Identification and characterization of biomarkers reflecting extracellular matrix remodeling in cancer

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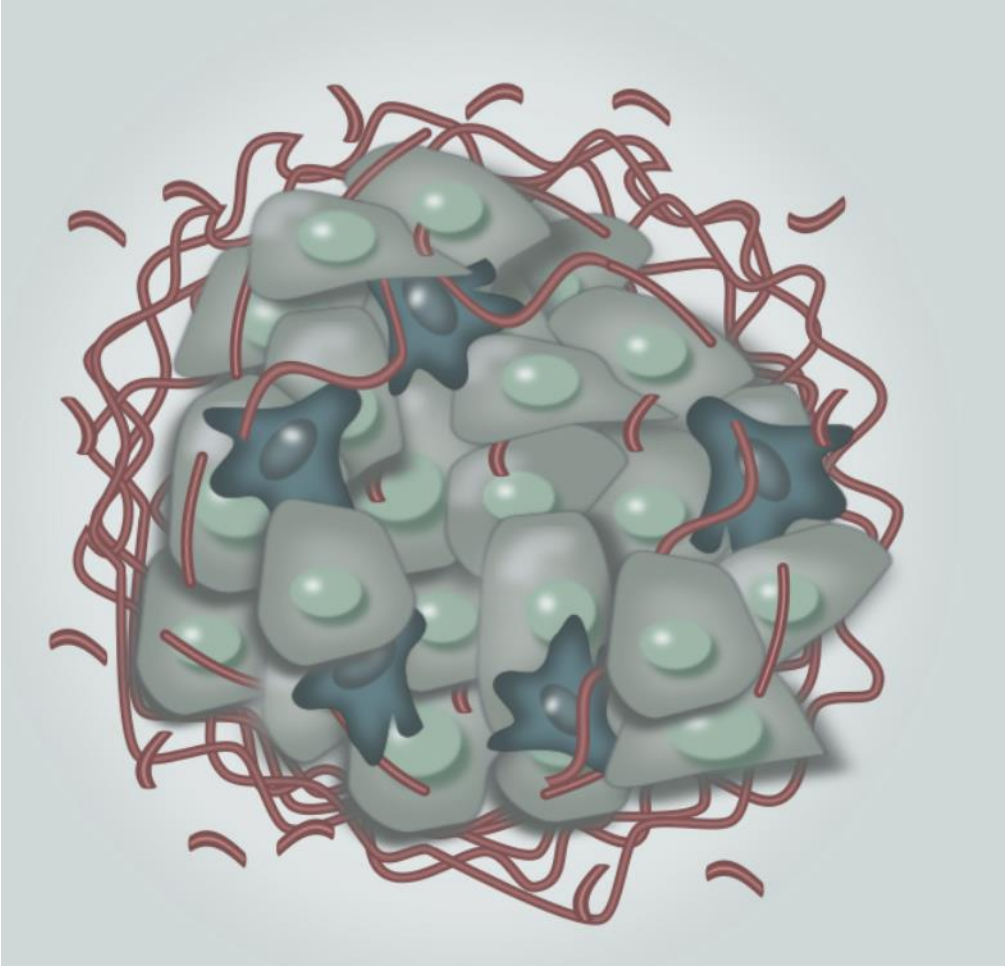
Using the tumor microenvironment as a source of protein biomarkers



PhD Thesis

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Stephanie Kehlet
September 2018

Preface

The work presented in this thesis was carried out as a collaboration between The Technical University of Denmark and Nordic Bioscience. The work was initiated in December 2014 and finalized in September 2018, including a nine month's maternal leave. The thesis is based on four original research papers and two unpublished studies (additional results 1 and 2).

Published papers

Paper I — **Age-related collagen turnover of the interstitial matrix and basement membrane: Implications of age- and sex-dependent remodeling of the extracellular matrix.** Stephanie N. Kehlet, Nicholas Willumsen, Gabriele Armbrecht, Roswitha Dietzel, Susanne Brix, Kim Henriksen and Morten A. Karsdal. *PLoS One. 2018 Mar 29;13(3).*

Paper II — **Excessive collagen turnover products are released during colorectal cancer progression and elevated in serum from metastatic colorectal cancer patients.** Stephanie N. Kehlet, Rebeca Sanz-Pamplona, Susanne Brix, Diana J. Leeming, Morten A. Karsdal and Victor Moreno. *Sci Rep. 2016 Jul 28;6:30599.*

Paper III — **Cathepsin-S degraded decorin are elevated in fibrotic lung disorders – development and biological validation of a new serum biomarker**. Stephanie N. Kehlet, Cecilie L. Bager, Nicholas Willumsen, Bidisha Dasgupta, Carrie Brodmerkel, Mark Curran, Susanne Brix, Diana J. Leeming and Morten A. Karsdal. *BMC Pulm Med. 2017 Aug 9;17(1):110.*

Paper IV — **A fragment of SPARC reflecting increased collagen affinity shows pathological relevance in lung cancer – implications of a new collagen chaperone function of SPARC.** Stephanie N. Kehlet, Tina Manon-Jensen, Shu Sun, Susanne Brix, Diana J. Leeming, Morten A. Karsdal and Nicholas Willumsen. *Cancer Biol Ther. 2018 Aug 1:1-9.*

The research in paper IV has contributed to one patent: SPARC assay - UK Patent Application No. 1721308.3

Unpublished studies

Additional results 1 — **Prognostic evaluation of a new class of liquid biopsy biomarkers reflecting type III and VI collagen formation in patients with metastatic colorectal cancer.** Stephanie N. Kehlet, Anette Høye, Mogens K. Boisen, Julia S. Johansen, Morten A. Karsdal, Nicholas Willumsen and Janine Erler

Additional results 2 — **Validation of a novel serum immunoassay targeting the pro-peptide of type XI collagen.** Stephanie N. Kehlet, Tina Manon-Jensen, Shu Sun, Susanne Brix, Morten A. Karsdal, Nicholas Willumsen and Yi He

Co-authored papers

Remodeling of the Tumor Microenvironment Predicts Increased Risk of Cancer in Postmenopausal Women: The Prospective Epidemiologic Risk Factor (PERF I) Study. Bager CL, Willumsen N, Kehlet SN, Hansen HB, Bay-Jensen AC, Leeming DJ, Dragsbæk K, Neergaard JS, Christiansen C, Høgdall E, Karsdal M.
Cancer Epidemiol Biomarkers Prev. 2016 Sep;25(9):1348-55

Matrix Metalloproteinase Mediated Type I Collagen Degradation - An Independent Risk Factor for Mortality in Women. Dragsbæk K, Neergaard JS, Hansen HB, Byrjalsen I, Alexandersen P, Kehlet SN, Bay-Jensen AC, Christiansen C, Karsdal MA.
EBioMedicine. 2015 Apr 30;2(7):723-9

Cohort Profile: The Prospective Epidemiological Risk Factor (PERF) study. Neergaard JS, Dragsbæk K, Kehlet SN, Hansen HB, Hansen G, Byrjalsen I, Alexandersen P, Lindgren LM, Bihlet AR, Riis BJ, Andersen JR, Qvist P, Karsdal MA, Christiansen C.
Int J Epidemiol. 2017 Aug 1;46(4):1104-1104i

Excessive matrix metalloprotease-mediated degradation of interstitial tissue (type I collagen) independently predicts short-term survival in an observational study of postmenopausal women diagnosed with cancer. Willumsen N, Bager CL, Kehlet SN, Dragsbaek K, Neergaard JS, Hansen HB, Bay-Jensen AC, Leeming DJ, Lipton A, Christiansen C, Karsdal M.
Oncotarget. 2017 Feb 11;8(32):52501-52510

Unique insight into microenvironmental changes in colorectal cancer: Ex vivo assessment of matrix metalloprotease-mediated molecular changes in human colorectal tumor tissue and corresponding non-neoplastic adjacent tissue. Willumsen N, Bager CL, Bay-Jensen AC, Kehlet SN, Harling H, Leeming DJ, Karsdal MA, Jorgensen LN.
Oncol Lett. 2017 May;13(5):3774-3780

Co-authored book

Biochemistry of Collagens, Laminins and Elastin – Structure, function and Biomarkers.
Chapter 21, 22 and 23 (SN Kehlet and MA Karsdal). Published by Elsevier.

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Abstract

Despite extensive research efforts and validation of numerous new cancer drugs in clinical trials every year, cancer continues to be one of the major causes of death in the western world, emphasizing the need for novel biomarkers. During the last few years, increased attention has been drawn towards precision medicine as a new treatment tool in cancer which strives at matching the right drug(s) to the right patient. To enable this approach, informative biomarkers are needed that can identify patients early, predict who will respond to a certain treatment, give information regarding treatment efficacy and give information about a likely outcome in relation to survival, progression or recurrence. Mounting evidence points towards the extracellular matrix (ECM) playing an active role in cancer progression and not just being a passive bystander. During tumor development and progression, the normal ECM homeostasis is disturbed and excessive ECM remodeling occurs leading to a structure and organization of the tumor-associated ECM that is very different from that of normal tissue. A consequence of this altered remodeling is an increased production of turnover products that are released into the circulation. These small protein fragments hold post-translational modifications giving rise to neo-epitopes that represents unique tissue fingerprints of the combination of the involved proteases and the composition of the ECM. Protein fingerprint biomarkers could provide novel insights into the understanding of cancer pathology as well as to improve cancer treatment and strategy towards a more personalized approach.

The hypothesis of this thesis was that the pathologically driven turnover of the ECM results in the release of neo-epitope fragments into the circulation that can serve as diagnostic, prognostic and/or predictive tools in cancer.

The overall aim of this thesis was to identify, characterize and validate blood-based neo-epitope biomarkers reflecting ECM remodeling in cancer and their ability to identify patients with cancer and provide prognostic value for the future outcome in cancer patients. The specific aims were as follows:

Aim 1 - To investigate the diagnostic and prognostic potential of neo-epitope biomarkers reflecting increased collagen turnover in patients with colorectal cancer (paper I, II and additional results 1)

Aim 2 – To identify novel neo-epitope biomarker targets reflecting structural changes in the ECM during cancer progression and develop immunoassays against the neo-epitopes of interest (paper III, IV and additional results 2)

To investigate the aims of this thesis we measured the following well validated protein fingerprint biomarkers in serum from patients with different solid tumors: MMP-degraded type I collagen (C1M), MMP-degraded type III collagen (C3M), MMP-degraded type IV collagen (C4M), formation of type I collagen (PINP), formation

of type III collagen (PRO-C3), formation of type IV collagen (P4NP7S) and formation of type VI collagen (PRO-C6). In addition, three novel immunoassays were developed and validated, targeting protease-generated neo-epitopes originating from ECM proteins. The clinical use of the biomarkers were investigated in different clinical studies.

The main findings were as follows:

Aim 1: We demonstrated that neo-epitope biomarkers reflecting collagen turnover were elevated in patients diagnosed with colorectal cancer, particular in patients with advanced stages. It was not possible to differentiate between cancer patients or healthy controls and subjects with adenomas suggesting that these biomarkers cannot be used for early diagnosis. However, neo-epitope biomarkers reflecting collagen formation showed a great potential as prognostic biomarkers and as a measure of tumor activity.

Aim 2: Three highly specific and robust immunoassays were developed and a pathological relevance was shown for two of the assays. These data suggest that structural changes to the ECM plays a pathological role in tumorigenesis and biomarkers reflecting these changes have potential as novel liquid-biopsy biomarkers in cancer. Whether their use is diagnostic, prognostic or predictive needs further evaluation in larger clinical cohorts.

The overall conclusion is that neo-epitope fragments reflecting changes of the ECM and the tumor microenvironment, have potential as promising serological biomarkers in cancer. The results strongly indicate that ECM turnover is altered in patients with cancer and protease-generated fragments of proteins originating from the tumor microenvironment are released into the circulation as a result of tumorigenesis. These biomarkers might be able to define a certain ECM phenotype in patients with cancer which may contribute to personalized medicine. For all of the measured biomarkers, further validation are needed to elucidate their exact use in cancer.

Dansk resumé

På trods af en omfattende forskningsindsats, og mange kliniske studier der validerer nye kræft behandlinger hvert år, er kræft stadig en af de førende årsager til global morbiditet, og nye biomarkører er derfor en nødvendighed. I løbet af de sidste par år, er der kommet øget fokus på skræddersyet/personlig medicin, som et nyt behandlingsredskab til kræft. Skræddersyet medicin stræber efter at matche det/de rigtige lægemidler til den rigtige patient. For at muliggøre denne tilgang er der brug for informative biomarkører, der kan identificere patienter tidligt, forudsige hvem der responderer på en bestemt behandling, give oplysninger om behandlingseffektivitet, og give oplysninger om patienters prognose i forhold til overlevelse, progression eller tilbagefald. Forskning peger mod, at den ekstracellulære matrix (ECM) spiller en aktiv rolle i kræft. Under dannelsen og udviklingen af en tumor, forstyrres den normale ECM homeostase, og der vil opstå en øget ECM remodelering, hvilket fører til en anderledes struktur og sammensætning af den tumor-associerede ECM i forhold til det normale væv. En konsekvens af denne ændrede vævsbalance, er en øget produktion af protein nedbrydningsfragmenter, der frigives til blodet. Disse små proteinfragmenter indeholder post-translationelle modifikationer, som giver anledning til neo-epitoper, der repræsenterer unikke vævs/protein ”fingeraftryk”. Disse vævs/protein ”fingeraftryk” kan bruges som biomarkører og give nyt indblik i forståelsen af selve kræft patogenesen samt at forbedre kræftbehandling og strategi mod en mere personlig og skræddersyet tilgang.

Hypotesen for denne afhandling var, at den patologisk-drevet omsætning af den ekstracellulære matrix resulterer i frigivelsen af neo-epitop fragmenter til blodet, der kan bruges som nye diagnostiske, prognostiske og/eller prædiktive biomarkører i cancer.

Det overordnede formål var at identificere, karakterisere og validere blodbaserede neo-epitop biomarkører, der afspejler ECM remodeling i kræft og deres evne til at identificere patienter med kræft og give oplysninger om patienters prognose i forhold til overlevelse. De specifikke formål var følgende:

Formål 1 - at undersøge det diagnostiske og prognostiske potentiale for neo-epitop biomarkører, der afspejler øget kollagen produktion eller nedbrydning hos patienter med kolorektal kræft (artikel I, II og supplerende resultater 1)

Formål 2 - at identificere nye neo-epitoper, der afspejler strukturelle ændringer i ECM under tumor udvikling og udvikle immun-assays mod disse potentielle neo-epitoper (artikel III, IV og supplerende resultater 2)

For at undersøge dette, målte vi følgende validerede neo-epitop biomarkører i serum fra patienter med forskellige solide tumorer: MMP-nedbrudt type I kollagen (C1M), MMP-nedbrudt type III kollagen (C3M), MMP-nedbrudt type IV kollagen (C4M), dannelse af type I kollagen (PINP), dannelse af type III kollagen (PRO-

C3), dannelse af type IV kollagen (P4NP7S) og dannelse af type VI kollagen (PRO-C6). Derudover blev tre nye immun-assays udviklet og valideret, som måler protease-genererede neo-epitoper der stammer fra ECM proteiner. Den kliniske anvendelse af biomarkørerne blev undersøgt i forskellige kliniske kohorter.

De vigtigste opnåede resultater var følgende:

Formål 1: Neo-epitop biomarkører der afspejler kollagen dannelse eller nedbrydning var forhøjet hos patienter diagnosticeret med kolorektal kræft, især hos patienter med metastaser. Det var ikke muligt at skelne mellem kræftpatienter eller raske kontroller og individer med adenomer, hvilket tyder på, at disse biomarkører ikke kan anvendes til tidlig diagnose. Yderligere viste vi, at neo-epitop biomarkører, der afspejler kollagen dannelse, har et stort potentiale som prognostiske biomarkører, og kan bruges som et mål for tumoraktivitet.

Formål 2: Tre specifikke og robuste immun-assays blev udviklet, og vi viste en patologisk relevans i kræft for to af disse assays. Disse data tyder på at strukturelle ændringer i ECM spiller en patologisk rolle i tumorigenesen, og biomarkører der afspejler disse ændringer har potentiale som nye blodbaserede biomarkører i kræft. Hvorvidt deres anvendelse er diagnostisk, prognostisk eller prædiktiv, kræver yderligere evaluering i større kliniske kohorter.

Den overordnede konklusion for denne afhandling er, at neo-epitop fragmenter, der afspejler ændringer i ECM, har potentiale som nye blodbaserede biomarkører i kræft. Resultaterne tyder stærkt på, at ECM sammensætningen ændres hos patienter med kræft og at protease-genererede fragmenter fra proteiner omkring tumor-mikromiljøet frigives til cirkulationen som følge af tumorigenese. Disse biomarkører kan muligvis definere en bestemt ECM-fænotype hos patienter med kræft, som kan bidrage til personlig og skræddersyet medicin. Der er behov for yderligere validering af de målte biomarkører, for at identificere deres nøjagtige anvendelse indenfor kræft.

Abbreviations

α-smooth Muscle Actin	α-SMA
Area Under the ROC curve	AUROC
Biomarkers In Patients with Pancreatic Cancer	BIOPAC
Carbohydrate Antigen 19-9	CA19-9
Cancer-Associated Fibroblast	CAF
Chronic Obstructive Pulmonary Disease	COPD
Colorectal Cancer	CRC
Electro-Chemiluminescence Immuno Assay	ECLIA
Enzyme-Linked ImmunoSorbent Assay	ELISA
Epithelial-to-Mesenchymal Transition	EMT
Estrogen Receptor	ER
European Medicines Agency	EMA
Extracellular Matrix	ECM
Food and Drug Administration	FDA
Hazard Ratio	HR
Idiopathic Pulmonary Fibrosis	IPF
Immunoreceptor Tyrosine-based Inhibitory Motif	ITIM
Leukocyte-Associated Immunoglobulin-like Receptor 1	LAIR-1
Non-Small Cell Lung Cancer	NSCLC
Mass Spectrometry	MS
Matrix MetalloProtease	MMP
Overall survival	OS
Post-Translational Modifications	PTMs
Progression-free survival	PFS
Prostate specific antigen	PSA
Receiver-Operating Curve	ROC
Secreted proteome acidic and rich in cysteine	SPARC
Small-Cell Lung Cancer	SCLC
Tissue Inhibitors of MetalloProteases	TIMP

1. Introduction

In the 1970s US declared a "War on Cancer" with the aim of identifying cancers early and thereby treat the patients before the cancer cells spread to other parts of the body. But now, 40 years later and despite extensive research efforts, cancer continues to be one of the major causes of death in the western world (1). What has become clear for the last 40 years, is that cancer is a disease with countless different characteristics and causes which complicates treatment. For many years, cancer was considered as merely a genetic disease arising due to somatic mutations. We know now that the right microenvironment surrounding the tumor together with the right tumor genome, are a necessity for tumor cells to invade and progress (2–4). This thesis focuses on identification and investigation of biomarkers reflecting changes in the extracellular matrix (ECM) of the tumor microenvironment which could provide novel insights into the understanding of cancer pathology as well as to improve cancer treatment and strategy towards a more personalized approach. The pathology of specific cancer types are outside the scope of this thesis.

In this chapter, a short introduction to the importance of precision medicine and cancer in general will be given. The significance of the tumor microenvironment, with a special focus on cancer-associated fibroblasts, and the remodeling of the ECM in cancer will be described followed by an overview of which biomarker classes exist. Last, a description on how to exploit the tumor microenvironment as a new source of protein biomarkers will be introduced.

1.1 Cancer in numbers – why do we need precision medicine?

In Denmark, every third person are diagnosed with cancer and cancer has been the leading cause of death since 2000 (1). From 1980 to 2012 the number of new cases per year (incidence) has increased by 73% in men and by 62% in women (all cancer types except non-melanoma skin cancer), while the number of deaths from cancer per year has increased by about 14% in men and 16% in women (5). Table 1 summarizes key numbers in cancer statistics in Denmark. As age is the strongest risk factor for developing cancer and the elderly population is expected to increase due to large birth cohorts after World War II, the cancer incidence is also expected to increase by about 32% towards 2030 (5). Early detection and better treatment strategies are therefore urgently needed.

Cancer is a dynamic disease and comprises a group of more than 100 heterogeneous diseases. Tumor heterogeneity can be divided into two classes; intra-tumoral and inter-tumoral heterogeneity. During tumor development, the heterogeneity of the tumor cells will generally increase resulting in a tumor that consists

Table 1. Cancer statistics in Denmark from 2011-2015 (all cancer types except non-melanoma skin cancer)

	No. of new cases/year (2011-2015)	No. of subjects diagnosed with cancer (end of 2015)	1-year relative survival (%, 2011-2915)	5-year relative survival (%, 2011-2015)	No. of cancer deaths/year (2011-2015)
Men	19126	124288	78	60	8143
Women	18096	160683	80	63	7394

Numbers are adapted from NORDCAN, Association of the Nordic Cancer Registries

of diverse cells harboring different molecular gene signatures (intra-tumoral heterogeneity). Inter-tumoral heterogeneity refers to the heterogeneity between patients with the same histological tumors (6). Therefore, drugs targeting a single genetic driver may not be sufficient for treatment and multi-agent treatment is necessary. Furthermore, one patient may respond to a certain drug whereas other patients will be resistant due to inter-tumoral heterogeneity. This makes it very difficult to have a general treatment strategy and therefore we need to have a more personalized approach. During the last few years increased attention has been drawn towards precision medicine as a new treatment tool in cancer and in 2015 former US president Barack Obama announced a research initiative focusing on precision medicine in cancer (7). Precision medicine is matching the right drug(s) to the right patient. To enable this approach novel informative biomarkers are needed that can predict who will respond to a certain treatment, give information regarding treatment efficacy and give information about a likely outcome in relation to survival, progression or recurrence.

To overcome the challenge with tumor heterogeneity and get one step closer towards personalized medicine, we need to identify and understand common traits for all tumors. In 2000, Hanahan and Weinberg published the hallmarks of cancer, describing fundamental capabilities that must be acquired for tumor development, including sustained proliferation, evasion of growth suppression, death resistance, replicative immortality, induced angiogenesis and initiation of tissue invasion and metastasis (8). Eleven years later, Hanahan and Weinberg revisited the hallmarks and added two important factors: dysregulated cellular metabolism and evasion of immune destruction (9). Since then, it has become clear that the ECM regulates many of the cellular responses that characterize the cancer hallmarks (10), suggesting that the ECM has an influence on tumor development and should be considered in relation to biomarker discovery and identification of drug targets. In fact, the tumor microenvironment may be a better source of precision medicine biomarkers compared to the tumor itself due to the heterogeneity.

1.2 The extracellular matrix – the foundation of tumor growth and progression

A solid tumor is a system composed of tumor cells, resident and infiltrating non-tumor cells and molecules present in the surrounding area of these cells. This system can be described as the tumor microenvironment and both the tumor cells and the surrounding cells take part in establishing the right milieu. There is much focus on the ECM as the most abundant component of the tumor microenvironment and its involvement in cancer progression. The ECM is a key regulator of cell and tissue function. It is a non-cellular organic component that is synthesized and secreted by multiple cells. It consists of a meshwork of proteins to which soluble factors, such as growth factor and cytokines, can bind and interact with the surrounding cells (11). The ECM is divided into two main types; the basement membrane and the interstitial matrix (figure 1). The basement membrane is located right beneath the epithelium and endothelium cell layers dividing them from the underlying stroma. It is primarily composed of type IV collagen, laminins, nidogen and perlecan. The main function of the basement membrane is to mediate tissue compartmentalization and regulate cell behavior (12,13). The interstitial matrix makes up the bulk of the ECM and is mainly composed of collagens, fibronectin, proteoglycans and glycoproteins, which altogether provide tissue hydration, enable binding of growth factors and cytokines to the tissue and cross-link the matrix to enhance its integrity (3,11).

Extracellular matrix remodeling in cancer

In order to maintain healthy tissue, the ECM must regenerate itself by degrading old and damaged proteins and replace them with new ones. Degradation and formation of ECM proteins are tightly controlled to ensure homeostasis. The synthesis of ECM proteins by fibroblasts has a relatively slow turnover and the proteolytic activity is limited (figure 1). However, during cancer development and progression these processes are hijacked and the normal ECM homeostasis is disturbed resulting in excessive ECM remodeling (14).

Collagen fibers in the healthy interstitial matrix are curly and coiled, whereas tumor-associated collagens show increased linearization and crosslinking reflecting increased collagen deposition and posttranslational modifications (15,16). The excessive crosslinking and linearization leads to a denser and stiffened ECM in which cancer cells can exploit the linearized collagen fibers as invasion “highways” towards the vasculature thereby promoting invasion and metastasis. In fact, multiphoton microscopy has shown that linearized collagen fibers converge on blood vessels (17) (figure 1).

Although collagen fibers enhance cancer cell migration, up-regulation of interstitial collagens may also act as a barrier against invasion. Thus, proteolysis of the ECM is also an important part of tumorigenesis and regulates cellular migration by modifying the density of the ECM (18). The basement membrane works as a barrier against initial tumor cell migration and retains the cells *in situ*. In the early stages of cancer development, the cells typically undergo epithelial-to-mesenchymal transition (EMT) where the cells polarity

and cell-cell junctions are lost resulting in a migratory mesenchymal phenotype. This process involves an altered protein expression including loss of the transmembrane protein E-cadherin and acquisition of N-cadherin and vimentin (19,20). In addition, the tumor cells and other cells in the tumor microenvironment starts to overexpress a large number of proteases which breakdown the basement membrane and interstitial matrix driving the cellular invasion (21) (figure 1). Degradation of the ECM also plays a role in angiogenesis as migration of endothelial cells and vessel sprouting rely on matrix proteolysis. It has been shown that matrix metalloprotease (MMP)-14 is expressed at the sprouting tip of the developing vasculature where it regulates matrix remodeling (22,23). MMP-9 is required for the angiogenic switch (one of the hallmarks of cancer) as this protease is responsible for proteolytic release of the critical angiogenic factor VEGF (24).

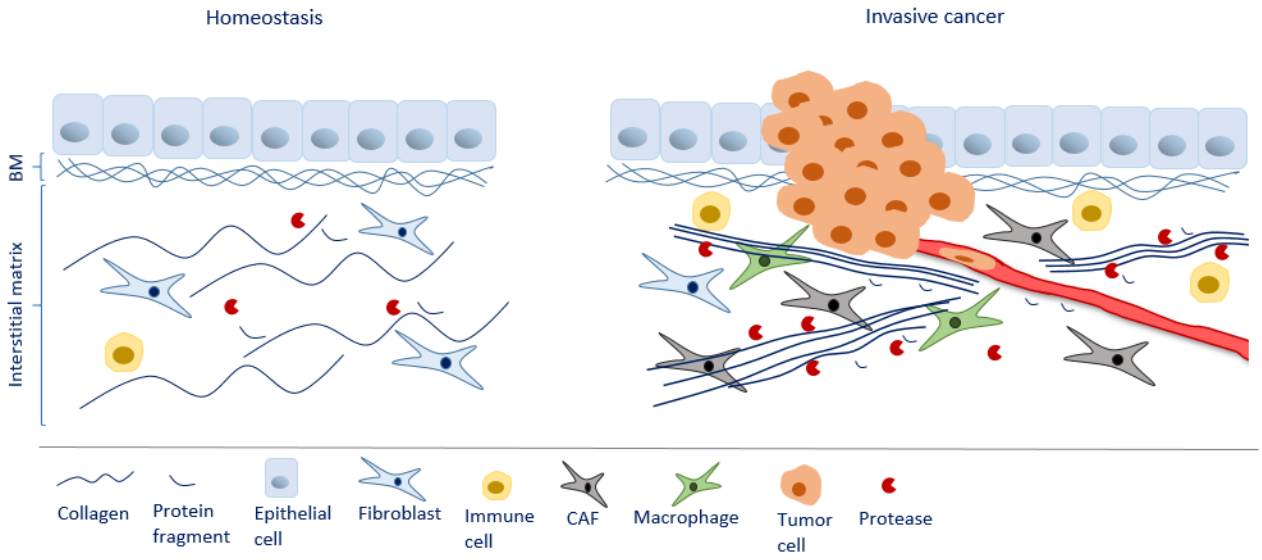


Figure 1. Extracellular matrix remodeling in cancer

To ensure homeostasis, extracellular matrix (ECM) remodeling is tightly controlled with a balanced protein degradation and formation ratio. This balance is disturbed upon localized abnormal growth of epithelial cells which is the initial development of cancer. The cancerous cells secrete factors that will activate fibroblasts leading to the formation of carcinoma-associated fibroblasts (CAFs) which promote excessive ECM remodeling and invasion of tumor cells into the interstitial matrix. ECM remodeling in cancer is characterized by desmoplasia - a fibrotic-like condition with increased collagen deposition, cross-linking and linearization. Tumor cells travels towards the vasculature using these linearized collagen fibers as "highways" enhancing metastasis. At the same time there is an increased expression of proteases resulting in the release of small degradation fragments into the blood. BM; basement membrane.

In addition to extracellular turnover of collagens, an intracellular process has also been identified, in which collagen is internalized through binding to collagen-specific receptors on the cell surface and delivered for lysosomal degradation. The urokinase plasminogen activator receptor–associated protein (uPARAP) receptor,

acts as an endocytic receptor internalizing collagen for lysosomal degradation and hence take significant part in remodeling of the ECM (25). uPARAP are found on malignant cells of various non-epithelial cancers and a subgroup of patients with epithelial cancer such as breast cancer. It may play a role in invasive tumor growth and has also been suggested as a novel target for antibody-drug conjugate mediated treatment of different types of cancers (26).

Another important consequence of increased ECM remodeling in cancer, is the release of bioactive fragments originating from altered processing and degradation of interstitial collagens. These fragments exert different functions which stimulate tumorigenesis (27). This will be elaborated in section "the good and the bad collagens".

Cancer-associated fibroblasts - the architects of the tumor microenvironment

Fibroblasts are the main cells of the ECM. They secrete ECM proteins including proteases to ensure correct remodeling and homeostasis of the matrix. In the 1970s it was discovered that fibroblasts within the tumor microenvironment acquire a modified phenotype which is similar to fibroblasts associated with wound healing, namely the ability to recruit cells, induce a fibrillar network and induce angiogenesis (28,29). This is in line with the view that tumors are "wounds that never heal" (30). These activated fibroblasts, residing in the tumor microenvironment, have been termed cancer-associated fibroblasts (CAFs) and play a key role in constructing a pro-tumorigenic microenvironment. It has been demonstrated that CAFs are functionally required for mediating tumor progression by promoting macrophage recruitment, neovascularization, inflammatory signaling and tumor growth (31–34).

CAFs are the most abundant cell type in the tumor microenvironment and known to dictate tumor outcome (35). CAFs have been shown to be associated with poor overall survival(36) and in breast carcinomas about 80% of the stromal fibroblasts acquire a CAF phenotype (37). Unlike normal fibroblasts, CAFs reside close to or within the tumor mass and facilitate tumor progression (38). The phenotype of CAFs isolated from malignant tissue is different from that of normal fibroblasts. CAFs are more metabolic active with a rapid proliferation rate, enhanced ECM production, with collagens being the main secreted proteins, and increased secretion of growth factors and other modulators (39–42). Furthermore, their morphology are different and CAFs express other or a higher amount of specific markers, such as α-smooth muscle actin (α-SMA) and vimentin, with α-SMA being the most commonly used (43). However, most of the well-used markers are not fibroblast-specific, thus not CAF specific. Recently, other markers have been identified whose expression is exclusively restricted to CAFs and exhibit prognostic value; aspirin (44), type XI collagen (COL11A1) (45) and microfibrillar-associated protein 5 (46). These new markers may have potential as candidate biomarkers and therapeutic targets due to their specificity.

As noted, the ECM regulates many of the hallmarks of cancer. As CAFs have been termed “the architects of cancer pathogenesis” (47) one may speculate that CAFs modulate many of these hallmarks. One of the most important hallmark is sustained and increased proliferation. Olumi *et al.* have shown that human prostatic CAFs grown with initiated human prostatic epithelial cells (carcinomas) dramatically stimulated growth and altered histology of the epithelial population (48). This effect was not detected when normal prostatic fibroblasts were grown with the initiated epithelial cells under the same experimental condition. In contrast, CAFs did not affect growth of normal human prostatic epithelial cells under identical conditions. These data suggest that CAFs stimulate progression of tumorigenesis, but not initiation of tumor development. The same study also showed that CAFs are able to render cells more resistant to apoptosis, another important cancer hallmark. For cancer cells to metastasize they need to induce angiogenesis (cancer hallmark). A study has shown that in the presence of CAFs, breast cancer tissue becomes more vascularized compared to cocultivation with normal fibroblasts, suggesting that CAFs can promote tumor neovascularization (49).

One of the alterations of the ECM during cancer progression is desmoplasia. Desmoplasia is known as tumor-associated fibrosis which is induced by CAFs. It results from overproduction of ECM, mainly collagens, which results in a dense and fibrous tissue. Desmoplasia can be observed in most solid tumors and is known to be tumor promoting. It has been shown that increased stromal collagen in mouse mammary tissue significantly increases tumor formation and results in a significantly more invasive phenotype (50). In addition, in patients with pancreatic cancer a high level of desmoplasia, measured by type I collagen content, was associated with poor overall survival (51). The reason for a poor outcome might be due to the formation of a fibrotic cap surrounding the tumor, thereby limiting cancer therapy delivery into the tumor, resulting in a lack of response to therapy.

While a majority of studies supports the fact that CAFs and desmoplasia has a tumor promoting effect, it has recently been suggested that desmoplasia is also involved in preventing tumor progression. By using a pancreatic mouse model, Rhim and colleagues (52) have shown that deletion of the sonic hedgehoc protein, a protein known to drive fibroblast-rich desmoplastic stroma, reduced the stromal content compared to wildtype mice. Surprisingly, these mice had a much more aggressive tumor compared to wildtype mice, suggesting a protective role of desmoplasia. Similar findings were shown by Özdemir *et al.* (53) who showed that depletion of CAFs in mice led to a much more invasive tumor phenotype and pancreatic cancer patients with low levels of CAFs around the tumor was associated with poor survival.

Taken together, CAFs and desmoplasia play a key role in cancer progression and are potential candidates for novel drug targets. However, the contradictory results suggest that the mechanisms involved in these

processes are very complex and not fully understood, highlighting the need for understanding which elements in the stroma are pro-tumorigenic and which are anti-tumorigenic.

The next section gives an overview of the main collagens within the ECM and their potential as pro/anti-tumorigenic peptides.

The" good and the bad" collagens

Collagens are the most abundant ECM molecules modulating cellular functions and physiological processes. To date, 28 collagens have been identified and is encoded by 42 different genes (54). Collagens are trimeric molecules composed of three α-chains which forms a characteristic triple helix. Collagens contain at least one triple-helical domain mainly containing the repeated sequence G-X-Y (with X being frequently proline and Y hydroxyproline) and non-triple helical domains which are used as building blocks by other ECM proteins. Based on their molecular assembly, collagens can be divided into six classes: fibrillary collagens, network forming collagens, fibril-associated collagens with interrupted triple helices (FACIT), transmembrane collagens, multiplexins and unclassified collagens (54,55) (table 2). Some collagens are localized differently in the basement membrane and the interstitial matrix whereas others are found in both areas linking the two compartments. It has been hypothesized that there are good and bad collagens in fibrosis depending on their localization and degradation as some collagen fragments have shown to have biological activity (56).

Table 2. Collagen classes and types (54,55)

Collagen class	Collagen type
Fibrillary collagens	I, II, III, V, XI, XXIV, XXVII
Network forming collagens	IV, VIII, X
FACIT's	IX, XII, XIV, XVI, XIX, XX, XXI, XXII
Transmembrane collagens	XIII, XVII, XXIII, XXV
Multiplexins	XV, XVII
Unclassified collagens	VI, VVI, XXVI, XXVIII

The amount of collagen changes dramatically during tumorigenesis (57). With lessons learned from fibrosis, some collagens could by themselves induce a reactive pro-tumorigenic stroma and thereby play a direct role in cancer development and progression. In this section, a description of type I, III, IV, VI and XI collagen will be given together with their active role in cancer (fragments from these collagens are investigated in this thesis).

Type I collagen

Type I collagen is a fibrillary collagen and the most abundant type of collagen found in almost all connective tissue. It is located in the interstitial matrix where it assembles into fibrils playing a key structural role (58). Due to the high level of type I collagen in different tissues, its well-documented turnover and role in a range of diseases (59), several biomarkers have been identified reflecting either type I collagen formation or degradation: PINP (N-terminal pro-peptide reflecting type I collagen synthesis), PICP (C-terminal pro-peptide reflecting type I collagen synthesis), CTX-I (C-terminal telopeptide reflecting type I collagen degradation), ICTP (cross-linked C-terminal telopeptide reflecting type I collagen degradation) and C1M (specific MMP-generated neoepitope reflecting type I collagen degradation) (60,61). PINP and CTX-I are primarily used as bone markers, whereas C1M has been shown to be significantly elevated in lung cancer (62), pancreatic cancer (63), ovarian- and breast cancer (64). These data suggests that MMP cleavage of type I collagen at this specific site, could play a pathological role in cancer. Another MMP-generated type I collagen fragment has been shown to regulate scar formation in vitro and in vivo by enhancing basement membrane protein generation, granulation tissue components and angiogenic factors (65). The amount and role of this fragment in cancer has not been established yet and whether these cleavages of type I collagen are a cause or consequence of cancer needs further investigations.

Type III collagen

Type III collagen is together with type I collagen the main component of the interstitial matrix and belongs to the fibril forming collagen family as well (58). It is often associated with type I collagen and essential for proper type I collagen fibrillogenesis (66). Type III collagen is secreted by fibroblasts and other mesenchymal cell types making it a major player in various inflammation-associated pathologies including cancer (67–71). During formation and incorporation into the ECM, N- and C-terminal pro-peptides are cleaved off leading to a mature collagen molecule (72). The pro-peptides can thereby work as surrogate biomarkers for type III collagen formation. The biomarker, PRO-C3 (N-terminal pro-peptide), has been shown to be associated with liver fibrosis (73,74), breast cancer (75) and pancreatic cancer (manuscript in preparation by our group).

Type IV collagen

Type IV collagen is the main component of the basement membrane and is part of the network-forming collagen family. After secretion to the extracellular space, the trimeric molecules self assembles into a lattice-shaped network providing structural support for the epithelial- and endothelial cells. In addition, type IV collagen binds growth factors and other basement membrane components including laminin, nidogen and perlecan (76,77). Several MMP-derived fragments have been identified: Arresten (located in the α1-chain within the non-collagenous 1 domain), canstatin (α2-chain), tumstatin (α3-chain), tetrastatin (α4-chain),

pentastatin (α5-chain) and hexastatin (α6-chain). These fragments that are released during type IV collagen processing have been shown to be inhibitors of angiogenesis, cell proliferation, apoptosis and tumor growth (78–82).

Type VI collagen

Type VI collagen is a beaded filament collagen found in the interface between the basement membrane and the interstitial matrix. Unlike other collagens, type VI collagen form large aggregates inside the cell. Upon secretion, the aggregates assembles into large beaded filaments forming a microfibrillar network (83). The main function of type VI collagen is to aid in cell attachment and connect related tissues to the surrounding matrix providing stability (84). During formation of the microfibrillar network, the triple helical core is proteolytically removed from its pro-peptide and further cleavage of the C-terminal pro-peptide of the α3-chain generates a fragment known as endotrophin (85,86). Endotrophin was first identified by Park and Scherer, 2012 as a bioactive molecule in breast cancer (87). The proposed mechanism of action in mice mammary tumors was induction of epithelial-to-mesenchymal transition (EMT) by stimulating TGF-β creating a fibrotic environment. The same study also showed that endotrophin possess chemoattractant properties infiltrating macrophages and endothelial cells within the tumor microenvironment.

Type XI collagen

Type XI collagen is a minor fibrillar collagen expressed by chondrocytes, osteoclasts and CAFs. The function of type XI collagen has been suggested to involve maintaining of proper fibril formation and diameter. Like all other collagens, type XI collagen is a heterotrimer consisting of α1-, α2- and α3-chains, which are synthesized as pro-collagens and proteolytically cleaved to yield mature type XI collagen (88). One of the most specific CAF genes that have been identified so far are COL11A1 which encodes the α1-chain of type XI collagen (89). Several studies have confirmed the presence of type XI collagen in a variety of cancerous tissues, including breast (90), head and neck (91,92), colon (93), lung (94) and pancreatic tissue (95). These data suggest that type XI collagen plays a critical role in cancer proliferation, invasion and metastasis. The exact mechanisms in which type XI collagen induces cancer needs further investigation, however its low expression in normal tissue and high expression in cancerous tissue suggests type XI collagen to be a potential therapeutic target.

To sum up, collagens can be bad or good depending on their localization and post-transcriptionally processing. Degradation of collagens during tumorigenesis leads to exposure of cryptic sites that are normally inactive. Exposure of these sites will lead to the transmission of unique signals to cells controlling their migration, proliferation and apoptosis and thereby play a direct role in cancer that being either protective or

pathological. Targeting (both positively and negatively) these sites and restore the natural ECM could provide novel treatment opportunities.

Proteases – the deconstructors of the extracellular matrix

When a malignant cell has undergone EMT and gained a mesenchymal phenotype, it can invade the basement membrane and the underlying interstitial matrix. Proteases play a significant role in this invasion by enhancing ECM destruction. Proteases represent a large family of enzymes that catalyze protein degradation by hydrolysis of peptide bonds at either the terminal ends of the protein chain (exo-peptidases) or within the protein chain (endo-peptidases) (96). They are divided into five groups depending on their catalytic mechanism, structure and protein targets; metalloproteases, serine proteases, cysteine proteases, aspartic acid proteases and threonine proteases (97). This thesis will focus on proteases within the metalloprotease family and cysteine protease family.

Matrix metalloproteases

Matrix metalloproteases (MMPs), the largest class of proteases, are zinc-dependent endo-peptidases and the main enzymes involved in ECM degradation. Their activity is low in normal conditions but increases during tissue repair and under pathological conditions (2). The MMP family comprise 23 members that are grouped according to their domain organization and substrate: collagenases (MMP-1, -8 and -13), stromelysins (MMP-3, -10 and -11), gelatinases (MMP-2 and -9), matrilysins (MMP-7 and MMP-26), membrane-bound (MMP-14, -15, -16, -17, -24 and -25) and others (MMP-12, -19, -20, -21, -23, -27 and -28) (98,99). MMPs are produced in an inactive form (zymogen). For their activation, the pro-peptide must be cleaved off either intracellularly or in the extracellular space. Once activated, MMPs can activate each other in a proteolytic cascade as it has been shown for MMP-2 that can be cleaved and thereby activated by MMP-14 (100). The balance between activation and inhibition of MMPs is critical as a skew towards increased activation can lead to ECM remodeling diseases, such as cancer (101). Therefore the activity of MMPs must be tightly controlled. This process is regulated by tissue inhibitors of metalloproteases (TIMPs) that are able to bind to the active site of MMPs at a ratio of 1:1 leading to an inactive state (102). During homeostasis the concentration of TIMPs exceeds that of MMPs keeping the ECM turnover in check whereas in cancer the balance is skewed towards increased expression and activation of MMPs. During cancer progression, MMPs can be secreted directly from tumor cells or cells from the tumor microenvironment (103). Increased expression of MMPs has been shown to be predictive of tumor invasion and poor patient survival in several different cancer types including, lung (104), prostate (105), gastric (106), colorectal (107), breast (108,109), ovarian (110) and pancreatic (111) highlighting the importance of ECM breakdown in cancer. Contradictory results have shown that cancer invasion can be protease-independent. Wolf *et al.* (112) have shown that cells *in vitro* can migrate through

the ECM when inhibiting different proteases, probably via amoeboid movement. A limitation of this study, however, was the use of pepsin-extracted collagen resulting in a less stabilized and dense network as the non-helical telopeptides in the collagen ends are removed during extraction. Using acid-extracted type I collagen gels, cell migration have been shown to be MMP-14 dependent (113).

Based on the many studies associating increased MMP expression with cancer invasion and poor outcome, several clinical trials have tested synthetic MMP inhibitors as novel drugs in cancer treatment. However, many of these trials have failed (114), suggesting that not all MMPs act as tumor promotors. In fact the effect of MMPs have been shown to depend on the cellular source. MMPs originating from the host cell may have suppressive activity whereas MMPs coming directly from the tumor cells will have tumor promoting effects (115). One example is the study reported by Kerkelä *et al.* (116) who demonstrated that MMP-12 expressed by tumor cells correlated with tumor aggressiveness while MMP-12 originating from macrophages had a tumor suppressive role.

Cysteine proteases - cathepsins

The family of cysteine cathepsins comprises cathepsin B, C, F, H, L, K, O, S, V, X and W (97). These proteases are synthesized as inactive precursors and become activated at acidic pH, except for cathepsin S which functions at a neutral pH (117). The activity of cysteine cathepsins are regulated by pH or binding of endogenous inhibitors such as cystatin (118). Cysteine cathepsins have for a long time been considered solely as intracellular proteases functioning in the endolysosomal pathways. However, this view has changed during the last years as studies have demonstrated an extracellular role, especially in relation to cancer. While being tightly controlled under normal physiological conditions, the acidic milieu in the tumor microenvironment dysregulate and increase their activity. Their main role in cancer development is establishing an increased ECM turnover favoring tumor progression and releasing biological active fragments (119).

Examples of non-collagenous proteins playing a role in cancer progression

Besides collagens, several other ECM proteins have been shown to play a role in cancer progression. This chapter will highlight the main functions of decorin, one of the most abundant proteoglycans in the interstitial matrix, and secreted proteome acidic and rich in cysteine (SPARC), a collagen chaperone known to regulate collagen fibrillogenesis.

Decorin

Decorin is the most studied member of the small leucine-rich proteoglycan family. It is a stromal proteoglycan mainly synthesized by fibroblasts, stressed vascular endothelial cells and smooth muscle cells (120). The main ECM binding partners are fibrillar collagens (type I, II, III and VI). Decorin has shown to play a role in the regulation of fibrillogenesis and stabilization of fibrils, and may act as a central player in collagen

assembly/turnover and consequently tissue homeostasis (121,122). Supporting this, decorin knock-out in mice results in abnormal collagen fibril formation and enhanced collagen degradation (123). Another important role of decorin is the ability to sequester multiple growth factors including TGF-β and directly antagonize several members of the receptor tyrosine kinase family (124–127). As a consequence, decorin regulates survival, migratory, proliferative and angiogenic signaling pathways. These many facets have made decorin to "the guardian from the matrix" recognizing the significance of decorin in tissue homeostasis (120).

Given the ability to modulate various signal transduction pathways decorin is a well-known tumor repressor that can counteract tumorigenic- and angiogenic growth (128). Genetic studies have shown that decorin knock-out mice are more prone to develop spontaneous intestinal tumors than wild-type mice, demonstrating that decorin deficiency is permissive for tumor development (129). Clinically, lack of decorin expression is associated with a poor prognosis in patients with breast cancer and soft tissue sarcoma (122,130). Several studies have shown a reduced expression of decorin (both at mRNA and protein level) in different cancer types, including prostate cancer (131), hepatocellular carcinomas (132), colon cancer (133) and multiple myeloma (134). In fact, tumor cells themselves produce less or no decorin compared to the surrounding stroma (122), which might be a mechanism for tumor cells to enhance growth.

SPARC

Secreted proteome acidic and rich in cysteine (SPARC), also referred to as osteonectin or basement membrane protein 40 (BM-40), is a 32-kDa matricellular protein regulating ECM assembly and deposition, growth factor signaling, and interactions between cells and their surrounding ECM (135,136). The expression of SPARC is elevated during embryonic development and is decreased in normal adult tissues. However, its expression is increased in epithelial cells with a high ECM turnover, during abnormal tissue growth associated with neoplasia, and during tissue injury and inflammation highlighting the importance of SPARC in tissue remodeling (137–139).

SPARC is known to have chaperone functions, both due to its ability to inhibit thermal aggregation of alcohol dehydrogenase in a concentration-dependent manner (140), but also due to its role in proper collagen assembly and incorporation into tissues (141). SPARC binds to the interstitial fibrillar collagens type I, III, and V and the basement membrane collagen type IV (142). Phenotypic characterization of SPARC-null mice has resulted in significant insight into its important role in collagen deposition and assembly. Several connective tissues in SPARC-null mice, including dermis, heart, bone, and periodontal ligament, have been shown to have less and smaller fibrillar collagens compared to wild-type mice (143–146). In addition, SPARC-null mutations in mice resulted in an altered morphology of collagen type IV located in the basement membrane of lens capsule, which resulted in an early onset of cataractogenesis (147).

Trombetta–eSilva and Bradshaw (141) and *Rentz et al*. (148) have proposed a model of collagen binding mechanisms of SPARC based on SPARC-null mice and cell studies. They hypothesize that SPARC will bind to pro-collagen as it is secreted from the cell or pro-collagen is secreted bound by SPARC. This binding prevents interaction of pro-collagen with cellular receptors like discoidin domain-containing receptor 2 and integrin α2β1 ensuring proper processing of pro-collagen and appropriate incorporation into fibrils. In the absence of SPARC, pro-collagen will accumulate at the cell surface hindering efficient assembly into the ECM, which will result in less total collagen and fewer thick collagen fibers. This might lead to increased collagen turnover on the cell surface at the expense of collagen deposition into the ECM. Supporting this hypothesis, overexpression of exogenous SPARC results in a more collagen rich ECM in the trabecular meshwork surrounding the eye (149).

The chaperone activity of SPARC may be regulated by different factors. Binding of SPARC to its ECM targets is Ca^{2+} -dependent. The extracellular concentration of Ca^{2+} is sufficient to bind SPARC and induce a conformational change resulting in increased affinity to collagens (150). This binding will be reversed once inside the cell due to low Ca^{2+} concentrations and collagens will be released and processed (151). Another important switch in the regulation of its chaperone activity is the presence of extracellular proteases. Studies have shown that cleavage of SPARC by different proteases increases the affinity to collagens up to 20-fold (152,153). Interestingly, SPARC has been shown to increase the expression and activity of different MMP's causing a positive feedback loop (154–156). If this feedback mechanism becomes uncontrolled, it might be involved in the pathology of ECM remodeling diseases with increased collagen deposition, such as cancer.

The role of SPARC in tumorigenesis appears to depend on its diverse functions in a given microenvironment. SPARC expressed by fibroblasts surrounding the tumor has been shown to be associated with a poor prognosis. High levels of SPARC expression in some cancer types have been reported to correlate with progression and poor prognosis, whereas in others SPARC shows anti-tumorigenic properties (157). However, as SPARC is a key player in proper collagen deposition, it may be a good candidate for keeping the microenvironment in check. For example, Brekken *et al*. (158) have shown that solid tumors in SPARC-null mice grow significantly larger than those in wild-type mice, in part due to a compromised tumor microenvironment.

1.3 Cancer biomarkers; from tissue biopsies to liquid biopsies – what, which and why?

In 1998, the National Institutes of Health Biomarkers Definitions Working Group defined a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes,

pathogenic processes, or pharmacologic responses to a therapeutic intervention" (159). Tumor tissue is the gold standard source of molecular cancer biomarkers. However, the past couple of years circulating molecular material has become an attractive alternative (59). A liquid biopsy is defined as "fluid biological samples (i.e. blood, cerebrospinal fluid, urine, saliva) that contain markers (i.e. circulating cells, cell-free circulating DNA, RNA, miRNA, proteins) that can provide information about the molecular characteristics of the tumor of the patient" (160). An ideal cancer biomarker should be able to be measured easily, reliably and cost-effectively by use of an assay with high analytical sensitivity and specificity. The main differences between tissue biopsies and liquid biopsies are listed in table 3 (161). The main disadvantage of tissue biopsies is its invasiveness and discomfort for the patients whereas a liquid biopsy can be easily obtained by a blood sample. Moreover, the clinical accuracy is often compromised due to intra-tumoral heterogeneity, i.e. the composition of tumor tissue area from which the biopsy is taken might differ from another area within the tumor (162).

Biomarkers can provide diverse information and are subdivided into four main groups according to their clinical application in the cancer field: Diagnostic biomarkers, prognostic biomarkers, predictive biomarkers and monitoring biomarkers.

Table 3. Differences between tissue biopsy and liquid biopsy (161)

Tissue biopsy	Liquid biopsy
Time-consuming procedure	Quick procedure
Localized sampling of tissue	Broad tissue profile
Difficult procedure	Easily obtained
Increased pain/risk	Minimal pain/risk
Invasive	Minimal invasive
Single snap-shot of the tumor	Real-time monitoring of the tumor
Multiple sampling are not feasible	High repeatability and reproducibility

Diagnostic biomarkers

One way to improve patient survival is to identify patients at an early stage. Biomarkers that are able to identify those in the general population with the disease from those not having the disease are termed diagnostic biomarkers. The benefit of identifying early stage cancers is clearly illustrated by the difference in the 5-year survival rate for early stage and advanced stage gastrointestinal tumors, being 90% for local tumors and 12% in advanced stages with metastasis (163). Thus, early detection and optimal population-screening programs are of paramount importance. The most commonly used non-invasive diagnostic

biomarker is prostate specific antigen (PSA). Even though this biomarker have specificity issues, it still remains the best screening tool on the market highlighting the need for better diagnostic tools (164).

Prognostic biomarkers

A prognostic biomarker is able to predict the future cancer outcome among persons without the disease or among cancer patients independent of treatment. The most common outcomes are survival, recurrence and progression. This class of biomarkers is used to classify patients into risk groups and thereby guide the direction of the treatment strategy (164). For example, patients with a poor prognosis need increased attention and monitoring whereas patients with a good prognosis can avoid overtreatment followed by side-effects.

Predictive biomarkers

A predictive biomarker is capable of predicting response to a certain treatment compared to effect of another treatment and these biomarkers enables precision medicine (164). Even though the field of cancer treatment is growing, especially with the advance in immunotherapy, a large percent of patients are still not benefitting from treatment. Study reports have shown that the response rates among lung cancer patients receiving immunotherapy varies from 21% to 67% (165). Without predictive biomarkers, oncologists treat patients in the blind and if the fraction of responders is too low, the drug may not be used even though it may be benefial for a fraction of patients. These facts highlight the importance of identifying novel predictive biomarkers. The estrogen receptor (ER) tissue biomarker was the first predictive recommended biomarker in breast cancer and is used to determine response to hormonal treatments. However, the predictive efficacy of this marker is not ideal as 1/3 of ER-positive patients are resistant to hormonal therapies (166).

Monitoring biomarkers

A monitoring biomarker is used to monitor patients after treatment to ensure that they remain disease free or are treated in case of relapse (164). A well-known monitoring biomarker is the carbohydrate antigen 19-9 (CA19-9) which was cleared by the FDA in 2002 as a patient monitoring tool in pancreatic cancer (167). CA19-9 has a relatively good diagnostic sensitivity but poor diagnostic specificity as this protein has been shown to be elevated in other cancer types as well as in benign pancreatic diseases (164). Therefore, CA19-9 is approved to monitor the therapeutic outcome in patients with pancreatic cancer.

Table 4 summarizes FDA-approved cancer biomarkers that are currently being used in the clinic and their application (168). Extensive research is focusing on identifying novel biomarkers and a high number of publication demonstrating the use of novel biomarkers are being published every year, but only a fraction of

Table 4. FDA-approved cancer biomarkers that are currently being used in the clinic and their application (168)

Name	Clinical application	Cancer type
Tissue biopsy		
ALK	Prognosis, prediction	NSCLC, lymphoma
BRAF V600	Prediction	Melanoma, colorectal
C-kit/CD117	Diagnosis, prediction	Gastrointestinal, melanoma
EGFR	Prognosis, prediction	NSCLC
ER/PR	Prediction	Breast
*HER2/*HER2	Prediction	Breast, gastric
KRAS	Prediction	Colorectal, NSCLC
PD-L1	Prediction	NSCLC
uPA/PAI-1	Monitoring	Breast
21-gene signature (Oncotype DX®)	Monitoring	Breast
70-gene signature (Mammaprint®)	Monitoring	Breast
Liquid biopsy		
5-protein signature (OVA1)	Diagnosis	Ovarian
Thyroglobulin	Monitoring	Thyroid
PSA	Diagnosis, monitoring	Prostate
Nuclear matrix protein 22	Monitoring	Bladder
NSE	Diagnosis, monitoring	SCLC, Neuroblastoma
Lactate dehydrogenase	Prognosis, monitoring	Germ cell tumors, lymphoma, leukemia, melanoma
Immunoglobulins	Diagnosis, monitoring	Myeloma, macroglobulinemia
HE4	Prediction, monitoring	Ovarian
Fibrin/Fibrinogen	Monitoring	Bladder
Cytokeratin fragment 21-1	Monitoring	Lung
Circulating tumor cells (CELLSEARCH®)	Prognosis	Breast, prostate, colorectal
Chromosomes 3, 7, 17 and 9p21	Monitoring	Bladder
CgA	Diagnosis, monitoring	Neuroendocrine tumors
CD20	Prediction	Lymphoma
CEA	Monitoring	Colorectal
Calcitonin	Diagnosis, monitoring	Thyroid
CA-125	Diagnosis, monitoring	Ovarian
CA19-9	Monitoring	Pancreatic, gallbladder, bile duct, gastric
CA15-3/CA27.29	Monitoring	Breast
BCR-ABL fusion gene	Diagnosis, prediction, monitoring	Leukemia
BRCA1 and *BRCA2*	Prediction	Ovarian
Beta-hCG	Prognosis, monitoring	Choriocarcinoma, germ cell tumors
B2M	Prognosis, monitoring	Multiple myeloma, leukemia, lymphoma
AFP	Diagnosis, prognosis, prediction, monitoring	Liver, germ cell tumors

ALK; anaplastic lymphoma kinase, EGFR; epidermal growth factor receptor, ER; estrogen receptor, PR; progesterone receptor, HER2; human epidermal growth factor receptor 2, PD-L1; programmed death-ligand 1, uPA; urokinase-type plasminogen activator, PAI-1; Plasminogen activator inhibitor-1, PSA; prostate-specific antigen, NSE; neuron specific enolase, HE4; human epididymis protein 4, CgA; Chromogranin A, CEA; carcinoembryonic antigen, BCR; breakpoint cluster region protein, ABL; Abelson tyrosine kinase, beta-hCG; beta-human chorionic gonadotropin, B2M; beta-2 microglobulin AFP; alpha-fetoprotein, NSCLC; non-small cell lung cancer

these are being FDA-approved. The reason why so many biomarkers fail to reach the clinic is due to inadequate performance in a clinical setting and the challenges in developing a reliable assay (false discovery) (169). To improve patient survival, there is a demanding need within the cancer field, for better biomarkers that can diagnose at an early stage, predict treatment response and cancer outcome. Many of the FDA-approved biomarkers are either genes or proteins involved in cell proliferation, enzymatic activities with the cancer-cells or abnormal secretion of proteins from the cancer cells. As none of these are "perfect", a combination strategy might be the way forward in order to target as many aspect of cancer development as possible. One way to overcome this could be by including the changes to the environment surrounding the tumor.

The diagnostic dilemma

The performance of a biomarker is most often evaluated by its sensitivity and specificity. Sensitivity is defined as the probability of being test positive when the disease is truly present (true positives) and is calculated by dividing the true positives with all of those who actually have the disease (true positives + false negatives). Specificity is the probability of being test negative when the disease is truly absent (true negatives) and is calculated by dividing the true negatives with all of those who actually are disease free (true negatives + false positives) (170). Both sensitivity and specificity are dependent on the choice of cut-off value which subjects are classified according to. An ideal biomarker should be able to completely distinguish between two groups at an optimal cut-off point (figure 2A), e.g. healthy and disease, high vs. low risk of progression and responders vs non-responders. Unfortunately, this is rarely the case and sometimes, as an example, healthy individuals are misclassified as diseased (false positives) and diseased as healthy (false negatives). A low cut-off point will most likely include all true positives, but also many false positives which results in a poor specificity (figure 2B). On the other hand, a high cut-off value will include all true negatives, but also many false negative and therefore have a poor sensitivity (figure 2B). Choosing the right cut-off value is a diagnostic dilemma. With a low cut-off value, a healthy individual will occasionally be misclassified as a diseased. This scenario is often discussed in relation to screening, where there may be a tendency to over-diagnose if the cut-off value is too low (171). This will lead to over-treatment and side-effects for otherwise healthy individuals. If the cut-off value is too high, patients who are actually diseased will be classified as healthy and in worst-case end up dying of an undetected disease. Accordingly, choosing the right cut-off value is of high importance as this number can determine the faith of patients. It is worth noticing that a diagnostic biomarker often needs to be more specific than a prognostic biomarker, as the misclassification of a diagnosis could cause more harm compared to misclassifying a prognosis of a patient (172).

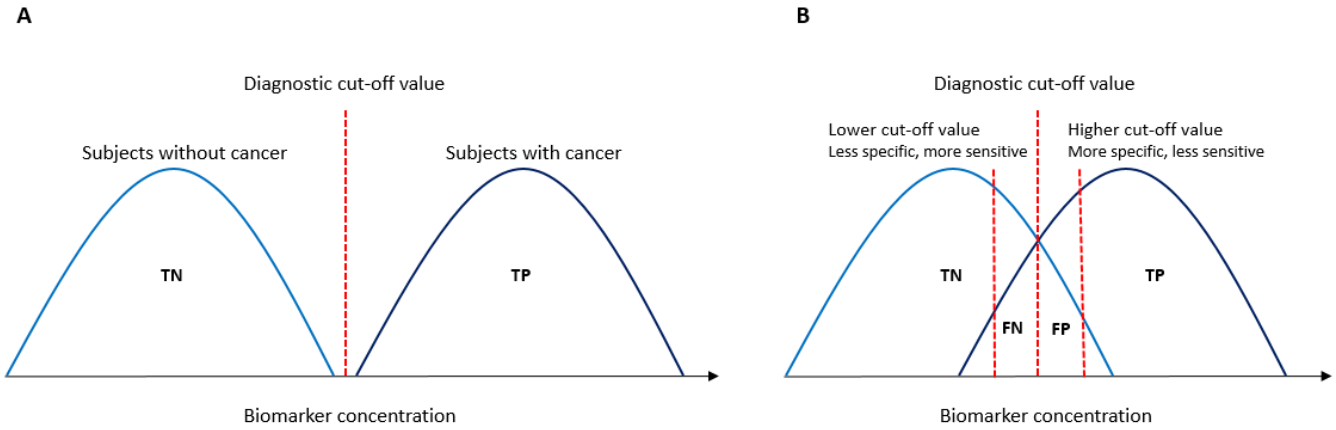


Figure 2. The diagnostic dilemma

(A) Illustration of an ideal biomarker being able to completely separate two groups at a defined cut-off value. **(B)** Illustration of how the cut-off value can affect specificity and sensitivity when there is not complete separation of the two groups. TN; True negatives, FN; false negatives, FP; false positives, TP; true positives.

The accuracy of a biomarker, interpreted by the sensitivity and specificity, is often reported by receiver-operating curve (ROC). A ROC curve is a plot of sensitivity vs. 1-specificty for all possible cut-off points (figure 3). The area under the ROC curve (AUROC) is a measure of how well the biomarker can separate the two groups of interest (170). An AUROC of 1 indicates perfect separation (high sensitivity and high specificity) of the two groups whereas an AUROC of 0.5 mean poor clinical accuracy, i.e. no separation of the two groups.

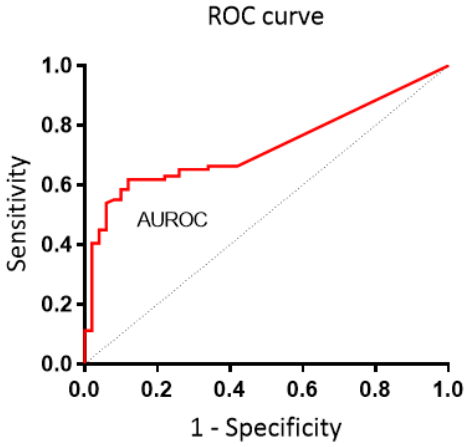


Figure 3. Illustration of a receiver-operating curve (ROC).

When calculating the area under the ROC (AUROC), a value approaching 1.0 indicates high clinical accuracy. In contrast, an AUROC of 0.5 (the dotted line) indicates a poor clinical accuracy.

The common confounders when evaluating serum biomarkers

When evaluating serum biomarkers, the pre-analytical procedures for blood sampling are essential and if not properly controlled, may have impact on the biomarker values. Put in a simple way, the best biomarker assay can be destroyed if the clinical samples are not controlled and vice versa, the best clinical samples may be worthless if the assay is not validated properly (the validation of an immunoassay will be described in the next chapter). Several parameters have been shown to influence biological variation or analytic performance of a given biomarker (173–175). As a starting point, the clinical samples should originate from the same study and collected and processed using the same procedures. In addition, the same sample matrix should be used, e.g. serum, plasma or urine, as the biomarker level may differ in the different matrices. The most common confounders influencing biological variation are age, gender, BMI, food intake, diurnal variation, physical activity and co-morbidities (176). As an example, it has been shown that the level of ECM-related biomarkers differ significantly in blood samples drawn from osteoarthritis patients before arising from bed compared to blood samples drawn after 1, 4 and 12 hours of daily activity (173). Furthermore, a simple thing as water intake has been shown to affect biomarker levels (177). Another study, evaluating collagen turnover as function of age in rats, has demonstrated that collagen turnover rates are consistently different in young vs old animals, up to 30-fold (178). Hence, it has been postulated that when evaluating biomarkers, subjects should optimally be matched for age, sex, weight (BMI) and ethnicity, as well as relevant lifestyle factors (e.g. smoking, alcohol intake and medical history) to rule out the contribution of these confounders (179). However, this is not always entirely possible and therefore the effects of these confounding factors should be investigated before initiating a clinical investigation or be included in the data analysis and evaluation.

1.4 The protein fingerprint technology – using the tumor microenvironment as a source of protein biomarkers

With a cancer incidence expected to increase by about 32% towards 2030 (5), there is an urgent need for novel biomarkers that can aid in early detection, prognosis and treatment response. As established in chapter 1.2, the ECM regulates many of the cellular responses that characterize the cancer hallmarks, hence influencing tumor development and progression. The tumor microenvironment may therefore be a new source of biomarkers and drug targets. Proteins originating from the ECM have been investigated as cancer biomarkers, however as seen in table 4 none of these have passed FDA approval. The methods used for quantifying these proteins in serum or plasma are more than two decades old and relies on quantifying total protein instead of looking at different parts or modifications of the proteins, which could provide more pathological specificity (180). By exploiting post-translational modifications (PTMs) in the ECM and the fact

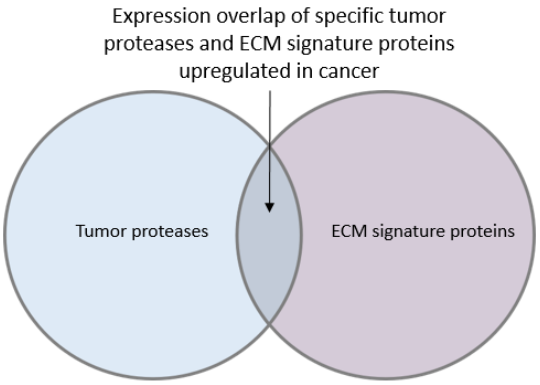


Figure 4. Combining tumor proteases and ECM signature proteins as novel cancer biomarkers

Schematic representation of the origin of an optimal extracellular matrix (ECM) cancer biomarker. The overlapping area represents a source of neo-epitope biomarkers that rely on the combination of a pathology specific ECM protein and a pathology specific protease.

that different tumor cells express certain proteases and different tissues contain signature proteins, an optimal biomarker may be identified. The overlapping areas in figure 4, represents the combination of specific proteases and specific ECM signature proteins that are needed to obtain high sensitivity and specificity. For example, a colorectal cancer cell expressing MMP-1 combined with an interstitial collagen involved in ECM remodeling may provide a possible neo-epitope biomarker of the initiation and progression of colorectal cancer. Nordic Bioscience has developed a technique to quantify different parts of a protein separately with each part providing different information. This technique is called “the protein fingerprint technology” and is based on immunoassays. The principle behind these assays is the use of monoclonal antibodies exclusively reacting with a specific fragment of a certain protein which become exposed after specific protease-mediated degradation, thus the antibody will only bind upon cleavage (figure 5). The generated neo-epitopes can provide a unique tissue fingerprint of the combination of the involved proteases and the composition of the ECM. As an example, pro-peptides of pro-collagens can serve as surrogate biomarkers for tissue formation whereas neo-epitopes on peptides derived from collagen degradation of the triple helical region reflects collagen degradation. By measuring tissue formation and degradation separately, a better understanding of the tissue homeostasis is obtained. As described previously, the tissue balance is in fact playing a direct role in tumor progression, both with increased formation and degradation being associated with a poor prognosis. The biomarkers used in this thesis all rely on the protein fingerprint technology. Table 5 summarizes the assays used in this thesis and which ECM process they represent.

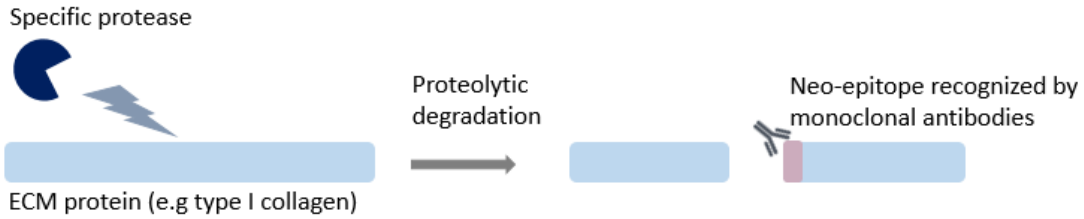
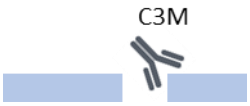
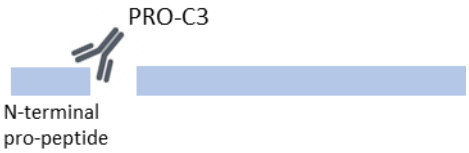
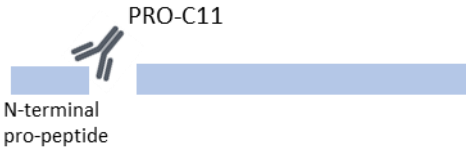
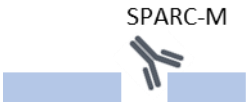


Figure 5. The protein fingerprint technology

A specific protease cleaves an ECM signature protein, e.g type I collagen, at a specific amino acid generating a neo-epitope. Monoclonal antibodies raised against this neo-epitoe will only recognize the cleaved fragment ant not the intact protein. Ultimately, immunoassays can be developed and optimized for targeting the protein fragments in the circulation. Modified from (11).

Molecular cancer biomarkers can be DNA, RNA or protein which are altered in response to tumorigenesis. The protein fingerprint assays reflect specific end-products of an altered tissue homeostasis (181). The fact that end-products are targeted may generate more pathological accuracy compared to DNA, RNA or total protein which are subject to numerous processes, i.e. transcription, translation and post-translational modifications, which will affect the final outcome. This is of course dependent on the medical need and biomarker usage.

Table 5. Schematic overview describing the protein fingerprint assays used in this thesis.

Biomarker	Specification	Process	Schematic
C1M	Neo-epitope of MMP-2,9,13 mediated degradation of type I collagen	Type I collagen degradation	
PINP	Internal epitope in the N-terminal pro-peptide of type I collagen	Type I collagen formation	
C3M	Neo-epitope of MMP-9 mediated degradation of type III collagen	Type III collagen degradation	
PRO-C3	Released N-terminal pro-peptide of type III collagen	Type III collagen formation	
C4M	Neo-epitope of MMP-2,9,12 mediated degradation of type IV collagen alpha 1	Type IV collagen degradation	
P4NP7S	Internal epitope in the 7S domain of type IV collagen	Type IV collagen formation	
PRO-C6	C-terminal of released C5 domain of type VI collagen α3 chain (endotrophin)	Type VI collagen formation	
PRO-C11	Released N-terminal pro-peptide of type XI collagen	Type XI collagen formation	
DCN-CS	Neo-epitope of cathepsin S mediated degradation of decorin	ECM remodeling; Decorin degradation	
SPARC-M	Neo-epitope of MMP mediated degradation of SPARC	ECM remodeling; SPARC degradation	

From biomarker target identification to clinical utility

As one of the aims in this thesis was to identify novel neo-epitope biomarker targets and develop immunoassays against the neo-epitopes of interest, the different steps/processes from target identification to clinical utility at Nordic Bioscience will be described in the following section. An overview is given in figure 6.

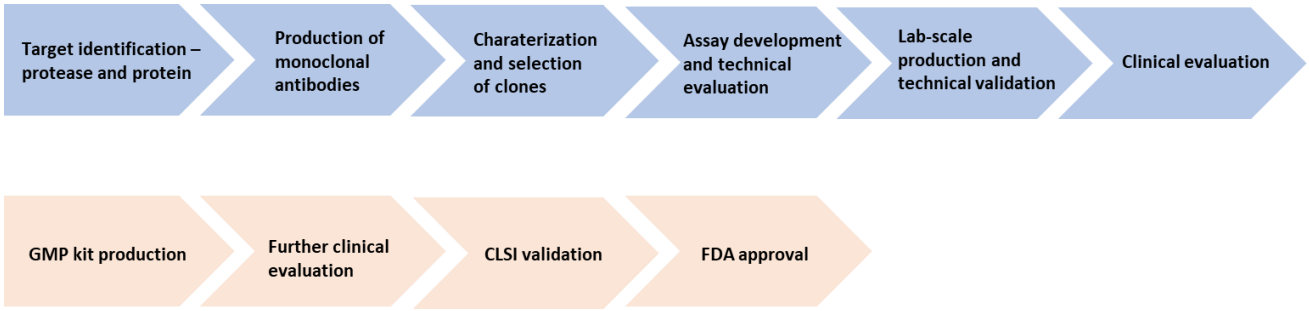


Figure 6. From biomarker target to clinical utility

The blue steps is performed at the RnD Department at Nordic Bioscience and comprises steps from biomarker target identification to lab-scale production and clinical evaluation. If the biomarker/assay of interest shows high potential, the assay will be transferred to the Production Unit at Nordic Bioscience. The orange steps shows the steps necessary to get an FDA approval of the biomarker.

Target identification

A neo-epitope biomarker target can be identified either by a literature search in which a target sequence is stated or by *in vitro/ex vivo* cleavage of the protein of interest by pathological relevant proteases. The target sequence can then be identified by mass spectrometry (MS). After having selected a target of interest, the most important parameter to evaluate is the uniqueness of the epitope recognized by the monoclonal antibody. Sequence homology should therefore be investigated by blasting the target sequence against other proteins. Other considerations are homology to other species enabling translational science and cleavage of the epitope by other proteases which will lead to loss of antigenicity.

Monoclonal antibody development

The production of monoclonal antibodies is a crucial step in assay development as non-monoclonality could interfere with assay validation. The monoclonal antibody is produced by consecutive immunizations of Balb/C mice with the antigen of interest until stable sera titer levels are reached. Hybridoma cells are then produced by fusion of spleen cells with myeloma cells. To secure monoclonal growth these cells are further subcloned using standard limited dilution. The clones with the best reactivity towards the calibrator peptide in a

preliminary enzyme-linked immunosorbent assay (ELISA) setting are selected for further clone characterization.

Immunoassay development – optimization and validation

After clone characterization, the most promising monoclonal antibody is selected based on native reactivity and specificity against the neo-epitope and purified following competitive immunoassay development and validation. The main steps include:

1. Determination of optimal coater and antibody ratio to obtain an OD of 2 (for ELISA).
2. Optimization of buffer and temperature/incubation time.
3. Native reactivity is tested in the selected settings and the setting with the best sensitivity and reactivity is selected for further assay validation.
4. The following validation tests are included: Specificity, precision, linearity (dilution recovery), accuracy (spiking), analyte stability, freeze/thaw and interference.
5. Assessment of biological relevance

Clinical evaluation and utility

If the biomarker is shown to have clinical relevance in a preliminary study cohort, the clinical utility needs to be further evaluated using relevant clinical cohorts. It is important to stress that a bad diagnostic biomarker may show promise as a prognostic/predictive biomarker, so different clinical cohorts are necessary for optimal evaluation.

2. Hypothesis and aims

Despite extensive research efforts and validation of numerous new cancer drugs in clinical trials every year, cancer continues to be one of the major causes of death in the western world, emphasizing the need for novel biomarkers. During the last few years, increased attention has been drawn towards precision medicine as a new treatment tool in cancer which strives at matching the right drug(s) to the right patient. To enable this approach, informative biomarkers are needed that can identify patients early, predict who will respond to a certain treatment, give information regarding treatment efficacy and give information about a likely outcome in relation to survival, progression or recurrence.

Mounting evidence points towards the ECM playing an active role in cancer progression and not just being a passive bystander. During tumor development and progression, the normal ECM homeostasis is disturbed and excessive ECM remodeling occurs leading to a structure and organization of the tumor-associated ECM that is very different from that of normal tissue. A consequence of this altered remodeling is an increased production of turnover products that are released into the circulation. These small protein fragments hold post-translational modifications giving rise to neo-epitopes that represents unique tissue fingerprints of the involved proteases and the composition of the ECM. Protein fingerprint biomarkers could provide novel insights into the understanding of cancer pathology as well as to improve cancer treatment and strategy towards a more personalized approach.

2.1 Hypothesis

The pathologically driven turnover of the ECM results in the release of neo-epitope biomarkers into the circulation that can serve as diagnostic, prognostic and/or predictive tools in cancer.

2.2 Aims

The overall aim of this thesis was to identify, characterize and validate blood based neo-epitope biomarkers reflecting ECM remodeling in cancer and their ability to identify patients with cancer and provide prognostic value for the future outcome in cancer patients.

Specific aims

Aim 1 - To investigate the diagnostic and prognostic potential of neo-epitope protein fingerprint biomarkers reflecting increased collagen turnover in patients with colorectal cancer (paper I, II and additional results 1)

Aim 2 – To identify novel neo-epitope protein fingerprint biomarker targets reflecting structural changes in the ECM during cancer progression and develop immunoassays against the neo-epitopes of interest (paper III, IV and additional results 2)

3. Neo-epitope biomarkers reflecting increased collagen turnover are elevated in patients with metastatic colorectal cancer – potential as novel prognostic biomarkers rather than diagnostic tools in cancer

This chapter summarizes the findings from paper I and II and furthermore describes an additional study which has been presented as a poster at ASCO 2018, Chicago and published in the Danish medical newspaper "Dagens Medicin" the 5th of June 2018 (aim 1).

3.1 Summary of paper I, II and additional results 1

Rationale

During cancer progression, the homeostasis of the ECM becomes imbalanced due to excessive collagen remodeling by matrix metalloproteases. As a consequence, small protein fragments of degraded collagens are released into the circulation which may be useful as novel diagnostic and prognostic tools in cancer.

As described in section 1.3, common confounders in clinical studies are age and gender, which needs to be accounted for when measuring biomarkers in clinical studies. The influence of these factors on collagen turnover biomarkers must therefore be established before being used in clinical trials. In paper I, we have investigated age- and sex-dependent ECM turnover as function of age in healthy men and women by measuring biomarkers of formation and degradation of the most abundant collagens of the interstitial matrix (type I and III collagen) and basement membrane (type IV collagen) in serum.

In paper II, we monitored the levels of protein fragments originating from type I, III or IV collagen in order to examine their potential use as novel diagnostic biomarkers in colorectal cancer.

The additional result section describes a study investigating the prognostic potential of biomarkers reflecting type III and VI collagen formation.

Methods and patient cohorts

By using validated competitive ELISAs, we assessed specific fragments of degraded and formed type I, III, IV and VI collagen in serum from healthy individuals and patients with colorectal cancer. The patient cohort in paper I consisted of 617 healthy men and women ranging in ages from 22 to 86. Paper II included patients with different stages of colorectal cancer (n=196), subjects with adenomas (n=99) and age matched healthy controls (n=99). The third study cohort (additional results 1) consisted of 40 healthy subjects and 40 patients with metastatic colorectal cancer.

Main findings and conclusions – aim 1

The first paper in this chapter shows that collagen turnover is affected by age and sex with the interstitial matrix (type I and III collagen) and the basement membrane (type IV collagen) being differently regulated in men and women. These results are important to consider when conducting clinical studies focusing on ECM-related disorders as these biomarkers have been shown to associate with various connective tissue disorders where the ECM balance is skewed. The second paper and the additional results demonstrate that neo-epitope biomarkers reflecting collagen turnover are elevated in patients diagnosed with colorectal cancer, particular in patients with advanced stages. It was not possible to differentiate between cancer patients or healthy controls and subjects with adenomas (paper II) suggesting that these biomarkers cannot be used for early diagnosis. However, neo-epitope biomarkers reflecting collagen formation showed a great potential as prognostic biomarkers (additional results 1) and as a measure of tumor activity (paper II). Larger studies are needed to further validate their prognostic and monitoring use.

3.2 Paper I, II and additional results 1

Paper I - Age-related collagen turnover of the interstitial matrix and basement membrane: Implications of age- and sex-dependent remodeling of the extracellular matrix

PLOS ONE

RESEARCH ARTICLE

Age-related collagen turnover of the interstitial matrix and basement membrane: Implications of age- and sex-dependent remodeling of the extracellular matrix

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Abstract

The extracellular matrix (ECM) plays a vital role in maintaining normal tissue function. Collagens are major components of the ECM and there is a tight equilibrium between degradation and formation of these proteins ensuring tissue health and homeostasis. As a consequence of tissue turnover, small collagen fragments are released into the circulation, which act as important biomarkers in the study of certain tissue-related remodeling factors in health and disease. The aim of this study was to establish an age-related collagen turnover profile of the main collagens of the interstitial matrix (type I and III collagen) and basement membrane (type IV collagen) in healthy men and women.

By using well-characterized competitive ELISA-assays, we assessed specific fragments of degraded (C1M, C3M, C4M) and formed (PINP, Pro-C3, P4NP7S) type I, III and IV collagen in serum from 617 healthy men and women ranging in ages from 22 to 86. Subjects were divided into 5-year age groups according to their sex and age. Groups were compared using Kruskal-Wallis adjusted for Dunn's multiple comparisons test and Mann-Whitney t-test. Age-specific changes in collagen turnover was most profound for type I collagen. PINP levels decreased in men with advancing age, whereas in women, the level decreased in early adulthood followed by an increase around the age of menopause (age 40–60). Sex-specific changes in type I, III and IV collagen turnover was present at the age around menopause (age 40–60) with women having an increased turnover. In summary, collagen turnover is affected by age and sex with the interstitial matrix and the basement membrane being differently regulated. The observed changes needs to be accounted for when measuring ECM related biomarkers in clinical studies.

preparation of the manuscript. MK, KH and NW are full-time employees at Nordic Bioscience A/S, and MK holds stocks in Nordic Bioscience A/S. Nordic Bioscience is a privately-owned, small-medium size enterprise partly focused on the development of biomarkers, including the ELISAs used in this study. None of the authors received fees, bonuses or other benefits for the work described in the manuscript. The specific roles of the authors are articulated in the 'author contributions' section.

Competing interests: M. Karsdal, K. Henriksen and N. Willumsen are employed at Nordic Bioscience A/S which is a company involved in discovery and development of biochemical biomarkers. M. Karsdal owns stocks at Nordic Bioscience. This does not alter our adherence to all PLOS ONE policies on sharing data and materials. S. Kehlet, G. Armbrecht, R. Dietzel and S. Brix report no conflict of interest.

Introduction

The extracellular matrix (ECM) is the backbone of all tissues. It is composed of several structural proteins, including collagens, which play a vital role for the function and maintenance of normal tissue function. Collagen type I and III are the most abundant collagens of the interstitial matrix and essential for its structure. The basement membrane, underlying epithelial or endothelial cells, primarily consist of collagen type IV which ensure optimal cell polarization and function [1,2].

ECM tissue turnover, i.e. the tight balance between protein degradation and formation can be classified as two processes: 1) tissue modeling, occurring during development and growth where new tissue is being generated; 2) tissue remodeling, where functional tissue is being maintained by replacing old and damaged proteins with new ones [3,4]. Every healthy organ is undergoing continuous remodeling with a tight control between degradation and formation. However, this delicate balance might be disturbed, leading to connective tissue disorders such as fibrosis and cancer [5]. Measurements of ECM turnover products in blood have shown that circulating components of the ECM, especially collagens, are elevated in fibrotic diseases [6–10] and cancer [11,12]. We have developed a panel of serum-based assays specifically measuring collagen fragments that reflect either degradation or formation separately [13–18]. The principle behind these assays is the use of monoclonal antibodies exclusively reacting with a specific fragment of a certain protein which become exposed after specific protease-mediated degradation. Antibodies raised against pro-peptides of pro-collagens reflect collagen formation whereas antibodies recognizing small neo-epitopes on peptides derived from collagen degradation of the triple helical region represent collagen degradation [5,19,20]. Table 1 summarizes the assays we have used in this study and which ECM process they represent.

PINP, which measures the N-terminal pro-peptide released during collagen formation, have been showed to primarily reflect synthesis of bone matrix [21]. C1M, which measures a MMP-degraded fragment of type I collagen released during tissue remodeling, is closely related to chronic inflammation with high levels being present in various inflammatory diseases [14,22–25]. Pro-C3 measures the pro-peptide of type III collagen, i.e. synthesis, and C3M measures a MMP-generated type III collagen fragment, i.e. degradation. Increased levels of Pro-C3 and C3M have also been linked to inflammatory diseases and especially fibrosis [25–30]. P4NP7S, which reflects type IV collagen formation by measuring the 7S domain of type IV collagen, has been associated with fibrosis of the liver [31] and C4M, which reflects MMP-mediated degradation of the basement membrane, is elevated in patients with diseases displaying chronic inflammation [18,24,32–34].

While it has been shown that levels of ECM proteins vary significantly with age [35–39], a collagen turnover profile in men and women of varying age using a well-defined panel of markers directly measuring degradation and formation of interstitial matrix and basement membrane collagens has never been publihsed. In this study, we investigated age- and sex-

Table 1. Description of the collagen degradation and formation assays used in this study.

Biomarker	Specification	Process	Surrogate measure
C1M[14]	Neo-epitope of MMP-2,9,13 mediated degradation of type I collagen	Type I collagen degradation	Chronic inflammation
PINP[13]	Internal epitope in the N-terminal pro-peptide of type I collagen	Type I collagen formation	Primarily bone synthesis
C3M[16]	Neo-epitope of MMP-9 mediated degradation of type III collagen	Type III collagen degradation	Chronic inflammation
Pro-C3[15]	Released N-terminal pro-peptide of type III collagen	Type III collagen formation	Fibrosis
C4M[18]	Neo-epitope of MMP-2,9,12 mediated degradation of type IV collagen alpha 1	Type IV collagen degradation	Chronic inflammation
P4NP7S[17]	Internal epitope in the 7S domain of type IV collagen	Type IV collagen formation	Fibrosis

https://doi.org/10.1371/journal.pone.0194458.t001

dependent ECM turnover as function of age in healthy men and women by measuring biomarkers of formation and degradation of the most abundant collagens of the interstitial matrix (collagen type I and III) and basement membrane (collagen type IV) in serum.

Materials and methods

Serum samples

The serum samples used in this study originated from a population-based cross-sectional study where subjects were recruited in 2007–2011 from a random sample of all districts in Berlin provided by the resident registration office. A total number of 617 subjects, comprising 303 healthy men and 314 healthy women aged 22–86, were included in this study. Characteristics of the study population are presented in Table 2.

Venous blood samples were collected by trained medical technologists between 08:00 and 10:00 AM after a 12 h fasting period. 30 min. after blood drawing, samples were centrifuged at 3500 rpm for 10 min, serum was obtained and samples were stored at -80˚C until analysis.

The study was approved by the local ethics committee (Ethikkommission der Charité, Charité –Universitätsmedizin Berlin, EA4/095/05) as well as the German Radiation Protection Ordinance (Z5-22462/2-2005-063). Written informed consent was obtained from all participants before they were included into the study and the study was carried out in accordance with ICH-GCP and according to the Declaration of Helsinki.

Collagen turnover biomarker analysis

All biomarker assays were manufactured by Nordic Bioscience (Herlev Denmark) and performed according to the manufacturer's specifications. The assays are competitive ELISA's which have been thoroughly validated for their use in human serum samples and technically characterized with regards to linearity, accuracy and reproducibility (the original assay reference for each biomarker is listed in Table 1). To eliminate/reduce inter-assay variation, all biochemical markers were measured using a single lot of reagents and serum controls were included on each plate. Females and males, as well as age-groups were randomly distributed on the different ELISA plates. All samples were analyzed by a CAP/CLIA-accredited laboratory (Nordic Bioscience Laboratory, Herlev, Denmark).

The neo-epitope biomarkers of matrix metalloproteinase (MMP) degraded type I, type III and type IV collagen (C1M, C3M, C4M) and type I, type III and type IV collagen formation products (PINP, Pro-C3, P4NP7S) were assessed in serum as previously described [13–18]. Briefly, 96-well pre-coated streptavidin plates were coated with biotinylated synthetic peptides specific for the protein of interest and incubated for 30 minutes at 20˚C. 20 μL of standard peptide or pre-diluted serum sample were added to designated wells followed by the addition of peroxidase-conjugated specific monoclonal antibodies and incubated for 1 h at 20˚C or overnight at 4˚C. Finally, tetramethylbenzinidine (TMB) (cat. 438OH, Kem-En-Tec Diagnostics, Denmark) was added to each well and the plates were incubated for 15 minutes at 20˚C. All incubation steps included shaking at 300 rpm and after each incubation step, the plates were washed five times with wash buffer (20 mM Tris, 50 mM NaCl, pH 7.2). The enzymatic reaction was stopped by adding 0.18 M H_2SO_4 and absorbance was measured at 450 nm with 650 nm as reference. A calibration curve was plotted using a 4-parameter logistic curve fit.

Statistical analysis

Subjects were stratified into 5-year interval age groups. To investigate if the biomarkers changed with age, the levels of the individual biomarkers in each 5-year age group were

Table 2. Basal characteristics of the study population.

Men (n = 303)				
Age group	**Age (median, years) Range (min.-max.)**	**Weight (median, kg) Range (min.-max.)**	**Height (median, cm) Range (min.-max.)**	**BMI (median, kg/m^2) Range (min.-max.)**
20–24 (n = 19)	24 22–24	75 58–128	181 170–197	23.1 19.8-37-4
25–29 (n = 25)	26 25–29	76 61–104	179 168–202	23.8 20.5–30.0
30–34 (n = 24)	31 30–34	78 64–120	178 164–189	24.7 18.7–35.8
35–39 (n = 24)	36 35–37	83 64–120	180 167–193	26.1 19.0–38.0
40–44 (n = 21)	43 40–44	87 53–122	179 164–195	26.5 19.7–36.4
45–49 (n = 18)	48 45–49	85 69–101	178 169–199	26.2 22.0–30.4
50–54 (n = 24)	52 50–54	83 57–106	181 171–188	25.5 17.1–32.4
55–59 (n = 28)	57 55–59	54 57–101	177 159–192	27.0 20.7–34.5
60–64 (n = 23)	63 60–64	82 70–109	176 166–197	26.1 22.9–34.9
65–69 (n = 30)	67 65–69	83 50–126	172 161–183	27.1 17.8–38.7
70–74 (n = 20)	72 70–74	84 67–102	175 158–184	29.0 22.2–33.6
75–79 (n = 17)	79 77–79	77 60–118	169 159–183	27.0 23.9–36.4
80+ (n = 30)	82 80–86	78 52–99	175 161–182	25.8 16.1–30.8
Women (n = 314)				
Age group	**Age (median, years) Range (min.-max.)**	**Weight (median, kg) Range (min.-max.)**	**Height (median, cm) Range (min.-max.)**	**BMI (median, kg/m^2) Range (min.-max.)**
20–24 (n = 12)	23 22–24	63 48–105	173 158–184	21.7 18.6–33.1
25–29 (n = 29)	26 25–29	62 52–74	166 157–180	21.4 18.5–27.9
30–34 (n = 22)	32 30–34	65 46–90	168 154–186	22.2 19.4–31.9
35–39 (n = 25)	36 35–37	72 51–93	164 155–185	25.1 18.8–34.0
40–44 (n = 26)	43 40–44	69 50–89	168 157–183	23.7 17.7–33.6
45–49 (n = 24)	48 45–49	76 51–95	166 146–180	26.8 18.5–36.1
50–54 (n = 25)	52 50–54	66 52–104	165 156–173	23.1 20.8–36.4
55–59 (n = 26)	57 55–59	66 50–87	163 155–175	25.4 19.1–32.0
60–64 (n = 32)	62 60–64	67 53–109	161 154–172	26.1 20.5–38.9
65–69 (n = 29)	67 65–69	64 47–97	161 152–171	24.9 19.2–33.6
70–74 (n = 20)	72 70–74	69 53–79	159 146–175	27.0 21.6–31.7

(*Continued*)

Table 2. (Continued)

75–79 (n = 21)	78 75–79	70 56–108	160 147–171	26.5 21.7–38.3
80+ (n = 23)	81 80–86	64 46–79	159 147–177	25.3 20.1–31.7

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compared using a Kruskal—Wallis ANOVA (S1 Table). The p-values were adjusted to account for multiple comparisons using Dunnett´s method. An increase or decrease is defined as a significant difference between two following age groups, e.g. age group 25–29 vs. 30–34 (two adjacent age groups).

To investigate if the biomarkers varied according to sex, the level of the individual biomarkers between men and women in the five-year age groups was compared using the Mann-Whitney test. Serum biomarker data can be found in S1 Data. Graph design and statistical analyses were performed using GraphPad Prism version 7.03 (GraphPad Software, Inc., CA, USA).

Results

Age specific changes in collagen remodeling

Type I collagen. Type I collagen formation and degradation were measured with the PINP and C1M assays, respectively. In men, the highest level of PINP was found at age 20–24 (Fig 1A).

The level declined with increasing age. Comparing the level at age 20–24 with the rest of the age-groups, a significant decrease was observed from age 45–49 to 80+ (S1 Table). In women, the highest level of PINP was found at age 20–24 and 80+. PINP levels decreased from age 20–24 until age 35–39, but not significantly (Fig 1A). Around menopause (average age 40–60 [40]) the level significantly increased until age 60–64 (S1 Table).

The level of C1M in men was relatively stable with no significant changes (Fig 1B). The level of C1M in women was also relatively stable with only a significant increase between age group 40–44 and 70–74 (Fig 1B) (S1 Table).

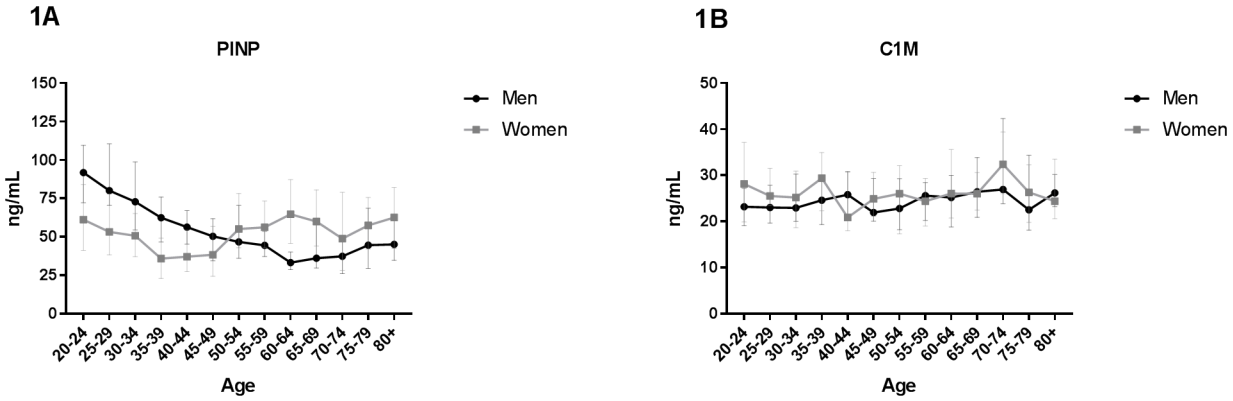

Fig 1. Type I collagen turnover as function of age. Biomarkers reflecting degradation and formation of type I collagen were measured in serum from 303 healthy men and 314 healthy women aged 22–86 divided into 5-year age groups. (A) Formation of interstitial type I collagen (PINP) and (B) degradation of interstitial type I collagen (C1M). Statistical significance of C1M and PINP between each age group was calculated using ANOVA comparing the mean of each group with the mean of every other group and is presented at the different age groups in S1 Table. All data are shown as median and interquartile range.

https://doi.org/10.1371/journal.pone.0194458.g001

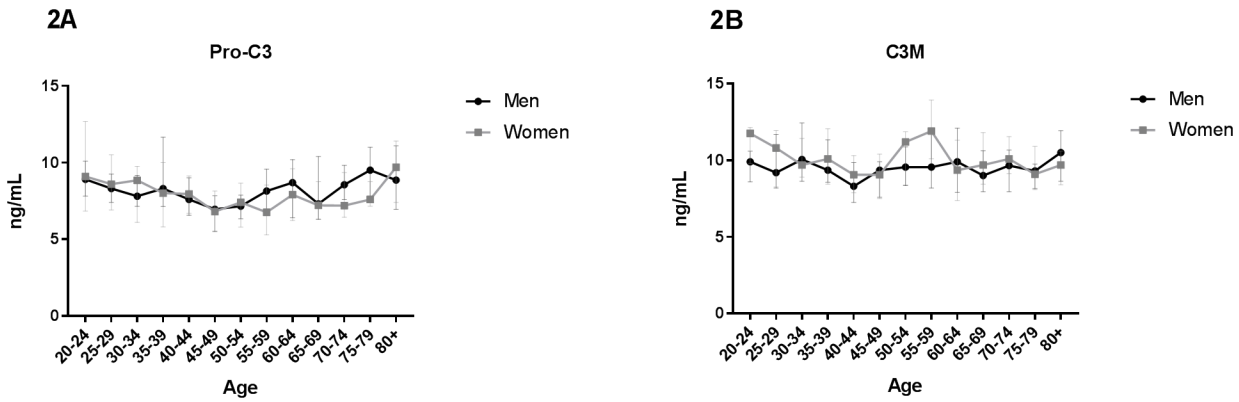

Fig 2. Type III collagen turnover as function of age. Biomarkers reflecting degradation and formation of type III collagen were measured in serum from 303 healthy men and 314 healthy women aged 22–86 divided into 5-year age groups. (A) Formation of interstitial type III collagen (Pro-C3) and (B) degradation of interstitial type III collagen (C3M). Statistical significance of C3M and Pro-C3 between each age group was calculated using ANOVA comparing the mean of each group with the mean of every other group and is presented at the different age groups in S1 Table. All data are shown as median and interquartile range.

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Type III collagen. The ELISA assays Pro-C3 and C3M were used to measure type III collagen formation and degradation, respectively. Pro-C3 levels in men showed a significant decrease between age 20–24 and 45–49 followed by a significant increase until age 80+ (Fig 2A) (S1 Table).

The level of Pro-C3 was relatively stable in women with no significant changes until age 45–49. A significant increase from age 45–49 and 55–59 until age 80+ was observed (Fig 2A) (S1 Table).

The level of C3M in men was relatively stable from age 20–80+ with no significant changes (Fig 2B). The same pattern was seen in women (Fig 2B). However, around menopause (average age 40–60 [40]) the level significantly increased until age 55–59 (S1 Table) followed by a steep significant decline (S1 Table).

Type IV collagen. Type IV collagen formation and degradation were measured with the P4NP7S and C4M assays, respectively. The level of P4NP7S in men and women was relatively stable with no significant changes across the age span (Fig 3A).

The same pattern was observed with C4M in men (Fig 3B). In women, a significant increase was observed from age 25–29 reaching a maximum at age 55–59 (Fig 1F) (S1 Table). The level significantly decreased reaching its starting level at age 80+ (S1 Table).

Sex specific changes in collagen remodeling

Type I collagen. For PINP, the starting level was significantly higher in men compared to women until the age of 45–49 (Table 3).

Around this age, the level starts to increase in women, whereas the level in men continues to decrease. In the postmenopausal period (age 60–80+), the level was significantly higher in women compared to men, except at the age of 70–79 (Table 3).

For C1M, no significant difference between the sexes was observed.

Type III collagen. The level of Pro-C3 was significantly different in women compared to men in specific age groups in the end of the menopausal period (age 55–59) and the postmenopausal period (70–74 and 75–79) with men having the highest level (Table 3).

For C3M, a significantly higher level was observed in women as compared to men at the age of 55–59 (menopausal period) (Table 3).

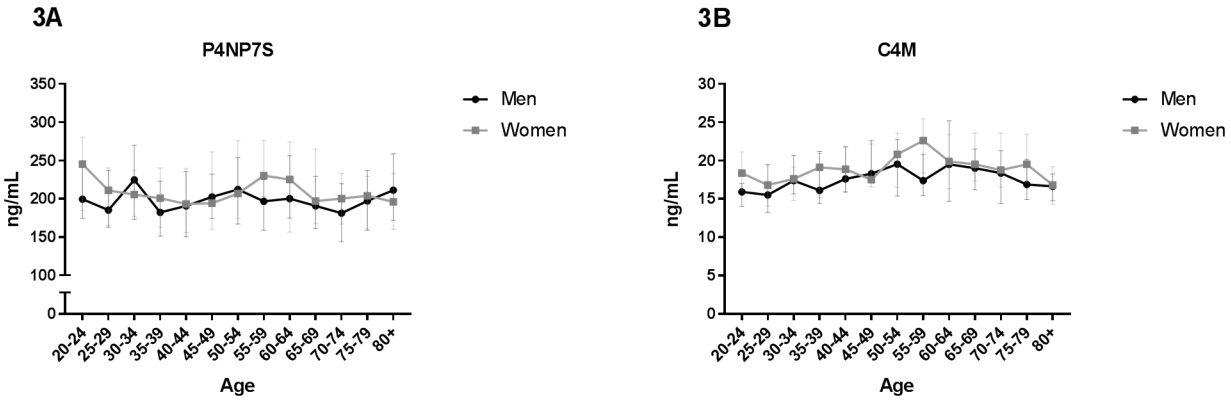

Fig 3. Type IV collagen turnover as function of age. Biomarkers reflecting degradation and formation of type IV collagen were measured in serum from 303 healthy men and 314 healthy women aged 22–86 divided into 5-year age groups. (A) Formation of basement membrane type IV collagen (P4NP7S) and (B) degradation of basement membrane type IV collagen (C4M). Statistical significance of C4M and P4NP7S between each age group was calculated using ANOVA comparing the mean of each group with the mean of every other group and is presented at the different age groups in S1 Table. All data are shown as median and interquartile range.

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Type IV collagen. As with C3M, a significantly higher level of P4NP7S in women at age 55–59 was observed (Table 3). The level of C4M was significant different in men and women at age 20–24 and 55–59 with women having the highest level (Table 3).

Discussion

The present study defines a collagen turnover profile of the main collagens of the interstitial matrix (type I and III collagen) and basement membrane (type IV collagen) as function of age in healthy men and women. The main findings of the study were: 1) age specific changes in collagen turnover was most profound for type I collagen with its formation being strongly age-dependent and 2) sex specific changes in collagen turnover was most apparent during the menopausal and post-menopausal periods with the interstitial matrix and basement membrane being differently regulated. To our knowledge, this is the first time a collagen turnover profile measuring collagen formation and degradation separately, has been established in healthy men and women across the age span of adults.

Type I collagen is the main ECM component of bone, composing 90% of the organic matrix [41] and its formation seems to be strongly age-dependent (Fig 1A). The level of PINP was higher in young men and women compared to older subjects with a progressive decrease until the age around 50. This observation is in accordance with previous studies showing a decrease in women between age 30–35, stable level from age 35–45 followed by an increase until the age of 65 [35,37]. In men, a significant decrease with age has also been observed [37]. The decline in PINP levels until middle-age may reflect a shift from bone modeling (formation) in childhood and early adulthood to bone remodeling (maintenance). As seen in Fig 1B, degradation of type I collagen (C1M) is relatively stable throughout the study period. As C1M is a marker of tissue inflammation and does not represent bone degradation [14], this may explain why C1M is not age-dependent and PINP is. One may argue that elder subjects have more inflammation than younger, hence a rise in C1M late in life was expected. However, the observed results may reflect that the elder subjects in this study are "super" healthy compared to the "normal" elder population.

Type III collagen is one of the main interstitial collagens and structurally similar to type I collagen. Despite being often associated with type I collagen, the formation of type III collagen showed a different pattern with an increase in both men and women after age 45–49 and

Table 3. Sex-specific changes in collagen remodeling.

	PINP				C1M			
Age group	**Median (men)**	**Median (women)**	**Significant**	**P-value**	**Median (men)**	**Median (women)**	**Significant**	**P-value**
20–24	91.8	61.2	Yes	0.02	23.2	28.2	No	0.18
25–29	80.1	53.2	Yes	0.0004	23.0	25.5	No	0.05
30–34	72.9	50.7	Yes	0.001	23.0	25.2	No	0.84
35–39	62.5	35.8	Yes	0.0004	24.6	29.4	No	0.11
40–44	56.3	37.0	Yes	0.001	25.8	20.9	No	0.25
45–49	50.3	38.4	No	0.18	22.0	24.9	No	0.38
50–54	46.7	55.0	No	0.34	22.8	26.0	No	0.82
55–59	44.4	56.2	Yes	0.01	25.6	24.4	No	0.85
60–64	33.2	64.7	Yes	<0.0001	25.2	26.1	No	0.66
65–69	36.1	59.9	Yes	0.001	26.5	26.0	No	0.62
70–74	37.3	48.9	No	0.19	27.0	32.4	No	0.27
75–79	44.6	57.4	No	0.10	22.5	26.3	No	0.67
80+	45.1	62.5	Yes	0.02	26.2	24.4	No	0.90
	PRO-C3				**C3M**			
Age group	**Median (men)**	**Median (women)**	**Significant**	**P-value**	**Median (men)**	**Median (women)**	**Significant**	**P-value**
20–24	8.9	9.1	No	0.80	9.9	11.8	No	0.07
25–29	8.3	8.6	No	0.66	9.2	10.8	No	0.21
30–34	7.8	8.9	No	0.85	10.1	9.7	No	0.68
35–39	8.3	8.0	No	0.25	9.4	10.1	No	0.30
40–44	7.6	8.0	No	0.87	8.3	9.1	No	0.33
45–49	7.0	6.8	No	0.94	9.4	9.1	No	0.78
50–54	7.2	7.4	No	0.70	9.6	11.2	No	0.24
55–59	8.2	6.8	Yes	0.03	9.6	11.9	Yes	0.02
60–64	8.7	7.9	No	0.38	9.9	9.4	No	0.49
65–69	7.3	7.2	No	0.31	9.0	9.7	No	0.20
70–74	8.9	7.2	Yes	0.04	9.7	10.1	No	0.25
75–79	9.5	7.6	Yes	0.01	9.3	9.1	No	0.64
80+	8.9	9.7	No	0.29	10.5	9.7	No	0.34
	P4NP7S				**C4M**			
Age group	**Median (men)**	**Median (women)**	**Significant**	**P-value**	**Median (men)**	**Median (women)**	**Significant**	**P-value**
20–24	199.5	245.2	No	0.20	15.9	18.4	Yes	0.04
25–29	185.3	210.9	No	0.49	15.5	16.8	No	0.56
30–34	224.9	205.6	No	0.52	17.4	17.6	No	0.89
35–39	182.3	200.8	No	0.27	16.1	19.1	No	0.50
40–44	190.6	193.3	No	0.82	17.6	18.9	No	0.70
45–49	202.5	194.2	No	0.72	18.3	17.5	No	0.36
50–54	212.3	206.8	No	0.77	19.6	20.8	No	0.58
55–59	196.7	230.3	Yes	0.02	17.4	22.6	Yes	0.001
60–64	200.3	225.2	No	0.88	19.5	19.9	No	1.00
65–69	190.7	196.9	No	0.31	19.0	19.5	No	0.25
70–74	199.5	245.2	No	0.20	15.9	18.4	Yes	0.04
75–79	185.3	210.9	No	0.49	15.5	16.8	No	0.56
80+	224.9	205.6	No	0.52	17.4	17.6	No	0.89

The level of the individual biomarkers between men and women in the five-year age groups was compared using the Mann-Whitney test. P-values are indicated and represent significant difference ($p \leq 0.05$) between men and women.

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55–59 respectively. As Pro-C3 is a marker of fibrosis [15,29,42], these results suggest that tissue fibrosis increases with advancing age.

Type IV collagen is one of the most abundant components of the basement membrane. No association between age and biomarker level (P4NP7S and C4M) was observed, however C4M in women was significantly increased with time comparing age 25–29 with age 55–59 (Fig 3B, S1 Table). These data suggest that basement membrane degradation is increased in the menopausal period.

Karsdal *et al.*[3] have conducted a study similar to this in rats. They found that type I collagen had an increased turnover in younger rats compared to old rats consistent with our findings. Type III collagen turnover was not significantly influenced by age while type IV collagen degradation was slightly upregulated in younger animals. These results can be translated into the present data in humans which is of importance when conducting pre-clinical and clinical studies in ECM remodeling diseases.

To investigate sex-specific changes in collagen turnover, the level of the individual biomarkers between men and women in the five-year age groups was compared. C3M, P4NP7S and C4M showed a significant difference between men and women in the menopausal period (age 40–60) with women having a higher level of collagen remodeling. PINP and Pro-C3 displayed an increase in women compared to men in the post-menopausal period (after the age of 60). PINP showed a significant lower level in women throughout the pre-menopausal period (before the age of 40). Together, these data suggest that hormonal status might affect collagen remodeling. Whether this is related to estrogen levels or other menopausal mechanisms needs further investigations. However, the fact that the biomarkers decreases again during the post-menopausal period indicates that a specific event during menopause is somehow influencing the collagen turnover. These sex-specific changes in collagen turnover could be associated with diseases that is more prevalent in postmenopausal women, as with osteoporosis and PINP [43].

One limitation of this study is the lack of younger subjects. Studies measuring the amino-terminal pro-peptide of procollagen type III have shown a high level in young children which decreased with advancing age with a short increase around puberty [38,39]. These data may reflect the process of modeling where new tissue is being formed during growth whereas the results of the current study only reflects the end of this process involving a shift towards ECM remodeling. Another limitation is the lack of information on the subject's renal function. We are currently investigating the association between renal function and the collagen turnover biomarkers. Unpublished data from our group show no association between renal function and C1M, PINP, C3M, PRO-C3 and C4M.

Subjects with ECM remodeling disorders, such as fibrosis and cancer, have been shown to have a different ECM turnover compared to healthy individuals [18,22,25,29,33,44] and attention has been drawn towards the involvement of matrix proteins in ECM remodeling disorders [45,46]. For example in cancer, it is now becoming evident that the tumor milieu, i.e. the ECM, is just as important as the tumor itself for tumor progression and metastasis [47,48]. The ability to measure changes in ECM turnover in ECM-involved pathologies, could be an important step towards a better personalized medicine [49,50].

The presented data are important to consider when conducting clinical studies focusing on ECM-related disorders as these biomarkers have been shown to associate with various connective tissue disorders where the ECM balance is skewed [5,15,18,22,25,29,33,44].

Conclusions

In conclusion, collagen turnover is affected by age and sex with the interstitial matrix (type I and III collagen) and the basement membrane (type IV collagen) being differently regulated.

We have established an age- and sex-dependent collagen turnover profile in healthy men and women and the observed changes needs to be accounted for when measuring ECM related biomarkers.

Supporting information

S1 Table. Age-related changes in collagen remodeling. Statistical significance (p-value) for each biomarker calculated using ANOVA comparing the mean of each group with the mean of every other group. Ns refers to no significance. The following biomarkers showed no significance and are therefore not presented: C1M (men), C3M (men), P4NP7S (men and women) and C4M (men).
(DOCX)

S1 Data. Serum biomarker data for each subject.
(XLSX)

Author Contributions

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Writing – original draft: Stephanie N. Kehlet.

Writing – review & editing: Stephanie N. Kehlet, Nicholas Willumsen, Gabriele Armbrecht, Roswitha Dietzel, Susanne Brix, Kim Henriksen, Morten A. Karsdal.

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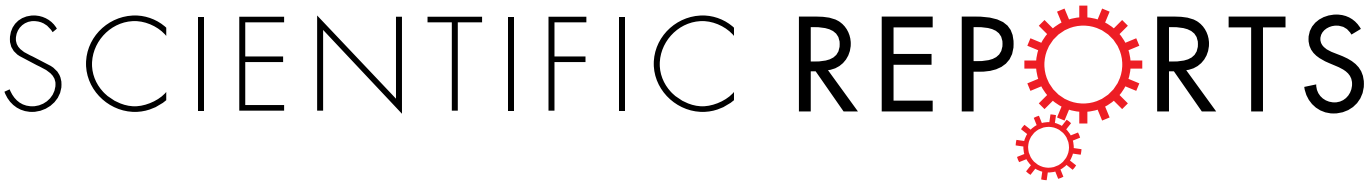
Paper II - Excessive collagen turnover products are released during colorectal cancer progression and elevated in serum from metastatic colorectal cancer patients



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Excessive collagen turnover products are released during colorectal cancer progression and elevated in serum from metastatic colorectal cancer patients

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During cancer progression, the homeostasis of the extracellular matrix becomes imbalanced with an excessive collagen remodeling by matrix metalloproteinases. As a consequence, small protein fragments of degraded collagens are released into the circulation. We have investigated the potential of protein fragments of collagen type I, III and IV as novel biomarkers for colorectal cancer. Specific fragments of degraded type I, III and IV collagen (C1M, C3M, C4M) and type III collagen formation (Pro-C3) were assessed in serum from colorectal cancer patients, subjects with adenomas and matched healthy controls using well-characterized and validated ELISAs. Serum levels of the biomarkers were significantly elevated in colorectal cancer patients compared to subjects with adenomas (C1M, Pro-C3, C3M) and controls (C1M, Pro-C3). When patients were stratified according to their tumour stage, all four biomarkers were able to differentiate stage IV metastatic patients from all other stages. Combination of all markers with age and gender in a logistic regression model discriminated between metastatic and non-metastatic patients with an AUROC of 0.80. The data suggest that the levels of these collagen remodeling biomarkers may be a measure of tumour activity and invasiveness and may provide new clinical tools for monitoring of patients with advanced stage colorectal cancer.

Colorectal cancer (CRC) is the third most common cancer and the fourth most common cause of cancer-related death worldwide, accounting for roughly 600.000 deaths per year[1]. The 5-year survival rate decreases drastically with advanced stages, being 90% for local tumours in initial stages and 12% in advanced stages with metastasis[2]. Clinical symptoms often appear at the late stages when the tumour has started to metastasize resulting in late diagnosis and lower survival rates[3]. CRC screening has resulted in a reduction in mortality[4] but the optimal diagnostic test has not yet been found. The standard fecal occult blood test has a low sensitivity as not all tumours cause bleeding, giving rise to many false negatives. Colonoscopy, though it has a high sensitivity (92–99%) for both pre-malignant lesions and tumours, is invasive, inconvenient for the patients and associated with high-cost[5], consequently inappropriate as screening tool to identify early stages of CRC. Thus alternative diagnostic tools have to be identified.

Serological biomarkers have the advantages of being easy to collect, non-invasive, typically low-cost, and have the ability to be followed over the course of the disease. Identification of serological biomarkers that can aid in early detection, diagnosis, disease monitoring and in individual treatment selection of CRC patients could have a high impact on the patient outcome.

The local microenvironment of a tumour has been shown to play important roles in tumour pathogenesis and there is much focus on the extracellular matrix (ECM) and its remodeling as contributor to malignancy[6,7]. The ECM is constantly being remodeled and in the healthy tissue there is a balanced ratio between degradation

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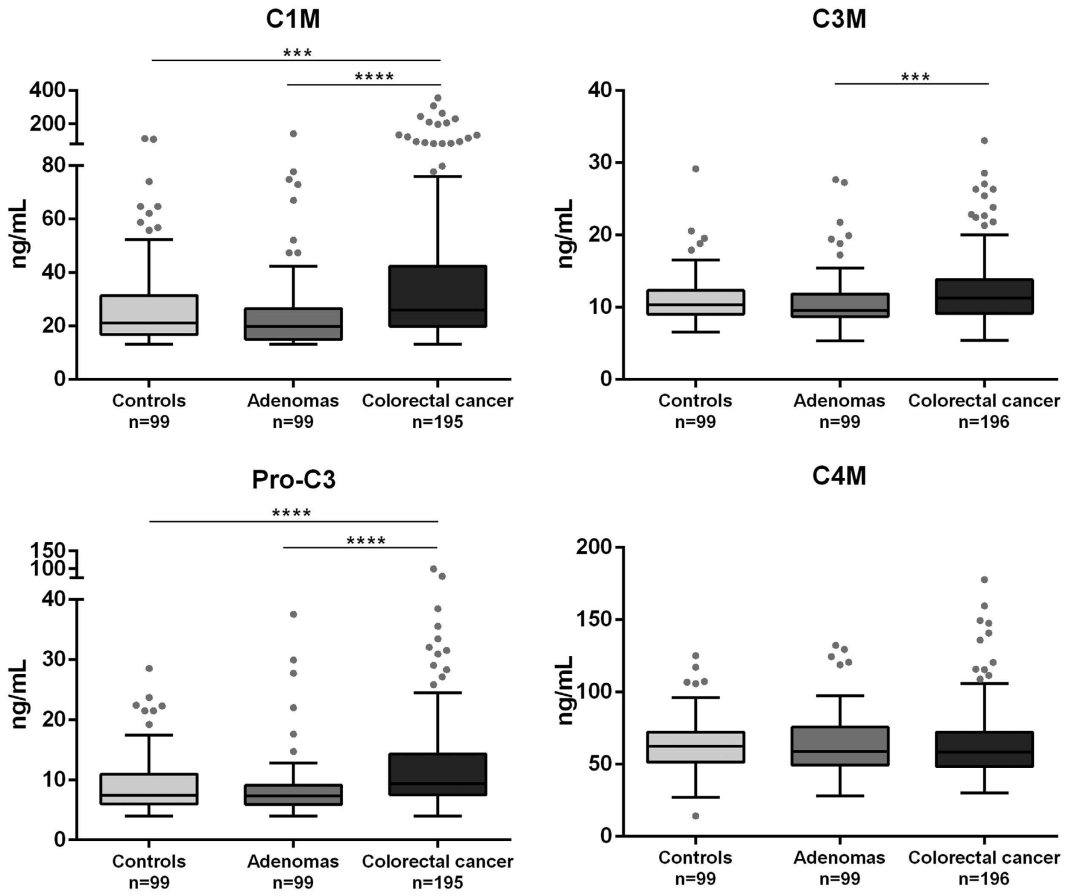

Figure 1. Serum levels of MMP-mediated degradation of type I collagen (C1M), type III collagen (C3M) and type IV collagen (C4M) and formation of type III collagen (Pro-C3). Groups were compared using a Kruskal–Wallis test. The p-values were adjusted to account for multiple comparisons using Dunnett's method. Significance levels: **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

and formation of ECM proteins. Disruption of this homeostasis may act as a driver of cancer development and invasion. Excessive ECM remodeling is characterized both by an increased collagen deposition (desmoplasia) and crosslinking leading to tissue stiffening as well as an increased expression of matrix metalloproteinases (MMPs)[6,8]. As a consequence, increased levels of tissue - and cancer-specific ECM turnover products, so-called neo-epitopes, are released into the circulation making them potential as novel blood-based biomarkers. Many of these proteins have been uniquely modified by the pathology, such as the generation of a unique degradation site by a cancer dependent protease (e.g. MMP) in a signature protein (e.g. collagen) providing a so-called protein fingerprint. This combination is more related to pathogenesis than unmodified proteins and therefore potential candidates for novel biomarkers in cancer[9].

Neo-epitope biomarkers have been shown to have a diagnostic potential in lung cancer[10], breast cancer[11], ovarian cancer[11] and pancreatic cancer[12]. In this study, we investigated whether serum biomarkers reflecting collagen remodeling could differentiate between colorectal cancer patients, subjects with adenomas and healthy controls. Furthermore, we examined if the individual biomarkers or combinations hereof could be used to stratify patients according to their tumour stage.

Results

Collagen remodeling biomarkers are elevated in colorectal cancer patients compared to subjects with adenomas and healthy controls. Serum levels of biomarkers specifically reflecting type I (C1M), type III (C3M) and type IV (C4M) collagen degradation and type III collagen formation (Pro-C3) were measured in healthy controls, subjects with adenomas and colorectal cancer patients. The data are presented in Fig. 1. In detail, the level of C1M and Pro-C3 were significantly elevated in colorectal cancer patients compared to healthy controls (C1M: $p < 0.001$, Pro-C3: $p < 0.0001$) and subjects with adenomas (C1M: $p < 0.0001$, Pro-C3: $p < 0.0001$). C3M was significantly elevated in colorectal cancer patients compared to subjects with adenomas ($p < 0.001$). No significant difference was observed between the groups for C4M.

These data suggest that altered type I and type III collagen remodeling is ongoing in colorectal cancer and not in the healthy tissue and pre-malignant stages. This reflects that an altered collagen turnover and the release of collagen protein fragments to the circulation is a pathological feature of colorectal cancer.

Group	No. of subjects	Gender, % females	Age (years) mean ± SD	Carcinoma *in situ* (n)	Tumour stage I (n)	Tumour stage II (n)	Tumour stage III (n)	Tumour stage IV (n)
Healthy controls	99	64.6	60.1 ± 6.3	–	–	–	–	–
Adenomas	99	26.3	59.8 ± 5.9	–	–	–	–	–
Colorectal cancer cases	196	32.1	63.1 ± 10.4	3	39	47	80	27

Table 1. Patients demographics and clinical profiles.

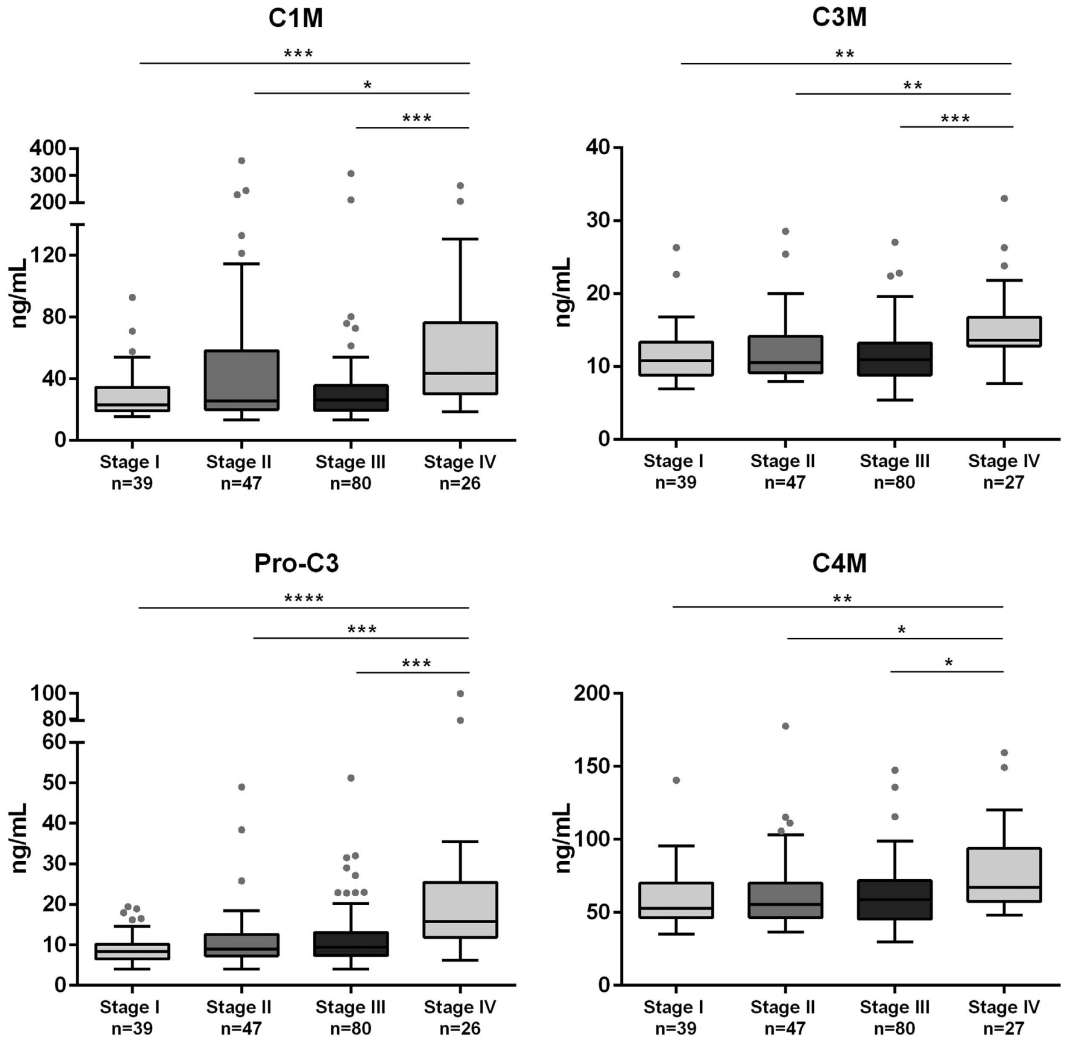

Figure 2. Serum levels of MMP-mediated degradation of type I collagen (C1M), type III collagen (C3M) and type IV collagen (C4M) and formation of type III collagen (Pro-C3) in colorectal cancer patients stratified according to their tumour stage. Groups were compared using a Kruskal–Wallis test. The p-values were adjusted to account for multiple comparisons using Dunnett´s method. Significance levels: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

Collagen remodeling biomarkers are elevated in stage IV colorectal cancer patients. To investigate if the collagen remodeling biomarkers could be used to stratify patients according to their tumour stage, we divided the colorectal cancer patients into four groups according to their tumour stage, excluding the three patients with carcinoma *in situ* (Table 1). The data are presented in Fig. 2. All four biomarkers were significantly elevated in patients with stage IV tumours compared to all other stages ($p < 0.05$ to <0.0001, dependent on the marker). No differences were observed among patients diagnosed with stage I-III. The high level of all markers in stage IV patients indicates that collagen remodeling takes part in colorectal cancer progression and invasion and that these four biomarkers are a measure of tumour activity in advanced stages.

Discriminative power of collagen remodeling biomarkers for metastatic and non-metastatic colorectal cancer. The area under the receiver operating characteristics (AUROC) and logistic regression

Biomarker	Cut-off value (ng/mL)	Sensitivity	Specificity	AUROC (95% CI)	p-value
C1M	30.7	77.8	67.9	0.74 (0.62–0.87)	<0.0001
C3M	11.9	85.7	65.5	0.78 (0.66–0.89)	<0.0001
C4M	52.3	96.4	43.0	0.74 (0.61–0.87)	<0.0001
Pro-C3	10.4	88.9	66.1	0.79 (0.65–0.91)	<0.0001

Table 2. Discriminative performance of collagen remodeling biomarkers for detecting metastatic colorectal cancer (stage IV) versus non-metastatic (stage I–III).

was used to evaluate the discriminative power of the four biomarkers for metastatic and non-metastatic colorectal cancer (Table 2). As shown in Table 2, Pro-C3 and C3M displayed the highest diagnostic accuracy in relation to identify metastatic tumours from non-metastatic tumours with an AUROC of 0.79 and 0.78, respectively. For each biomarker, the optimal cut-off value was determined by ROC curve analyses and the sensitivity and specificity are shown in Table 2.

We performed logistic regression analyses to calculate the best diagnostic value by combining all biomarkers and to correct for confounding factors (age and gender). Tumour stage (non-metastatic vs metastatic (stage IV)) was included as dependent variable and the four biomarkers, age and gender as explanatory variables. In this logistic regression model, C3M and Pro-C3 were found to be statistically significant (C3M: $p = 0.006$, Pro-C3: $p = 0.002$) and the diagnostic value for separating metastatic patients from non-metastatic patients increased to an AUROC of 0.80 (95% CI: 0.65–0.91), a sensitivity of 92.6% and a specificity of 67.3% when 0.097 was used as cut-off value. Based on the logistic regression model, we propose the following algorithm which could be used as an aid for clinicians to manage patients that have been diagnosed with colorectal cancer and to assess the severity and tumour progression/activity by collecting a blood sample and measure Pro-C3 and C3M concentrations in serum: Tumour activity score $= -4.96 + 0.16 \times \text{C3M} + 0.07 \times \text{Pro-C3}$. Applying this model to the data from the current study, an AUC of 0.83 is achieved for detecting advanced stage colorectal cancer patients. This model/algorithm needs to be further validated in large clinical studies in order to identify the appropriate score for advanced stage colorectal cancer.

Discussion

In the present study we measured a panel of four collagen remodeling biomarkers in serum from patients with colorectal cancer, subjects with adenomas and healthy controls. To our knowledge, this is the first study investigating the diagnostic potential of type I, III and IV collagen turnover biomarkers in colorectal cancer. The main finding was that type I, III and IV collagen remodeling biomarkers were significantly elevated in stage IV metastatic colorectal cancer compared to all other stages. Combination of all four biomarkers resulted in differentiation between metastatic and non-metastatic patients with an AUROC of 0.80, while Pro-C3 alone gave rise to an AUROC of 0.79. Together, the data demonstrate that these biomarkers, especially Pro-C3, can be used to assess tumour activity/invasiveness in patients diagnosed with colorectal cancer based on a blood sample. Moreover, the data indicate that the excessive collagen degradation (C1M, C3M and C4M) and formation (Pro-C3) observed in metastatic colorectal cancer patients could play a role in cancer pathogenesis, either as a driver of tumour progression or by being a consequence hereof.

Elevated levels of collagen-derived fragments have been detected in other cancer types, including lung-[10], breast-[11], ovary-[11] and pancreatic cancer[12]. Our results together with these data support that ECM remodeling is an important part of and/or contributes to cancer development and progression[6,7]. In fact, it is becoming widely accepted that ECM remodeling and dysregulation directly affect the hallmarks of cancer as defined by Hanahan and Weinberg in 2000[13–15].

C1M and C3M reflects interstitial matrix remodeling, where the C1M assay measures a type I collagen degradation fragment generated by cleavage with MMP-2, −9 and −13[16] and the C3M assay measures a type III collagen degradation fragment generated by cleavage with MMP-9[17], respectively. C4M reflects basement membrane remodeling and measures a type IV collagen α1- chain fragment generated by cleavage with MMP-12[18]. These four MMPs (2, 9, 12 and 13) have in fact been associated with colorectal cancer supporting an increased collagen degradation[19–21]. The disruption of the basement membrane and the interstitial matrix is an essential prerequisite for tumour invasion. For tumour cells to metastasize, they must not only degrade the matrices of the colon wall, but also the matrices of lymphatic system, blood vessels and at the secondary site. Degradation of the interstitial matrix paves the way for the tumour cells thereby enhancing migration across the matrix to nearby lymph nodes and blood vessels[19,22]. In the current study, C1M, C3M and C4M were significantly elevated in stage IV patients. In theory, we would expect the basement membrane and interstitial matrix of the colon wall to be degraded at early stages of invasion (stage I-III), however this was not detectable in serum by the current biomarker assays. This may be explained by a lower MMP activity at earlier tumour stages or perhaps by increased cellular uptake of collagen fragments in initial stages. Our results might rely on an ongoing degradation of both the basement membrane and interstitial matrix of the colon wall leading to tumour progression into deeper tissues and excessive release of collagen turnover products into the circulation as a consequence of metastasis. At this stage, the basement membrane and interstitial matrix of the lymphatic system, blood vessels and the secondary site might also have been breached, likewise resulting in enhanced levels of collagen fragments detectable in serum.

Pro-C3 measures the true formation of type III collagen as the antibody is directed against the cleavage site of the N-terminal collagen III pro-peptide[23] which are released during collagen formation and maturation. This marker has been extensively studied in liver fibrosis as a biomarker of progression and burden of disease[24–27]. Cox

and Erler[28] have recently reviewed the link between fibrosis and tumour metastasis. It was discussed that tumour cells can prepare a pre-metastatic niche in a manner that resembles the development of fibrosis. In order to convert an unfavorable tumour environment of a distant site into favorable surroundings, tumour cells secrete a lot of factors before and upon arrival at the metastatic niche. This includes factors that promote increased collagen deposition and crosslinking as observed in fibrotic tissue. The increased formation of type III collagen measured in stage IV patients may originate from the metastatic niche either as a result of tissue priming or upon tumour cell arrival. This is in line with several studies showing the importance of increased collagen deposition in the metastatic niche for further tumour progression[29–32].

Identification of neo-epitope biomarkers that directly reflect the changes of the extracellular matrix during cancer could be a new way handling the medical need in colorectal cancer. Each neo-epitope results from a specific pathological process giving rise to a very unique and specific biomarker[9]. The fact that the biomarkers were significantly elevated in stage IV patients and had a high discriminative value for metastatic patients, clearly indicates that the level of collagen remodeling biomarkers is a measure of tumour activity and severity. Therefore, it is likely that these biomarkers could be novel candidates as tools to monitor colorectal cancer patients with advanced stages. Such tools would be valuable since it is often the metastases rather than the primary tumour which cause the poor prognosis of cancer patients[33]. Blocking the invasion of cancer and its growth at the distant site could therefore improve the patient outcome. The ECM is in fact an emerging target for cancer drug therapy[34–36]. Several preclinical studies have investigated key ECM proteins as targets for novel cancer drugs (reviewed in ref. 34). The present biomarkers have a potential as treatment of efficacy biomarkers in relation to ECM modifying drugs. Since the four biomarkers investigated here reflect excessive ECM remodeling, they might be able to identify patients with a densely remodeled stroma who would benefit most from ECM targeting drugs. Furthermore, the biomarkers could be used to monitoring advanced stage patients after therapy, as we hypothesize that circulating levels would decrease after successful treatment of the tumour creating a homeostatic tissue environment and then increase again if there is recurrence. Carcinoembryonic antigen (CEA) is the most widely used serum biomarker in patients with colorectal cancer[37]. The poor sensitivity of this test for early stage tumours makes it unsuitable for screening and early detection and the main use of CEA in colorectal cancer is for surveillance and monitoring before and after surgery/treatment. However the usefulness of CEA is controversial[37–39]. CEA is an oncofetal antigen whereas the investigated biomarkers are designed as a measure of tumour activity, invasiveness and pathology specific which may increase specificity and sensitivity. However, this has to be validated in larger cohorts together with CEA measurements.

One limitation of this study is its cross-sectional nature. To fully elucidate the prognostic applicability and treatment efficacy of the collagen turnover biomarkers, larger longitudinal studies are needed. Another limitation is the absence of a replication/validation cohort which would have supported the findings. However, we find these preliminary results to be important steps towards identifying novel blood-based biomarker tools in colorectal cancer.

In conclusion, colorectal cancer is a field with an urgent need for biomarkers that can aid in diagnosis and prognosis. We have assessed a panel of biomarkers reflecting collagen turnover of the extracellular matrix in patients with colorectal cancer, subjects with adenomas and controls. All markers were significantly elevated in stage IV patients suggesting that excessive collagen turnover takes part in cancer progression and metastasis. As these markers are designed to measure tumour activity, they may increase the understanding of cancer pathology and, if validated in larger clinical studies, provide new clinical tools for patient monitoring and efficacy of treatment.

Methods

Patient samples. This study included serum samples from 394 individuals comprising 99 healthy controls, 99 patients with adenomas and 196 patients with colorectal cancer at different stages. For all individuals, 9 ml of blood was collected and centrifuged to separate serum. Then, samples were divided into single-use aliquots and preserved at −80 °C. All controls and patients were recruited at the Bellvitge University Hospital (Barcelona, Spain) and all samples were handled the exact same way. Written informed consent was obtained from all patients and the Ethics Committee of the Bellvitge University Hospital approved the protocol with reference PR073/11. The study was carried out in accordance with ICH-GCP and according to the Declaration of Helsinki. Table 1 shows the detailed characteristics of healthy controls, adenomas and colorectal cancer patients.

Protein Fingerprint biomarker analysis. The Protein Fingerprint biomarkers of matrix metalloproteinase (MMP) degraded type I, type III and type IV collagen (C1M, C3M, C4M) and type III collagen formation (Pro-C3) were assessed in serum as previously described[16–18,23]. Briefly, 96-well pre-coated streptavidin plates were coated with biotinylated synthetic peptides specific for the protein of interest and incubated for 30 minutes at 20 °C. 20 μL of standard peptide or pre-diluted serum sample were added to designated wells followed by the addition of peroxidase-conjugated specific monoclonal antibodies and incubated for 1 h at 20 °C or overnight at 4 °C. Finally, tetramethylbenzinidine (TMB) (cat. 438OH, Kem-En-Tec Diagnostics, Denmark) was added to each well and the plates were incubated for 15 minutes at 20 °C. All incubation steps included shaking at 300 rpm and after each incubation step, the plates were washed five times with wash buffer (20 mM Tris, 50 mM NaCl, pH 7.2). The enzymatic reaction was stopped by adding 0.18 M H_2SO_4 and absorbance was measured at 450 nm with 650 nm as reference. A calibration curve was plotted using a 4-parameter logistic curve fit.

Statistical analysis. The levels of the individual biomarkers in serum samples in each group (controls, adenomas and cases) were compared using a Kruskal–Wallis test (non-parametric test). The p-values were adjusted to account for multiple comparisons using Dunnett´s method.

The diagnostic power of individual and combined markers was investigated by the area under the receiver operating characteristics (AUROC). Sensitivity and specificity were determined for appropriate cut-off values based on the ROC curves. Logistic regression analyses were carried out to calculate the best diagnostic value when combining all biomarkers and correcting for age and gender. To correct for over fitting, an internal validation was conducted by calculating the bootstrap optimism-corrected AUC (the data were resampled 1000 times with the bootstrapping method).

Unless otherwise stated, data are shown as Tukey box plots, where the boxes represent the 25th, 50th and 75th percentiles. The whiskers represent the lowest and highest value, except outliers, which are higher than 1.5 times the 75th percentile or lower than 1.5 times the 25th percentile. P-values <0.05 were considered significant. Statistical analyses were performed using the R statistical computing software (http://www.r-project.org), MedCalc Statistical Software version 12 (MedCalc Software, Ostend, Belgium) and GraphPad Prism version 6 (GraphPad Software, Inc., CA, USA). Graphs were designed using GraphPad Prism version 6 (GraphPad Software, Inc., CA, USA).

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Author Contributions

S.N.K. was the main author of the manuscript. S.N.K. carried out the measurements, data analysis and statistical analysis in discussion with R.S. and V.M., R.S. and V.M. provided the samples and patient information. R.S., S.B., D.J.L., M.A.K. and V.M. critical revised the manuscript and approved the final manuscript.

Additional Information

Competing financial interests: D.J. Leeming and M.A. Karsdal are employed at Nordic Bioscience A/S which is a company involved in discovery and development of biochemical biomarkers. M.A. Karsdal owns stocks at Nordic Bioscience. S.N. Kehlet, R. Sanz-Pamplona, S. Brix and V. Moreno reports no conflict of interest.

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Additional results 1 - Prognostic evaluation of a new class of liquid biopsy biomarkers reflecting type III and VI collagen formation in patients with metastatic colorectal cancer

This study was presented as a poster at ASCO 2018, Chicago and published in the Danish medical newspaper "Dagens Medicin" the 5th of June 2018 (aim 1). The study is still ongoing with the aim of confirming the findings using a second larger cohort.

Introduction

The local microenvironment of a tumor plays an important role in colorectal cancer (CRC) progression (2,3). The tissue composition and expression of proteins changes during tumor development and invasion and as a result, unique ECM protein fragments are released to the circulation. These ECM turnover biomarkers have previously shown to be relevant biomarkers in CRC (182, paper II). A desmoplastic stroma surrounding the tumor, characterized by excessive collagen deposition, can result in reduced drug delivery into the tumor, leading to poor prognosis and lack of therapy response (183). Therefore, biomarkers reflecting an increased desmoplastic reaction hold a prognostic potential. In this study, we present a new class of liquid biopsy proteins, reflecting collagen formation (desmoplasia), and evaluate their prognostic use in metastatic CRC.

Study cohort and methods

Pro-peptides from type III collagen (PRO-C3) and type VI collagen (PRO-C6) were measured with ELISAs in pre-treatment (standard of care chemotherapy) serum from 40 patients with metastatic CRC and 40 healthy donors. Biomarker levels in patients and healthy donors were compared using unpaired, two-tailed Mann-Whitney test. The biomarkers were further evaluated by univariate Cox-regression analysis for their association with overall survival (OS) and progression-free-survival (PFS).

Results

To confirm previous findings, serum levels of PRO-C3 and PRO-C6 were compared in CRC patients and healthy donors. A seen in figure 7, significantly elevated levels of PRO-C3 and PRO-C6 in patients with metastatic CRC compared to healthy donors were demonstrated (PRO-C3: 11.5 ng/mL vs. 6.8 ng/mL, $p<0.0001$, PRO-C6: 8.0 ng/mL vs. 5.9 ng/mL, $p<0.0001$).

We next investigated the prognostic use of collagen formation biomarkers. The ability of PRO-C3 and PRO-C6 at baseline to predict OS and PFS is shown in Table 6, which summarizes hazard ratios (HR) with 95%CIs calculated from univariate Cox proportional-hazard regression. When divided into quartiles (Q1-Q4), a step-wise increase in hazard ratio was observed with increasing levels of PRO-C3 and PRO-C6. High levels (Q4) of PRO-C3 and PRO-C6 were most predictive of OS with a HR of 8.7 and 6.8, respectively. Levels of each

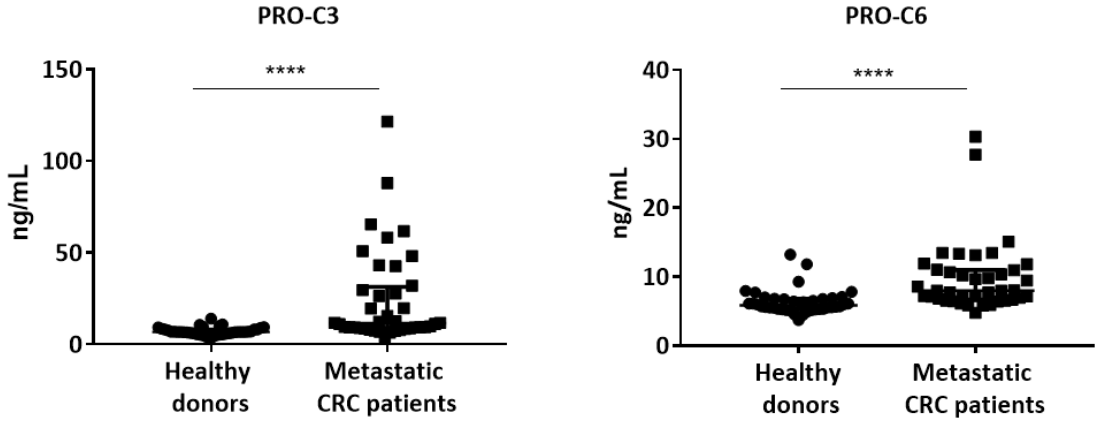

Figure 7. Serum levels of pro-peptides from type III collagen (PRO-C3) and type VI collagen (PRO-C6). Groups were compared using a Mann-Whitney test. Error bars are shown as median with interquartile range. Significance level: ****: p<0.0001.

biomarker in Q2-Q4 were also able to predict PFS, although not significantly for PRO-C3. This may be due to the low number of subjects in each group (n=10). Figure 8 shows Kaplan-Meier OS and PFS curves within the follow-up period according to baseline levels. The median OS was 266 or 213 days in biomarker high patients (75th percentile) vs. 1330 or 979 days in biomarker low patients (25th percentile) for PRO-C3 and PRO-C6, respectively. The median PFS was 251 or 267 days in biomarker high patients (75th percentile) vs. 329 or 496 days in biomarker low patients (25th percentile) for PRO-C3 and PRO-C6, respectively.

Table 6. Association between PRO-C3 and PRO-C6 at baseline and overall survival (OS) and progression free survival (PFS) for metastatic CRC patients. Hazard ratios (HR) were calculated by univariate Cox proportional-hazard regression. Biomarkers were divided into quartiles with the lowest quartile (Q1) used as a reference to calculate the HR for patients in the medium and upper quartiles (Q2-Q4).

Variable	Quartiles	HR (OS)	95% CI (OS)	p-value (OS)	HR (PFS)	95% CI (PFS)	p-value (PFS)
PRO-C3	Q1: 4.2 – 8.8 ng/mL						
	Q2: 8.9 – 11.2 ng/mL	2.4	0.8 – 7.1	0.12	1.8	0.6 – 5.4	0.3
	Q3: 11.8 – 29.6 ng/mL	3.6	1.2 – 10.6	0.02	1.4	0.5 – 3.8	0.5
	Q4: 32.2 – 121.6 ng/mL	8.7	2.7 – 28.2	0.0004	2.3	0.7 – 7.1	0.2
PRO-C6	Q1: 4.8 – 6.6 ng/mL						
	Q2: 6.8 – 8.0 ng/mL	2.9	1.0 – 8.5	0.05	3.1	1.0 – 9.3	0.048
	Q3: 8.0 – 10.9 ng/mL	2.7	0.9 – 7.5	0.07	3.6	1.2 – 10.8	0.02
	Q4: 11.0 – 30.3 ng/mL	6.8	2.3 – 20.3	0.0006	2.6	0.8 – 9.0	0.12

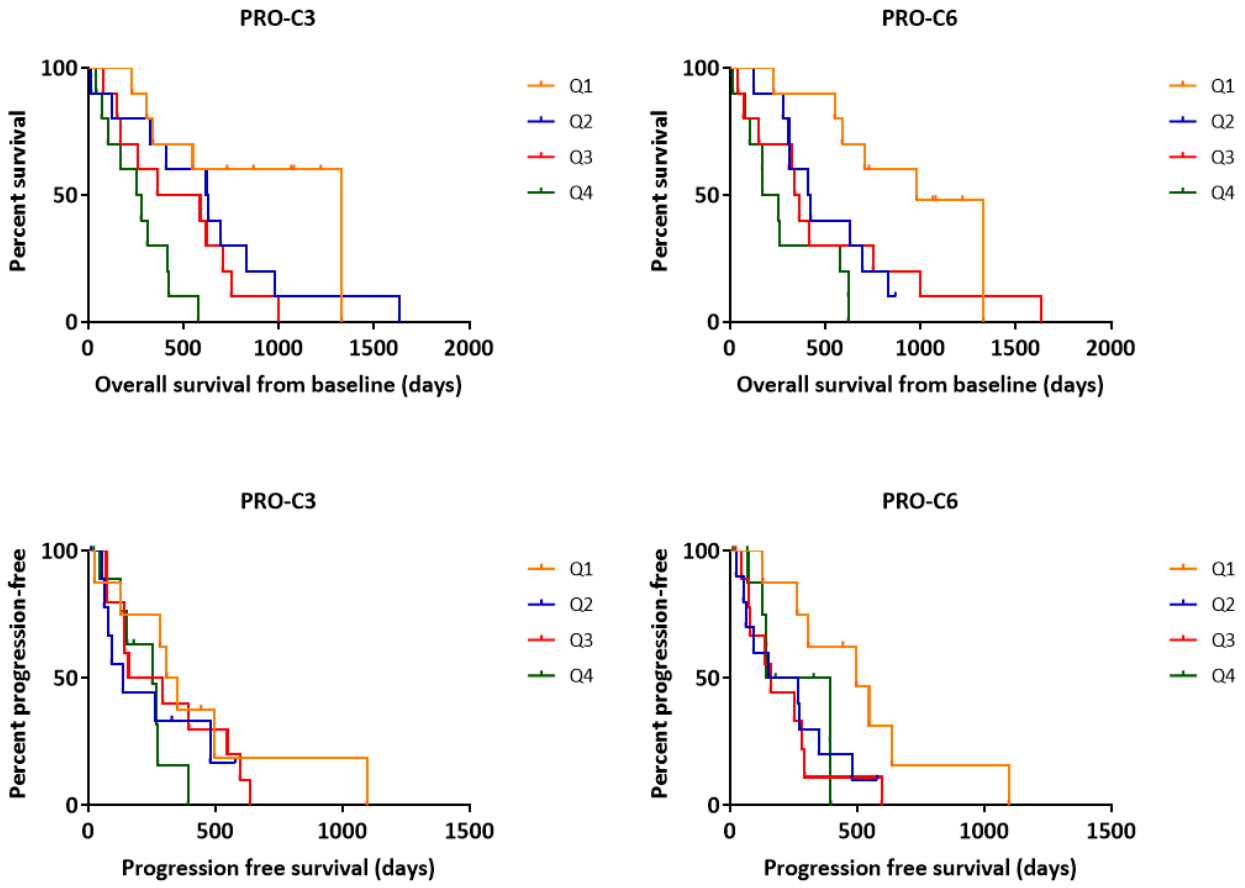

Figure 8. Medium and high levels of PRO-C3 and PRO-C6 at baseline is associated with shorter overall survival and progression free survival. Kaplan-Meier curves illustrate the overall survival and progression free survival for patients within the follow-up period according to baseline levels. Patients were divided into quartiles according to their biomarker levels.

Conclusion

This study evaluated the prognostic use of collagen pro-peptides (surrogate markers for collagen formation) that are released into the circulation as a consequence of tumorigenesis. High serum levels of PRO-C3 and PRO-C6 were significantly associated with poor OS and shorter PFS in patients with metastatic CRC indicating a prognostic potential. The results may suggests that increased collagen deposition around the tumor, limits cancer therapy delivery into the tumor, resulting in a lack of response to therapy.

4. Neo-epitope protein fragments reflecting structural changes in the ECM shows potential as novel liquid biopsy biomarkers in cancer – development of novel immunoassays

This chapter summarizes the findings from paper III, IV and additional results 2 that present the development of novel immunoassays targeting neo-epitope protein fragments and their potential as novel biomarkers in cancer (aim 2).

4.1 Summary of paper III, IV and additional results 2

Rationale

The pathological influence of increased ECM remodelling in cancer is well-established and several ECM proteins have been shown to play a direct role in tumor progression. The papers presented in this chapter focus on decorin, which is one of the most abundant proteoglycans of the extracellular matrix, SPARC, a collagen chaperone know to be essential for proper fibril formation and type XI collagen, a fibrillary collagen shown to be a highly specific marker for CAFs. In paper III, an ELISA targeting a degraded fragment of decorin was developed. The biomarker potential of this neo-epitope was investigated in fibrotic lung disorders, including lung cancer. Paper IV describes the development of an ELISA targeting a cleavage site of SPARC known to modulate collagen binding and its biological relevance in patients with lung cancer, idiopathic pulmonary fibrosis (IPF) and chronic obstructive pulmonary disease (COPD). In addition, the ability of SPARC to protect fibrillar collagens from proteolytic degradation was investigated *in vitro*, potentially adding a new collagen chaperone function to SPARC. In additional results 2, an electro-chemiluminescence immunoassay (ECLIA) was developed targeting the pro-peptide of type XI collagen.

Methods and patient cohorts

Monoclonal antibodies were raised against the three neo-epitopes and used in competitive immunoassay settings. The assays were optimized according to buffer, temperature and incubation time, choosing the conditions with the highest sensitivity and specificity. The assays were technically validated by determining specificity, inter- and intra-assay precision, dilution recovery, accuracy, analyte stability and interference.

The pathological relevance and biomarker potential of the decorin and SPARc assays were evaluated using pilot cohorts. In paper III, three cohorts were included. Cohort 1 consisted of 8 lung cancer patients, 8 IPF patients, 8 COPD patients, 8 colonoscopy-negative controls and 20 healthy controls. Cohort 2 included 12 non-small cell lung cancer (NSCLC) patients, 8 small-cell lung cancer (SCLC) patients and 43 healthy controls. Cohort 3 consisted of 116 IPF patients and 38 healthy controls. Paper IV included two cohorts. Cohort 1 consisted of patients with lung cancer (n=8), IPF (n=7), COPD (n=8) and healthy colonoscopy-negative

controls with no symptomatic or chronic disease (n=6). Cohort 2 included 40 patients with different stages of lung cancer, and 20 age- and gender-matched healthy colonoscopy-negative controls with no symptomatic or chronic disease.

Main findings and conclusions – aim 2

All three assays were technically robust and highly specific for the neo-epitope of interest. The decorin fragment was significantly elevated in lung cancer patients ($p<0.0001$) and IPF patients ($p<0.001$) when compared to healthy controls. The diagnostic power for differentiating lung cancer patients and IPF patients from healthy controls was 0.96 and 0.77. The SPARC fragment was significantly elevated in lung cancer patients when compared to healthy subjects (cohort 1: $p=0.0005$, cohort 2: $p<0.0001$). No significant difference was observed for IPF and COPD patients compared to healthy subjects. In addition, recombinant SPARC was able to completely inhibit interstitial collagen degradation adding a new collagen chaperone to SPARC.

In conclusion, three highly specific immunoassays were developed and a pathological relevance was shown for the decorin and SPARC fragment. These data suggest that structural changes to the ECM plays a pathological role in tumorigenesis and biomarkers, reflecting these changes have potential as novel liquid-biopsy biomarkers in cancer. Whether their use is diagnostic, prognostic or predictive needs further evaluation in larger clinical cohorts.

4.2 Paper III, IV and additional results 2

Paper III - Cathepsin-S degraded decorin are elevated in fibrotic lung disorders – development and biological validation of a new serum biomarker

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BMC Pulmonary Medicine

RESEARCH ARTICLE Open Access

Cathepsin-S degraded decorin are elevated in fibrotic lung disorders – development and biological validation of a new serum biomarker

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Abstract

Background: Decorin is one of the most abundant proteoglycans of the extracellular matrix and is mainly secreted and deposited in the interstitial matrix by fibroblasts where it plays an important role in collagen turnover and tissue homeostasis. Degradation of decorin might disturb normal tissue homeostasis contributing to extracellular matrix remodeling diseases. Here, we present the development and validation of a competitive enzyme-linked immunosorbent assay (ELISA) quantifying a specific fragment of degraded decorin, which has potential as a novel non-invasive serum biomarker for fibrotic lung disorders.

Methods: A fragment of decorin cleaved in vitro using human articular cartilage was identified by mass-spectrometry (MS/MS). Monoclonal antibodies were raised against the neo-epitope of the cleaved decorin fragment and a competitive ELISA assay (DCN-CS) was developed. The assay was evaluated by determining the inter- and intra-assay precision, dilution recovery, accuracy, analyte stability and interference. Serum levels were assessed in lung cancer patients, patients with idiopathic pulmonary fibrosis (IPF), patients with chronic obstructive pulmonary disease (COPD) and healthy controls.

Results: The DCN-CS ELISA was technically robust and was specific for decorin cleaved by cathepsin-S. DCN-CS was elevated in lung cancer patients ($p < 0.0001$) and IPF patients ($p < 0.001$) when compared to healthy controls. The diagnostic power for differentiating lung cancer patients and IPF patients from healthy controls was 0.96 and 0.77, respectively.

Conclusion: Cathepsin-S degraded decorin could be quantified in serum using the DCN-CS competitive ELISA. The clinical data indicated that degradation of decorin by cathepsin-S is an important part of the pathology of lung cancer and IPF.

Keywords: Decorin, Cathepsin-S, Extracellular matrix, Cancer, Idiopathic pulmonary fibrosis, Serum biomarker

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Background

Idiopathic pulmonary fibrosis (IPF), chronic obstructive pulmonary disease (COPD) and lung cancer are lung pathologies which are characterized by excessive accumulation of extracellular matrix (ECM) leading to loss of tissue homeostasis and progressive disease phenotype [1–3]. Biomarkers which reflect these processes may therefore play an important role in identifying patients with rapid disease progression. Decorin is a member of the small leucine-rich proteoglycan (SLRP) family and is one of the most abundant proteoglycans of the interstitial matrix. The protein is mainly secreted and deposited by fibroblasts. It consists of a single covalently attached N-terminal glycosaminoglycan (GAG) chain, composed of either dermatan or chondroitin sulfate, and 12 leucine-rich tandem repeats representing the protein core [4–6].

Due to its diverse ECM protein binding partners and its regulation of cell growth and cell differentiation, decorin has been named as "the guardian from the matrix" [7] recognizing the significance of decorin in tissue homeostasis. The main ECM binding partners are fibrillar collagens (type I, II, III and VI) and decorin has shown to play a role in the regulation of fibrillogenesis and stabilization of fibrils, and may act as a central player in collagen assembly/turnover and consequently tissue homeostasis [8, 9]. Supporting this, decorin knock-out in mice results in abnormal collagen fibril formation and enhanced collagen degradation [10].

In addition to playing a role in collagen fibril formation in the interstitial ECM, decorin sequesters multiple growth factors, such as TGF-beta and directly antagonizes several members of the receptor tyrosine kinase family, including the epidermal growth factor receptor (EGFR) and insulin-like growth factor receptor I (IGF-IR) [7, 11–14]. As a consequence, decorin regulates survival, migratory, proliferative and angiogenic signaling pathways.

Decorin's ability to modulate various signal transduction pathways has given it a valid reputation within cancer and several studies have revealed decorin as a tumor repressor which counteracts tumorigenic and angiogenic growth [15]. Furthermore, reduced decorin within the tumor stroma is a poor prognostic factor of invasive breast-, lung- and soft tissue cancers as well as in myeloma [5, 16–18].

Decorin appears to have a protective role in cancer and has also been shown to have anti-fibrotic properties. Fibrosis is characterized by an increased and disorganized deposition of ECM proteins resulting in loss of tissue and organ function. One of the key pro-fibrotic mediators is TGF-beta, a chemotactic factor for fibroblasts enhancing the synthesis of ECM proteins. As decorin is an inhibitor of TGF-beta, numerous studies have investigated the decorin's potential to block the fibrotic response and decorin has shown to reduce tissue fibrosis in kidney and lung in multiple disease models [19–21].

Increased ECM remodeling and protease-mediated degradation of ECM proteins is a well-documented and significant component of cancer pathology and lung fibrosis [1–3, 22]. We hypothesize that degradation of decorin may have biomarker potential in these pathologies as degradation of decorin might inactivate and disrupt its anti-tumor and anti-fibrotic capabilities.

A decorin fragment was previously identified in human knee articular cartilage using mass spectrometry (MS/MS) [23]. The aim of this work was to develop a competitive enzyme-linked immunosorbent assay (ELISA) targeting the specific degraded fragment of decorin, identify the protease generating this fragment, and to investigate the biomarker potential of this fragment in serum from patients with various lung pathologies.

Methods

Selection of peptides

The following cleavage site (↓) on decorin was previously identified in human knee articular cartilage using mass spectrometry and published by Zhen et al. [23]: $_{72}$LDK↓VPKDLPPDTT$_{84}$ located in the first leucine rich repeat of the protein.

In order to generate an antibody specific for the N-terminal of the cleavage fragment, a sequence of 10 amino acids adjacent to the site was chosen as the target: ↓$_{75}$VPKDLPPDTT$_{84}$. This amino acid sequence was used to design the standard peptide. The sequence was blasted for homology to other human secreted extracellular matrix proteins using NPS@: Network Protein Sequence Analysis with the UniprotKB/Swiss-prot database [24].

Synthetic peptides used for monoclonal antibody production and validation of the ELISA assay were purchased from Chinese Peptide Company (Hangzhou, China) and Genscript (Piscataway, NJ, USA) and shown in Table 2. A biotinylated peptide (VPKDLPPDTT-biotin) was included as a coating peptide on streptavidin-coated ELISA plates. The specificity of the antibody was tested by including an elongated standard peptide with an additional amino acid added to the N-terminal of the peptide sequence (KVPKDLPPDTT), as well as a non-sense standard peptide (DSSAPKAAQA) and a non-sense biotinylated coating peptide (biotin-DSSAPKAAQA) in the assay validation. The immunogenic peptide (VPKDLPPDTT-KLH) was generated by covalently cross-linking the standard peptide to Keyhole Limpet Hemocyanin (KLH) carrier protein using Succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate, SMCC (Thermo Scientific, Waltham, MA, USA, cat.no. 22336).

Monoclonal antibody production

Four to six week old Balb/C mice were immunized by subcutaneous injection of 200 μL emulsified antigen containing 50 μg immunogenic peptide (VPKDLPPDTT-KLH) mixed with Freund's incomplete adjuvant (Sigma-Aldrich, St. Louis, MO, USA). Consecutive immunizations were performed at 2-week intervals until stable sera titer levels were reached. The mouse with the highest titer rested for four weeks and was then boosted with 50 μg immunogenic peptide in 100 μL 0.9% NaCl solution intravenously. Hybridoma cells were produced by fusing spleen cells with SP2/0 myeloma cells as previously described [25]. The resultant hybridoma cells were then cultured in 96-well microtiter plates and standard limited dilution was used to secure monoclonal growth. The supernatants were screened for reactivity using the biotinylated peptide (VPKDLPPDTT-biotin) as coating agent in the competitive immunoassays.

Clone characterization

The reactivity of the monoclonal antibodies was evaluated by displacement using human serum samples and the standard peptide (VPKDLPPDTT) in a preliminary ELISA using 10 ng/mL biotinylated coating peptide on streptavidin-coated microtiter plates (Roche, Basel, Switzerland, cat. #11940279) and the supernatant from the antibody producing monoclonal hybridoma cells. The clone with the best reactivity towards the standard peptide was purified using protein-G-columns according to the manufacturer's instructions (GE Healthcare Life Sciences, Little Chalfont, UK, cat. #17–0404-01).

Cleavage of decorin in vitro

Reconstituted human recombinant decorin (ACRO Biosystems, Newark, DE, USA, cat. # DE1-HS223) was diluted to a final concentration of 100 μg/mL in cathepsin buffer (100 mM sodium phosphate, 2 mM DTT, 0.01% Brij-35, pH 7.4), MMP-buffer (50 mM Tris-HCL, 200 mM NaCl, 10 mM $CaCl_2$, 100 μM ZnAc, pH 7.5) or ADAMTS-5 buffer (50 mM Tris, 100 mM NaCl, 5 mM $CaCl_2$, 0.05% Brij-35, pH 7.5). The solutions incubated at 37 °C for 1 h, 24 h and 72 h with or without the addition of the following proteases: Cathepsin-S (Merckmillipore, cat. # 219343), cathepsin-L (Merckmillipore, cat. # 219402), APMA activated MMP-2 (Biocol, cat. # II.5), MMP-9 (Giotto, cat. # G04MP09C) and ADAMTS-5 (R&D systems, cat. # 2198-AD). Cathepsins and MMPs were added to a final concentration of 2 μg/mL and ADAMTS-5 to a final concentration of 10 μg/mL. A positive control protein with known cleavage by the above proteases was included. The reaction was stopped by adding E-64 (final concentration of 1 uM) to cathepsins solutions and EDTA to the MMP solutions (final concentration of 1 uM) and ADAMTS-5 solutions (final concentration of 5 uM). Cathepsin-, MMP- and ADAMTS-5 buffer with relevant proteases were included as controls. Samples were stored at −80 °C until analysis. The cleavage of decorin was confirmed by silver-staining according to the manufacturer's instructions (SilverXpress®, Invitrogen, cat. #LC6100) and coomassie blue (data not shown).

DCN-CS (decorin degraded by cathepsin-S) ELISA protocol

Optimal incubation -buffer, –time and -temperature, as well as the optimal concentrations of antibody and coating peptide were determined and the finalized DCN-CS competitive ELISA protocol was as follows:

A 96-well streptavidin-coated microtiter plate was coated with 2.5 ng/mL biotinylated coating peptide dissolved in assay buffer (50 mM Tris-BTB, 4 g/L NaCl, pH 8.0) and incubated for 30 min. at 20 °C in darkness shaking (300 rpm). Twenty μL standard peptide or pre-diluted serum (1:4) were added to appropriate wells, followed by the addition of 100 μL monoclonal antibody dissolved in assay buffer to a concentration of 30 ng/mL to each well and incubated 20 h at 5 °C in darkness shaking (300 rpm). One hundred μL of goat anti-mouse POD-conjugated IgG antibody (Thermo Scientific, Waltham, MA, USA, cat. #31437) diluted 1:6000 in assay buffer to obtain a final concentration of 130 ng/mL was added to each well and incubated 1 h at 20 °C in darkness shaking. All incubation steps were followed by five washes in washing buffer (20 mM Tris, 50 mM NaCl, pH 7.2). Finally, 100 μL tetramethylbenzidine (TMB) (cat. 438OH, Kem-En-Tec Diagnostics, Denmark) was added to each well and the plate was incubated for 15 min at 20 °C in darkness shaking. The enzymatic reaction was stopped by adding 0.18 M H2SO4 and absorbance was measured at 450 nm with 650 nm as reference. A calibration curve was plotted using a 4-parameter logistic curve fit. Data were analyzed using the SoftMax Pro v.6.3 software.

Technical evaluation of the DCN-CS ELISA

To evaluate the technical performance of the DCN-CS ELISA, the following validation tests were carried out: Inter- and intra-assay variation, linearity, lower limit of detection, upper limit of detection, lower limit of quantification, analyte stability (freeze/thaw and storage) and interference.

The inter- and intra-assay variation was determined by ten independent runs on different days using ten quality control samples covering the detection range, with each run consisting of double-determinations of the samples. The ten quality control samples consisted of: two human serum samples, one sheep serum sample, one fetal calve serum sample, four human serum samples spiked with

standard peptide and two samples with standard peptide in buffer. Intra-assay variation was calculated as the mean coefficient of variance (CV%) within plates and the inter-assay variation was calculated as the mean CV% between the ten individual runs. To assess linearity of the assay, two-fold dilutions of human serum samples were performed and dilution linearity was calculated as a percentage of recovery of the un-diluted sample. The lower limit of detection (LLOD) was determined from 21 measurements using assay buffer as sample and was calculated as the mean + three standard deviations. The upper limit of detection (ULOD) was determined from ten independent runs of the highest standard peptide concentration and was calculated as the mean back-calibration calculation + three standard deviations. The lower limit of quantification (LLOQ) was determined from three independent runs of a serum sample diluted stepwise and determined as the highest DCN-CS level quantifiable in serum with a coefficient of variation below 30%. Analyte stability was first determined by the effect of repeated freeze/thaw of serum samples by measuring the DCN-CS level in three human serum samples in four freeze/thaw cycles. The freeze/thaw recovery was calculated with the first cycle as reference. Second, analyte stability in relation to storage was determined by a 24 h study performed at 4 °C or 20 °C. The DCN-CS level in three human serum samples was measured after 0 h, 2 h, 4 h and 24 h of storage and recovery was calculated with samples stored at −20 °C as reference. Interference was determined by adding a low/high content of hemoglobin (0.155/0.310 mM), lipemia/lipids (4.83/10.98 mM) and biotin (30/90 ng/mL) to a serum sample of known concentration. Recovery percentage was calculated with the normal serum sample as reference.

Clinical validation of DCN-CS

Patient serum samples consisted of three different cohorts. Cohort 1 was obtained from the commercial vendor ProteoGenex (Culver City, CA, USA) and included patients with non-small cell lung cancer (NSCLC), IPF, COPD and colonoscopy-negative controls with no symptomatic or chronic disease. A panel of healthy donors acquired from the commercial vendor Valley Biomedical (Winchester, VA, USA) were included as controls (Table 1).

Cohort 2 consisted of lung cancer patients acquired from the commercial vendor Asterand (Detroit, MI, USA) and healthy control serum samples obtained from a Danish study population.

Cohort 3 was a combination of serum samples from patients diagnosed with IPF (baseline samples, CTgov reg. NCT00786201) and healthy control serum samples acquired from the commercial vendor Valley Biomedical (Winchester, VA, USA) (Table 1).

Statistical analysis

The level of DCN-CS in serum samples was compared using one-way ANOVA adjusted for Tukey's multiple comparisons test (parametric data), Kruskal-Wallis adjusted for Dunn's multiple comparisons test (non-parametric data) or unpaired, two-tailed Mann-Whitney test. D'Agostino-Pearson omnibus test was used to assess the normality of the data. The diagnostic power was investigated by the area under the receiver operating characteristics (AUROC). Sensitivity and specificity were determined for cut-off values based on the ROC curves. The cut-off values should be regarded as a preliminary estimated cut-off point applied to achieve the reported maximized sensitivity and specificity.

Unless otherwise stated, data are shown as Tukey box plots, where the boxes represent the 25th, 50th and 75th percentiles. The whiskers represent the lowest and highest value, except outliers, which are higher than 1.5 times the 75th percentile or lower than 1.5 times the 25th percentile. *P*-values <0.05 were considered significant. Statistical analyses were performed using GraphPad Prism version 6 (GraphPad Software, Inc., CA, USA) and MedCalc Statistical Software version 12 (MedCalc Software, Ostend, Belgium). Graphs were designed using GraphPad Prism version 6 (GraphPad Software, Inc., CA, USA).

Results

Specificity of the DCN-CS ELISA assay

The target sequence, $_{75}$VPKDLPPDTT$_{84}$, was blasted for homology to other human secreted extracellular matrix proteins using NPS@: Network Protein Sequence Analysis with the UniprotKB/Swiss-prot database. The target sequence was found to be unique to human decorin when compared to other secreted ECM proteins. Allowing one amino acid mismatch, two secreted extracellular matrix proteins, Wnt-11 and Podocan, were identified with mismatches at the 6th and 4th position, respectively (Table 2). There was no reactivity against the sequence of Wnt-11, whereas some reactivity was observed against Podocan (data not shown). However, the affinity of the antibody was approximately 10 times higher for decorin than the podocan peptide. At the same time, it is unknown whether podocan will be cleaved in vivo between the exact two amino acids creating this peptide fragment. Furthermore, decorin has been shown to be the most abundant proteoglycan in human adult skin [6] decreasing the likelihood of reactivity towards podocan in biological samples.

The specificity of the competitive DCN-CS ELISA was evaluated by analyzing the reactivity towards the standard peptide, a non-sense peptide, an elongated peptide and using a non-sense biotinylated coating peptide. All peptide sequences are shown in Table 2 and results are shown in Fig. 1. The antibody only reacted with the standard peptide

Table 1 Clinical sample overview and patients demographics

Cohort	Samples	No. of subjects	Mean age (range)	Gender, % females	Tumor stage I	Tumor stage II	Tumor stage III	Tumor stage IV
1	NSCLC patients	8	61 (47–77)	12.8	1	2	3	2
1	IPF patients	8	74 (55–82)	62.5	-	-	-	-
1	COPD patients	8	75 (69–82)	50.0	-	-	-	-
1	Colonoscopy-negative controls	8	55 (44–65)	75.0	-	-	-	-
1	Healthy controls	20	34 (20–51)	10.0	-	-	-	-
2	NSCLC patients[a]	12	60 (47–80)	25.0	5	2	4	-
2	SCLC patients	8	61 (54–82)	25.0	3	1	4	-
2	Healthy controls	43	71 (60–82)	100.0	-	-	-	-
3	IPF patients	116	65 (43–80)	21.5	-	-	-	-
3	Healthy controls	38	34 (20–58)	10.5	-	-	-	-

[a]No tumor stage information of one patient

and the standard peptide clearly inhibited the signal in a dose-dependent manner compared to the other peptides. No detectable signal was observed when using the non-sense biotinylated coating peptide. These data suggest that the selected antibody exhibits high epitope specificity.

Degradation by Cathepsin-S

The ability of different proteases to generate the specific decorin fragment was investigated by incubating recombinant human decorin with cathepsin-S (Cat-S), Cathepsin-L (Cat-L), MMP-2, MMP-9 and ADAMTS-5. As shown in Fig. 2, Cat-S was able to generate decorin fragments in a time-dependent manner. Almost 6-fold higher decorin fragments were detected after incubating recombinant decorin with Cat-S for 24 h. No cleavage was observed with Cat-L, MMP-9, MMP-2 and ADAMTS-5 up to 72 h of incubation (data not shown).

Together, these results show that Cat-S can generate the target peptide recognized by the antibody.

Technical evaluation of the DCN-CS ELISA assay

A series of technical validations were performed to further evaluate the DCN-CS ELISA. The different validation steps and DCN-CS performance are shown in Table 3. The measuring range (LLOD to ULOD) of the assay was determined to 1.2–345.3 ng/mL and the lower limit of quantification (LLOQ) was 5.3 ng/mL. The intra- and inter-assay variation was 3 and 13%, respectively. The acceptance criterion was below 10% for the intra-assay variation and below 15% for the inter-assay variation and therefore acceptable. Human serum needed to be diluted 1:4 to obtain linearity and mean dilution recovery for pre-diluted human serum was 100%. The analyte recovery in serum was 94% after 4 freeze/thaw cycles and after storage at 4 °C for 24 h the recovery was 87%. The acceptance criterion was a recovery within 100% ± 20%. Analyte stability was also tested at 20 °C for 2, 4 and 24 h. The recovery after 2 and 4 h was 93% and 78%, respectively. However after 24 h the analyte could not be recovered within the acceptance range (53% recovery). These data indicate that the analyte in serum is stable at 4 °C and serum samples to be analyzed for DCN-CS should not be stored above this temperature for more than four hours. No interference was detected from either low or high contents of biotin, lipids or hemoglobin with recoveries ranging from 86 to 107%. The acceptance criterion was a recovery within 100% ± 20%.

Table 2 Synthetic peptides used for development and validation of the DCN-CS ELISA assay

Peptide name	Amino acid sequence
Selection/standard peptide	VPKDLPPDTT
Immunogenic peptide	VPKDLPPDTT-KLH
Biotinylated coating peptide	VPKDLPPDTT-biotin
Elongated peptide	KVPKDLPPDTT
Non-sense selection peptide	DSSAPKAAQA
Non-sense coating peptide	biotin-DSSAPKAAQA
Wnt-11 peptide	VPKDLDIRPV
Podocan peptide	VPKHLPPALY

Clinical evaluation – DCN-CS as a biomarker for fibrotic lung disorders

DCN-CS were measured in serum samples from three independent cohorts including patients with lung cancer, IPF, COPD and healthy controls. The data are presented in Fig. 3. Results from cohort 1 show that DCN-CS was significantly elevated in serum from NSCLC ($p < 0.0001$) and IPF ($p < 0.001$) patients as compared to healthy controls. No significance was observed for COPD patients. The mean level of DCN-CS was also significantly higher in NSCLC patients compared to colonoscopy-negative

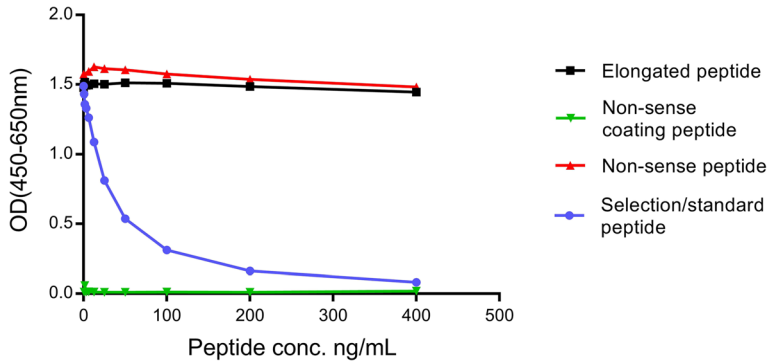

Fig. 1 Specificity of the DCN-CS monoclonal antibody. Monoclonal antibody reactivity towards the standard peptide (VPKDLPPDTT), the elongated peptide (KVPKDLPPDTT), a non-sense peptide (DSSAPKAAQA) and a non-sense coating peptide (biotin-DSSAPKAAQA) was tested for in the competitive DCN-CS ELISA assay. Signals are shown as optical density (OD) at 450 nm (subtracted the background at 650 nm) as a function of peptide concentration

controls, IPF and COPD patients. Data from cohort 2 confirmed the findings from cohort 1: DCN-CS was significantly elevated in NSCLC and SCLC patients as compared to healthy controls ($p < 0.0001$). Cohort 3 included patients with IPF and confirmed the results observed in cohort 1; IPF patients had a significantly higher level of DCN-CS ($p < 0.0001$) as compared to healthy controls.

The area under the receiver operating characteristics (AUROC) was used to evaluate the discriminative power of DCN-CS in relation to NSCLC and IPF. NSCLC patients, IPF patients and healthy controls from all cohorts were pooled and grouped into 'NSCLC', 'IPF' and 'healthy controls'. As shown in Table 4, DCN-CS was able to discriminate between NSCLC patients and healthy controls with an AUROC of 0.96 (95%CI: 0.90–0.99), $p < 0.0001$) with a specificity of 100% and sensitivity of 90% for an estimated optimal cut-off value. Similarly, DCN-CS was able to identify IPF patients from healthy controls with an AUROC of 0.77 (95%CI: 0.71–0.83), $p < 0.0001$) with a specificity of 83% and sensitivity of 63% for an estimated optimal cut-off. The ROC curves are presented in Fig. 4.

These findings indicate that DCN-CS levels are able to separate patients with NSCLC and IPF from healthy controls with high diagnostic accuracy. Thus this specific fragment in serum has biomarker potential in fibrotic lung disorders such as lung cancer and IPF.

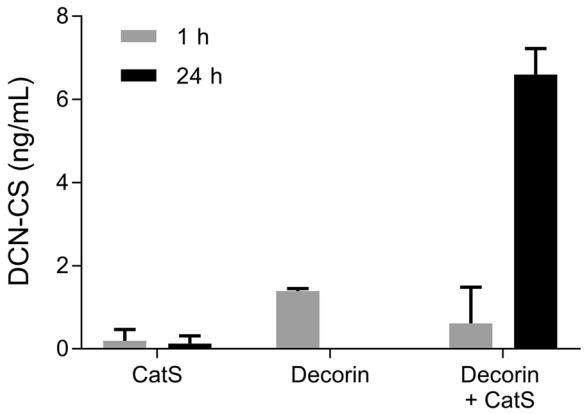

Fig. 2 Cleavage of decorin by Cathepsin-S. Degraded decorin levels were measured after 1 h and 24 h incubation of human recombinant decorin with Cathepsin-S. Data were normalized by subtracting the background measured in buffer alone. The experiment was repeated twice and data are shown as the mean of the two replicates with standard deviation

Discussion

The present study describes the development and biological validation of a technically robust competitive ELISA assay quantifying a Cat-S degraded fragment of decorin in serum. The main findings of this study were: 1) the fragment was significantly elevated in lung cancer and IPF patients compared to healthy controls 2) the fragment was detectable in serum and 3) the assay was technically robust and specific towards a unique Cat-S degraded fragment of decorin, DCN-CS. To our knowledge

Table 3 Technical validation data of the DCN-CS ELISA assay

Tecnical validation step	DCN-CS performance
Detection range (LLOD-ULOD)	1.2–345.3 ng/mL
Lower limit of quantification (LLOQ)	5.3 ng/mL
Intra-assay variation	3%
Inter-assay variation	13%
Dilution of serum samples	1:4
Dilution recovery (1:4 pre-dilution)[a]	100% (82–113%)
Freeze/thaw recovery (4 cycles)[a]	94% (90–97%)
Analyte stability up to 24 h, 4 °C[a]	87% (86–90%)
Interference Lipids, low/high	107%/86%
Interference Biotin, low/high	100%/100%
Interference Hemoglobin, low/high	100%/100%

[a]Percentages are reported as mean with range shown in brackets

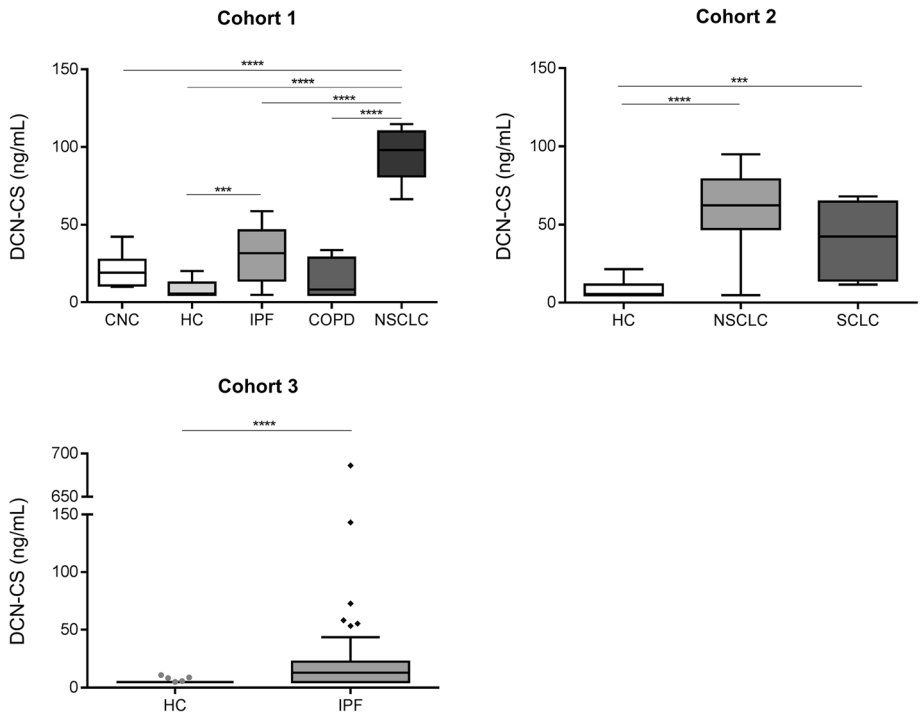

Fig. 3 Serum DCN-CS levels in patients with fibrotic lung disorders. Serum DCN-CS was assessed in three independent cohorts: Cohort 1 included patients with NSCLC ($n = 8$), IPF ($n = 8$), COPD ($n = 8$), colonoscopy-negative controls (CNC) ($n = 8$) and a panel of healthy controls (HC) ($n = 20$). Data were compared using one-way ANOVA adjusted for Tukey's multiple comparisons test. Cohort 2 consisted of patients with NSCLC ($n = 12$), SCLC ($n = 8$) and healthy controls (HC) ($n = 43$). Data were compared using Kruskal-Wallis adjusted for Dunn's multiple comparisons test. Cohort 3 comprised serum samples from patients diagnosed with IPF ($n = 116$) and healthy controls (HC) ($n = 38$). Groups were compared using unpaired, two-tailed Mann-Whitney test. Data are shown as Tukey box plots. Significance levels: ***: $p < 0.001$ and ****: $p < 0.0001$

this is the first biological validation of a specific decorin fragment in fibrotic lung disorders.

Decorin has been shown to play a protective role in cancer and fibrosis due to its ability to modulate various signal transduction pathways and sequester TGF-beta via direct binding [5]. This has led to the speculation that degradation of decorin may induce the development of cancer and fibrotic diseases by disrupting the binding to its binding partners. The lungs are an organ with a large amount of interstitial matrix and we have shown that patients with fibrotic lung disorders, such as cancer and IPF, have an increased level of degraded decorin. Cat-S is produced in both tumor cells and tumor-associated macrophages and has been associated with growth, angiogenesis and metastasis in different cancer types [26–28]. The interaction between these two proteins might give rise to a certain pathology where the interstitial matrix is involved and this interaction can be measured by the assay presented here.

The data suggest that Cat-S specific degradation of decorin has a relevant role in the pathology of lung cancer and IPF. We hypothesize that increased degradation of decorin triggers a fibrotic response both by inhibiting binding of TGF-beta to decorin which will result in the release of excessive amounts of TGF-beta but also by disrupting proper collagen fibril formation leading to loss of homeostasis in the interstitial matrix. Several studies have shown that disrupting the normal collagen turnover balance leads to fibrosis and cancer [1–3, 22]. Why the specific decorin fragment was not significantly elevated in patients with COPD is to be investigated further.

Based on the high elevated level of degraded decorin in patients compared to healthy controls, the present assay can provide a novel non-invasive clinical tool in

Table 4 Discriminative performance of DCN-CS in NSCLC and IPF

	Cut-off value (ng/mL)	Sensitivity	Specificity	AUROC (95% CI)	*p*-value
NSCLC vs. healthy controls	21.5	90.0	100.0	0.96 (0.90–0.99)	<0.0001
IPF vs. healthy controls	8.9	62.9	82.7	0.77 (0.71–0.83)	<0.0001

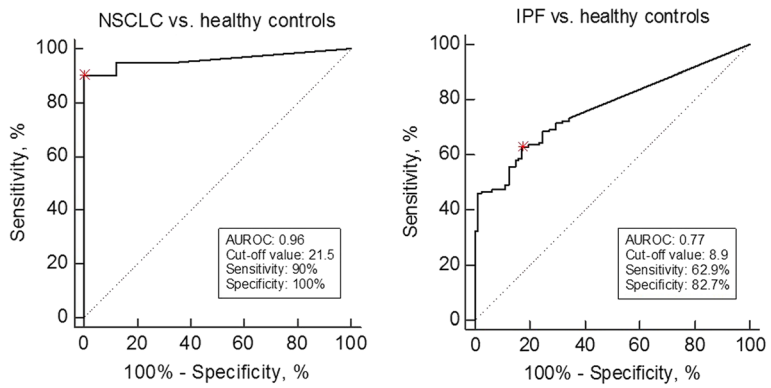

Fig. 4 ROC curve analysis. Roc curve analysis was used to evaluate the ability of DCN-CS to discriminate between patients and healthy controls. The preliminary estimated cut-off values for the reported sensitivity/specificity are marked with a red asterix

lung cancer and IPF. The fact that the AUC was higher for lung cancer compared to IPF, suggests that this pathological event seems to be more associated with lung cancer than IPF and DCN-CS could serve as a potential diagnostic biomarker for lung cancer. In relation to IPF, the data suggests other clinical uses of this biomarker, such as prognosis and/or prediction. This needs to be further investigated in larger clinical studies. Evidence suggests that decorin fragments can function as pro-inflammatory signaling molecules, so-called damage-associated molecular patterns (DAMPs), capable of inducing an inflammatory response [5, 29]. High levels of degraded decorin might therefore indicate a severe inflammatory state. However further studies are needed to investigate whether the DCN-CS fragment functions as a DAMP.

The DCN-CS assay was shown to be technically robust, with low values of LLOD, intra- and inter variation and acceptable dilution recovery, interference and analyte stability at 4 °C. The fact that the assay did not detect the elongated peptide nor a non-sense peptide indicates that the monoclonal antibody is specific towards the cleavage site between amino acid 74 and 75 located in the first leucine-rich repeat of decorin. This was supported by data showing that DCN-CS was able to quantify high levels of the fragment after in vitro cleavage of decorin with Cat-S. Reactivity towards intact decorin was minimal further demonstrating that this assay does not measure total protein but a specific degraded fragment.

The target peptide fragment was originally identified by Zhen et al. [23] in human articular cartilage digested with ADAMTS-5. We have shown that this fragment is generated by Cat-S and not by Cat-L, MMP-2/9 or ADAMTS-5 in vitro using recombinant decorin. The fact that ADAMTS-5 degradation could not generate the target peptide fragment under our conditions, indicates that other proteases may have to cleave the protein before ADAMTS-5 can generate this fragment. Imai et al. [30] have examined the ability of different MMP'-s to cleave decorin and found that MMP-2, MMP-3 and MMP-7 were able to generate degradation fragments. None of these fragments correspond to our target fragment which supports our findings that the DCN-CS fragment is specifically generated by Cat-S and not MMP's. This is important since different protein fragments may reflect different pathological events [31, 32], i.e. Cat-S degraded decorin might reflect one disease state whereas MMP-degraded decorin reflects other disease activity patterns. As the present assay enables quantification of a specific neo-epitope it might be superior to other commercial assays in which decorin is quantified but the precise epitope is not known. These quantification capabilities also increases the biomarker potential as it may reflect a direct pathological event, such as fibrosis.

The diagnostic validation of DCN-CS in the present study is limited by relatively small population sizes and cross-sectional designs and clinical information was limited. In addition, it was not easy to match cases and controls according to age, gender and tobacco consumption in this preliminary study which therefore could be confounding factors. However the fact that we could confirm the findings in independent cohorts, increases the validity. Larger longitudinal studies are needed to fully validate the potential of DCN-CS as a diagnostic and/or prognostic biomarker in fibrotic lung diseases.

Conclusion

In conclusion, we have developed a technically robust competitive ELISA assay targeting a specific Cat-S degraded fragment of decorin (DCN-CS). The level of DCN-CS was significantly higher in patients with lung cancer and IPF compared to healthy controls, suggesting a pathological role of degraded decorin in these lung disorders.

Abbreviations

ADAMTS: A disintegrin and metalloproteinase with thrombospondin motifs; AUROC: Area under the receiver operating characteristics; Cat-L: Cathepsin L;

Cat-S: Cathepsin-S; COPD: Chronic obstructive pulmonary disease; DAMP: Damage-associated molecular pattern; DCN-CS: Decorin degraded by cathepsin-S; ECM: Extracellular matrix; EGFR: Epidermal growth factor receptor; ELISA: Enzyme-linked immunosorbent assay; GAG: Glycosaminoglycan; IPF: Idiopathic pulmonary fibrosis; IGF-IR: Insulin-like growth factor receptor; LLOD: Lower limit of detection; LLOQ: Lower limit of quantification; NSCLC: Non-small cell lung cancer; MMP: Matrix metalloproteinase; SLRP: Small leucine-rich proteoglycan; ULOD: Upper limit of detection

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Availability of data and material
The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

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Authors' contributions
SNK was the main author of the manuscript. SNK carried out the measurements, data analysis and statistical analysis in discussion with CLB and NW. BD, CB and MC were responsible for designing and conducting the clinical IPF study. SB, MK and DJL supervised the entire project and experimental work and had a significant role in defining the hypotheses. All authors participated in data interpretation and critical revised and approved the final manuscript.

Ethics approval and consent to participate
For the clinical IPF study (CTgov reg. NCT00786201) the following ethic committee approved the study: Sterling Institutional Review Board, Sterling Independent Services, Inc. (Atlanta). The healthy control serum samples (cohort 2) were obtained from a Danish study population approved by The National Committee on Health Research Ethics (Denmark). According to Danish law additional ethical approval for measuring biochemical biomarkers in previously collected samples is not required. For the samples obtained from the commercial vendors Proteogenex, Asterand and Valley Biomedical, appropriate Institutional Review Board/Independent Ethical Committee approved sample collection and all patients filed informed consent.

Consent for publication
Not applicable.

Competing interests
C.L. Bager, N. Willumsen, D.J. Leeming and M.A. Karsdal are employed at Nordic Bioscience A/S which is a company involved in discovery and development of biochemical biomarkers. M.A. Karsdal owns stocks at Nordic Bioscience. M. Curran, B Dasgupta and C. Brodmerkel are employees of Janssen R&D LLC. and own stock in Johnson & Johnson. S.N. Kehlet and S. Brix reports no conflict of interest.

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Paper IV - A fragment of SPARC reflecting increased collagen affinity shows pathological relevance in lung cancer – implications of a new collagen chaperone function of SPARC

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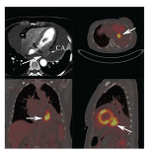

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RESEARCH PAPER

A fragment of SPARC reflecting increased collagen affinity shows pathological relevance in lung cancer – implications of a new collagen chaperone function of SPARC

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ABSTRACT

The matricellular protein SPARC (secreted proteome acidic and rich in cysteine) is known to bind collagens and regulate fibrillogenesis. Cleavage of SPARC at a single peptide bond, increases the affinity for collagens up to 20-fold. To investigate if this specific cleavage has pathological relevance in fibrotic disorders, we developed a competitive ELISA targeting the generated neo-epitope on the released fragment and quantified it in serum from patients with lung cancer, idiopathic pulmonary fibrosis (IPF), chronic obstructive pulmonary disease (COPD) and healthy subjects. Furthermore, the ability of SPARC to protect fibrillar collagens from proteolytic degradation was investigated *in vitro*, potentially adding a new collagen chaperone function to SPARC. The fragment was significantly elevated in lung cancer patients when compared to healthy subjects measured in a discovery cohort ($p = 0.0005$) and a validation cohort ($p < 0.0001$). No significant difference was observed for IPF and COPD patients compared to healthy subjects. When recombinant SPARC was incubated with type I or type III collagen and matrix metalloproteinase-9, collagen degradation was completely inhibited. Together, these data suggest that cleavage of SPARC at a specific site, which modulates collagen binding, is a physiological mechanism increased during pathogenesis of lung cancer. Furthermore, inhibition of fibrillar collagen degradation by SPARC adds a new chaperone function to SPARC which may play additional roles in the contribution to increased collagen deposition leading to a pro-fibrotic and tumorigenic environment.

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Introduction

Fibrosis is a part of the pathology and/or an end-point in many diseases such as cancer, liver cirrhosis and fibrotic lung disorders. Fibrosis is characterized by an increased deposition of extracellular matrix (ECM), including collagens, which interferes with normal tissue function leading to organ failure. There is a persuasive amount of data showing that 'secreted proteome acidic and rich in cysteine' (SPARC), also referred to as osteonectin or basement membrane protein 40 (BM-40) is an important factor for fibrogenesis,[1-5] and SPARC expression has been shown to be upregulated in fibrosis and cancer.-[6–9] SPARC is a 32-kDa matricellular protein known to regulate ECM assembly and deposition, growth factor signaling and interactions between cells and their surrounding ECM.[10,11] The expression of SPARC is increased in epithelial/endothelial cells with a high ECM turnover, during abnormal tissue growth associated with neoplasia and during tissue injury and inflammation, highlighting the importance of SPARC in tissue remodeling.[12-14]

The SPARC protein is divided into three different structural and functional modules. Studies have shown that these modules contains bioactive peptides with different biological functions (Figure 1).[15,16,17] For example, small synthetic peptides with sequences derived from module II (follistatin-like domain) are able to regulate proliferation of endothelial cells, stimulate fibroblast proliferation and promote angiogenesis. Module III (extracellular calcium binding domain) contains collagen binding sites and peptide domains that are able to induce MMP production, stimulate angiogenesis and inhibit endothelial cell proliferation. These data suggest that the activity of SPARC is modulated upon cleavage leading to unmasking of domains with biological functions that are distinct from those observed for the native protein. SPARC binds multiple ECM proteins in a calcium-dependent manner within module III, with collagens being the best characterized binding partners. It has been suggested that SPARC acts as an extracellular chaperone due to its many chaperone-like properties. Several studies have shown that SPARC binds different collagens (collagen type I, II, III, IV and V) in the ECM and is important for correct collagen deposition and assembly.[18-24] The cleavage of a single peptide bond by metalloproteinases (MMP's) increases the affinity for collagens up to 20-fold[25,26,] (Figure 1). Cleavage of SPARC at this specific site has been detected in mouse tissues, suggesting a physiological mechanism of modulating collagen binding.

Even though SPARC is considered of importance in collagen processing, oncology and fibrosis, the exact pathological function of the different subparts of the molecule remains to

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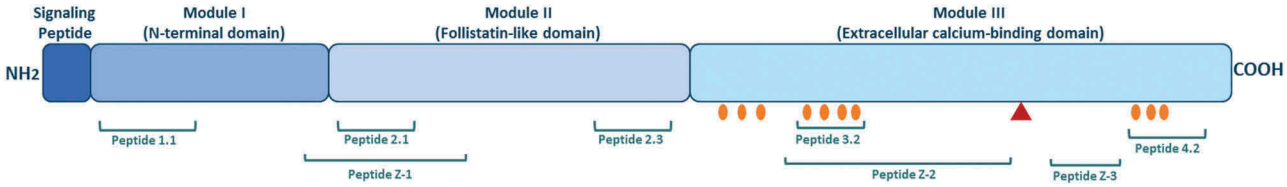

Figure 1. The structure of SPARC and bioactive peptides.

The SPARC protein is divided into three different modules containing bioactive peptides. Peptide 1.1 inhibits spreading of endothelial cells and fibroblasts and potentiates MMP-2 activation. Peptide 2.1 inhibits proliferation of endothelial cells but stimulates proliferation of fibroblasts. Peptide 2.3 stimulates endothelial cell proliferation and angiogenesis. Peptide 3.2 induces MMP production. Peptide 4.2 inhibits cell spreading of endothelial cells and fibroblasts, but stimulates endothelial cell migration. Peptide Z-1 has biphasic effect on endothelial cell proliferation and stimulates vascular growth. Peptides Z-2 and Z-3 inhibit endothelial cell proliferation, but stimulate their migration. Collagen binding sites are shown with orange circles. The red triangle represents the cleavage site associated with increased collagen affinity.

be understood. In the present study, we investigated if MMP-cleavage of SPARC at a specific site known to be involved in increased collagen affinity, has pathological relevance in fibrotic disorders. We developed and validated a competitive enzyme-linked immunosorbent assay (ELISA) quantifying this specific fragment in the circulation. Additionally, we examined if binding of SPARC to fibrillar collagens (type I and III collagen) interfered with their degradation by MMP-9 and MMP-13, proteases known to play important roles in tumor progression.[27,28]

Results

Specificity of the SPARC-M ELISA assay

The target sequence, LLARDFEKNY, was blasted for homology to other human secreted extracellular matrix proteins using NPS@: Network Protein Sequence Analysis with the UniprotKB/Swiss-prot database. The target sequence was found to be unique to human SPARC when compared to other secreted ECM proteins. Allowing one amino acid mismatch, four secreted extracellular matrix proteins, Von Willebrand factor, glucagon, SPARC-like protein 1 and ADAMTS15, were identified with mismatches at the 6th, 2nd, 3rd and 6th position, respectively (Table 1). There was no reactivity against the sequence of these four peptides (Figure 2A) suggesting high specificity of the antibody for the target sequence. The specificity of the competitive SPARC-M ELISA was further evaluated by analyzing the reactivity towards the calibrator peptide, a non-sense peptide, an elongated peptide, a truncated peptide and using a non-sense biotinylated coating peptide. All peptide sequences are shown in Table 1 and results are shown in Figure 2B. The antibody only reacted with the calibrator peptide and the calibrator peptide clearly inhibited the signal in a dose-dependent manner compared to the other peptides. No detectable signal was observed when using the non-sense biotinylated coating peptide.

Together, these data suggest that the selected antibody exhibits high neo-epitope specificity.

Degradation of SPARC by MMP-8 and MMP-13

To further evaluate the specificity of the antibody and to investigate which proteases generate SPARC-M, different gelatinases (MMP-2 and MMP-9) and collagenases (MMP-8

Table 1. Synthetic peptides used for development and validation of the SPARC-M ELISA assay.

Peptide name	Amino acid sequence
Calibrator peptide	LLARDFEKNY
Immunogenic peptide	LLARDFEKNY-GGC-KLH
Biotinylated coating peptide	LLARDFEKNY-K-biotin
Elongated peptide	ELLARDFEKNY
Truncated peptide	LARDFEKNY
Non-sense calibrator peptide	VPKDLPPDTT
Non-sense coating peptide	VPKDLPPDTT-biotin
Von Willebrand factor (VWF)	LLARDCQDHS
Glucagon (GCG)	LAARDFINWL
SPARC-like protein 1 (SLP1)	LLLRDFKKNY
ADAMTS15 (A15)	LLARDQCNLH

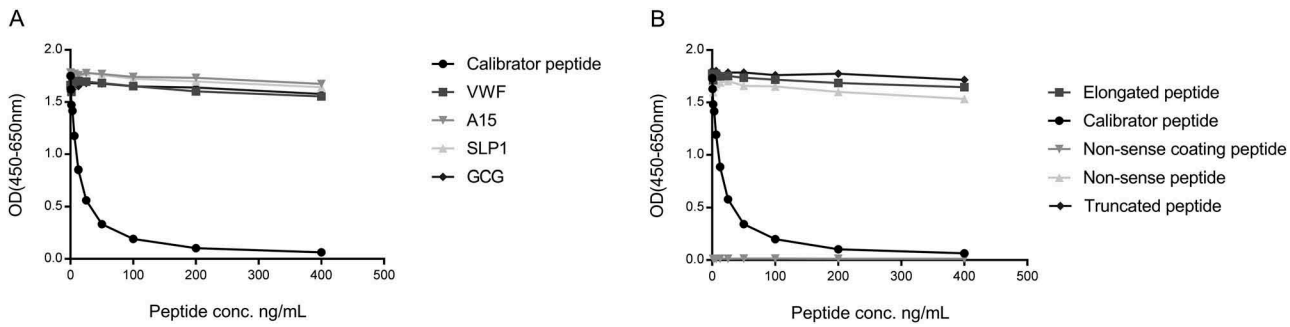

Figure 2. Specificity of the SPARC-M monoclonal antibody.

Monoclonal antibody reactivity towards (A) the calibrator peptide (LLARDFEKNY), the elongated peptide (ELLARDFEKNY), the truncated peptide (LARDFEKNY) a non-sense peptide (VPKDLPPDTT) and a non-sense coating peptide (VPKDLPPDTT-biotin) and (B) Von Willebrand factor (VWF), ADAMTS15 (A15), SPARC-like protein 1 (SLP1) and glucagon (GCG), was tested for in the competitive SPARC-M ELISA. Signals are shown as optical density (OD) at 450 nm (subtracted the background at 650 nm) as a function of peptide concentration.

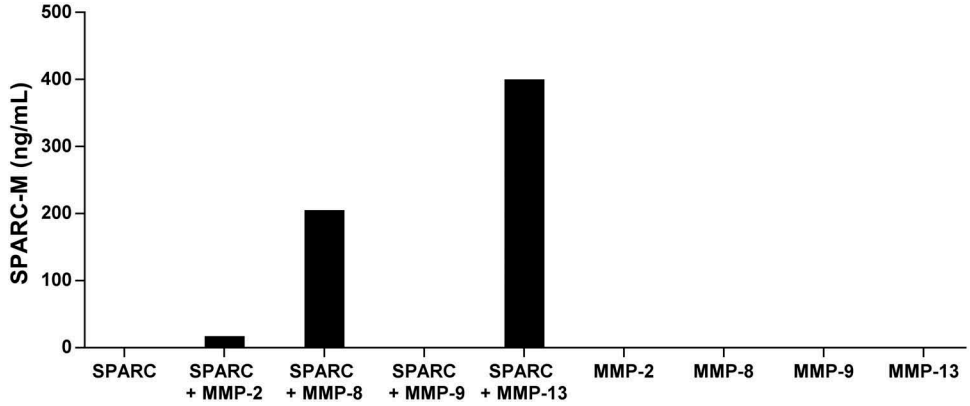

Figure 3. Cleavage of SPARC by MMP-8 and MMP-13.

SPARC was incubated with different MMP's and SPARC-M levels were measured after 24 hours. Data were normalized by subtracting the background measured in buffer alone. The graph below is representative of two experiments.

and MMP-13) were incubated with recombinant full-length SPARC. As shown in Figure 3, the collagenases were able to generate the fragment, with MMP-13 giving the highest level of SPARC-M. In contrast, no SPARC-M was detected without the collagenases or when incubated with MMP-9. MMP-2 was able to generate a small amount of SPARC-M as compared to the collagenases.

These results indicate that the antibody is specific for the cleavage site and that collagenases compared to gelatinases have a higher preference for SPARC at this specific site.

Technical evaluation of the SPARC-M ELISA

The technical performance of the SPARC-M ELISA was further evaluated according to inter – and intra-assay variation, linearity, lower limit of detection, upper limit of detection, analyte stability (freeze/thaw and storage) and interference. The results from the different validation steps and SPARC-M performance are summarized in Table 3. The measuring range (LLOD to ULOD) of the assay was determined to 2.7–300.7 ng/mL. The intra- and inter-assay variation was 6% and 10%, respectively. The acceptance criterion was below 10% for the intra-assay variation and below 15% for the inter-assay variation and therefore acceptable. To obtain linearity, human serum needed to be diluted 1:4. The mean dilution recovery for human serum was 96% calculated with 1:4 pre-diluted samples as references. The analyte stability was analyzed according to freeze/thaw cycles and storage stability at 4°C and 20°C with an acceptance criterion of the recovery within 100% ± 20%. The analyte recovery in serum was 92% after 4 freeze/thaw cycles. After storage at 4°C for 48 hours the recovery was 84%. Analyte stability was also tested at 20°C for 4, 24 and 48 hours. The recovery after 4 hours was 88%. However after 24 hours the analyte could not be recovered within the acceptance range (50% recovery). These data indicate that the analyte in serum is stable at 4°C up to 48 hours, however upon analysis serum samples should not be stored above 20°C for more than four hours. No interference was detected from either low or high contents of biotin, lipids or hemoglobin with recoveries ranging from 80–98%. The acceptance criterion was a recovery within 100% ± 20%.

Clinical evaluation of SPARC-M

To investigate whether SPARC-M had clinical disease relevance and biomarker potential, SPARC-M was measured in patients with different fibrotic lung disorders and healthy controls. The discovery cohort (cohort 1) consisted of patients with lung cancer, IPF, COPD and healthy controls (Table 2). As shown in Figure 4A, SPARC-M was significantly elevated in lung cancer patients compared to healthy controls ($p = 0.0005$) and COPD patients ($p = 0.0003$). IPF patients also had an increased level of SPARC-M compared to healthy controls although not significant ($p = 0.66$). To validate the findings in lung cancer patients, SPARC-M was measured in a validation cohort (cohort 2) including 40 lung cancer patients and 20 healthy controls (Table 2). A

Table 2. Clinical sample overview and patients demographics.

Cohort	Samples	No. of subjects	Mean age (range)	Gender, % females	Mean BMI (range)	Tumor stage I	Tumor stage II	Tumor stage III	Tumor stage IV
1	Lung cancer patients	8	61 (47–77)	13	-	-	-	-	-
1	IPF patients	7	73 (55–81)	57	-	-	-	-	-
1	COPD patients	8	75 (69–82)	50	-	-	-	-	-
1	Healthy controls	6	55 (44–65)	83	-	-	-	-	-
2	Lung cancer patients	40	62 (55–66)	50	25 (16–35)	10	10	10	10
2	Healthy controls	20	62 (60–65)	50	26 (22–32)	-	-	-	-

Table 3. Technical validation data of the SPARC-M ELISA assay.

Tecnical validation step	SPARC-M performance
Detection range (LLOD-ULOD)	2.7–300.7 ng/mL
Intra-assay variation	6%
Inter-assay variation	10%
Dilution of serum samples	1:4
Dilution recovery (1:4 pre-dilution)	96% (77–102%)
Freeze/thaw recovery (4 cycles)	92% (86–103%)
Analyte stability up to 48 h, 4°C and 4 h, 20°C	88% (84–96%)
Interference Lipids, low/high	96%/97%
Interference Biotin, low/high	96%/98%
Interference Hemoglobin, low/high	96%/80%

Percentages are reported as mean with range shown in brackets

significant increase in SPARC-M in lung cancer patients as compared to healthy controls was observed in this cohort as well ($p < 0.0001$) (Figure 4B).

The area under the receiver operating characteristics (AUROC) was used to evaluate the discriminative power of SPARC-M in relation to lung cancer patients and healthy controls (cohort 2). SPARC-M was able to discriminate between patients and healthy controls with an AUROC of 0.87 (95%CI: 0.78–0.96).

To examine if the level of SPARC-M was different in patients with metastasis (high tumor burden) compared to patients with localized tumors, patients from cohort 2 were stratified according to their tumor stage (stage I-IV). A significantly higher level of SPARC-M was found in metastatic patients (stage IV) compared to stage I patients (Figure 4C). Moreover, the discriminative accuracy increased with tumor stage with an AUC of 0.71 for stage I, an AUC of 0.87 for stage II, an AUC of 0.91 for stage III and an AUC of 0.99 for stage IV.

Together, these data demonstrate that the investigated cleavage site, which modulates collagen binding and measured by SPARC-M, is a physiological mechanism that is increased during progression and invasion of lung cancer.

Inhibition of fibrillar collagen degradation by SPARC

To investigate if the binding of SPARC to collagens interfered with and inhibited fibrillar collagen degradation, type I collagen or type III collagen was incubated together with MMP-9 alone or together with MMP-9 and SPARC. The degradation of collagens was measured by ELISAs measuring type I collagen degradation by MMP-9 and MMP-13 (C1M) and type III collagen degradation by MMP-9 (C3M). As shown in Figure 5, MMP-9, degraded both collagens in a time-dependent manner illustrated by an increase in C1M (Figure 5A) and C3M (Figure 5B) concentration. The addition of SPARC to collagen completely inhibited both type I and type III collagen degradation by MMP-9.

To examine if SPARC also had a protective function in collagenase-mediated degradation of collagens, type I collagen and MMP-13 was incubated with or without SPARC and degradation was measured by C1M. Interestingly, no change in type I collagen degradation was observed by the addition of SPARC (Figure 5C).

These data suggest a new chaperone function of SPARC, i.e. protecting fibrillar collagens from degradation by gelatinases but not by collagenases.

Discussion

The present study validates a new serum biomarker reflecting increased collagen binding by SPARC and demonstrates a new collagen chaperone function of SPARC. The main findings of this study were: 1) the investigated fragment, SPARC-M, was detectable in serum and significantly elevated in lung cancer patients compared to healthy controls, 2) the SPARC-M ELISA was technically robust and specific towards a MMP-degraded fragment of SPARC and 3) SPARC was able to inhibit MMP-9-mediated degradation of fibrillar collagens. To our knowledge, this is the first biological validation of this specific fragment in human serum and the first study to show that SPARC acts by preventing collagen degradation.

Studies have shown that the collagen binding function of SPARC can be modulated by extracellular proteolytic processing.[25,26,29] We found that MMP-8 and MMP-13 had preference for the investigated cleavage site compared to MMP-2 and MMP-9. The fact that some MMPs show preference for this site over others, suggest a way for the stroma to regulate collagen binding to SPARC and thereby fibril formation. Previous studies using SDS-gel electrophoresis

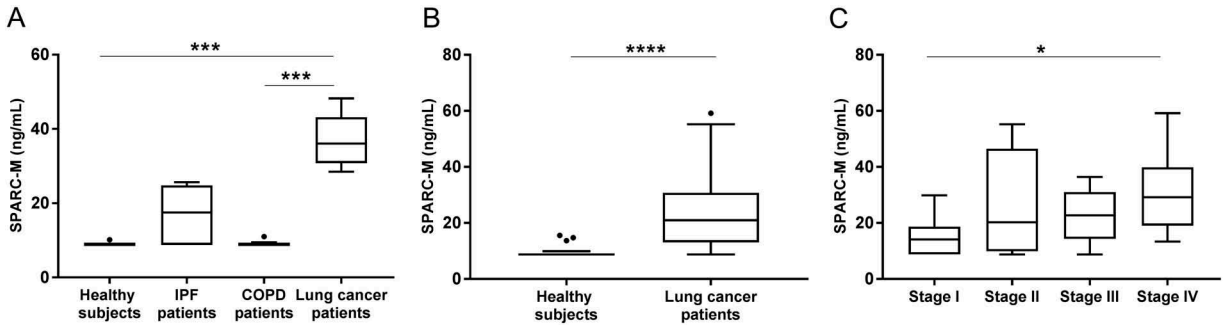

Figure 4. Serum SPARC-M levels in patients with fibrotic disorders and healthy controls.

(A) Cohort 1: Serum SPARC-M was assessed in healthy controls (n = 6), IPF patients (n = 7), COPD patients (n = 8) and lung cancer patients (n = 8). Groups were compared using Kruskal-Wallis adjusted for Dunn's multiple comparisons test. (B) Cohort 2: Serum SPARC-M was assessed in healthy controls (n = 20) and lung cancer patients (n = 40). Groups were compared using unpaired, two-tailed Mann-Whitney test. (C) Lung cancer patients (from cohort 2) were stratified according to their cancer stage (stage I-IV, n = 10 in each group). Data were compared using Kruskal-Wallis adjusted for Dunn's multiple comparisons test. All Data are shown as Tukey box plots. Significance level: *: $p < 0.05$, ***: $p < 0.001$, ****: $p < 0.0001$.

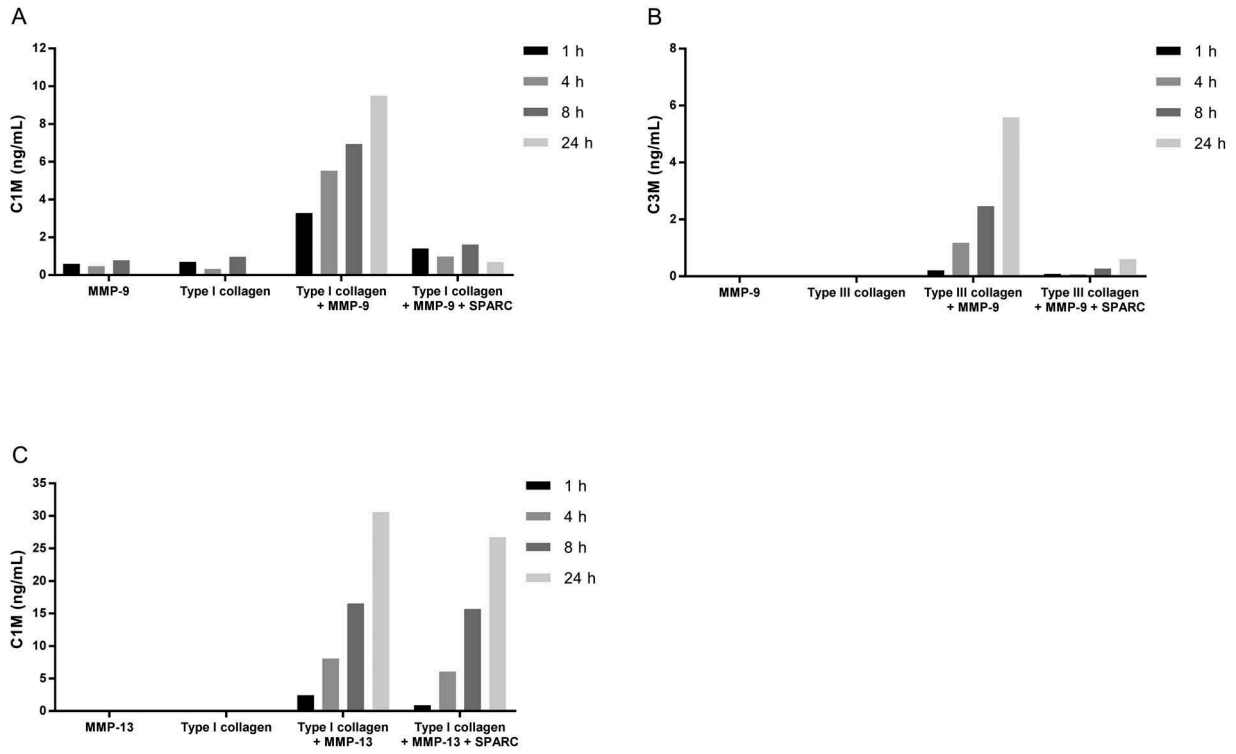

Figure 5. SPARC inhibits fibrillar collagen degradation by MMP-9.

(A) Type I collagen or (B) type III collagen was incubated with MMP-9 alone or together with MMP-9 and SPARC. (C) Type I collagen was incubated with MMP-13 alone or together with MMP-13 and SPARC. The solutions incubated at 37°C for 1 h, 4 h, 8 h and 24 h. The reaction was stopped by adding 1 μM EDTA to the solutions. Collagen degradation was measured with ELISAs targeting MMP-9 and MMP-13 degraded type I collagen, C1M (A)(C) and MMP-9 degraded type III collagen, C3M (B). MMP-buffer with either MMP's or collagen alone were included as negative controls. Data were normalized by subtracting the background measured in buffer alone. The graphs below are representative of two experiments.

are in concordance with our findings, showing that MMP-13 is able to cleave SPARC at this site.[25,29] However, Sasaki *et al.*[25] demonstrated cleavage by MMP-9 and MMP-2, although to a lesser extent than MMP-13. As shown in Figure 3, MMP-2 is able to generate a small amount of the fragment whereas MMP-9 is negative. The discrepancy between our data and the data presented by Sasaki *et al.* with MMP-9 might be due to different detection methods (ELISA vs. SDS-gel electrophoresis) and warrants further investigations.

The investigated cleavage site of SPARC has been shown to be present in mouse tissue quantified by immunohistochemistry using polyclonal antibodies against the cleavage site,[26] however this is the first time this cleavage is demonstrated in humans. SPARC-M was significantly elevated in patients with lung cancer compared to healthy controls. An increase of SPARC-M was also observed in IPF patients, although it was found not to be significantly elevated. We hypothesize that the SPARC-M fragment is released to the circulation upon MMP-cleavage and here represents a surrogate measure of the bioactive part of SPARC which is retained within the matrix, and have increased collagen affinity. Interestingly, SPARC itself has been shown to increase the expression of MMP's in fibroblasts [30–32] causing a positive feedback loop with MMP-cleavage of SPARC which may, if uncontrolled, be involved in the pathology of ECM remodeling diseases with increased collagen deposition, such as lung cancer and IPF. The fact that patients with IPF and lung cancer, and not COPD, had elevated levels of SPARC-M, supports this hypothesis. In accordance with our findings, several studies have shown an increased expression of SPARC in cancer and fibrosis. [6-9] As SPARC-M was elevated in stage IV patients and the discriminative power increased with tumor stage support that this cleavage is in fact a pathological mechanism in lung cancer that increases with tumor burden. These results indicate a prognostic value of SPARC-M, although further studies are needed to evaluate this.

The limitations of the present clinical studies are the relatively small population sizes and limited clinical information. However, as we could confirm the findings in two independent cohorts, increases their validity. Larger longitudinal studies are needed to validate the potential of SPARC-M as a biomarker in fibrotic lung diseases.

This study also demonstrates a new collagen chaperone function of SPARC. In general, the chaperone function of SPARC has been linked to its ability to inhibit thermal aggregation of alcohol dehydrogenase in a concentration-dependent manner[33] and its importance for correct collagen deposition and assembly.[18-24] Here, we show that SPARC is able to interfere with the degradation of fibrillar collagens by MMP-9 but not MMP-13. These findings may indicate that SPARC plays a chaperone role in maintaining a collagen structure that does not enable gelatinolytic (MMP-9) processing, but collagenolytic (MMP-13) processing. How this translates to physiological conditions remains to be established.

The observed collagen chaperone function of SPARC could be involved in the pathogenesis of fibrotic disorders by

contributing to increased collagen deposition. We hypothesize that stress, such as malignant transformation or tissue injury, causes activation of fibroblast and increased SPARC expression which induces MMP expression resulting in a positive feedback mechanism with cleavage of SPARC by MMP's. Cleavage at this specific site will enhance binding of SPARC to collagens, preventing collagen degradation by MMP's. This will result in increased collagen deposition and thereby play a role in fibrogenesis and tumorigenesis.

In summary, we have shown that SPARC is able to inhibit degradation of fibrillar collagens and that cleavage of SPARC at a specific site, known to modulate collagen binding, is a pathological mechanism in lung cancer. Whether this is a cause or consequence of lung cancer needs further investigation.

Materials and methods

Development of SPARC-M (SPARC degraded by mmp's) ELISA

Selection of peptides

The selection of target peptide for ELISA development was based on the following cleavage site (↓) on SPARC previously identified by Edman degradation and published by Sasaki *et al.*:[25] $_{211}$HPVE ↓ LLARDFEKNYNMYIFP$_{230}$.

To generate an antibody specific for the N-terminal of the cleavage fragment, a sequence of 10 amino acids adjacent to the site was chosen as the target: ↓$_{215}$LLARDFEKNY$_{224}$. The sequence was blasted for homology to other human secreted extracellular matrix proteins using NPS@: Network Protein Sequence Analysis with the UniprotKB/Swiss-prot database [34].

Synthetic peptides used for monoclonal antibody production and validation of the ELISA were purchased from Genscript and shown in Table 1. The target sequence was used as the calibrator peptide (LLARDFEKNY). A biotinylated peptide (LLARDFEKNY-K-biotin) was included as a coating peptide with addition of a lysine residue to the C-terminal end to ensure biotin linking. The specificity of the antibody was tested by including an elongated calibrator peptide with an additional amino acid added to the N-terminal of the target peptide sequence (ELLARDFEKNY), a truncated calibrator peptide with a removal of the first N-terminal amino acid (LARDFEKNY) as well as a non-sense calibrator peptide (VPKDLPPDTT) and a non-sense biotinylated coating peptide (VPKDLPPDTT-biotin) in the assay validation. To screen for any potential cross-reactivity to other ECM proteins and further test the antibody specificity, four peptides (derived from Von Willebrand factor, glucagon, SPARC-like protein 1 and ADAMTS15) with one amino acid mismatch compared to the first six amino acids in the target sequence were also included (Table 1). The immunogenic peptide (LLARDFEKNY-GGC-KLH) was generated by covalently cross-linking the standard peptide to Keyhole Limpet Hemocyanin (KLH) carrier protein using Succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate, SMCC (Thermo Scientific, cat.no. 22336). Glycine and cysteine residues were added at the C-terminal end to ensure right linking of the carrier protein.

Monoclonal antibody production

Six week old Balb/C mice were immunized by subcutaneous injection of 200 µL emulsified antigen containing 100 µg immunogenic peptide (LLARDFEKNY-GGC-KLH) mixed with Stimune Immunogenic Adjuvant (Thermo fisher, cat. no. 7925000). Consecutive immunizations were performed at 2-week intervals until stable sera titer levels were reached. The mouse with the highest titer rested for four weeks and was then boosted with 100 µg immunogenic peptide in 100 µL 0.9% NaCl solution intravenously. Hybridoma cells were produced by fusing spleen cells with SP2/0 myeloma cells as previously described.[35] The resultant hybridoma cells were then cultured in 96-well microtiter plates and standard limited dilution was used to secure monoclonal growth.

Clone characterization

The reactivity of the monoclonal antibody from different clones was evaluated by displacement using human serum samples and the calibrator peptide (LLARDFEKNY) in a preliminary ELISA using 10 ng/mL biotinylated coating peptide on streptavidin-coated microtiter plates (Roche, cat. no. 11940279) and the supernatant from the antibody producing monoclonal hybridoma cells. The clone with the best reactivity towards the calibrator peptide was purified using protein-G-columns according to the manufacturer's instructions (GE Healthcare Life Sciences, cat. no. 17–0404-01).

SPARC-M ELISA protocol

Optimal incubation buffer, -time and -temperature, as well as the optimal concentrations of antibody and coating peptide were determined and the finalized SPARC-M competitive ELISA protocol was as follows:

A 96-well streptavidin-coated microtiter plate was coated with 1.1 ng/mL biotinylated coating peptide dissolved in assay buffer (50 mM Tris-BTB, 4 g/L NaCl, pH 8.0) and incubated for 30 min. at 20°C with shaking (300 rpm) in darkness shaking. Twenty µL calibrator peptide or pre-diluted serum (1:4) were added to appropriate wells, followed by the addition of 100 µL monoclonal antibody dissolved in assay buffer to a concentration of 14 ng/mL per well and incubated 1 hour at 20°C in darkness with shaking (300 rpm). One hundred µL of goat anti-mouse horse-radish peroxidase (POD)-conjugated IgG antibody (Thermo Scientific, cat. no. 31437) diluted 1:6000 in assay buffer was added to each well and incubated 1 hour at 20°C in darkness with shaking. All incubation steps were followed by five washes in washing buffer (20 mM Tris, 50 mM NaCl, pH 7.2). Finally, 100 µL tetramethylbenzidine (TMB) (Kem-En-Tec Diagnostics, cat. no. 438OH) was added to each well and the plate was incubated for 15 minutes at 20°C in darkness with shaking. The enzymatic reaction was stopped by adding 0.18 M H2SO4 and absorbance was measured at 450 nm with 650 nm as reference. A calibration curve was plotted using a 4-parameter logistic curve fit. Data were analyzed using the SoftMax Pro v.6.3 software.

Technical evaluation of the SPARC-M ELISA

To evaluate the technical performance of the SPARC-M ELISA, the following validation tests were carried out: Inter-

and intra-assay variation, linearity, lower limit of detection, upper limit of detection, analyte stability (freeze/thaw and storage) and interference.

The inter- and intra-assay variation was determined by ten independent runs on different days using seven quality control samples covering the detection range, with each run consisting of double-determinations of the samples. The seven quality control samples consisted of: two human serum samples and five samples with standard peptide in buffer. Intra-assay variation was calculated as the mean coefficient of variance (CV%) within plates and the inter-assay variation was calculated as the mean CV% between the ten individual runs analyzed on different days. To assess linearity of the assay, two-fold dilutions of human serum samples were performed and dilution linearity was calculated as a percentage of recovery of the un-diluted sample. The lower limit of detection (LLOD) was determined from 21 measurements using assay buffer as sample and was calculated as the mean + three standard deviations. The upper limit of detection (ULOD) was determined from ten independent runs of the highest standard peptide concentration and was calculated as the mean back-calibration calculation + three standard deviations. Analyte stability was first determined by the effect of repeated freeze/thaw of serum samples by measuring the SPARC-M level in three human serum samples in four freeze/thaw cycles. The freeze/thaw recovery was calculated with the first cycle as reference. Second, analyte stability in relation to storage was determined by a 48 hour study performed at 4°C or 20°C. The SPARC-M level in three human serum samples was measured after 0 h, 4 h, 24 h and 48 h of storage, and recovery was calculated with samples stored at −20°C as reference. Interference was determined by adding a low/high content of hemoglobin (0.155/0.310 mM), lipemia/lipids (4.83/10.98 mM) and biotin (30/90 ng/mL) to a serum sample of known concentration. Recovery percentage was calculated with the serum sample as reference.

Cleavage of SPARC *in vitro*

Recombinant human SPARC (PeproTech, cat. no. 120–36) was reconstituted to a final concentration of 1000 ug/mL in MMP-buffer (50 mM Tris-HCl, 150 mM NaCl, 10 mM CaCl2, 10uM ZnCl, 0.05%Brij35, pH 7.5). MMP-2, MMP-8, MMP-9 and MMP-13 (Giotto, cat. no. G04MP02C, G04MP08C, G04MP09C, G04MP13C) were added 1:10 (1 µg MMP and 10 µg SPARC). Digestion of carboxymethylated transferrin (a natural substrate of MMP's) was included as a positive control. The solutions incubated at 37°C for 24 h. The reaction was stopped by adding 1 µM EDTA to the solutions. MMP-buffer added the different proteases alone were included as negative controls. Samples were stored at −80°C until analysis. The activity of the proteases was confirmed by silverstaining according to the manufacturer's instructions (SilverXpress®, Invitrogen, cat. no. LC6100) and coomassie blue (data not shown).

Clinical validation of SPARC-M

Patient serum samples were obtained from the commercial vendor ProteoGenex. The discovery cohort (cohort 1) consisted of patients with lung cancer, idiopathic pulmonary fibrosis (IPF), chronic obstructive pulmonary disease (COPD) and healthy colonoscopy-negative controls with no symptomatic or chronic disease (Table 2). The validation cohort (cohort 2) included 40 patients with different stages of lung cancer, and 20 age- and gender-matched healthy colonoscopy-negative controls with no symptomatic or chronic disease (Table 2). Appropriate Institutional Review Board/Independent Ethical Committee approved sample collection and all subjects filed informed consent.

Effect of SPARC on fibrillar collagen degradation

Recombinant human SPARC (PeproTech, cat. no. 120–36) was reconstituted to a final concentration of 1000 ug/mL in MMP-buffer. Natural human type I collagen (Abcam, cat. no. ab7533) and type III collagen (Abcam, cat. no. ab7535) was dialyzed for 2 days to remove the acetic acid, against MMP buffer using Slide-A-Lyzer™ Dialysis Cassettes, 3.5 K MWCO, 0.5 mL (Thermofisher, cat. no. 66333) according to the manufacturer's instructions. The collagens were either incubated with MMP-9 (Giotto, Firenze, cat. no. G04MP09C) alone (MMP:collagen ratio of 1:17) or together with MMP-9 and SPARC (collagen:SPARC molar ratio of 1:10). In addition type I collagen was also incubated with MMP-13 (Giotto, cat. no. G04MP13C) with or without SPARC. The solutions incubated at 37°C for 1 h, 4 h, 8 h and 24 h. The reaction was stopped by adding 1 µM EDTA. MMP-buffer with either collagen or MMP's alone were included as negative controls. Digestion of carboxymethylated transferrin (a natural substrate of MMP's) was included as a positive control and this reaction was stopped after 24 h. Samples were stored at −80°C until analysis. MMP-9 and −13 mediated degradation of type I collagen was investigated by an ELISA measuring type I collagen degradation (C1M) (Nordic Bioscience) and type III collagen was investigated by an ELISA measuring MMP-9 mediated degradation of type III collagen (C3M) (Nordic Bioscience). The C1M analyte has previously been shown to be generated by MMP-9 and MMP-13, and the C3M analyte by MMP-9, and the assays do not react to non-cleaved collagen[36,37]. The activity of the MMP's was confirmed by Coomassie blue staining (data not shown).

Statistical analysis

The level of SPARC-M in serum samples was compared using unpaired, two-tailed Mann-Whitney test or Kruskal-Wallis adjusted for Dunn's multiple comparisons test. Patients in cohort 2 were stratified according to their tumor stage and the level of SPARC-M in each group was compared using Kruskal-Wallis adjusted for Dunn's multiple comparisons test. The discriminative power was investigated by the area under the receiver operating characteristics (AUROC) comparing patients with lung cancer and healthy controls. Graph design and statistical analyses were performed using GraphPad Prism version 7 (GraphPad Software, Inc.).

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Disclosure statement

T. Manon-Jensen, S. Sun, D.J. Leeming, M.A. Karsdal and N. Willumsen are employed at Nordic Bioscience A/S which is a company involved in discovery and development of biochemical biomarkers. M.A. Karsdal owns stocks at Nordic Bioscience. S.N. Kehlet and S. Brix reports no conflict of interest.

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Additional results 2 - Validation of a novel serum immunoassay targeting the pro-peptide of type XI collagen

This section is a draft manuscript describing assay validation of an Electro-ChemiLuminescence ImmunoAssay (ECLIA) targeting the pro-peptide of type XI collagen. To investigate the pathological relevance and prognostic potential, this biomarker is going to be measured in a cohort of 1200 patients with pancreatic cancer, where survival outcome is available (the BIOPAC (biomarkers in patients with pancreatic cancer) cohort, https://clinicaltrials.gov/ct2/show/NCT03311776).

Introduction

The tumor microenvironment is often characterized by an extensive amount of cancer-associated fibroblasts (CAFs) which are fibroblasts that are irreversibly activated due to persistent injurious stimuli from the surrounding stroma. They are the most abundant cell type in the tumor microenvironment and known to dictate tumor outcome (35). CAFs have been shown to be associated with poor overall survival and in breast carcinomas about 80% of the stromal fibroblasts acquire a CAF phenotype (37). Activated CAFs secrete growth factors, enzymes and other ECM proteins that promote tumor growth, angiogenesis, tumor invasion and metastasis and they are therefore thought of as a rich reservoir of tumor-promoting factors (29). Recognizing this important role of CAFs and a desmoplastic stroma in tumor development and progression, identifying and targeting stromal components of the tumor is a field of extensive research in cancer.

One of the main proteins secreted by CAFs are collagens and studies have focused on identifying CAF-specific genes/proteins that could serve as novel biomarkers. One of the most specific CAF genes that have been identified so far are COL11A1 which encodes the α1-chain of type XI collagen (45). Type XI collagen is a minor fibrillar collagen expressed by chondrocytes, osteoclasts, CAFs, but not quiescent fibroblasts. The function of type XI collagen has been suggested to involve maintaining of proper fibril formation and diameter. Like all other collagens, type XI collagen is a heterotrimer consisting of α1-, α2- and α3-chains, which are synthesized as pro-collagens and proteolytically cleaved to yield mature type XI collagen. Cleaved pro-peptides released to the circulation could serve as surrogate biomarkers of type XI collagen formation and provide novel diagnostic and/or prognostic information in patients with a stroma-rich cancer.

In the present study, we developed an antibody targeting the N-terminal pro-peptide of type XI collagen and technically validated a competitive Electro-ChemiLuminescence ImmunoAssay (ECLIA) quantifying this specific fragment in the circulation.

Methods

Selection of peptides and monoclonal antibody production

A sequence of 10 amino acids next to the cleavage site in the N-terminal pro-peptide of the α1-chain of type XI collagen was chosen as the target: $_{244}$DSSAPKAAQA$_{253}$↓. The first six amino acids was blasted for homology

to other human secreted extracellular matrix proteins using NPS@: Network Protein Sequence Analysis with the UniprotKB/Swiss-prot database (184).

To generate a monoclonal antibody and technical validate the ECLIA, synthetic peptides were purchased from Genscript (Piscataway, NJ, USA) and shown in table 7. The target sequence was used as the calibrator peptide (DSSAPKAAQA). A biotinylated peptide (biotin-DSSAPKAAQA) was included as a coating peptide. The specificity of the antibody was tested by including an elongated calibrator peptide with an additional amino acid added to the C-terminal of the target peptide sequence (DSSAPKAAQAQ), a truncated calibrator peptide with a removal of the first C-terminal amino acid (DSSAPKAAQ) and a non-sense calibrator peptide (LLARDFEKNY). To screen for any potential cross-reactivity to other proteins and further test the antibody specificity, three peptides (derived from cystatin-M, Lysyl oxidase homolog 1 and Keratin-like protein KRT222) with one amino acid mismatch compared to the first six amino acids in the target sequence were also included (table 7).

Table 7. Synthetic peptides used for development and validation of the PRO-C11 ECLIA

Peptide name	Amino acid sequence
Calibrator peptide	DSSAPKAAQA
Immunogenic peptide	KLH-CGG-DSSAPKAAQA
Biotinylated coating peptide	biotin-DSSAPKAAQA
Elongated peptide	DSSAPKAAQAQ
Truncated peptide	DSSAPKAAQ
Non-sense calibrator peptide	LLARDFEKNY
Cystatin-M (CYS-M)	DPQVQKAAQA
Lysyl oxidase homolog 1 (LOXH-1)	PDPGPEAAQA
Keratin-like protein KRT222 (KRT222)	DEEALKAAQA

The immunogenic peptide (KLH-CGG-DSSAPKAAQA) was generated by covalently cross-linking the calibrator peptide to Keyhole Limpet Hemocyanin (KLH) carrier protein using Succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate, SMCC (Thermo Scientific, Waltham, MA, USA, cat.no. 22336). Glycine and cysteine residues were added at the N-terminal end to ensure right linking of the carrier protein. Monoclonal antibodies were generated by subcutaneous immunization of six week old Balb/C mice with 200 µL emulsified antigen containing 50 µg immunogenic peptide (KLH-CGG-DSSAPKAAQA) mixed with Freund's incomplete adjuvant (Sigma-Aldrich, St. Louis, MO, USA). Consecutive immunizations were performed at 2-week intervals until stable sera titer levels were reached. The mouse with the highest titer rested for four weeks and was then boosted with 50 µg immunogenic peptide in 100 µL 0.9% NaCl solution intravenously. Hybridoma cells were produced by fusing spleen cells with SP2/0 myeloma cells as previously described (185).

The resultant hybridoma cells were then cultured in 96-well microtiter plates and standard limited dilution was used to secure monoclonal growth.

The best clone was selected based on reactivity towards the above mentioned synthetic peptides and the monoclonal antibody was purified using protein-G-columns according to the manufacturer's instructions (GE Healthcare Life Sciences, Little Chalfont, UK, cat. #17-0404-01).

PRO-C11 ECLIA protocol

The competitive PRO-C11 ECLIA was performed accordingly, after determination of optimal incubation buffer, -time and -temperature, as well as the optimal ratio of antibody and coating peptide:
A 96-well streptavidin-coated MSD GOLD SECTOR plate (Meso Scale Diagnostics, Maryland, USA, cat. #L15SA) was blocked with 150 µL blocking buffer/well (10mM PBS + 5% BSA, pH 7.4) for 1 hour at 20°C in darkness with shaking (300 rpm). After three times of washing (200mM PBS + 10% Tween-20, pH 6.5) the plate was coated with 0.5 ng/mL biotinylated coating peptide (25 µL/well) dissolved in assay buffer (100 mM PBS-BTB, 4 g/L NaCl, pH 7.4) and incubated for 1 hour at 20°C in darkness with shaking (300 rpm). After washing, 25 µL calibrator peptide or serum sample were added to appropriate wells, followed by the addition of 25 µL monoclonal antibody dissolved in assay buffer to a concentration of 15 ng/mL per well and incubated 20 hours at 4°C in darkness with shaking (300 rpm) followed by washing. Twenty-five µL of 2 ug/mL SULFO-TAG goat anti-mouse secondary antibody (Meso Scale Diagnostics, Maryland, USA, cat. #R32AC) was added to each well and incubated 40 min. at 20°C in darkness with shaking (300 rpm). The plate was washed three times and 150µl/well of MSD GOLD Read Buffer (Meso Scale Diagnostics, Maryland, USA, cat. #R92TG) was added immediately prior to reading of the plate. The intensity of the signal was measured and the raw signal values were used to generate a calibration curve using a 4-parameter logistic curve fit. Data were analyzed with the Meso Scale Workbench v. 4.0 software.

PRO-C11 technical evaluation

The technical performance of the PRO-C11 ECLIA was evaluated with the following validation tests: Lower Limit of Measuring Range (LLMR), Upper Limit of Measuring Range (ULMR), Inter- and intra-assay variation, linearity, accuracy (spiking), analyte stability (freeze/thaw and storage) and interference.
The analytical measurement range was defined as the concentrations from LLMR to ULMR (the linear part of the standard curve) estimated from ten independent runs. The inter- and intra-assay variation was determined by ten independent runs on different days using seven quality control samples covering the detection range, with each run consisting of double-determinations of the samples. The seven quality control samples included three human serum samples and four samples with calibrator peptide in buffer. Intra-assay

variation was calculated as the mean coefficient of variance (CV%) within plates and the inter-assay variation was calculated as the mean CV% between the ten individual runs analyzed on different days. Linearity (dilution recovery) was determined with 2-fold dilutions of three human serum samples and calculated as percentage recovery of the un-diluted samples. Accuracy (spiking recovery) was assessed by combining eight human serum samples of known concentration and spiking recovery was calculated as the measured PRO-C11 amount percentage recovery of the theoretical amount. In addition cleaved recombinant type XI collagen was spiked into serum. Analyte stability was determined by the effect of repeated freeze/thaw and in relation to temperature storage. Three serum samples were thawed and frozen four times followed by PRO-C11 measurement. The freeze/thaw recovery was calculated with the first cycle as reference. A 48 hour study was performed to determine analyte stability at 4°C and 20°C using three human serum samples. The level of PRO-C11 was measured after 4h, 24h and 48 h of storage, and recovery was calculated with non-stressed samples stored at -20°C as reference. Interference was determined by adding a low/high content of hemoglobin (2.5/5 mg/mL), lipemia/lipids (1.5/5 mg/mL) and biotin (30/90 ng/mL) to a serum sample of known concentration. Recovery percentage was calculated with the serum sample as reference.

In vitro cleavage of the N-terminal pro-peptide

To further investigate the specificity of the antibody, recombinant human type XI collagen (synthesized by Genscript, Piscataway, NJ, USA) was cleaved by BMP-1 (R&D Systems, MN, USA) , the known protease responsible for cleavage of the pro-peptide. Type XI collagen and BMP-1 were added 1:10 (2 µg BMP and 20 µg type XI collagen) and the cleavage was performed in BMP-1 cleavage buffer (50 mM TrisHCL, 150 mM NaCl, 5 mM CaCL2, 0.1% Brij-35 pH 7.5). The solutions incubated at 37°C for 6h and 24h. The reaction was stopped by adding 1 µM EDTA to the solutions. BMP-1 and type XI collagen alone were included as negative controls. Samples were stored at -80°C until analysis. The cleavage was confirmed by measuring the samples in the PRO-C11 ECLIA.

Statistical analysis

Graphs were designed using GraphPad Prism version 7 (GraphPad Software, Inc., CA, USA).

Results

Specificity of the PRO-C11 ECLIA

To ensure antibody specificity against the N-terminal pro-peptide of type XI collagen, the first six amino acids in the target sequence, PKAAQA, was blasted for homology to other human secreted extracellular proteins using NPS@: Network Protein Sequence Analysis with the UniprotKB/Swiss-prot database. The target sequence was found to be 100% unique to human type XI collagen when compared to other human proteins.

Allowing one amino acid mismatch, three proteins, cystatin-M, Lysyl oxidase homolog 1 and Keratin-like protein KRT222, were identified with mismatches at the 6th, 5th, and 6th position, respectively (table 7). The antibody showed minimal reactivity against the three peptides as compared to the calibrator peptide within the measurement range (figure 9A). The specificity of the competitive PRO-C11 ECLIA was further evaluated by analyzing the reactivity of the antibody towards different synthetic peptides (table 7, figure 9B). There was no reactivity towards the elongated, the truncated and the non-sense peptide and the calibrator peptide clearly inhibited the signal in a dose-dependent manner compared to the other peptides.

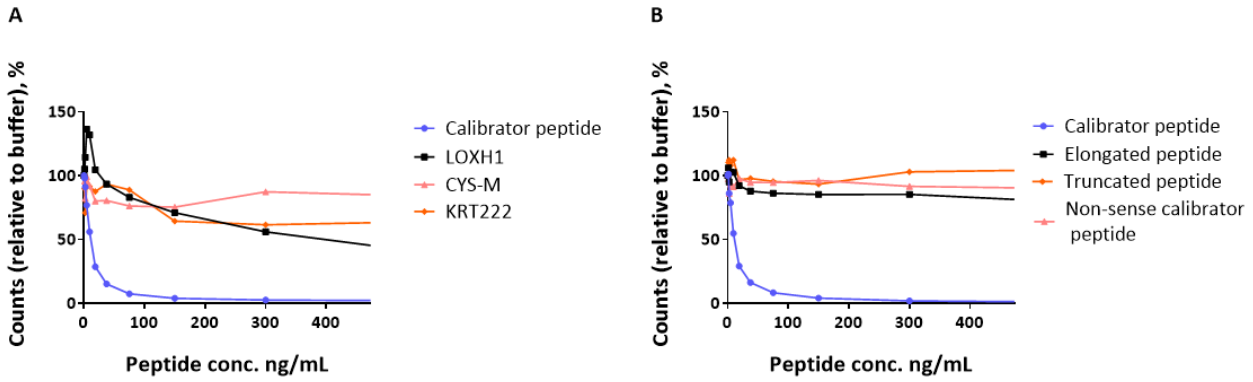

Figure 9. Specificity of the PRO-C11 monoclonal antibody

The percentage of inhibition relative to buffer at given concentrations in competitive PRO-C11 ECLIA with calibrator peptide (DSSAPKAAQA) and (A) three deselection peptides: Lysyl oxidase homolog 1 (LOXH-1, PDPGPEAAQA), Cystatin-M (CYS-M, DPQVQKAAQA), Keratin-like protein KRT222 (KRT222, DEEALKAAQA) and (B) displacement peptides: elongated peptide (DSSAPKAAQAQ), truncated peptide (DSSAPKAAQ) and non-sense calibrator peptide (LLARDFEKNY).

To further evaluate the specificity of the antibody, BMP-1, which is the protease known to cleave off the pro-peptide of the α1-chain of type XI collagen, was incubated together with recombinant type XI collagen and the samples were measured in the PRO-C11 ECLIA. As shown in figure 10, BMP-1 was able to generate the pro-peptide fragment in a time dependent manner.

Together, these data suggest that the monoclonal antibody exhibits high neo-epitope specificity towards the N-terminal pro-peptide when cleaved off by BMP-1.

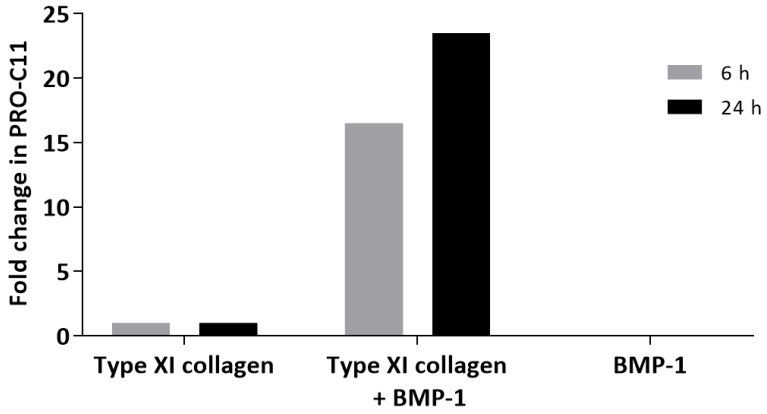

Figure 10. Cleavage of type XI collagen by BMP-1

Type XI collagen was incubated with BMP-1 and PRO-C11 levels were measured after 6 hours and 24 hours. Data were normalized by subtracting the background measured in buffer alone. The graph shows the fold change increase in PRO-C11 between type XI collagen and type XI collagen cleaved with BMP-1.

PRO-C11 technical evaluation

The technical performance of the PRO-C11 ECLIA is summarized in table 8. The measuring range (LLMR to ULMR) of the assay was determined to 5.6 – 473.8 ng/mL. The intra- and inter-assay variation was 7% and 9%, respectively. The mean dilution recovery for human serum was 101% observed from undiluted to a 1:4 dilution or until the measured value was below LLMR. Mean spiking recovery for human serum spiked with different serum samples was determined to 94%. Serum spiked with cleaved protein gave a recovery of 95% indicating high accuracy of this assay. The analyte stability was analyzed according to freeze/thaw cycles and sample stability at 4°C and 20°C. The analyte recovery in serum was 118% after 4 freeze/thaw cycles. The PRO-C11 analyte was recovered after prolonged storage of human serum at 4˚C for 24 or 48 hours, resulting in a 104% and 90% recovery, respectively. Storage at 20˚C for 24 or 48 hours resulted in a 104% and 125% recovery, respectively. These data indicate that the analyte in serum is stable at 4°C up to 48 hours, however upon analysis serum samples should not be stored above 20°C for more than 24 hours. No interference was detected from either low or high contents of lipids or hemoglobin with recoveries ranging from 86-102%. Low levels of biotin (30 ng/mL) gave a recovery of 109%, however very high levels of biotin (90 ng/mL) resulted in a recovery of 189%. The normal range of biotin is 0.2-3 ng/mL, thus at concentration 10 times higher than the physiological concentration of biotin, no interference is observed.

Table 8. Technical validation data of the PRO-C11 ECLIA

Technical validation step	PRO-C11 performance	Acceptance criterion
Measuring range (LLMR-ULMR)	5.6 - 473.8 ng/mL	NA
Intra-assay variation	7%	10%
Inter-assay variation	9%	15%
Dilution of serum samples	Undiluted	NA
Dilution recovery	101%	100% ± 20%.
Spiking recovery (cleaved material in serum)	95%	100% ± 20%.
Spiking recovery (serum in serum)	94%	100% ± 20%.
Freeze/thaw recovery (after 4 cycles)	118%	100% ± 20%.
Analyte stability at 4°C, 24h/48h	104% / 90%	100% ± 20%.
Analyte stability at 20°C, 24h/48h	104%/125%	100% ± 20%.
Interference Lipids, low/high	86%/102%	100% ± 20%.
Interference Biotin, low/high	109%/189%	100% ± 20%.
Interference Hemoglobin, low/high	93%/101%	100% ± 20%.
Percentages are reported as mean		

Conclusion

The present study validated a new serological ECLIA quantifying the pro-peptide of type XI collagen. The assay was found to be technically robust with an antibody highly specific for the neo-epitope. Further studies are needed to evaluate the diagnostic/prognostic use of PRO-C11 in cancer.

5. Discussion

The hypothesis of this PhD thesis was that pathologically driven turnover of the ECM results in the release of neo-epitope biomarkers into the circulation that can serve as diagnostic, prognostic and/or predictive tools in cancer. To investigate this, biomarkers reflecting ECM remodeling was measured in different patient cohorts using robust and technically validated immunoassays. These studies resulted in four original published papers and two additional unpublished studies (additional results 1 and 2). Paper I was included to provide information about common confounders and their association with some of the protein fingerprint biomarkers studied in this thesis. Paper II and additional results 1 investigated the diagnostic and prognostic use of protein fingerprint biomarkers in cancer. Paper III, IV and additional results 2 introduced the development and validation of novel protein fingerprint biomarkers reflecting structural changes of the ECM. Each of the included papers discuss their respective findings. The discussion in this section are therefore of a more general nature and the following headlines will guide the discussion:

- Considerations when developing biomarker assays and evaluating serological biomarkers (chapter 5.1)
- Are ECM remodeling and ECM-derived neo-epitopes the cause or consequence of cancer? (chapter 5.2)
- Can protein fingerprint biomarkers be used as novel diagnostic/prognostic tools in cancer? (chapter 5.3 and 5.4)

Table 5 (page 32) gives an overview of the protein fingerprint biomarkers used in this thesis.

5.1 Considerations when developing biomarker assays and evaluating serological biomarkers

Technical and clinical evaluation

The technical evaluation described in paper III, IV and additional results 2 illustrates a typical assay development process for the protein fingerprint biomarkers. The assays developed in this thesis were robust and highly specific for the neo-epitopes of interest. As described in section 1.4 several steps are needed to ensure a well-validated robust assay. First, a pathologically relevant biomarker target must be identified. The target of interest can be identified by *in vitro/ex vivo* proteolytic digestion followed by mass spectrometry (MS). The decorin target in paper III was identified by MS after *ex vivo* proteolytic cleavage of human knee articular cartilage (186). In paper IV, the SPARC cleavage site was identified by *in vitro* cleavage of SPARC by different metalloproteases (152). An important consideration is that targets identified by these methods may not be generated *in vivo* and native circulating molecules may have a certain conformation masking the neo-

epitope of interest. However, during assay validation, native reactivity and biological relevance were investigated and the fragments were found to be present in serum from patients with different lung disorders suggesting that these fragments are generated *in vivo*. One way to improve target identification and the diagnostic utility could be to perform *ex vivo* proteolytic cleavage in a model system using normal and diseased human material and conduct a direct comparison of the generated peptides. In this way, only fragments that are generated or increased in the diseased samples will be used as targets. Second, one must assure that the assay is specific, reproducible and accurately measures the neo-epitope of interest. Specificity was evaluated by testing the antibody reactivity towards peptides similar to the calibrator peptide. In addition, reactivity towards the cleaved protein and not the intact protein confirmed high specificity towards the neo-epitope. Accuracy was evaluated by spiking the target peptide (calibrator) or cleaved protein into serum and calculating the percentage recovery. It is worth noting, that the monoclonal antibodies used in this thesis are raised against small peptides and the affinity of the antibody can change when used in native material. This could be improved by affinity maturation using native material. Precision and reproducibility were analyzed by inter- and intra-precision variation and dilution recovery (linearity).

As described in section 1.3, several common confounders exist that can influence biomarker measurements. Thus, when assessing biomarkers, it is extremely important that the subjects in each group are matched. If populations are not matched an observed difference in biomarker level may not be pathological relevant, but merely due to differences in e.g. age. Paper I highlights the importance of age- and sex-matching when evaluating collagen turnover biomarkers in clinical studies. This paper demonstrated that healthy men and women have different levels of circulating collagen turnover fragments and across the biomarkers, the level increased in women around the age of menopause. The cohort in paper II was age-matched but not gender matched. The healthy control group consisted of 65% women whereas the colorectal cancer group consisted of 32% with a mean age around menopause. We observed increased biomarker levels in the colorectal cancer group, which contains less women, suggesting that this increase is in fact a pathological increase and not just a difference between men and women. Furthermore, when adjusting for age and gender in a logistic regression model, C3M and PRO-C3 were independent predictors of metastasis. The cohorts in paper III, IV and additional results 2 were not age- and gender matched. As these papers include “proof-of-concept” cohorts, larger and more optimal studies are needed to elucidate the exact clinical applicability of these biomarkers.

An important consideration when measuring serological biomarkers is that a systemic pool of the protein of interest is measured. As tissue remodeling is a physiological process taking place in every organ and becomes increased as a result of a pathological response, one cannot be certain that the protein of interest measured

in serum is in fact from the diseased organ of interest. As cancer is an age-related disease (5) and the number of co-morbidities increase with age (187), the contribution of co-morbidities from other organs becomes an important factor when evaluating biomarker results. Unfortunately, medical history was not available for the cohorts included in this thesis, which is a limiting factor.

The gold standard for protein-based biomarker quantification is the ELISA (188). The recent years several other immunoassays have emerged, such as AlphaLISA and ECLIA. In this thesis, two different immunoassays were used for the assay development; the ELISA (paper III and IV) and ECLIA (additional results 2). The ELISA is based on measuring light absorbance reflecting enzyme activity whereas the ECLIA measures light intensity stimulated by electricity. The main advantage of ECLIA compared to ELISA is its higher sensitivity, however this kind of assay have a much higher cost compared to standard ELISA. PRO-C11 was tested on both platforms, and the ECLIA had the highest sensitivity, thus the PRO-C11 assay was optimized using this assay platform.

The protein fingerprint biomarkers vs total proteins – What do we gain?

A protein is not just a protein and assessment of different parts/PTMs of a protein, instead of a pool of total protein, may provide essential information in biomarker measurement. A standard example is hemoglobin where the intact protein is a necessity in life but PTM-modified glycosylated hemoglobin is the gold standard marker of diabetes (11). The protein fingerprint biomarkers investigated in this thesis derive from ECM proteins. It has been well-established that collagen neo-epitopes provide more pathology relevant information compared to total protein (189–191). An example is the measurement of total type I collagen vs. its degradation fragments in postmenopausal women with osteoporosis (189). Post-menopausal women with osteoporosis had increased levels of total serum type I collagen compared to age-matched individuals, which is peculiar as osteoporosis is characterized by bone degradation. The use of protein fingerprint assays able to separate type I collagen formation and degradation, revealed that osteoporotic women had an increase in both degradation and formation, but with a balance skewed towards degradation leading to bone loss. These results show that measuring serological proteins with an assay using polyclonal antibodies directed towards the intact protein, can provide information that is misleading and therefore misinterpreted (figure 11).

The protein fingerprint technology relies on combining a pathology-dependent protease and a signature protein. This combination potentially increase the specificity of the biomarkers. The proteins evaluated in this thesis are not tissue/cancer specific, however the combination of pathology-relevant proteins and proteases compared to total protein, may reduce the systemic background noise and thereby increase specificity for the local remodeling process.

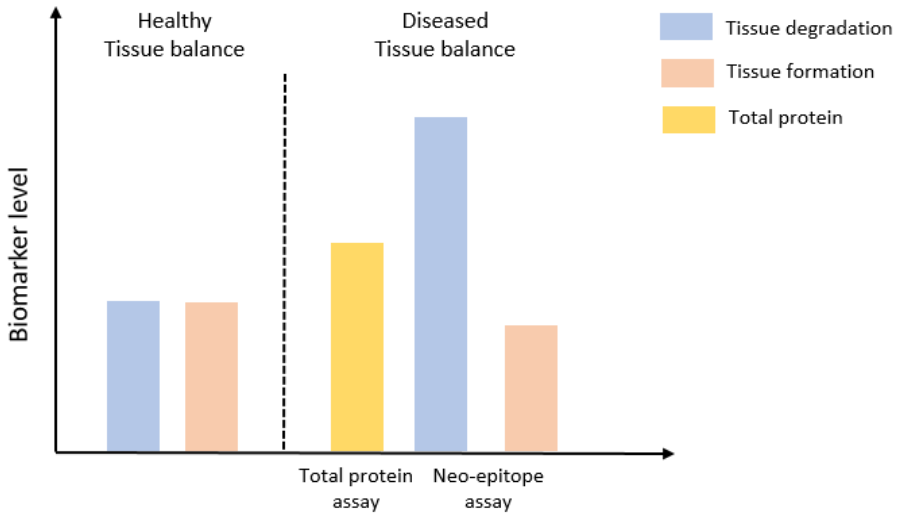

Figure 11. Total protein assays vs neo-epitope assays

Neo-epitope assays separating tissue degradation and formation may provide more pathological relevant information compared to standard commercial ELISAs using polyclonal antibodies measuring total protein.

5.2 Are ECM remodeling and ECM-derived neo-epitopes the cause or consequence of cancer?

In paper II, III, IV and additional results 1, ECM-derived neo-epitopes were elevated in cancer patients with different solid tumors compared to healthy controls. These data suggest that the ECM remodeling process is more active in cancer giving rise to increased release of small ECM peptides into the circulation. The fact that the collagen derived neo-epitopes were elevated with an increase in tumor stage (paper II) suggests that the fragments are secreted directly from the tumor microenvironment. Whether ECM remodeling and thus, ECM-derived neo-epitopes are a cause or consequence of cancer will be discussed in this section.

The important role of the right tumor microenvironment was proposed in 1889 with the seed and soil hypothesis describing how cancer cells (the seed) need a certain microenvironment (the soil) to progress (192). This was later confirmed by showing that normal tissue can revert a malignant phenotype (193–195). These studies showed that cancer cells need altered malignant surroundings to proliferate and progress. Another example, is the pre-metastatic niche. Tumor cells can convert an unfavorable tumor environment of a distant site into favorable surroundings by secreting factors before and upon arrival at the metastatic niche. These factors promote, among other things, increased collagen deposition and crosslinking and in this way

an optimal microenvironment is established (196,197). The increased levels of PRO-C3 and PRO-C6 (observed in paper II and additional results 1), which are surrogate markers of collagen formation, might originate from the priming of the metastatic niche in these patients and thereby contribute to a pro-tumorigenic environment. It has also been shown that dormant tumor cells, which are residual tumor cells that are inactive for a prolonged period of time, can become malignant in response to changes of the ECM (198).

Thus, extensive research suggest that altered ECM remodeling is a pre-requisite for cancer development and cancer progression. Supporting this, Bager *et al.* have shown that protein fingerprint biomarkers reflecting collagen and vimentin degradation are significantly elevated in subjects prior to cancer diagnosis compared to subjects with no cancer diagnosis within the follow-up period. C1M (type I collagen degradation) and VICM (citrullinated and MMP-degraded vimentin) independently predicted an increased risk of cancer (199). These results indicate that a certain ECM phenotype is important for cancer development and subjects with an early sign of an increased ECM turnover have a higher chance of developing cancer compared to subjects with a low turnover. Bager *et al.* (Nordic Bioscience) are currently investigating if levels of collagen-derived biomarkers further increase upon diagnosis and as function of cancer progression in subjects with an elevated ECM turnover prior to diagnosis. Consequently, the discriminative power of these biomarkers increases as the separation between healthy and diseased increases. In paper II, we did not observe a significant difference in biomarker levels between healthy controls and subjects with adenomas which was anticipated on the basis of the results generated by Bager *et al.* (199). This might be due to the fact that no follow-up data were available on whether the subjects with adenomas were actually diagnosed with cancer later on and if so when they were diagnosed. Furthermore, the subject number was lower in our cohort and we only investigated colorectal cancer patients whereas the Bager cohort included several cancer types which might have a more aggressive ECM remodeling phenotype. The findings by Bager *et al*., and the fact that a portion of both the healthy controls and adenomas in paper II had high levels of the biomarkers reflecting an altered increased ECM turnover, could lead to the hypothesis that subjects with an altered ECM remodeling have an increased risk of cancer. This needs to be further evaluated.

In addition to structural changes of the ECM being a driver of tumorigenesis, it is also possible that the ECM-derived neo-epitopes have tumorigenic properties themselves and are not only turnover products released as a consequence of tumorigenesis. As mentioned in the introduction (chapter 1.2), proteolysis of collagens can result in the release of cryptic sites with signaling functions. Biomarkers playing an active role in cancer could be more specific with a higher diagnostic/prognostic/predictive value. Whether the investigated collagen-derived neo-epitopes play an active role is beyond the scope of this thesis. However, as shown in the additional results 1 section, patients with high levels of PRO-C3 and PRO-C6 (collagen formation) have a

worse prognosis compared to patients with low levels. These results suggest that excessive collagen deposition around the tumor may limit cancer therapy delivery and immune cell access into the tumor, resulting in a lack of response to therapy and/or potentially that the pro-peptides have an active biological function resulting in tumor progression. Studies have shown that different collagen fragments can have biological activity by binding to integrins and immunoreceptor tyrosine-based inhibitory motif (ITIM) bearing receptors resulting in pro-tumorigenic responses. The group of Mina Bissel have shown that blocking of β1-integrin on malignant cells reverts a malignant phenotype to a phenotype similar to normal human breast epithelial cells (193) suggesting that ECM components, that are able to activate integrins, are dynamic players in tumorigenesis. The leukocyte-associated immunoglobulin-like receptor 1 (LAIR-1) is an ITIM receptor. This receptor is expressed on the majority of human peripheral blood mononuclear leukocytes. Collagen fragments have been shown to activate this receptor resulting in a negative regulation of an immune response (200–202). This mechanism could maintain tumor infiltrating immune cells in a pro-tumorigenic state explaining why patients with high levels of collagen remodeling biomarkers have a shorter survival. In addition, the PRO-C6 assay also targets the bioactive fragment of type VI collagen, endotrophin. Endotrophin is known to have pro-tumorigenic properties by augmenting fibrosis via induction of TGF-β, angiogenesis and inflammation through recruitment of macrophages (87). Thus, patients with a high level of PRO-C6 (endotrophin) may have a worse prognosis due its pro-tumorigenic function.

Paper IV revealed a biomarker potential of a fragment of SPARC reflecting increased affinity for collagens. This study also demonstrated a new chaperone function of SPARC, namely the ability of SPARC to protect interstitial collagens from being degraded by MMPs. Cleavage of SPARC might generate a fragment with pro-tumorigenic properties when produced in excess. We hypothesize that increased cleavage of SPARC at this specific site known to increase the affinity to collagens, is involved in increasing the collagen deposition by inhibiting degradation of collagens. This mechanism could play an active role in fibrotic disorders with increased collagen deposition, such as cancer and deserves further evaluation.

5.3 Can protein fingerprint biomarkers be used as diagnostic tools in cancer?

There is a need for biomarkers that can aid in early detection and diagnosis of cancer. The aim of paper II was to investigate the diagnostic potential of serological neo-epitope biomarkers reflecting collagen turnover in colorectal cancer. Colorectal cancer develops from a benign precursor lesion called an adenoma. The progression from an adenoma into cancer can easily take 10 years (203) providing a large time-span during which screening and curative intervention can take place. In the present study, the subjects with adenomas showed no significant difference in biomarker levels when compared to healthy controls. Comparing healthy

controls with the colorectal cancer patients, an increased level of C1M and PRO-C3 was observed. The highest level of the biomarkers were observed in the stage IV patients. These data suggests that collagen-derived fragments cannot be used as tools for early detection of colorectal cancer. In line with this, several of these collagen-derived neo-epitope biomarkers have been shown to be increased in other cancer types as well as other ECM-remodeling diseases and therefore they are not specific for cancer or one specific cancer type (62,63,204,205). C1M and C3M may reflect a general inflammatory state and PRO-C3 a fibrotic state.

The discriminative performance of a biomarker can be evaluated by the AUC. In paper II, PRO-C3 and C3M had the best discriminative performance for detecting metastatic colorectal cancer vs non-metastatic, with an AUC of 0.8 when combining the biomarkers. These data suggest that the ELISAs used in this study, are able to measure the systemic tumor activity (when the tumor has spread to other organs of the body) whereas when the tumor is localized, the sensitivity of these assays are not high enough to discriminate between the tumor stages and healthy controls. Based on these data, the diagnostic potential of these collagen-derived biomarkers is low for early detection and diagnosis, whereas the biomarkers can be used to evaluate the burden of disease and tumor activity (metastasis vs non-metastasis).

In the technical papers (paper III and IV), the pathological relevance of the investigated decorin- and SPARC fragments was demonstrated using “proof-of-concept” cohorts. We found that the fragments were elevated in different lung pathologies. DCN-CS was significantly elevated in patients with NSCLC compared to patients with COPD, IPF and healthy controls. IPF patients also had an increased level of DCN-CS when compared to healthy controls in another cohort. SPARC-M was significantly increased in lung cancer patients when compared to healthy controls and patients with COPD. These data indicates that these biomarkers may be specific for some lung disorders compared to others including healthy individuals suggesting a diagnostic potential, however this needs to be validated in larger cohorts as well as in other diseases.

Additional results 2 describes the validation of an ECLIA targeting the pro-peptide of type XI collagen. Type XI collagen is one of the genes that has been shown to be most specific to CAFs. García-Pravia *et al.* demonstrated by immunohistochemistry that the pro-peptide of type XI collagen was only present in tissue from pancreatic cancer patients whereas no or only weak staining were observed in healthy subjects and patients with chronic pancreatitis (89). These results suggest that the pro-peptide of type XI collagen is a specific candidate marker for CAFs compared to the most used markers, α-SMA and vimentin, which are also expressed in normal fibroblasts and other cell types. Unfortunately, the time frame of this thesis did not allow us to investigate the clinical use of this biomarker in cancer but PRO-C11 has the potential as a new serological diagnostic tool. In addition, CAFs are also being considered as a therapeutic target (29,206).

Finding the right patient population with the right CAF phenotype, is crucial and the level of PRO-C11 could have the potential to guide the selection of patients for such treatments in the future.

To sum up, as the protein fingerprint biomarkers originate from ECM proteins and the ECM is a common denominator in multiple disorders, the diagnostic potential is low. However, as the right tumor microenvironment is a necessity for tumor progression, the prognostic potential will be discussed in the next section.

5.4 Can protein fingerprint biomarkers be used as prognostic tools in cancer?

There is a need for novel prognostic biomarkers that can provide information on survival and cancer recurrence. In the additional results 1 section we demonstrated that high levels of biomarkers reflecting interstitial collagen formation are significantly associated with poor overall survival in patients with metastatic colorectal cancer. This is in line with several other studies investigating the prognostic use of collagens in cancer (207–212). In addition, another study from our group measuring C1M, C3M, C4M and PRO-C3 in two independent metastatic breast cancer cohorts, found that high levels of these collagen fragments were associated with shorter overall survival and time to progression (75). In the presented study, we found that patients with the highest level of PRO-C3 and PRO-C6 had a 9- and 7-fold increased risk of dying compared to patients with the lowest biomarker levels, respectively. The hazard ratio (HR) reported in some of the cited studies (210–212) are lower compared to our reported values. The fact that the protein fingerprint biomarkers used in this study reflect true collagen formation, might provide a better prognostic value as compared to degradation products and/or total collagen used in the other studies. In addition, we have measured type III and type VI collagen, whereas the cited papers measure type I, III, IV and XVIII collagens. As highlighted in the introduction, different collagens can be bad or good depending on their localization and post-transcriptionally processing, thus identifying the right collagen (fragment) in the right patient population is important. The difference in HR can of course also be due to differences in patient characteristics (stage, treatment ect.), patient number and cancer type. In order to conclude which biomarker provides the best prognostic value, they should be compared head-to-head in the same study.

It is well-established that the tumor stroma promotes growth and progression (9). Wang *et al.* (213) have shown that a high stroma density is associated with a significantly longer survival in patients with pancreatic cancer stage I and II, but not in patients with tumors that have spread to other parts of the body (stage III and IV). These data suggest that a dense stroma creates a host defense mechanism which restricts inflammation and progression in localized tumors. Whereas if the cancer cells have metastasized, a dense

stroma and increased collagen deposition become a defense mechanism of the cancer cells, maybe by establishing the pre-metastatic niche and limiting anti-cancer therapy and immune cell delivery into the tumor. As an extension of the study presented in the additional results 1 section, our group has just received samples from patients with different stages of colorectal cancer. Unfortunately, it was not possible to measure the protein fingerprint biomarkers before submission of this thesis, but it will be interesting to investigate the prognostic value of the collagen formation biomarkers in these patients with different tumor stages.

6. Conclusion

The overall aim of this thesis was to identify, characterize and validate blood based neo-epitope biomarkers reflecting ECM remodeling in cancer and their ability to identify patients with cancer and provide prognostic value for the future outcome in cancer patients. Based on the results presented in this thesis, the overall conclusion is that neo-epitope fragments reflecting changes of the ECM and the tumor microenvironment, have potential as promising serological biomarkers in cancer.

More specifically, the main conclusions are as follows:

- The level of collagen-derived fragments reflecting collagen turnover are significantly elevated in patients with colorectal cancer. The diagnostic potential is low as these biomarkers are not specific for a certain type of cancer as well as cancer-specific. However, they are able to identify metastatic patients from non-metastatic patients indicating a potential as burden of disease biomarkers.
- The prognostic potential of neo-epitope biomarkers reflecting collagen formation is promising. We showed that a high baseline level of PRO-C3 and PRO-C6 in patients with metastatic colorectal cancer is associated with a poor prognosis compared to patients with a low level of these biomarkers.
- Three novel immunoassays were developed and technically robust. These assays target specific fragments of proteolytically degraded decorin (DCN-CS), SPARC (SPARC-M) and type XI collagen (PRO-C11). DCN-CS and SPARC-M are released into the circulation and elevated in patients with different lung pathologies, including lung cancer, indicating biomarker potential.

The results strongly indicate that ECM turnover is altered in patients with cancer and protease-generated fragments of proteins originating from the tumor microenvironment are released into the circulation as a result of tumorigenesis. This thesis thereby support existing literature showing that alterations of the microenvironment surrounding the tumor is an important part of tumor pathology. Furthermore, these biomarkers might be able to define a certain ECM phenotype in patients with cancer which may contribute to personalized medicine. For all of the measured biomarkers, further validation are needed to elucidate their exact use in cancer.

7. Perspectives

Today, there is no optimal tools to identify cancer patients whom will benefit from a certain treatment and a large percentage of patients included in clinical trials do not respond to their treatment. Hence, there is an important and unmet medical need for predictive biomarkers in the cancer field. The neo-epitope protein fingerprint biomarkers show a potential as novel non-invasive biomarkers that can contribute to personalized medicine in cancer. Future work should focus on further validation of these biomarkers in larger cohorts in relation to prognosis and prediction. As noted, a large percentage of patients included in clinical trials do not respond to certain types of treatment. As an example, reported response rates range from 23% to 67% in clinical trials with non-small cell lung cancer (NSCLC) patients receiving anti-PD-1 or anti-PD-L1 antibodies (214). Our group has shown that the ratio of C3M vs PRO-C3 (C3M/PRO-C3), is able to identify stage IV pancreatic cancer patients that benefit significantly from an ECM modifying compound in combination with chemotherapy (unpublished data); hereby providing true predictive value of the protein fingerprint biomarkers.

The last decades, chemo- and targeted therapies have been the standard therapy in cancer treatment. Recently, immuno-therapy has begun to revolutionize the field, and cancer patients may benefit from durable long-lasting responses. Some patients have an immune-excluded phenotype, in which a dense stroma with an excessive amount of collagen in the tumor microenvironment restricts the access of tumor infiltrating lymphocytes into the tumor, resulting in poor responses (215,216). Our group have demonstrated that PRO-C3, C1M, C3M and C4M are predictors of poor overall survival in metastatic melanoma patients receiving immuno-therapy. Furthermore, the same study has also shown that these biomarkers is significantly elevated in patients with progressive disease at baseline indicating an association with treatment response (unpublished data). These results indicate a prognostic/predictive potential of the neo-epitope biomarkers in relation to immunotherapy which should be further investigated in other cohorts.

If the neo-epitope protein fingerprint biomarkers are validated in larger cohorts and able to truly predict who will respond to a certain therapy, the next step would be to seek Food and Drug Administration (FDA)/European Medicines Agency (EMA) approval as a companion/complementary diagnostics (217). This would require a validation of the biomarker of interest according to Clinical & Laboratory Standards Institute (CLSI) guidelines which ensure a highly robust and accurate assay. The CLSI guidelines focus primarily on reproducibility, analytical specificity, stability and linearity. Based on the results presented in this thesis, PRO-C3 is the biomarker with the highest prognostic and predictive potential. If the PRO-C3 assay is further validated in specific cancer patients according to CLSI guidelines and an FDA/EMA approval is obtained, the assay should be transferred to a world-wide platform developed by e.g. Roche, Abbott or Siemens, to ensure

direct global clinical utility. Nordic Bioscience, in collaboration with BMS and Roche, is currently performing a CLSI validation of PRO-C3 in NASH patients supporting a high potential of this biomarker in fibrotic disorders.

The results generated in this thesis, indicates that increased collagen deposition is associated with a bad prognosis. As pancreatic cancer is the most fibrotic cancer type, the neo-epitope biomarkers reflecting collagen formation, should be measured in clinical studies including patients with pancreatic cancer. PRO-C11, which has the potential as a new CAF specific biomarker, is scheduled to be measured in a cohort of 1200 patients with pancreatic cancer, where survival outcome is available cancer (the BIOPAC cohort, https://clinicaltrials.gov/ct2/show/NCT03311776). This study will provide information regarding the prognostic potential of this biomarker.

As mentioned in the introduction and discussion, the ECM fragments that are being targeted by the protein fingerprint ELISAs, may have a biological active function and be directly involved in tumorigenesis. This gives these fragments a potential as novel drug targets. Based on the results generated in this thesis and the fact that the PRO-C6 assay targets endotrophin, we are currently investigating if the PRO-C6 antibody are able to inhibit the formation of metastasis in mice with colon cancer, possibly by blocking the function of endotrophin.

The cleavage of SPARC at a specific site, measured by the SPARC-M assay developed during this PhD thesis, generates a conformational change of SPARC which increases the affinity to collagens. This cleavage might therefore be involved in increased collagen deposition, which we know, based on the results from the additional results 2, equals a bad prognosis for patients with metastatic colorectal cancer. It would therefore be interesting to investigate if blocking of this cleavage/fragment of SPARC using specific antibodies could decrease the collagen deposition, indicating drug target potential.

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