed a non-significant trend of association. Of the cytokines, tKRT18, aKRT18 and nKRT18 correlated with IL6, CXCL8 and CXCL10; the strongest correlation was between tKRT18 and CXCL8 ($\beta = 0.522$, $p < 0.001$).

Based on the univariate analyses, the factors included in stepwise multiple linear regression models were invasion through muscularis propria, nodal metastasis, distant metastasis, mGPS, BMI, Klintrup-Mäkinen Score, and tumor necrosis. Of these factors, distant metastasis was the most prominent indicator of serum tKRT18 ($\beta = 0.250$, $p < 0.001$) and aKRT18 ($\beta = 0.363$, $p < 0.001$), whereas the main predictor of serum nKRT18 was mGPS ($\beta = 0.180$, $p = 0.002$). However, when IL6, CXCL8, and CXCL10 were included in the models, CXCL8 was the major determinant of all KRT18 forms and explained alone approximately 34% of the variation in tKRT18 levels, 27% in nKRT levels, and 26% in aKRT levels.

5.1.2 MMP8 (II)

Increased serum MMP8 level associated with higher mGPS ($p < 0.001$). The median MMP8 level was 59.2 ng/mL in patients with mGPS 0, 81.8 ng/mL in patients with mGPS 1, and 280.9 ng/mL in patients with mGPS 2.

In univariate analysis, serum MMP8 had strong positive correlations with CRP ($r = 0.324$), NLR ($r = 0.436$) and blood neutrophil count ($r = 0.467$) ($p < 0.001$ for all). In addition, MMP8 level correlated positively with the levels of several cytokines; the strongest correlations were with IL1RN ($r = 0.609$), IL7 ($r = 0.491$), and CXCL8 ($r = 0.550$) ($p < 0.001$ for all). MMP8 did not show any association with blood lymphocyte count or CCL11.

In multiple linear regression analysis, tumor stage variables, patient age, and patient gender were taken into account and enter method was used. The positive correlations between MMP8 and CRP ($\beta = 0.220$), NLR ($\beta = 0.379$), IL1RN ($\beta = 0.545$), IL7 ($\beta = 0.402$), and CXCL8 ($\beta = 0.433$) remained significant also in this analysis ($p < 0.001$ for all).

5.1.3 APOA1 (III)

Patients with mGPS 1–2 had lower APOA1 levels compared to those with mGPS 0 (median 1.22 g/L vs. 1.32 g/L, respectively, $p=0.001$).

In univariate analysis, serum APOA1 levels correlated inversely with several markers of systemic inflammation. The strongest correlations were seen between APOA1 and serum CRP ($r=-0.436$), blood neutrophil count ($r=-0.413$) and serum CXCL8 ($r=-0.425$) ($p<0.001$ for all). APOA1 concentration also correlated negatively with blood leukocyte count, blood monocyte count and serum IL1RN, IL6, IL7, CXCL10 and CCL4 levels. APOA1 level did not correlate with blood lymphocyte count, IL4, IFNG, CCL2 and CCL11. Multivariable linear regression analyses were not performed for APOA1.

5.1.4 Glutamine, histidine, and phenylalanine (IV)

Of the studied amino acids, glutamine and histidine levels were lower in patients with mGPS 1–2 (mean 445.0 $\mu\text{mol/L}$ and 49.5 $\mu\text{mol/L}$, respectively) compared to patients with mGPS 0 (mean 499.5 $\mu\text{mol/L}$ and 59.8 $\mu\text{mol/L}$, respectively) ($p<0.001$ for both). On the contrary, the levels of phenylalanine were higher in patients with mGPS 1–2 (mean 97.3 $\mu\text{mol/L}$) compared to patients with mGPS 0 (mean 81.3 $\mu\text{mol/L}$) ($p<0.001$). When the analyses were performed separately for patients with stage I–III and stage IV disease, significant associations between mGPS 1–2 and low histidine, low glutamine, and high phenylalanine level remained in both patient subgroups. The levels of alanine, glycine, isoleucine, leucine, tyrosine and valine did not show significant associations with mGPS.

Multiple linear regression analyses were performed using the enter method, and the correlations between amino acids and other continuous variables were adjusted for tumor location, tumor stage variables, and preoperative RT/CRT. In these analyses, glutamine level correlated negatively with serum CRP ($\beta=-0.260$, $p<0.001$), blood neutrophil count ($\beta=-0.171$, $p=0.003$), and CXCL8 ($\beta=-0.341$, $p<0.001$). Histidine level correlated inversely with serum CRP ($\beta=-0.388$, $p<0.001$), NLR ($\beta=-0.153$, $p=0.006$), IL6 ($\beta=-0.252$, $p=0.003$), and CXCL10 ($\beta=-0.261$, $p=0.002$). Phenylalanine level correlated positively with CRP ($\beta=0.406$, $p<0.001$), NLR ($\beta=0.191$, $p=0.001$), blood leukocyte count ($\beta=0.207$, $p<0.001$), and blood neutrophil count ($\beta=0.270$, $p<0.001$). Of the cytokines, positive correlations were observed between serum phenylalanine level and IL1RN, IL6, IL7, CXCL8, IL12 and CXCL10.

5.2 Serum KRT18 and the extent of tumor necrosis (I)

Tumor necrosis index, an estimate of the total area of necrotic tissue in the primary tumor assessed by the pathologist, had a significant positive association with tKRT18 ($r=0.264$, $p<0.001$), aKRT18 ($r=0.224$, $p<0.001$) and nKRT18 ($r=0.195$, $p=0.001$) in the univariate analysis. In multivariable analysis (correlations adjusted for tumor stage variables, BMI and preoperative RT/CRT), tumor necrosis index had a significant correlation with tKRT18 ($\beta=0.206$, $p=0.001$) and showed a non-significant trend of positive association with nKRT18 ($\beta=0.165$, $p=0.012$), but did not associate with aKRT18.

5.3 Serum MMP8 and tumor-infiltrating immune cells (II)

Tumor-infiltrating inflammatory cells were analyzed using tissue microarrays. In univariate analysis, MMP8 showed a negative correlation with CD3 T cells (invasive margin: $r=-0.162$, $p=0.008$), FoxP3 T cells (invasive margin: $r=-0.198$, $p=0.001$; tumor center: $r=-0.187$, $p=0.002$) and mast cells (invasive margin: $r=-0.245$, $p<0.001$; tumor center: $r=-0.179$, $p=0.003$), but did not correlate with the densities of CD8 T cells, CD68 macrophages, or neutrophils. In the multivariable analysis, only the correlation with mast cells remained significant (invasive margin: $\beta=-0.167$, $p=0.005$; tumor center: $\beta=-0.149$, $p=0.010$).

5.4 Correlation network (I–IV)

To illustrate the intercorrelations between the studied serum markers (KRT18, MMP8, APOA1, and amino acids), systemic inflammatory regulators (CRP, albumin and cytokines) and tumor necrosis index, a 2D visualization was created (Fig. 7). In this illustration, cytokines formed a cluster on the right, indicating strong intercorrelations, while branched-chain amino acids (valine, leucine, and isoleucine) formed a cluster on the left with alanine and tyrosine. tKRT18, aKRT18 and nKRT18 correlated negatively with APOA1 and positively with phenylalanine, and tKRT18 and aKRT18 correlated negatively with glutamine. APOA1 showed an inverse correlation with MMP8. Tumor necrosis index correlated positively with phenylalanine, CRP, MMP8, tKRT18, aKRT18, CXCL8, and IL6 levels, and negatively with APOA1 levels.

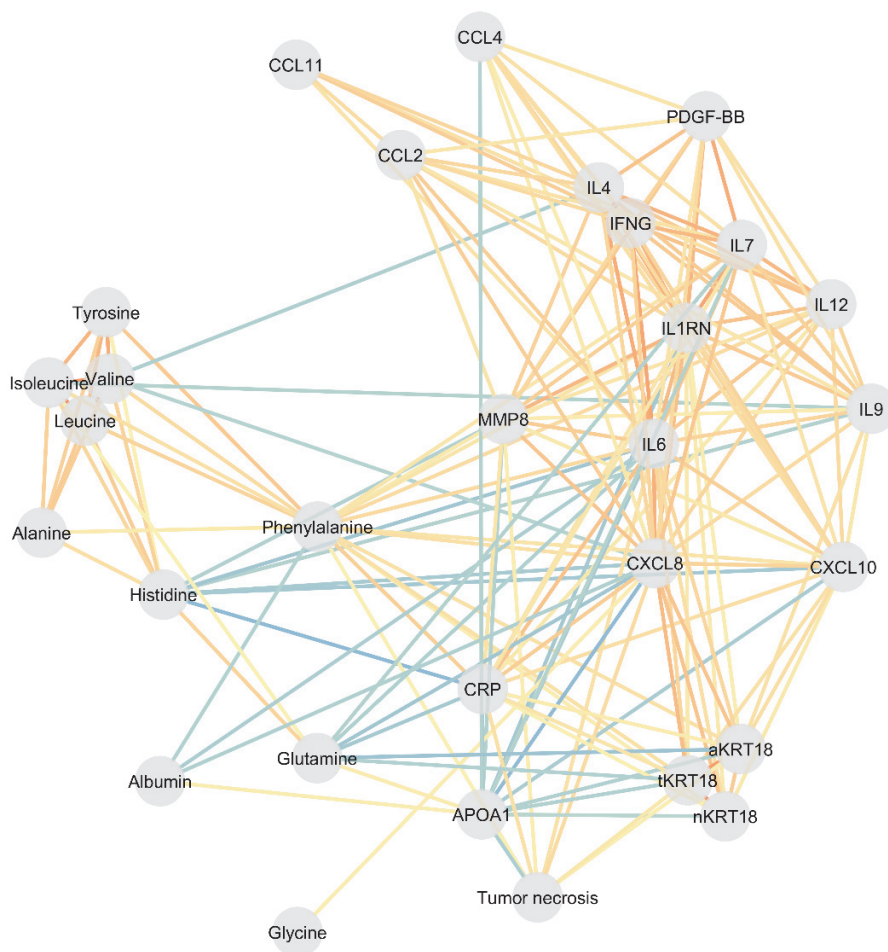


Fig. 7. Correlation network of the interrelationships between tumor necrosis index and serum levels of KRT18, MMP8, metabolites (APOA1 and amino acids), and the markers of systemic inflammation (CRP, albumin and cytokines). Individual variables are represented by nodes and their associations are represented by edges (connecting lines). Only the associations with $p < 0.01$ and Pearson $r > 0.200$ or < -0.200 are shown, and the edge length illustrates the significance of the association. Blue edges indicate negative correlation and orange edges positive correlation, with darker color indicating stronger correlation. Abbreviations: APOA1: Apolipoprotein A1, CCL: Chemokine (C-C motif) ligand, CRP: C-reactive protein, CXCL: Chemokine (C-X-C motif) ligand, IFN: interferon, IL: interleukin, aKRT18: keratin 18 released during apoptosis, nKRT18: keratin 18 released during necrosis, tKRT18: total keratin 18, MMP8: matrix metalloproteinase 8, PDGF: platelet-derived growth factor.

5.5 Serum tKRT18, nKRT18, MMP8, and APOA1 associate with survival independent of stage (I–IV)

In the survival analyses, serum levels of tKRT18, nKRT18, MMP8, and APOA1 had stage-independent prognostic value in the Cox regression model, whereas aKRT18 and amino acid levels did not independently associate with survival. Defined cut-off points based on the ROC analysis were: tKRT18 680 U/L, nKRT18 420 U/L, MMP8 100 ng/mL, and APOA1 1.235 g/L.

In the univariate Cox regression model, elevated serum levels of tKRT18 and nKRT18 correlated significantly with worse outcome. For patients with elevated and low serum tKRT18 level, the 10-year CSS rate was 63% and 84%, respectively ($p < 0.001$, Fig. 8A) and the 10-year OS rate was 48% and 74%, respectively ($p < 0.001$, Fig. 8B). For patients with elevated and low serum nKRT18 level, the 10-year CSS rate was 66% and 84%, respectively ($p < 0.001$, Fig. 8C) and the 10-year OS rate 51% and 74%, respectively ($p < 0.001$, Fig. 8D). tKRT18 and nKRT18 levels did not significantly associate with DFS. In multivariate analysis, both tKRT18 and nKRT18 levels were independent prognostic factors in their own models for OS (tKRT18: HR 1.19, 95% CI 1.19–2.89, $p = 0.007$; nKRT18: HR 1.80, 95% CI 1.16–2.78, $p = 0.008$), but not for CSS (tKRT18: $p = 0.061$; nKRT18: $p = 0.064$) (Tables 8 and 9).

Increased serum MMP8 level associated with decreased CSS and OS in the univariate analysis. For patients with elevated and low serum MMP8 level, the 10-year CSS rate was 60% and 80%, respectively ($p < 0.001$, Fig. 9A) and the 10-year OS rate was 50% and 73%, respectively ($p < 0.001$, Fig. 9B). In multivariate analysis, MMP8 was an independent prognostic factor for CSS (HR 2.16, 95% CI 1.24–3.78, $p = 0.007$) but not for OS ($p = 0.066$) (Table 10).

Elevated serum APOA1 level correlated with favorable prognosis. For patients with elevated and low serum APOA1 level, the 5-year CSS rate was 84% and 62%, respectively ($p < 0.001$, Fig. 9C) and the 5-year OS rate was 79% and 45%, respectively ($p < 0.001$, Fig. 9D). In the multivariate model, increased APOA1 level associated with better OS (HR 0.32, 95% CI 0.17–0.60, $p < 0.001$) and showed a non-significant trend of association with better CSS ($p = 0.012$) (Table 11).

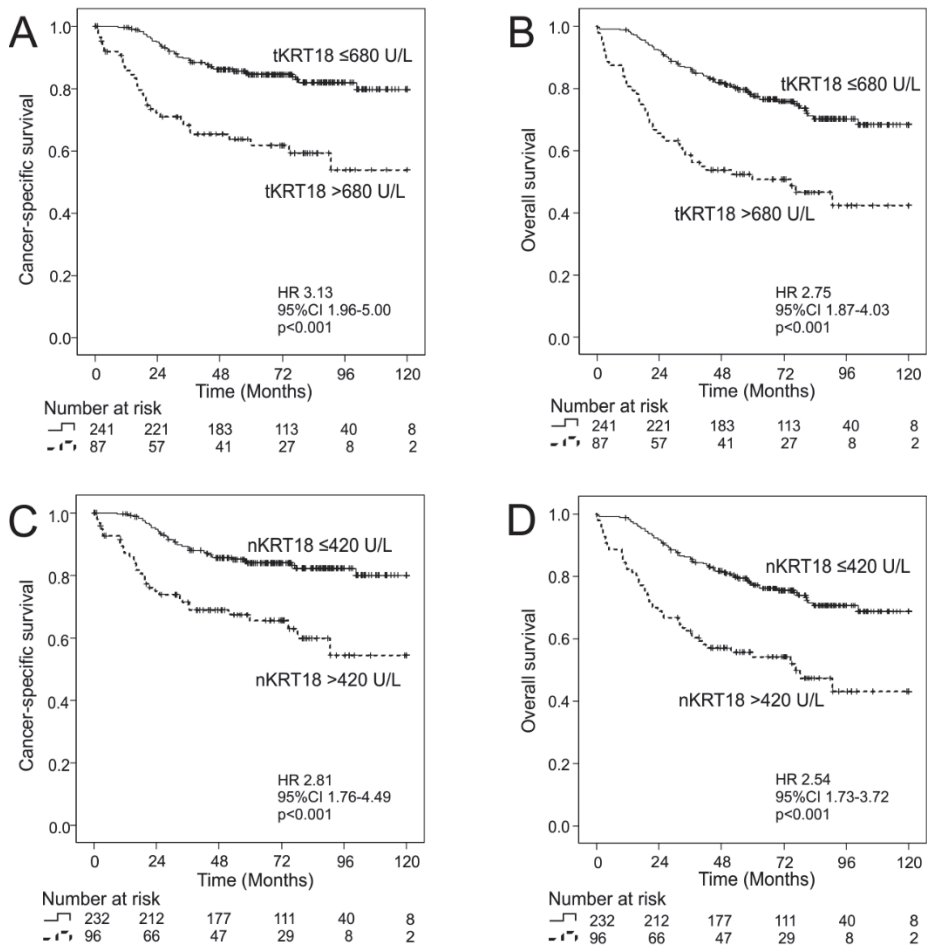


Fig. 8. Kaplan-Meier survival curves. (A) tKRT18 and CSS. (B) tKRT18 and OS. (C) nKRT18 and CSS. (D) nKRT18 and OS.

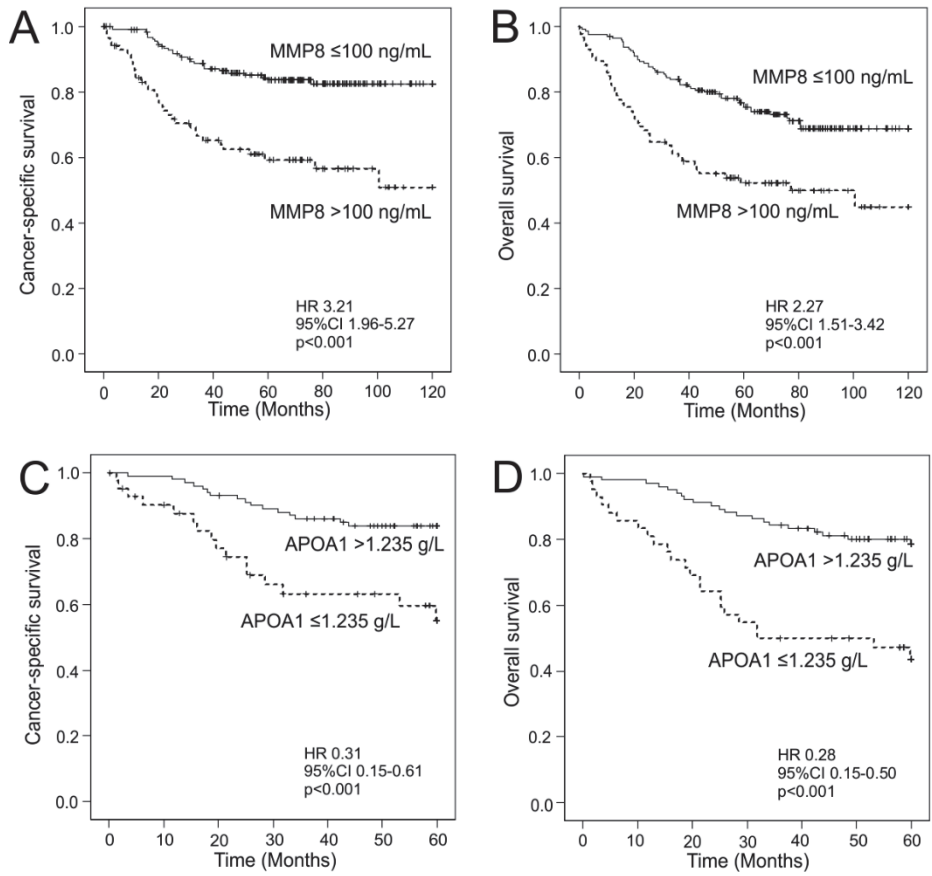


Fig. 9. Kaplan-Meier survival curves. (A) MMP8 and CSS. (B) MMP8 and OS. (C) APOA1 and CSS. (D) APOA1 and OS.

Table 8. Cox regression model with enter method for the independent prognostic significance of serum tKRT18 level.

Variables	CSS			OS		
	HR	95%CI	p value	HR	95%CI	p value
Age (<65 vs. ≥65)	1.83	1.08–3.11	0.024	1.99	1.28–3.08	0.002
Tumor invasion (T1–T2 vs. T3–T4)	1.00	0.50–2.03	0.994	0.97	0.58–1.63	0.914
Nodal metastases (N0 vs. N1–N2)	3.43	1.68–7.03	0.001	2.21	1.34–3.65	0.002
Distant metastases (M0 vs. M1)	5.74	3.08–10.68	3.5E-8	3.59	2.11–6.11	3.0E-6
Preoperative radiotherapy or chemoradiotherapy (No vs. Yes)	1.17	0.59–2.34	0.653	1.02	0.60–1.73	0.946
Lymphatic invasion (No vs. Yes)	2.10	1.06–4.15	0.034	1.45	0.89–2.37	0.135
mGPS (0 vs. 1-2)	1.26	0.70–2.28	0.448	1.37	0.85–2.18	0.194
Serum tKRT18 (≤680 U/L vs. >680 U/L)	1.74	0.98–3.11	0.061	1.19	1.19–2.89	0.007

Abbreviations: CSS: cancer-specific survival, OS: overall survival, CI: confidence interval, HR: hazard ratio, mGPS: modified Glasgow Prognostic Score, tKRT18: total keratin 18.

Table 9. Cox regression model with enter method for the independent prognostic significance of serum nKRT18 level.

Variables	CSS			OS		
	HR	95%CI	p value	HR	95%CI	p value
Age (<65 vs. ≥65)	1.82	1.07–3.08	0.026	1.97	1.27–3.05	0.002
Tumor invasion (T1–T2 vs. T3–T4)	1.02	0.50–2.05	0.964	1.00	0.60–1.68	0.996
Nodal metastases (N0 vs. N1–N2)	3.43	1.68–7.03	0.001	2.21	1.34–3.65	0.002
Distant metastases (M0 vs. M1)	5.80	3.12–10.78	<0.001	3.65	2.14–6.20	<0.001
Preoperative radiotherapy or chemoradiotherapy (No vs. Yes)	1.15	0.58–2.30	0.692	1.00	0.59–1.71	0.990
Lymphatic invasion (No vs. Yes)	2.12	1.07–4.19	0.032	1.46	0.90–2.38	0.126
mGPS (0 vs. 1–2)	1.28	0.71–2.30	0.417	1.39	0.87–2.21	0.171
Serum nKRT18 (≤420 U/L vs. >420 U/L)	1.71	0.97–3.01	0.064	1.80	1.16–2.78	0.008

Abbreviations: CSS: cancer-specific survival, OS: overall survival, CI: confidence interval, HR: hazard ratio, mGPS: modified Glasgow Prognostic Score, nKRT18: keratin 18 released during necrosis.

Table 10. Cox regression model with enter method for the independent prognostic significance of serum MMP8 level.

Variables	CSS			OS		
	HR	95%CI	p value	HR	95%CI	p value
Age (<65 vs. ≥65)	1.65	0.90–3.00	0.104	2.19	1.30–3.68	0.003
Tumor invasion (T1–T2 vs. T3–T4)	0.62	0.30–1.26	0.188	0.72	0.42–1.25	0.243
Nodal metastases (N0 vs. N1–N2)	4.30	1.97–9.35	2.4E-4	2.30	1.33–3.96	0.003
Distant metastases (M0 vs. M1)	7.09	3.72–13.51	2.6E-9	4.36	2.50–7.60	2.0E-7
Lymphatic invasion (No vs. Yes)	1.80	0.85–3.85	0.127	1.33	0.77–2.29	0.302
Grade (1–2 vs. 3)	2.07	1.32–3.24	0.002	1.76	1.22–2.52	0.002
mGPS (0 vs. 1–2)	0.93	0.50–1.75	0.832	1.27	0.76–2.11	0.362
Serum MMP8 (≤100 ng/mL vs. >100 ng/mL)	2.16	1.24–3.78	0.007	1.54	0.97–2.43	0.066

Abbreviations: CSS: cancer-specific survival, OS: overall survival, CI: confidence interval, HR: hazard ratio, mGPS: modified Glasgow Prognostic Score, MMP8: matrix metalloproteinase 8.

Table 11. Cox regression model with enter method for the independent prognostic significance of serum APOA1 level.

Variables	CSS			OS		
	HR	95%CI	P value	HR	95%CI	P value
Age (<65 vs. ≥65)	3.31	1.34–8.15	0.009	2.48	1.20–5.13	0.015
Tumor invasion (T1–T2 vs. T3–T4)	1.46	0.39–5.45	0.571	1.25	0.48–3.30	0.649
Nodal metastases (N0 vs. N1–N2)	4.94	2.02–12.12	<0.001	2.84	1.44–5.61	0.003
Distant metastases (M0 vs. M1)	4.73	1.81–12.32	0.001	3.00	1.33–6.74	0.008
Tumor location (colon vs. rectum)	2.01	0.77–5.29	0.157	1.64	0.73–3.67	0.232
Preoperative radiotherapy or chemoradiotherapy (No vs. Yes)	0.31	0.08–1.21	0.092	0.38	0.13–1.12	0.079
mGPS (0 vs. 1–2)	1.20	0.47–3.07	0.699	1.24	0.58–2.69	0.580
Serum APOA1 (≤1.235 vs. >1.235 g/l)	0.37	0.17–0.81	0.012	0.32	0.17–0.60	<0.001

Abbreviations: CSS: cancer-specific survival, OS: overall survival, CI: confidence interval, HR: hazard ratio, mGPS: modified Glasgow Prognostic Score, APOA1: apolipoprotein A1.

6 Discussion

Inflammation is an important element of normal responses to infection and injury. However, it can also damage normal tissues, and in situations where there are no pathogens, it may do more harm than good. Inflammation is associated with metabolic alterations that are beneficial in the short term, but when sustained, can lead to tissue breakdown. In cancer patients, systemic inflammation impairs the utilization of nutrients and promotes catabolism, leading to muscle wasting. Malnutrition is often seen in colorectal surgery patients due to poor appetite, bowel obstruction and malabsorption, and it negatively affects postoperative recovery and patient outcomes (Burden et al., 2010; Daniele et al., 2017; Gillis & Wischmeyer, 2019). Sarcopenia is also prevalent in CRC patients having elective surgery, and preoperative sarcopenia predicts worse DFS, CSS, and OS in resectable stage I–III CRC (Miyamoto et al., 2015).

In CRC patients, systemic inflammation is associated with muscle loss, impaired nutritional status, increased comorbidity, complications on treatment and worse survival (Richards et al., 2012b; McMillan, 2013). Even among patients with metastatic disease, elevated mGPS serves as a marker of worse prognosis (Køstner et al., 2016). Thus, it is important to understand better the mechanisms and metabolic consequences of cancer-related systemic inflammation.

6.1 Serum biomarkers of systemic inflammation beyond mGPS in colorectal cancer

6.1.1 *tKRT18*, *aKRT18*, and *nKRT18*

Systemic inflammation is activated in a subset of CRC patients. It is more common in patients with distant metastasis but is also present in some patients with local disease, and the trigger remains poorly understood. In study I, we investigated the potential relationship between cell death and systemic inflammation. Necrotic cells may release intracytoplasmic fragments and DAMPs to the circulation and stimulate inflammation (Zhang et al., 2010), and it can be hypothesized that cell injury has a role in eliciting systemic inflammation in CRC patients. Indeed, study I supports a link between cell death and systemic inflammation in CRC, especially in metastatic disease. Serum levels of cell death marker KRT18 were increased in patients with an elevated mGPS, and CXCL8 was identified as the most important

determinant of KRT18 levels. CXCL8 is a key mediator in inflammation, and neutrophils have been shown to synthesize and release CXCL8 in response to injury-released intracellular DAMPs (Zhang et al., 2010). On the other hand, cell injury may also be a consequence of systemic inflammation. In patients with systemic inflammation, many cells and tissues in addition to the tumor may suffer energy and nutrient deficiency, potentially leading to increased cell death and KRT18 release.

The origin of circulating KRT18 in CRC patients is unclear. Necrosis often occurs inside both primary tumors and metastases due to hypoxia and metabolic stress, which attracts inflammatory cells to tumor sites. In this study, we evaluated the extent of necrotic tumor tissue in the primary tumor, and used a rather imprecise estimate, tumor necrosis index, in the analyses. Tumor necrosis index had a significant positive association with all KRT18 forms in the univariate analysis. In multivariable analysis, it showed a statistically significant correlation with tKRT18 and a non-significant trend of association with nKRT18. Previously, extensive tumor necrosis in the primary tumor has been shown to associate with elevated mGPS and IL6 levels in CRC (Richards et al., 2012a; Guthrie et al., 2013b), and also in this study, tumor necrosis index associated with several markers of systemic inflammation (Fig. 7). Thus, these findings support a possible role of necrosis in the primary tumor releasing KRT18 into the blood of CRC patients and participating in the activation of systemic inflammation.

However, in our subgroup analyses KRT18 levels correlated significantly with mGPS only in stage IV CRC, but not in stage I–III CRC. Distant metastasis was a major predictor of KRT18 levels, and metastasis may be an important source of circulating KRT18. It has also previously been shown that tKRT18 and aKRT18 levels are more elevated in patients with stage IV CRC compared to stage I–III CRC (Greystoke et al., 2012). Metastatic spread may result in cell damage both in the metastasis and at the metastatic site. The liver is the most common site of metastasis in CRC patients and a potential source of blood KRT18 fragments. Hepatocytes express KRT18, and circulating KRT18 levels have been shown to increase in several liver diseases (Strnad et al., 2008; Feldstein et al., 2009; Li et al., 2010; Joka et al., 2012).

A minority of CRC patients have evidence of systemic inflammation before potentially curative surgery, but in some of those who have it, the systemic inflammatory response does not resolve after surgery (McMillan et al., 2003; Guthrie et al., 2013a). The mechanism underlying the persistent inflammation remains unclear. It has been suggested that in these patients, the continuing

presence of systemic inflammation could reflect micrometastatic disease or non-malignant disease invoking inflammation and/or tissue injury/necrosis (Guthrie et al., 2013a). Interestingly, also tKRT18 levels remain elevated or even increase after removal of the tumor in some CRC patients, which was suggested to represent remaining tumor cell dissemination (Ausch et al., 2009). In the present studies, only preoperative samples were analyzed, and it is unknown whether systemic inflammation and/or elevated KRT18 levels persisted in these patients after surgery.

In healthy individuals, there seems to be a wide range in blood tKRT18 and aKRT18 levels (Scott et al., 2009), but based on Swedish blood donors the reference value for tKRT18 is ≤ 450 U/L and for aKRT18 ≤ 150 U/L (M30Apoptosense[®] and M65[®] ELISA kit instructions, Peviva AB). According to these cut-off values, 57% of CRC patients in this study cohort had elevated tKRT18 levels and 70% had elevated aKRT18 levels. Thus, many CRC patients, and especially those with systemic inflammation, seem to have significantly increased circulating KRT18 levels. Further studies are required to investigate the mechanisms and origin of KRT18 release and the clinical implications of this finding.

6.1.2 MMP8

In study II, elevated serum MMP8 levels were observed in CRC patients with systemic inflammation. MMP8 is mainly expressed by neutrophils, but the source of circulating MMP8 in CRC is undetermined. MMP8 level did not correlate with the density of tumor-infiltrating neutrophils in this study, and it has previously been shown that only few CRC cells express MMP8 (Väyrynen et al., 2012b). On the contrary, blood neutrophil count strongly correlates with serum MMP8 levels (Väyrynen et al., 2012b), suggesting that circulating neutrophils are the main source of serum MMP8 in CRC. In this study, MMP8 level also showed a strong correlation with CXCL8, which is an important chemoattractant for neutrophils.

Mitochondrial DAMPs have been shown to induce MMP8 release from neutrophils (Zhang et al., 2010). Thus, a potential mechanism of MMP8 elevation during systemic inflammation is that injury-released DAMPs elicit systemic inflammation and lead to neutrophil activation and MMP8 secretion. MMP8 participates in regulating inflammatory reactions by cleaving and activating inflammatory mediators (Cox et al., 2010; Solan et al., 2012). In concordance with the results of this study, increased MMP8 levels have previously been reported in several chronic inflammatory conditions like sepsis (Solan et al., 2012), rheumatoid

arthritis (Tchetverikov et al., 2004), periodontal disease (Marcaccini et al., 2009), and metabolic syndrome (Gonçalves et al., 2009). However, MMP8 has also been reported to harbor anti-inflammatory actions (Van Lint & Libert, 2006), and its functions probably vary between different tissues, cell types and disease processes. Interestingly, MMP8 may also have a role in regulating metabolic alterations, as it has been shown to cleave insulin receptor and APOA1 *in vitro* (Salminen et al., 2015; Lauhio et al., 2016). In this study cohort, MMP8 level correlated inversely with APOA1 level (Fig. 7).

6.1.3 APOA1

Serum APOA1 levels associated negatively with systemic inflammation in CRC patients. Previously, abnormal levels of lipids have been linked with cancer risk and progression in several malignancies (Borgquist et al., 2016). In addition, decreased serum APOA1 levels have been reported in CRC patients (Engwegen et al., 2006; Peltier et al., 2016) and low HDL levels in inflammatory diseases like inflammatory bowel disease and rheumatoid arthritis (Sappati Biyyani et al., 2010; Liao et al., 2015).

Potential mechanisms leading to low circulating APOA1 levels are decreased synthesis of APOA1 and/or increased APOA1/HDL clearance and catabolism. APOA1 is expressed mainly by the intestine and the liver, and in response to cytokines, its expression in a human liver cancer cell line has been shown to decrease (Haas et al., 2003). Moreover, inflammation can induce HDL composition changes and structure modifications conferring pro-inflammatory capacity to HDL particles (Khovidhunkit et al., 2004).

The clinical consequences of these lowered APOA1 levels are unidentified, but APOA1 is known to possess important anti-inflammatory, anti-apoptotic and antioxidant properties (Zamanian-Daryoush & DiDonato, 2015) that may be disturbed. The mechanism by which APOA1 exerts its anti-inflammatory effects in macrophages, endothelial cells and adipocytes appears to be via cholesterol depletion of lipid rafts on cell membranes via ABCA1 (Tang et al., 2009; Umemoto et al., 2013). Disruption of the lipid rafts attenuates proinflammatory signaling, as many receptors with key immunological functions are localized within these structures (Jahangiri, 2010). Moreover, APOA1 can modulate the function of immune cells by several mechanisms, such as attenuating their activation (Murphy et al., 2011), chemotaxis (Iqbal et al., 2016), and chemokine expression (Bursill et al., 2010).

6.1.4 Glutamine, histidine and phenylalanine

According to the results of study IV, the activation of systemic inflammation in CRC is associated with changes in serum levels of glutamine, histidine and phenylalanine. The alterations in circulating amino acid concentrations derive from changes in their input and/or use. Histidine and phenylalanine are essential amino acids in humans, and glutamine is conditionally essential. Considering that essential amino acids cannot be synthesized *de novo* in the human body, their pool is supplied by dietary proteins and degradation of tissue proteins. The amino acid pool is depleted by synthesis of new proteins, synthesis of nitrogen containing compounds, and conversion to glucose, glycogen, fatty acids, ketone bodies or $\text{CO}_2 + \text{H}_2\text{O}$.

Patients with high mGPS had decreased serum levels of glutamine, which may reflect increased use of glutamine during systemic inflammation. The demand of glutamine is known to increase during catabolic states like infection, injury and cancer (Fürst et al., 1989; Labow & Souba, 2000). In these situations it is mainly released from the skeletal muscle and used by rapidly dividing cells such as enterocytes, immune cells and fibroblasts (Labow & Souba, 2000). In cancer cachexia, reduced glutamine levels may reflect increased demand of glutamine by the tumor or by the immune system (Parry-Billings et al., 1991). The liver may also increase its glutamine utilization in response to proinflammatory cytokines (Fischer et al., 1995).

Glutamine is an important nutrient as it can donate its nitrogen and carbon into macromolecular synthesis, energy formation, and signaling (Reitzer et al., 1979; Hensley et al., 2013). Normally, most of the consumption of glutamine occurs in the gut and kidney, and glutamine plays an important role in maintaining intestinal barrier function (Hensley et al., 2013; Wang et al., 2015). In cancer cells, glutaminolysis is a key element for energy production besides glycolysis, and it has been suggested that in cancer-associated wasting, glutamine is mainly used by the tumor (Kuhn et al., 2010; Hensley et al., 2013; Choi & Park, 2018). However, according to the results of this study, immune system activation and immune cells may be important consumers of glutamine in CRC patients with systemic inflammation.

Serum phenylalanine levels were elevated in patients with high mGPS. The mechanism of this elevation is unknown but might be related to oxidative stress resulting from inflammation. Normally, most of phenylalanine is converted to tyrosine by phenylalanine hydroxylase (PAH) in the liver. PAH activity, which can

be estimated from the phenylalanine to tyrosine ratio, is downregulated in situations of oxidative stress, such as chronic inflammation (Fuchs et al., 2012). PAH needs the cofactor 5,6,7,8-tetrahydrobiopterin, which is highly sensitive to oxidation (Dumitrescu et al., 2007). Comparable to our results, previous studies have reported increased blood concentrations of phenylalanine or phenylalanine to tyrosine ratio in inflammatory conditions like trauma (Ploder et al., 2008), sepsis (Rath et al., 1987), and HIV-infection (Ollenschläger et al., 1988). In ovarian cancer, serum phenylalanine levels correlate positively with immune activation markers (Neurauter et al., 2008). Moreover, increased phenylalanine level in both skeletal muscle and serum has been shown to correlate with body mass loss in tumor-bearing mice (Lautaoja et al., 2019). The clinical consequences of increased phenylalanine levels in CRC patients are not clear.

Serum histidine levels were decreased in CRC patients with elevated mGPS. Histidine is known to exhibit antioxidant and anti-inflammatory effects as it can scavenge toxic oxygen species (Cai et al., 1995) and suppress pro-inflammatory cytokine production in different cell types (Son et al., 2005; Andou et al., 2009). Previously, lower histidine levels compared to healthy controls have been reported in the serum and urine of patients with stage I–IV CRC (Qiu et al., 2010; Leichtle et al., 2012; Tan et al., 2013). Low serum histidine levels have also been reported in patients with rheumatoid arthritis, a chronic inflammatory disease (Sitton et al., 1986). In patients with chronic kidney disease, plasma histidine deficiency has been shown to associate with inflammation and protein-energy wasting (Watanabe et al., 2008).

Histidine is decarboxylated to histamine by the enzyme histidine decarboxylase (HDC). Histamine regulates multiple physiological and pathophysiological processes, including allergic reactions, gastric acid secretion, neurotransmission, and immune responses. In the gut, mast cell-derived histamine may contribute to increased intestinal permeability that has been described in many inflammatory diseases (Bischoff, 2009; Fukui, 2016; Potts et al., 2016). Histamine can also decrease appetite and stimulate lipolysis (Sakata et al., 1997; Tsuda et al., 2002). Mast cells and basophils are the main cells that synthesize and store histamine, but HDC can also be induced in other types of cells in response to cytokines and lipopolysaccharides (Endo et al., 1986; Gutowska-Owsiak et al., 2014). In CRC, increased HDC activity and histamine content in the neoplastic tissue have been reported (Cianchi et al., 2005; Kennedy et al., 2012). Thus, increased HDC activity and consumption of histidine in histamine synthesis during systemic inflammation could contribute to the observed low serum histidine levels

in this study. Although only four patients had histidine levels outside the laboratory reference range, and even though this finding probably has no clinical relevance, histidine and histamine have many functions that support their possible role in cancer-related malnutrition, which should be addressed by further studies.

6.2 Serum biomarkers with prognostic value in colorectal cancer

Currently, the prognostic classification of CRC is largely based on TNM staging. However, additional prognostic markers would be valuable, and the markers of inflammation can be useful. In these studies, we found several prognostic markers that maintain association with clinical outcome after accounting for standard prognostic variables including stage, patient age, and mGPS.

6.2.1 *tKRT18* and *nKRT18*

Study I indicated that high serum levels of *tKRT18* and *nKRT18* associate with worse survival. This finding is consistent with two previous studies that have shown an association between high plasma *tKRT18* and *aKRT18* levels and worse prognosis in both early and advanced CRC (Koelink et al., 2009, Greystoke et al., 2012). It has also been reported that high blood *aKRT18* levels predict worse survival in lung cancer and advanced gastric carcinoma (Ulukaya et al., 2007; Yaman et al., 2010), and high *tKRT18* levels associate with worse progression-free survival in advanced non-small-cell lung cancer (Oven Ustaalioglu et al., 2012). Previous studies have not included *nKRT18* in their analyses. In the future, the prognostic value of different *KRT18* fragments should be more extensively investigated in CRC in different subgroups as well as in other epithelial cancers.

6.2.2 *MMP8*

In study II, high serum *MMP8* level associated with adverse survival. Previous studies have shown that high circulating levels of several *MMPs*, including *MMP7*, *MMP10*, *MMP11* and *MMP12*, associate with poor prognosis in CRC (Maurel et al., 2007; Kushlinskii et al., 2013; Klupp et al., 2016; Pang et al., 2016), but the prognostic significance of *MMP8* level has been unknown. Shortly after study II was published, also another study reported the association between high serum *MMP8* level and poor prognosis in CRC (Böckelman et al., 2018). Further studies are warranted to confirm the prognostic value of serum *MMP8* level in CRC.

6.2.3 APOA1

In study III, elevated serum APOA1 levels associated with better prognosis. Similar findings have been reported in various malignancies in both local and metastatic disease (Table 12). However, our study is the only one conducted with a European cohort; in all other studies the participants were mostly Chinese. In metastatic CRC, low APOA1 has been shown to significantly associate with inferior OS (Quan et al., 2017). Moreover, it has been shown that CRC patients with increased APOA1 levels after chemotherapy have better DFS and OS than those without (Wang et al., 2016b). In this study, the ROC analysis indicated 1.235 g/L as an optimal cut-off value for APOA1 for discriminating survivors from non-survivors, which is compatible with previously reported cut-off points (0.95-1.56 g/L). Our cut-off point is also very close to the reference value published by Contois et al. (1996), who found that APOA1 levels <1.20 g/L were associated with increased risk for coronary heart disease. However, in this study APOA1 showed stronger association with OS than CSS. APOA1 has a protective role in cardiovascular diseases, the leading cause of death in Finland (Official Statistics of Finland 2017), which may partly explain the positive association between APOA1 level and OS. Altogether, the results suggest that APOA1 is a potential prognostic marker of CRC, but more large-scale studies with different populations are needed to validate the results and determine the optimal cut-off point.

Table 12. Prognostic significance of circulating apolipoprotein A1 in cancer.

Cancer	N	Cut-off value	OS	CSS	DFS	Independent prognostic value	Reference
Gastric cancer (stage I–IV)	1201	1.4 mM	+			No	Ma et al. 2018
Breast cancer without distant metastases	1044	1.56 g/L	0		0	No	Li et al. 2017
Invasive ductal breast cancer	299	1.12 g/L	+		+	Yes	Lin et al. 2017
Esophageal squamous cell carcinoma (stage I–IV)	210	1.21 g/L	+			Yes	Wang et al. 2016a
Locally advanced cervical squamous cell carcinoma with concurrent CRT	331	1.2 g/L	+		+	Yes	Chang et al. 2018a
Surgical renal cell carcinoma (stage I–IV)	786	1.04 g/L	+		+	Yes	Guo et al. 2016
Nasopharyngeal carcinoma (stage I–IVB)	1927	1.125 mmol/L	+		+	Yes	Chang et al. 2018b
Non-metastatic nasopharyngeal carcinoma	1196	1.025 g/L			+	Yes	Luo et al. 2015
Metastatic nasopharyngeal carcinoma	807	1.065 g/L	+			Yes	Jiang et al. 2014
Non-muscle-invasive bladder cancer	470	1.19 g/L	+	+		Yes	Shang et al. 2018
Hepatocellular carcinoma patients undergoing curative resection	433	1.04 g/L	+		+	Yes	Ma et al. 2016
Metastatic colorectal cancer	508	1.105 g/L	+			Yes	Quan et al. 2017
Extranodal natural killer/T-cell lymphoma, nasal type	236	0.95 g/L	+			Yes	Quan et al. 2016
Non-small cell lung cancer (stage I–IV) with chronic hepatitis B viral infection	141	1.17 g/L	+			Not evaluated	Chen et al. 2018

Abbreviations: OS: overall survival; CSS: cancer-specific survival; DFS: disease-free survival; CRT: chemoradiotherapy.

6.3 Potential therapeutic agents

As MMPs are involved in tumor invasion, angiogenesis and metastasis, they represent a promising target for cancer therapy (Winer et al., 2018). However, the development of MMP-inhibitors has been full of challenges and the results of human clinical trials have been disappointing (Coussens et al., 2002). The initial trials were improperly designed and used broad-spectrum inhibitors that were ineffective and had severe side effects (Coussens et al., 2002). More specific inhibitors are now available, and MMP targeting remains desirable. A MMP8-specific inhibitor has not been tested in humans or in clinical trials, but in septic rodents it has been shown to mitigate myocardial injury and suppress neuroinflammation (Lee et al., 2014; Zhou et al., 2014).

APOA1 mimetic peptides (D-4F, FX-5A) are potential therapeutic agents that were designed to mimic the anti-inflammatory and antioxidant functionalities of APOA1 (Reddy et al., 2014). They do not have sequence homology to APOA1, but are similar in their lipid associating structural motifs (Anantharamaiah et al., 2007). APOA1 mimetics have been shown to attenuate atherosclerosis, intestinal inflammation and tumor growth in mouse models (Su et al., 2012; Cedó et al., 2016; Meriwether et al., 2019). In a human randomized controlled trial, D-4F was well tolerated and lowered the HDL inflammatory index (Dunbar et al., 2017), but its use in clinical trials after that has not been reported.

Another group of drugs that could mediate the beneficial effects of APOA1 are statins. Statins are inhibitors of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase and they lower the levels of cholesterol. In addition, they possess various other effects such as improving endothelial function, decreasing oxidative stress and inflammation, and preventing platelet aggregation (Liao & Laufs, 2005). Statins can increase APOA1 levels (Yeh et al., 2016; Khera et al., 2017) and reduce CRP levels (Albert et al., 2001). Several studies have shown that statin therapy associates with slightly improved survival in CRC (Gray et al., 2016; Lash et al., 2017; Voorneveld et al., 2017), although it has also been suggested that the observed association has been due to selection bias and immortal-time bias (Emilsson et al., 2018). The mechanisms responsible for the antitumor effects of statins are not well understood. Proposed mechanisms include reduction in cholesterol levels (Nielsen et al., 2012) and direct effects against cancer cells, such as induction of apoptosis and inhibition of cell proliferation (Rao et al., 1998; Kodach et al., 2007). The results of this study suggest that the beneficial activity of statins on cancer could at least partly be mediated through the reduction of systemic

inflammation. Although the anti-inflammatory properties render statins an interesting candidate for the treatment of systemic inflammation and cancer-associated malnutrition, there are no reports on malnourished cancer patients treated with statins, and the few studies using animal models of cancer cachexia have yielded conflicting results (Muscaritoli et al., 2003; Palus et al., 2013).

In study IV, CRC patients with systemic inflammation were found to have lowered serum glutamine levels, suggesting that these patients could benefit from glutamine supplementation. Indeed, it has been reported that glutamine intake among CRC patients may reduce some side effects of chemotherapy (Daniele et al., 2001; Wang et al., 2007) and improve nitrogen balance, immune system and wound healing after surgery (Jolfaie et al., 2015). A review by Kuhn and coworkers reported that in various clinical studies, glutamine supplementation in cancer patients improves host metabolism and clinical status without increasing tumor growth (Kuhn et al., 2010). However, not all studies have shown significant benefit (Bozzetti et al., 1997; Pan et al., 2005), and there is still a concern that glutamine intake might stimulate tumor growth (Kuhn et al., 2010). According to the ESPEN guidelines on nutrition in cancer patients, the role of glutamine supplementation remains controversial (Arends et al., 2017a).

Study IV also indicates that patients with systemic inflammation have decreased serum histidine levels, but the possible effects of histidine supplementation are undetermined. In obese women with metabolic syndrome, histidine supplementation has been reported to improve insulin resistance through suppressed inflammation (Feng et al., 2013), whereas in patients with rheumatoid arthritis no advantage from histidine treatment was shown (Pinals et al., 1977).

6.4 Limitations

There are some possible limitations concerning the collection and use of serum samples. Both plasma and serum are commonly used matrices in clinical and biological studies, but different collecting procedures and the clotting process influence the levels of proteins and metabolites in these matrices (Yu et al., 2011). Previous studies have indicated that serum samples may have higher MMP levels than plasma samples due to molecules generated during coagulation (Zhang et al., 2010; Jonsson et al., 2016), and plasma is the preferred medium to measure analytes that are released or consumed during the clotting process (Webb et al., 1998). However, we did not have plasma samples, and serum MMP8 levels have been shown to correlate positively with plasma MMP8 levels in the immunofluorometric

assay that was used in study II (Tuomainen et al., 2008). M30 and M65 assays have been shown to be more reliable in serum compared with plasma (Greystoke et al., 2008), and the reproducibility of metabolite measurements has been reported to be good in both plasma and serum (Yu et al., 2011). Moreover, blood collection tube components and the processing of samples may affect the results (Bowen & Remaley, 2014).

These studies are mostly based on unselected CRC patient material of stages I–IV, which is a major limitation. Further studies are required to evaluate the prognostic performance of these serum markers in more strictly defined and therapeutically relevant subgroups such as stage I–III and stage IV patients separately. Other limitations are the combination of colon and rectal cancer into one entity and excluding patients who received RT/CRT in study II. Moreover, one possible limitation is that some tumors were classified according to TNM6 and others to TNM7 in studies II and III. However, the changes from TNM6 to TNM7 have been rather small.

These studies are limited by their observational nature, as observational studies are vulnerable to influences by confounding factors. To reduce the effects of potential confounders, multiple linear regression and multivariate survival analyses were performed. Moreover, the observed correlations between variables do not indicate causation. Still, observational studies are important as they can help to inform about probable cause and effect associations and guide further research.

In these studies, data on some important patient characteristics and prognostic factors, such as adjuvant therapy, are lacking. For patients with stage IV CRC, information on the location of metastases was not collected. Moreover, no information regarding patients' diet or lifestyle was available, and the fasting status of the patients could not be confirmed. Although diet could affect serum metabolite levels, it has been shown that APOA1 concentrations are not affected by fasting/non-fasting status (Nordestgaard et al., 2016). Furthermore, no information about the patients' weight loss was available, and the presence of sarcopenia was not evaluated. Previously, CRP >10 mg/L and coinciding albumin <30 g/L have been used to determine the presence of "laboratory cachexia" (Gray & Axelsson, 2018), and elevated mGPS has been suggested to indicate the presence of precachexia, a potential early stage of cachexia (MacDonald, 2012). However, there are many factors and conditions in addition to cancer-related systemic inflammation that can affect CRP and albumin levels. Persistent elevations in CRP are seen in chronic inflammatory diseases like rheumatoid arthritis, but in those situations CRP level is generally less than 10 mg/dL. Most patients with marked

elevation (>10 mg/dL) have bacterial infections or have suffered major trauma (Fors Nieves et al., 2017). Hepatic synthesis of albumin is primarily affected by osmotic colloid pressure and inflammatory states, but also by nutritional status and hormones (Nazha et al., 2015). Hypoalbuminemia may also reflect increased catabolic rate of albumin or its loss into the urine or intestine (Levitt & Levitt, 2016).

Studies I–III suggest that measuring blood levels of tKRT18, nKRT18, MMP8, and APOA1 in CRC patients can provide valuable prognostic information. REMARK guidelines (REporting recommendations for tumor MARKer prognostic studies) have been developed to encourage investigators to report prognostic marker research accurately (McShane et al., 2005), and the REMARK guidelines were taken into account in these studies. However, according to the guidelines, the ideal approach is to confirm findings on completely independent data, and one limitation is that we did not have a validation cohort.

We analyzed biomarkers as continuous variables in all tests except survival analyses. Markers are often dichotomized to simplify the analysis and to make it easier for clinicians to use marker information in decision making (Sauerbrei et al., 2018). However, it has been argued that categorization of continuous data is unnecessary for statistical analysis, and especially, should not be applied to explanatory variables in multiple regression, as it may lead to loss of power, residual confounding and bias (Royston et al., 2006).

The present studies are also limited by multiple hypothesis testing, which increases the probability of finding a difference just by chance. However, these studies are mostly exploratory and hypothesis generating, and further research is needed to confirm the results.

7 Conclusions

This study found serum markers of systemic inflammation beyond mGPS in CRC patients and evaluated the prognostic significance of these determinants. Figure 10 summarizes the main findings.

CRC patients with systemic inflammation	Cell death-related KRT18 fragments ↑*
	MMP8 ↑*
	APOA1 ↓*
	Glutamine ↓
	Histidine ↓
	Phenylalanine ↑
*Predicts worse survival	

Fig. 10. Summary of the results.

Based on the results, the following conclusions were made:

1. During systemic inflammation in CRC, serum levels of MMP8 and phenylalanine are increased. On the contrary, serum levels of APOA1, glutamine and histidine are decreased. Elevated serum KRT18 levels associate with systemic inflammation, especially in stage IV CRC, suggesting a link between cell death and systemic inflammation in CRC. Further investigation is required to confirm these findings and to clarify the mechanisms, possible cause-effect relationships and clinical significance of these alterations.
2. Tumor necrosis index, a measure of the extent of necrotic tissue in the primary tumor, correlates with high serum KRT18 level. However, serum CXCL8 level and distant metastasis are more important determinants of KRT18 levels than the tumor necrosis index. The tissues releasing KRT18 to the circulation of CRC patients remain to be determined.
3. Serum MMP8 levels do not correlate with local immune cell infiltrates in primary CRC tumors, suggesting that other cells than tumor-infiltrating immune cells are the main source of circulating MMP8 in CRC patients.
4. High serum concentrations of tKTR18, nKRT18 and MMP8, and low serum levels of APOA1 are independently associated with impaired survival in CRC. Further studies are required to evaluate the prognostic performance of these markers in relevant subgroups such as stage I–III and stage IV patients separately.

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