The microenvironment in human ovarian carcinoma - characterization through proteomic analysis of tissue interstitial fluid

Hanne Haslene-Hox

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Scientific environment

The present study was carried out in the Cardiovascular Research Group at the Department of Biomedicine, University of Bergen during the years 2009 – 2013. Sample collection was done in collaboration with the Department of Gynecology and Obstetrics at Haukeland University Hospital. Professor Helge Wiig, professor Olav Tenstad and professor Helga Salvesen were supervisors. Financial support was given by Locus on Cardiovascular research, University of Bergen and the University of Bergen.

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Hanne Haslene-Hox

Contents

Abbreviations

BST2	Bone marrow stromal antigen 2
COP	Colloid osmotic pressure
EC	Endometrial cancer
ECM	Extracellular matrix
GAG	Glucosaminoglycan
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GO	Gene ontology
HA	Hyaluronic acid
HPLC	High performance liquid chromatography
HSA	Human serum albumin
IF	Interstitial fluid
LC	Liquid chromatography
LC-MS/MS	Tandem mass spectrometry
LTQ	Linear iontrap
m/z	Mass-to-charge ratio
MIDAS	MRM-initiated detection and sequencing
MRM	Multiple reaction monitoring
MS	Mass spectrometry
OC	Ovarian cancer
OH	Healthy ovarian tissue
PLCO	Prostate, Lung, Colorectal and Ovarian cancer study
PBS	Phosphate-buffered saline
QQQ	Triple quadropole
RPC	Reversed-phase chromatography
SEC	Size-exclusion chromatography

sGAG	Sulphated glucosaminoglycan
SIS	Stable isotope standards
SRM	Selected reaction monitoring
TIF	Tumor interstitial fluid
UKCTOCS	United Kingdom Collaborative Trial of Ovarian Cancer Screening
V_A	Available volume
V_E	Excluded volume
VEGF	Vascular endothelial growth factor

Abstract

The interstitial fluid of tissues and that of tumors in particular, represents the secretome and is thus a valuable source of tissue-specific proteins. The differential expression of proteins that are secreted in tumor tissue can reflect specific characteristics of the tumor biology. To determine changes in the tumor secretome, access to fluid that reliably reflects the local microenvironment is essential, and can enable us to identify proteins that can be used to monitor or treat disease.

Substantial efforts have gone into the search for tumor-specific proteins, mainly by using proteomic strategies, but it remains a challenge to go beyond lists of candidate proteins to clinical relevant biomarkers. The ability to translate proteomic findings to the clinic is hampered by the low capacity in verification of suggested biomarker candidates.

To be able to characterize changes in the tissue microenvironment of human tumors, access to the tumor interstitial fluid (TIF) is necessary. We investigated whether the fluid isolated from ovarian carcinoma tissue by centrifugation was representative for interstitial fluid. Determination of creatinine and Na^+ in isolated fluid and plasma showed that the *ex vivo* admixture from the intracellular compartment is limited, and we concluded that the isolated fluid was representative for undiluted TIF.

Although the tumor microenvironment has received increased attention in the recent years, studies on TIF from human tissue samples are limited. We summarized the recent progress in the use of TIF for proteomic analysis. When comparing data from studies using TIF as a substrate there were substantial differences between proteomes of the same cancer, as well as between proteomes from different cancer types. These differences may be due to the choice of isolation technique as well as proteomic strategy. Major challenges reside in the lack of available techniques for validating the origin of the isolated fluid and hence of proteins in the sample.

The assumption that IF has a higher concentration of proteins produced in the tumor

compared to plasma is widespread. By assessing the known biomarker CA-125 in TIF and corresponding plasma we could demonstrate that this assumption is true. This underlines the advantage of using TIF as a substrate for proteomic analysis. Furthermore, the gradient between TIF and plasma was dependent on stage, and the concentration of CA-125 in TIF may harbor additional information of relevance for differential diagnosis or prognosis for ovarian cancer.

Using IF as a substrate we have analyzed the proteomes of both healthy and malignant gynecological tissues. By extensive fractionation before mass spectrometry analysis, we were able to detect low abundance proteins that are up-regulated in the tumor microenvironment. Furthermore, variation in protein concentration in individual samples was assessed, and we found substantial heterogeneity between individual tumors. Validation by targeted MS and antibody-based techniques gave similar results, and label-free quantification in unfractionated individual samples indicated that the actin-related protein WD repeat-containing protein 1 can have a central role in tumor progression. The extensive Orbitrap proteomes produced from individual samples of both healthy and malignant gynecological tissues can harbor a number of other tumor-specific proteins.

The exclusion of proteins from parts of the tumor microenvironment has been studied in animal models, but has not earlier been determined in human tumors. We quantified abundant plasma proteins by mass spectrometric analysis of unfractionated and undiluted interstitial fluid. Fifteen proteins with varying molecular weight and pI were used as probes to determine the relation of available distribution volume with molecular weight and charge in healthy and malignant gynecological tissues. We found that the fractional available distribution volume of albumin was significantly increased in ovarian carcinomas compared with healthy ovarian tissue. Furthermore, the available distribution volume of large plasma proteins was dependent on molecular weight in healthy ovarian and endometrial carcinoma tissues, but not in ovarian carcinoma tissue. Subsequently, we quantified the composition of extracellular matrix components, showing a high concentration of collagen, and low

concentration of hyaluronan in healthy compared with malignant gynecological tissues.

In conclusion, we have demonstrated the advantage of using TIF for biomarker discovery in humans, as well as to characterize the tumor interstitium by measuring the available distribution volume of proteins. Proteomic analysis of TIF can have the potential to improve differential diagnosis, prognosis and choice of treatment, and reveal possible targets for therapeutic intervention for ovarian and endometrial cancer.

List of papers

This thesis is a summary of the following papers, which are referred to by their roman numerals in the text[1].

I **Haslene-Hox H, Oveland E, Berg KC, Kolmannskog O, Woie K, Salvesen HB, Tenstad O & Wiig H (2011).** A new method for isolation of interstitial fluid from human solid tumors applied to proteomic analysis of ovarian carcinoma tissue. *PLoS ONE* **6**(4):e19217

II **Haslene-Hox H, Tenstad O & Wiig H (2013).** Interstitial fluid – A reflection of the tumor cell microenvironment and secretome. *Biochim Biophys Acta* pii: S1570-9639(13)00039-3. doi: 10.1016/j.bbapap.2013.01.028. [Epub ahead of print]

III **Haslene-Hox H, Oveland E, Woie K, Salvesen HB, Wiig H & Tenstad O (2013).** Increased WD-repeat containing protein 1 in interstitial fluid from ovarian carcinomas shown by comparative proteomic analysis of malignant and healthy gynecological tissue. *Biochim Biophys Acta* pii: S1570-9639(13)00208-2. doi: 10.1016/j.bbapap.2013.05.011. [Epub ahead of print]

IV **Haslene-Hox H, Madani A, Berg KCG, Woie K, Salvesen HB, Wiig H & Tenstad O (2013).** Demonstration of a stage dependent gradient in CA-125 between tumor interstitial fluid and plasma. *submitted*

V **Haslene-Hox H, Oveland E, Woie K, Salvesen HB, Tenstad O & Wiig H (2013).** Distribution volume of macromolecules in malignant and healthy human gynecological tissue – effects of extracellular matrix structure. *manuscript*

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1 Introduction

1.1 Interstitium

The interstitium is located between the cells of a tissue and the vascular and lymphatic vessels, and is comprised of two compartments: the extracellular matrix (ECM) built of a collagen fiber framework containing a gel-phase made up of glucosaminoglycans (GAG), and the interstitial fluid (IF) that is composed of interstitial water and soluble components like ions and proteins [1, 2]. The ECM, IF and stroma (i.e. vasculature, lymphatics, fibroblasts and immune cells) make up the tissue microenvironment [3].

1.2 ECM

1.2.1 Collagen

Collagens are a diverse group of proteins, and there are 28 known types of collagen at present. Common for all collagens are that they consist of three polypeptide chains that form right-handed triple helical collagenous domains. The amino acid sequence of the polypeptides is given as $(X\text{-}Y\text{-}Gly)_n$, where the glycine is necessary to facilitate packaging of the triple helix. The X and Y positions of the polypeptide chains are often occupied by proline and hydroxyproline [4]. The lengths of the collagenous and non-collagenous domains of the different collagens are highly variable between collagen types [2, 4].

The collagens are the main structural proteins of tissues, and have a dominant role in scaffolding of various tissues. The different types of collagen are present in variable amounts in different tissues and organs, with collagen types I, II and III as the most abundant [4]. Collagens are mainly present with no or low net charge at physiological pH [2].

1.2.2 GAGs

GAGs are polyanionic polysaccharide chains of variable length that consist of repeating disaccharide units of hexosamine and uronic acid or galactose [1, 2]. There are four main classes of GAGs, where three of them bind covalently to a protein backbone to form proteoglycans, namely heparin/heparan sulphate, chondroitin/dermatan sulphate and keratin sulphate. The fourth GAG is hyaluronan/hyaluronic acid (HA), which is present as a soluble component [2]. As the GAGs have a high negative charge density and hydrophilic character at physiological pH, they govern mass transfer characteristics in the interstitium, and contribute to osmotic pressure and hydration of the interstitium by attracting counterions [2, 5].

1.3 The tumor interstitium

The tumor interstitium differs from normal tissue interstitium in several aspects, related to composition and features of both stromal cells and the ECM components [6]. As this work mainly focuses on the structural and soluble components of the tumor microenvironment (ECM and IF), the tumor stroma is only mentioned briefly. The stroma of tumors differs from the stroma of normal tissue in that it often contains increased number of fibroblasts (cancer associated fibroblasts) and has deposition of collagen I and fibrin [3]. The vasculature of tumors is usually irregular and with an increased permeability due to the increased presence of vascular endothelial growth factor (VEGF), and tumors mostly lack functional lymphatics [2, 6]. The tumor can contain increased amounts of collagens, proteoglycans and GAGs (especially chondroitin sulphate and hyaluronan) [2], which can contribute to the increased interstitial fluid pressure observed in tumors [2, 5, 7].

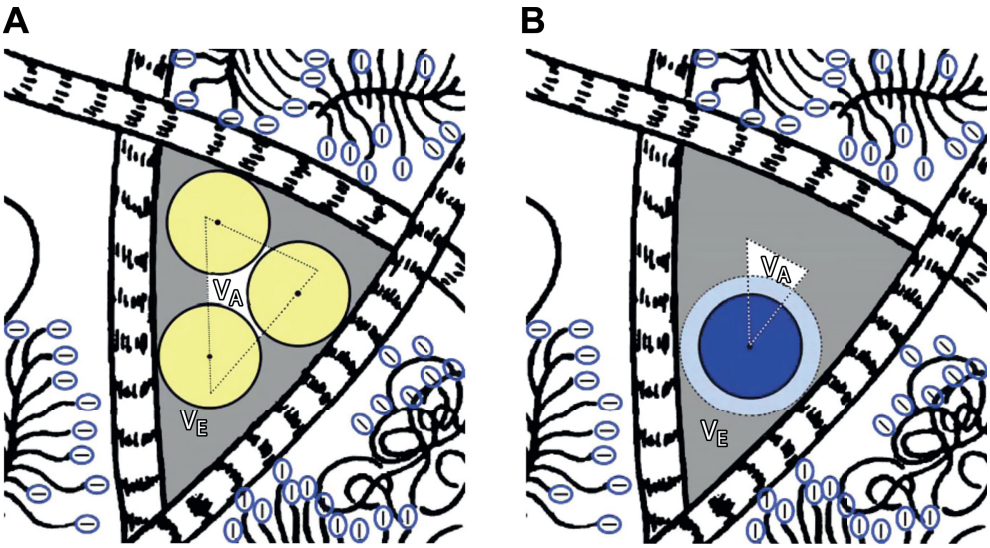

Figure 1: Schematic models of the interstitium and the exclusion phenomenon. **A:** Sterical exclusion. The presence of structural molecules, e.g., neutral collagen (cross-striped bars) and negatively charged hyaluronan and proteoglycans, results in macromolecular crowding of the interstitial space. Consequently, the fluid space available for other species diffusing through the interstitial media is less than the total interstitial fluid volume. The center of the spherical molecule (yellow) can only access the area inside the dotted line, the available volume V_A, and is excluded from the area outside the dotted line, V_E. **B:** Sterical and charge exclusion. GAGs have negative charge at physiological pH that may add to its steric exclusion effect. Electrostatic factors are involved in selectively excluding negatively charged macromolecules that are distributed in and transported through the interstitium. A negatively charged probe will accordingly have a higher apparent radius (light blue) than an uncharged one. The case illustrated shows that, when the combined effects of steric and electrostatic factors are considered, V_E is higher than in the case where only steric factors were accounted for (A). From [8].

1.4 Volume exclusion

A distinct effect of the ECM components, mainly collagen and GAGs, on the IF composition, is the effect of volume exclusion. Exclusion of proteins takes place because two molecules cannot occupy the same space, and thus part of the interstitial space volume is restricted for proteins (Figure 1) [1, 2, 8].

The amount of interstitial volume accessible to a protein is dependent both on molecular weight and charge of the protein, and will affect both the concentration of a protein in the interstitium and its ability to penetrate the tissue. The available distribution volume,

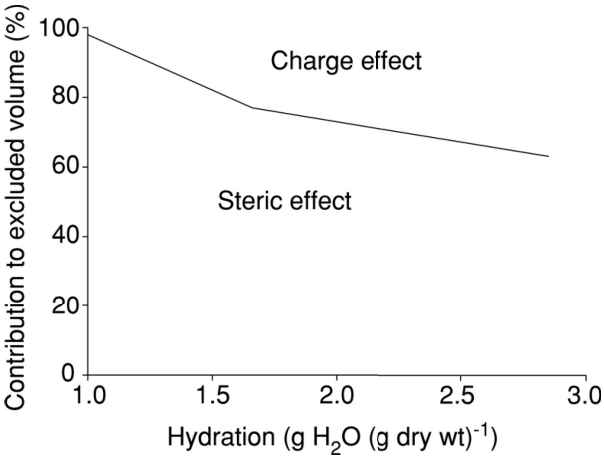

Figure 2: Relative contribution of steric and charge effects to excluded volume of native human serum albumin (HSA, pI = 4.9) related to hydration of dermis. Calculations based on data for equilibration in phosphate buffered saline (PBS, sum of steric and charge effects) and 1 M NaCl (steric effect only). Area below division line represents steric whereas area above line represents charge effect. We notice an increased importance of steric and a reduced importance of charge for exclusion of HSA with reduced hydration. From [9].

V_A, or excluded interstitial volume, V_E, for a protein can be determined if the amount of the protein in the interstitium along with the concentration of the protein in the available volume, are known [2, 8].

It has been suggested that the interstitium can be characterized as a gel chromatography column, where large molecules have a smaller distribution volume (high exclusion) compared to smaller molecules [8]. Collagen is the major excluding agent that accounts for sterical (geometrical) exclusion, while GAGs contribute to electrostatical exclusion due to their negative charge [8]. The grade of exclusion varies with hydration and the contribution of the charge effect increase with increasing hydration (Figure 2) [9].

1.4.1 Resistance to drug uptake in tumors

In tumors, both hyaluronan [10, 11] and collagen [12, 13] have been shown to restrict therapeutic agents from uptake into the tissue, an effect that was shown to diminish by using hyaluronidase or collagenase to degrade the ECM, respectively, and thus increase drug uptake [6]. The ECM is thus important to consider when developing personalized medicine [14]. Krol et al. [15] showed that the available volume fraction of dextrans in gels, as representative for the ECM, decreased linearly with increasing dextran molecular

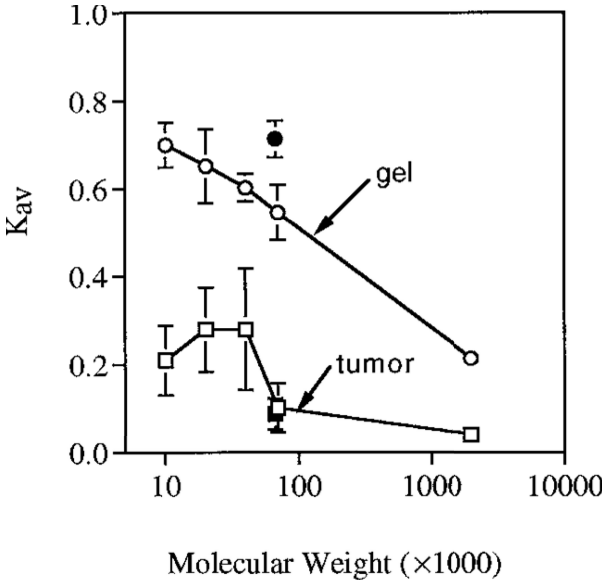

Figure 3: The available volume fraction of dextrans (white) and bovine serum albumin (black) in fibrosarcomas (squares) and polymer gels (circles). Data points are means of 24 – 35 measurements in six to eight fibrosarcomas (tumor) or means of 12 measurements in four different gel preparations (gel); bars, SD. From [15].

weight. In tumors on the other hand, the available volume fraction was independent of molecular weight between 10 and 40 kDa, had a significant decrease between 40 and 70 kDa, and only small reductions at higher molecular weights (Figure 3). Thus, the exclusion phenomenon must be attributed to not only the concentration of collagen and GAGs, but also the orientation of fibers in the ECM.

1.5 Interstitial fluid

The fluid phase of the interstitium, IF, contains electrolytes and plasma proteins in addition to other substances either produced locally or originating from plasma [2]. The tumor interstitial fluid (TIF) have been shown to have high H^+, CO_2, and lactic acid, and low glucose and O_2, compared with normal IF [16]. Although the characterization of TIF have received little focus until now [3], the interest for the study of proteins in TIF is increasing, in part due to the rapid development in the field of proteomics, as will be discussed below.

1.6 Proteomics

In 1995, Wasinger and Wilkins defined the term proteome as the entire PROTEin complement expressed by a genOME, or by a cell or tissue type [17, 18, 19]. While the genome in an organism is finite, the proteome is dynamic and changes between compartments and tissues as well as under different conditions. Further, the number of proteins is much larger than the number of genes, due to alternative splicing, protein processing and post-translational modifications [19, 20].

The main goal of proteomics – the study of the proteome, is to identify and quantify all the proteins in a certain compartment, e.g. cell, tissue or body fluid, under conditions of interest [20, 21]. Mass spectrometry (MS) has become the method of choice for analysis of complex protein samples, and dominates the field of proteomics today [20, 21].

1.6.1 Clinical proteomics

In clinical proteomic experiments the goal is to characterize the proteomes of normal or diseased tissues or biological fluids, thus identifying and quantifying the protein differences that associate with, define or cause the diseased state. This to illuminate pathobiology, improve disease classification or identify new therapeutic targets [22, 23]. Biomarker discovery is an integral part of clinical proteomic research in which MS-based proteomic approaches are used to identify peptides, proteins or post-translational modifications that support early disease detection, facilitate diagnosis, inform prognosis, guide therapy or monitor disease activity.

1.6.2 Secretome

The term ‘secretome’ was first used by Tjalsma et al. [24], describing the proteins released by a cell, a tissue or organism through different secretion mechanisms [25]. The cancer secretome include all the proteins in the interstitial fluid of a tumor mass, secreted from

both cancer and stromal cells.

The two main sources for cancer secretome studies have been cancer cell line supernatants and proximal biological fluids (e.g. IF). The majority of reported studies utilize the conditioned media collected from *in vitro* cell cultures [26, 27], but issues have been raised as to whether the cell cultures are able to replicate the complexity of the tumor microenvironment *in vivo* [25]. Studies of the secretome *in vitro* have the advantage of being able to simulate disease models and changes in the secretome as a result of changed physiological parameters (e.g. hypoxia or hyperoxia) or as a response to secreted factors [28]. However, one of the main challenges is to distinguish between secreted proteins, the proteins released into the conditioned media by cell death and proteolysis and proteins from the bovine serum in culture media. Since the concentration of secreted proteins is low, lysis of only a small number of cells can have a contaminating effect [28].

In vivo secretome studies are more complex and reflect the true physiological state of the tissue, but because of the obstacles in the isolation of interstitial fluid, containing the *in vivo* secretome, few studies have been performed [26, 28]. Tissue secretomics, where interstitial fluid is collected from tumor tissue, constitutes a potent approach to bridge the gap between proteomics and tumor biology.

1.6.3 Biomarkers

Biomarkers can be defined as molecular indicators whose presence or metabolism correlates with important disease-related physiological processes and/or disease outcomes [29], and can indicate the presence of disease or provide information of its behavior [30]. A successful biomarker should be measured reproducibly, be specific to a disease or treatment and possess high sensitivity and specificity [22]. Although the discovery of markers for early diagnosis receives considerable attention, biomarkers can contribute to improved cancer staging and grading, as well as to select and monitor therapy and detecting recurrence (Figure 4) [31].

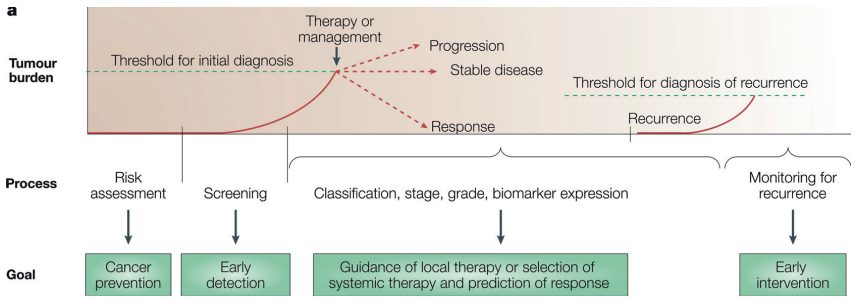

Figure 4: Schematic representation of the uses of biomarkers at different stages in the clinical evolution of cancer. Before diagnosis, markers might be used for risk assessment and screening. At diagnosis, markers can assist with staging, grading, and selection of initial therapy. Later, they can be used to monitor therapy, select additional therapy, or monitor for recurrent disease. Modified from [31].

In the infancy of proteomic biomarker discovery, most efforts employed blood in comparative analyses of patients versus healthy controls, with the expectation that the differential proteins could include new biomarkers [32]. Unfortunately, blood harbors an enormous complexity when considering the sheer number of protein constituents and the enormous dynamic range of expression level, making the discovery of biomarkers challenging [22, 26, 32, 33]. As a result, alternative clinical sources to the secretome, as tissues and TIF, proximal fluids and surrogate proximal fluids (e.g. cell culture media) are being employed [22, 26, 33, 34].

This development is based on the assumption that tumor-specific proteins are present at much higher concentrations in the diseased tissue, and thus facilitates identification of the highly tumor-expressed proteins that are most likely to be released into the blood in detectable amounts [35, 36, 37]. Many acute phase proteins, present because of a global inflammatory response to cancer, have been suggested as biomarker candidates. This can be due to the use of control samples from healthy individuals that do not have the same global inflammation response. Accordingly, the systemic inflammatory response in a group of cancer patients can appear to have diagnostic or prognostic potential, but are not specific to the disease in question, but rather a change common for many conditions that initiate an inflammatory response in the body [37, 38].

It remains a challenge to go beyond lists of candidate proteins and convert candidates into validated plasma markers [32], as few of the differentially detected proteins stand out as superior for diagnostic and prognostic purposes. The limited translational potential biomarkers have had so far can be attributed to the low capacity to verify and validate suggested biomarker candidates, as well as the challenging task of separating real tumor-specific proteins from secondary protein changes and finding the locally produced proteins that can be detected in blood [38, 39].

1.7 Ovarian cancer

Ovarian cancer (OC) is the second most common gynecologic cancer in western countries, and the most lethal, with an incidence of 225 500 new cases and 140 200 deaths worldwide in 2008 [40, 41]. Most patients with early stage disease are asymptomatic, and when presenting, symptoms are usually nonspecific and includes abdominal fullness, abdominal pain, early satiety or bloating [40, 42]. More than 70 % of patients are diagnosed with advanced disease, with a 5-year survival rate of 10 to 30 %. This is opposed to patients diagnosed when the cancer is limited to the ovaries, with long term survival rates as high as 85 to 95 % [40, 42].

Epithelial ovarian cancer accounts for over 90 % of all ovarian malignancies, and can be further divided into histological subtypes, with the most common being serous (75 %), mucinous (10 %), endometrioid (10 %) and clear cell carcinomas, each resembling different analog cell types, with different prognosis and molecular characteristics [40, 42, 43].

1.7.1 Development of ovarian cancer

A clear etiological factor for development of ovarian cancer has not yet been identified [42]. Since nulliparity, early menarche and late menopause have been found to be associated with an increased risk of OC, while pregnancy, lactation and the use of oral contraceptives are associated with a reduced risk, there seems to be a correlation between the number of ovu-

lations and the risk of developing ovarian cancer. Accordingly, the risk of ovarian cancer is proportional to the number of lifetime ovulations, and is suggested to be related to the repeated rupture of the ovarian epithelium, and possible formation of epithelial inclusion cysts, which can undergo malignant transformation [40, 42].

Recently, there have been presented experimental and clinical evidence that cancerous cells that arise in the fimbriated end of the fallopian tube can implant on the ovary causing high-grade serous ovarian carcinomas [44, 45, 46], and that high grade serous ovarian carcinomas may actually be fallopian tube serous carcinomas that have spread to the ovaries [45, 46]. Thus, a new classification of ovarian cancer has been suggested, consisting of two subgroups: type I, which are low grade and slow growing and arise from epithelial cells at the ovarian surface or in inclusion cysts, and type II carcinomas that are high grade and disseminates rapidly from the fallopian tubes [44, 47].

1.7.2 Treatment

When ovarian cancer is suspected, an exploratory laparotomy is usually performed, with tumor debulking, total abdominal hysterectomy and bilateral salpingo-oophorectomy, omentectomy, lymph node sampling, peritoneal washings and inspection of all peritoneal surfaces [40, 42].

Following initial surgery, treatment recommendations vary with stage and extent of surgical debulking. In high-risk early stage patients and advanced stage disease, the standard of care is combination therapy including taxane and a platinum compound, usually paclitaxel and carboplatin [40, 42]. Approximately 80 % with early disease, and 50 % with advanced disease have complete clinical remission after initial cytoreductive surgery and chemotherapy [40, 42], however, the majority of patients with advanced disease recur and 25 % never have remission, but develop drug resistance [48]. Recurrent or drug-resistant disease is generally not curable today, and further treatment focuses on palliation of symptoms and prevention of complications. More effective treatment options for patients with

relapsed disease, as molecular targeted agents [48] are underway in clinical trials [40], with the greatest success being agents that target VEGF [42].

1.7.3 Biomarkers

The search for biomarkers in ovarian cancer begun with the identification of CA-125 in 1983 [49], and in the following years substantial efforts have been invested in detecting biomarkers for early diagnosis of ovarian cancer, with numerous proteins proposed as biomarkers [37, 50, 51, 52, 53]. CA-125 is found up-regulated in 50 % of early stage and 80 % of advanced stage ovarian cancer, and is approved for use in monitoring of recurrent disease, but not for detection. Nevertheless, CA-125 still remains the single best biomarker for early detection in ovarian cancer [30, 37].

In addition to single biomarkers, panels of biomarkers have been proposed, but none of the panels or analytic approaches revealed improvement compared with CA-125 alone [54, 55, 56, 57, 58, 59]. This may in part be due to the use of samples from advanced stage disease, taken at time of diagnosis, for biomarker discovery. Such biomarkers may increase sensitivity and specificity near time of diagnosis, rather than extend the time before clinical diagnosis, when the cancer could be detected by screening [30, 60, 61, 62].

Another challenge is the heterogeneity in ovarian cancer, with biomarker expression differing significantly between the different histological subtypes [43], as well as the evidence that a significant proportion of ovarian cancers might originate in premalignant lesions in the distal fallopian tube [30, 44, 45]. These new insights need to be taken into account when searching for and evaluating new biomarkers for ovarian cancer.

Several screening trials, where the Prostate, Lung, Colorectal and Ovarian (PLCO) cancer study and the United Kingdom Collaborative Trial of Ovarian Cancer Screening (UKCTOCS) are the largest, look at the use of CA-125 in combination with transvaginal ultrasound in screening of the general population. No mortality effect could be identified in the ovarian screening arm of PLCO [63]. Results on mortality from the ongoing UKC-

TOCS are yet to be published. However, preliminary results on sensitivity and specificity showed that serial CA-125 measurements with ultrasound as a second screening option significantly improved the specificity compared to screening only by transvaginal ultrasound, greatly reducing the number of patients that underwent surgery with benign abnormalities [64].

In proteomic biomarker studies, the use of samples proximal to the tumor is increasingly important, and ascites [65, 66], peritoneal fluid [67], cultured cells [68, 69], secreted proteins in cell culture media [70, 71, 72], laser microdissected cells [73], whole tissue homogenate [36, 74] and interstitial fluid [75, 76, 77] have been studied to some extent for ovarian cancer. Nevertheless, the lack of native IF results in biomarker discovery that is solely qualitative, and new strategies are needed for sample purification and analysis, to produce biomarkers that can be translated into the clinic.

1.8 Endometrial cancer

Endometrial cancer (EC) is the most frequent gynecological cancer, and the fourth most common cancer in women, with about 142 000 annual incidences and 42 000 annual deaths worldwide, with the majority of cases occurring in postmenopausal women. The most frequent symptom of EC is abnormal uterine bleeding at early stage disease, and therefore EC is usually diagnosed early, with an overall 5-year survival rate of 80 % [78].

The main type of EC is the endometrioid type, which accounts for 80 % of cases. The remaining cases include types as mucinous, serous, clear-cell and squamous-cell carcinomas. ECs can be grouped into two clinicopathological subgroups. The estrogen-related endometrioid type I carcinomas with a good prognosis, and the non-estrogen related type II carcinomas, including low differentiation endometrioid, serous and clear cell ECs that have a poor prognosis [78, 79, 80]. There is, however, often overlap between the clinical, histopathological, immunohistochemical and genetic characteristics of the tumors [78, 79, 80].

The two types of EC also differ in genetic alterations. Whereas most type II ECs have *p53* mutations, type I carcinomas can be characterized with several genetic alterations, as microsatellite instability, *PTEN* alterations, and mutations of *PIK3CA*, *K-Ras* and *CTNNB1* [79].

1.8.1 Treatment

The primary treatment for EC is surgery, and the procedures include total hysterectomy and bilateral salpingo-oophorectomy, acquisition of peritoneal fluid or washings for cytology, and in selected cases omentectomy and retroperitoneal lymph node dissection [78, 81]. The extent of lymphadenectomy is controversial because of undocumented therapeutic effect and benefit, as well as increased complication rates among patients with pelvic lymphadenectomy.

Because of the overlap between type I and II endometrial cancers, with cancer recurring in up to 20 % of type I, and only half of type II cancers [81], it is difficult to separate between patients that can be cured by surgery alone, and patients that need an aggressive clinical course, including lymphadenectomy [79, 82].

After surgery, adjuvant radiotherapy can be applied to treat pelvic lymph-node regions that can contain microscopic disease. In general, patients with lesions of surgical stage IA and IB and high differentiation (grade 1 or 2) can be treated without postoperative radiotherapy [78]. In addition to radiotherapy, adjuvant cytotoxic chemotherapy is applied when considered appropriate, usually by cisplatin and doxorubicin chemotherapy [78, 81].

1.8.2 Biomarkers

Prognostic markers to assist in the choice of treatment alternatives in endometrial cancer patients are needed, as well as markers for early discovery. Several genetic markers to aid in the classification of Type I and II endometrial carcinomas have been suggested [79, 82, 83, 84]. Stathmin has been proposed as marker for high-risk endometrial cancer patients

from genomic studies [84] and has been verified in immunohistochemical tissue analysis [85].

Compared to the massive effort in characterizing the ovarian cancer proteome, the focus on the endometrial cancer proteome has been limited. A few proteomic studies have been undertaken on endometrial carcinoma, all using tumor homogenate, either from clinical tissue samples or cultured cells. DeSouza et al. [86, 87] have utilized iTRAQ to find differentially expressed proteins in whole tissue samples from healthy and malignant endometrial tissue, and detected candidate biomarkers have been further investigated in a larger study including both type I and type II EC [88], by tissue microarrays [89], in secretomes from endometrial and cervix cancer cell lines [90] and quantified by targeted MS [91]. The differential expression of chaperonin 10 and pyruvate kinase M2 along with several other proteins were confirmed, but not investigated in blood, and the biomarker potential remains to be determined.

The differential proteome of endometrial cancer cells, isolated by laser microdissection, has recently been reported [92]. Multiple proteins were found elevated in stage I endometrial cancer, including annexin 2 and peroxiredoxin 1, which were verified in tissue microarrays.

By utilizing biotinylation to select for membrane proteins, bone marrow stromal antigen 2 (BST2) was identified as significantly up-regulated in endometrial cancer cell lines, and the use of anti-BST2-antibodies effectively inhibited tumor growth in a xenograft model, suggesting that BST2 may be a target in molecular therapy [93].

In addition to proteomic discovery studies on tumor homogenate, a couple of studies have used serum to determine the diagnostic and prognostic values of selected biomarker candidates. This applies to human epididymis protein 4, which was shown to correlate with an aggressive EC phenotype [80]. In addition apolipoprotein-1, prealbumin and transferrin have been suggested as diagnostic markers of early disease [94].

There seems to be no characterization of IF from endometrial carcinomas, but endome-

trial fluid from healthy premenopausal women have been characterized [95], and can act as a valuable comparison in the interpretation of endometrial cancer proteomic data.

2 Aims of study

Overall aim of study: Study the tumor microenvironment and tumor-specific proteins of human ovarian carcinomas by using interstitial fluid as a substrate to reveal new knowledge on the tumor biology of ovarian cancer.

Specific aims of the study:

1. Investigate if fluid can be isolated from excised human ovarian carcinoma tissue by centrifugation, and determine whether the isolated fluid is representative for native interstitial fluid.

2. Explore the protein contents of interstitial fluid from healthy and malignant gynecological tissue using a proteomic approach, and if possible identify tumor-specific proteins.

3. Test the assumption that proteins present in interstitial fluid can be detected in plasma, and determine if there is a concentration gradient from interstitial fluid to plasma, using CA-125 as a model protein.

4. Determine exclusion in healthy and malignant gynecologic tissue by using plasma proteins as probes, and correlate the distribution volume of proteins to the collagen and GAG contents.

3 Methods

3.1 Biopsies

Tissue samples of ovarian and endometrial carcinomas as well as healthy ovarian tissue (OH) that were obtained from patients undergoing hysterectomy and oophorectomy as primary action for gynecologic cancer, were used for all papers. Tumor samples were excised from an area without apparent necrosis at the tumor surface, and placed on ice for transportation to the laboratory. The surgeries were done at Department of Gynecology and Obstetrics at Haukeland University Hospital between 2008 and 2012. The research protocol was approved by the Norwegian Data Inspectorate (Protocol # 961478-2), Norwegian Social Sciences Data Services (Protocol # 15501) and the local ethical committee (Protocol ID REKIII nr. 052.01). All samples were collected after obtaining the patients' written informed consent.

3.2 Tissue centrifugation

We isolated IF from tissue samples by utilizing the centrifugation method developed for mammary tumors in rats by Wiig et al. [96], and this method was adapted and validated for human tissue samples in Paper I. Tissue samples were placed on a nylon mesh with pore size 15 – 20 μm and subsequently centrifuged in an Eppendorf tube at 106 *g* for 10 minutes. The fluid that collected in the bottom of the tube was sampled and frozen at –80 °C for further analysis. All handling of the tissue was done in 100 % humid atmosphere to prevent evaporation from the samples.

3.3 Validation of isolated tissue fluid as IF

When applying tissue centrifugation for isolation of IF we needed to determine if the fluid derived solely from the extracellular fluid compartment without admixture of intracellular

fluid. The extracellular origin of the centrifugate was validated in skin and tumor in rats by Wiig et al. by using the extracellular tracer ^{51}Cr-EDTA [96]. The extracellular tracer was in equilibrium in IF and plasma, and the addition of tracer-free intracellular fluid caused by the centrifugation would result in a centrifugate-to-plasma ratio lower than 1.

To be able to validate the centrifugation method in human tissue samples, we needed to use endogenous substances that are predominantly present in the extracellular space and can diffuse freely over the capillary membrane, but with low intracellular concentrations. Creatinine and Na^+ were used to validate the isolated fluid as IF in Paper I.

3.3.1 Creatinine

Creatinine is mainly synthesized in muscle tissue and circulate freely in the extracellular space [97]. Creatinine was determined by high-performance liquid chromatography (HPLC) [98], using three strong cation exchange columns in series to separate creatinine from other co-eluting substances and utilizing mobile phases of different pH to focus the creatinine signal, as extensively described in Paper I.

3.3.2 Sodium

Na^+ is kept at a low intracellular concentration of approximately 5 mM and extracellular at approximately 145 mM by the Na^+/K^+-pump in the cell membrane [16, 99]. Na^+ was determined by dilution of paired centrifugate and plasma samples in 0.65 % HNO_3 and analysis by flame spectrometry, as described in Paper I. Na^+-measurements in paired centrifugate and eluate samples were also used to determine the interstitial fluid volume in Paper V.

3.4 Tissue elution

To determine the total mass of interstitial proteins for calculations of available distribution volume in Paper V, tissue samples were incubated in a potassium buffer for 48 hours with

continuous rotation. Samples were then centrifuged, the supernatant was collected and the tissue was freeze-dried for subsequent determination of ECM constituents.

3.5 Colloid osmotic pressure

Colloid osmotic pressure (COP) was determined in IF and plasma with a colloid osmometer using a membrane with cutoff 30 kDa and equipped with a transducer as described in detail by Aukland and Johnsen [100].

3.6 High-performance liquid chromatography

In liquid chromatography (LC), a liquid mobile phase containing the sample is moved through a column filled with packing material that has specific sites for binding or retention, and the differential equilibration in the packing material of the analytes results in separation. The object of any chromatographic technique is to separate, or resolve, the species of interest from other compounds, and the solid and mobile phases selected depend on the nature of samples and separation target.

3.6.1 Immunodepletion

Immunoaffinity depletion, or immunodepletion, is the removal of selected proteins by the binding to specific antibodies. The 22 most common plasma proteins make up 99 % of the total protein mass in plasma [33], and the same proteins dominate IF to a similar degree. To be able to investigate lower abundance proteins in IF and plasma, the 14 most abundant plasma proteins were removed from plasma and IF samples prior to mass spectrometric analysis in Paper I and III.

3.6.2 Size-exclusion chromatography

Size-exclusion chromatography (SEC) separates the proteins in a sample according to molecular weight, or rather hydrodynamic radii, and was used to characterize the macromolecular composition of IF, plasma and ascites before and after immunodepletion in Paper I. SEC in combination with reversed-phase chromatography (RPC) were utilized to determine the albumin concentration of IF, eluate and plasma in Paper V.

3.6.3 Reversed-phase chromatography

RPC separate proteins in a sample according to hydrophobicity, and was used for separation of proteins prior to LC coupled to tandem mass spectrometry (MS/MS) in paper I and III. Further, RPC is used in the separation of peptides in LC-MS/MS on linear iontrap (LTQ) or LTQ-Orbitrap.

3.7 Mass spectrometry

The basic principle of MS is to generate ions from e.g. peptide mixtures and to detect them qualitatively and quantitatively by their respective mass-to-charge ratio (*m/z*) and abundance [20, 21, 101]. A mass spectrometer consists of an ion source that produces ions from the injected peptides, a mass analyzer that separates the peptides based on *m/z*, and a detector that creates mass spectra. To generate sequence specific information, making protein identification possible, an MS/MS needs to be recorded. The first stage involves reading all peptide ions that are introduced into the instrument at any given time resulting in an MS spectrum of precursor ions. Selected precursor ions are isolated and fragmented in the gas phase of a collision cell, producing product ions. The product ions are separated in a mass analyzer and the *m/z* of each ion is registered in the detector. The acquired MS/MS spectrum is thus a record of *m/z* (x-axis) and intensities (y-axis) of all the resulting product ions generated from an isolated precursor ion. The fragmentation pattern encoded

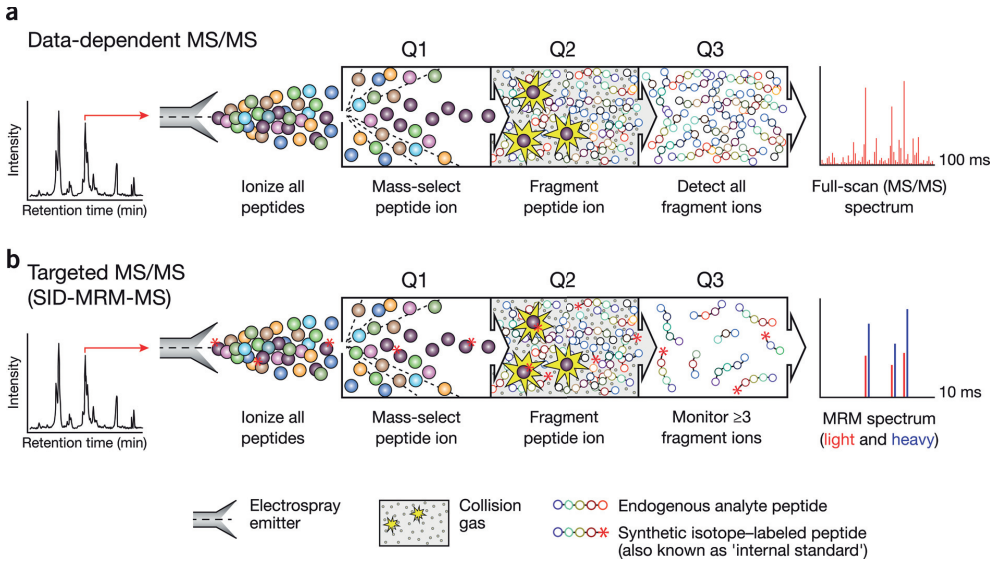

Figure 5: Comparison of conventional data-dependent analysis to targeted MRM-MS on a triple quadrupole mass spectrometer. **A:** In a data-dependent MS experiment, digested proteins are loaded on a reversed-phase column attached to a liquid chromatography setup and eluted via electrospray to yield gas-phase ions. At any given point in the chromatographic separation many tens to hundreds of peptides are eluting nearly simultaneously. A full-scan MS spectrum is acquired and informs collection of subsequent MS/MS scans in which 4-10 ions observed in the MS spectrum are automatically selected on the basis of their signal intensity (Q1) for fragmentation by collision with inert gas (Q2). The complete array of fragment ions is detected (Q3), which constitutes the full-scan MS/MS spectrum (far right). **B:** In a stable isotope dilution-MRM-MS analysis, proteotypic peptides uniquely representing proteins of interest are predefined together with their most informative fragment ions. Peptides are selected for fragmentation (Q1 and Q2), and fragment ions are selected for detection (Q3) based on a user-specified list of targeted precursor-fragment pairs ('transitions'). Synthetic peptides containing stable-isotope labels can be spiked in as standards (asterisks). Comparing labeled to unlabeled peak area (far right) provides precise relative quantification of the endogenous analyte. From [23].

by the MS/MS spectrum allows identification of the amino acid sequence of the peptide that produced it and the proteins present are inferred [20, 21, 101].

MS analysis was performed on IF and plasma in Paper I, IF in Paper III, and IF, plasma and eluate in Paper V. The proteins in the samples were digested by trypsin into peptides, and the peptide mixtures were analysed by LC-MS/MS on an iontrap (Paper I and III), triple quadrupole (QQQ, paper III) and a LTQ-Orbitrap (paper III and V), as described in detail in the respective papers.

3.7.1 Selected reaction monitoring

Selected reaction monitoring (SRM), also called multiple reaction monitoring (MRM), utilize a triple quadrupole as a dual mass filter selecting peptides with predefined masses for fragmentation and analysis (Figure 5). Peptides unique for the proteins of interest are used to create a *m/z* filter such that only the peptides fulfilling the defined criteria are fragmented and analyzed, filtering precursor ion masses (Q1) and product ion masses (Q3) with Q2 acting as a collision cell [102, 103]. Precursor ion/product ion pairs, referred to as transitions, are used for the quantification of a target peptide, and, by extension, the target protein. This can be done either semi-quantitatively through MRM-initiated detection and sequencing (MIDAS), or absolute, by spiking the sample with stable isotope standards (SIS) [103].

The SISs are synthetic versions of the targeted peptides containing an amino acid labeled with a stable isotope (e.g. ^{15}N or ^{13}C). The labeled internal standards separate in LC and fragment identically to their native counterparts but are distinguished in MS and MS/MS-spectra by the increased masses of the peptide and the product ions containing the labeled amino acid [23]. MIDAS and SRM with SISs were used for quantification of selected proteins in paper III based on the method described by Unwin et al. [104].

3.7.2 Spectral counting

For the label-free quantification of proteins in paper V, spectral counting was applied. Spectral counting has been shown to correlate strongly with the protein's abundance in a complex mixture [32], and can thus be used as a means of quantification in discovery proteomic analysis. The number of spectra identified for a protein was normalized by the total number of spectra identified within each individual sample. In iontrap analysis, each sample was run in six replicates, and the number of spectra for a protein in each technical replicate was pooled. For both iontrap and Orbitrap data, proteins where less than three spectra were identified in a biological sample were excluded from the analysis, to reduce the false

positive rate due to artificially high fold-changes in low abundance proteins [105].

3.8 Western blot

Western blot was used as a non-MS verification technique in paper III, to validate the presence of proteins identified by discovery proteomics, assess the variability in the selected proteins between individual patients, and compare to the label-free SRM verification technique employed on the same samples. Bis-Tris gels (4 – 12 %) and hydrophobic polyvinylidene difluoride membrane were chosen, as described in detail in paper III. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was chosen as a protein loading control, together with Coomassie Blue for total protein staining.

3.9 Determination of ECM constituents

3.9.1 Collagen

The content of collagen was determined in paper V by the method developed by Woessner [106], on supernatant from papain-digested fat-free dried tissue.

3.9.2 Sulphated glucosaminoglycans

Sulphated GAGs (sGAG) were measured in Paper V, with the commercial available Blyscan kit (Biocolor Ltd., Carrickfergus, United Kingdom) using the dye label 1, 9-dimethylmethylene blue, providing a specific label for dermatan/chondroitin sulphates, keratin sulphates, and heparin sulphates, including heparin.

3.9.3 Hyaluronic acid

The concentration of the soluble glucosaminoglycan HA was measured by an enzyme-linked binding protein assay (Corgenix, Broomsfield, CO), as described in paper V. As

the analyzed tissue had been eluted before determination of ECM constituents, HA was determined for both tissue eluate and supernatant from the papain-digested dried tissue.

4 Results

Paper I: A New Method for Isolation of Interstitial Fluid from Human Solid Tumors Applied to Proteomic Analysis of Ovarian Carcinoma Tissue In this paper we showed that the centrifugation technique developed for TIF isolation in rats [96] could be translated to tumors from human ovarian carcinomas. The admixture of intracellular fluid in the isolated fluid was estimated through the comparison of the small endogenous molecules creatinine and Na^+. Both molecules are not locally produced, have a predominantly extracellular origin, and are in equilibrium between plasma and interstitial fluid. We showed that the ratio between the optimally isolated fluid and plasma was not different from 1.0 (Figure 1), and thus concluded that the isolated sample was representative for native interstitial fluid without substantial admixture of intracellular fluid.

Furthermore, access to undiluted TIF enabled the quantification of COP, a determinant of transcapillary fluid exchange. We showed that COP was 79 % in TIF compared to plasma, higher than in human skin [107], but similar to mammary tumors in rats [96].

Proteomic analysis revealed that TIF isolated by centrifugation represented a valuable substrate for identification of tumor-specific proteins that may act as diagnostic or prognostic markers and as targets for molecular imaging or therapy.

Paper II: Interstitial fluid — A reflection of the tumor cell microenvironment and secretome TIF reflects the tumor cell microenvironment and secretome. The most central methods used for TIF isolation are microdialysis, capillary ultrafiltration, tissue elution and tissue centrifugation (Figure 2), and the proteomes that result from these samples have marked differences that may in part be caused by differences in fluid isolation techniques. By comparing six published TIF proteomes generated using different isolation and analytical techniques, we found that the common proteins between the proteomes were similar

to highly conserved proteins in exosomes, indicating that exosomes may be a considerable component of TIF. The comparison further highlighted the challenges in finding clinical relevant biomarkers, since the identified biomarker candidates overlapped between different cancers, and there are large differences between proteomes of the same cancer type in different studies.

Paper III: Increased WD-repeat containing protein 1 in interstitial fluid from ovarian carcinomas shown by comparative proteomic analysis of malignant and healthy gynecological tissue Comparative proteomic analysis of immunodepleted and fractionated TIF from ovarian and endometrial carcinoma as well as normal IF from healthy ovaries revealed a number of differently expressed proteins in ovarian carcinoma as compared with the two reference tissues (Figure 1). Several 20S proteasome proteins were up-regulated in both ovarian and endometrial carcinoma compared to healthy ovarian tissue, indicating an extracellular role for the 20S proteasome.

Further, selected proteins reflecting cancer-related traits were validated by SRM-MS (Figure 2 and 5) and Western blot (Figure 3). The antibody- and mass spectrometry-based methods reflected the same individual variations in ovarian cancer patients, and the actin-related protein WD repeat-containing protein 1 was shown by MS-analysis of additional IF samples to be up-regulated in ovarian carcinoma compared with healthy ovaries (Figure 6), and is suggested as a therapeutic target for ovarian carcinoma.

Paper IV: Demonstration of a stage dependent gradient in CA-125 between tumor interstitial fluid and plasma Central for the utility of TIF in proteomic biomarker discovery is the assumption that disease-specific proteins are present in the extracellular microenvironment of the tumor in high - and thus more easily detectable, concentrations.

Further, these proteins will be transported from the tumor to the blood circulation, and can serve as plasma biomarkers. By using undiluted TIF isolated by centrifugation we quantified the concentration of CA-125 in TIF and in paired plasma samples. We showed that the concentration gradient between TIF and plasma is substantial (Figure 1), verifying the common assumption of local up-concentration, using the known ovarian cancer biomarker CA-125 as a model.

We further demonstrated that the CA-125 gradient is stage dependent (Figure 2), and suggest that assessing this gradient may have clinical value in terms of determination of tumor mass and stage classification.

Paper V: Distribution volume of macromolecules in malignant and healthy human gynecological tissue – effects of extracellular matrix structure We measured the extracellular fluid volume of human ovarian tumors and healthy ovarian tissue as Na^+ distribution space. By utilizing label free quantitative proteomics on paired individual samples of native IF and eluate, we could use abundant plasma proteins as endogenous probes to determine available and excluded volume of macromolecules as a function of molecular size and charge. We found that the available distribution volume of albumin was increased in ovarian carcinoma compared with healthy ovarian tissue. Furthermore, the distribution volume of plasma proteins with a molecular weight between 40 and 190 kDa decreased with size for endometrial carcinoma and healthy ovarian tissue, but was independent of molecular weight for well-hydrated ovarian carcinoma tumors. Further, there was only effect of charge on macromolecular distribution volume in healthy ovaries, which has lower hydration and high collagen content, indicating that a condensed interstitium increases the influence of negative charges.

A number of earlier suggested biomarker candidates were significantly elevated in the cancerous samples compared to healthy ovarian tissue. The identification of known

biomarkers, like Spindlin-1 for ovarian cancer and Stathmin for endometrial cancer, show that the IF, even when unfractionated, can be a valuable source for tissue-specific proteins.

5 Discussion

The aim of this thesis work was to develop strategies for improved proteomic analysis of the cancer secretome by isolating a pure interstitial fluid sample as well as decreasing and quantifying disturbances of intracellular and plasma proteins. Overall, we demonstrate the importance of access to the tumor microenvironment in proteomic biomarker studies.

5.1 Methodological considerations

5.1.1 TIF isolation techniques

We used Na^+ for determination of cellular fluid admixture after centrifugation. This is possible due to the substantially higher concentration of Na^+ in the extracellular fluid phase. Because of this, Na^+ can be used for measurement of extracellular fluid volume, and has together with Cl^-, mannitol, ^{51}Cr-EDTA and inulin been used for this purpose [5]. Interestingly, there is no significant difference between total extracellular volume determined with the typical extracellular tracer inulin and Na^+-space [15, 108]. By using extracellular substances as creatinine and Na^+, the cellular leakage induced by sample handling can be assessed. Nevertheless, cell lysis *in vivo*, e.g. due to necrosis of the tissue, cannot be determined by this method, and thus non-secreted intracellular proteins may be found in the isolated IF.

In paper IV and V, eluate was collected in addition to centrifugate. In paper V, the tissue samples are eluted in buffer for 48 hours, substantially longer than in the proposed IF collection method by Celis et al. [109], and it is likely that intracellular proteins leaked out during this time period. In addition, we used a potassium-buffer without Na^+ to enable the measurement of tumor-derived total Na^+ that may disturb the integrity of the cellular membrane.

In paper IV we determined CA-125 concentration in eluate collected after one hour with

the protocol proposed by Celis et al. [109] and after 24 hours incubation. The amount of CA-125 in the eluate increased in the 24-hour compared to the 1-hour eluate, suggesting that eluate collected after one hour had not equilibrated with the buffer, and was thus not representative for the TIF concentration of CA-125. As shown by the excluded volume of albumin in paper V, it is likely that the large glycoprotein CA-125 does not distribute in the entire extracellular volume. In ovarian carcinoma tissue we found no relationship between pI or molecular weight and available volume in the molecular range investigated, and we assume that CA-125 has an available distribution volume similar to albumin, of approximately 40 % of the total extracellular volume. Using this assumption, the calculations of CA-125 concentration in TIF based on the measured values in eluate is comparable to the concentration obtained in fluid isolated by centrifugation. Thus, knowledge on the extracellular volume as well as exclusion in the tissue of interest can make the calculation of *in vivo* TIF protein concentrations possible, when using an elution protocol. By combining the centrifugation and elution techniques for TIF protein isolation it is possible to determine the concentration as well as distribution volume of proteins in the tumor microenvironment. The combined strengths of the two techniques reveal new insight into the tumor ECM that can be transferred to clinical applications, such as the development of targeted drugs.

5.1.2 The completeness of proteomes

The result from discovery proteomic analysis by MS is a long list of detected proteins, but repeated analyses of identical samples will identify different and partly overlapping subsets of the proteome [110]. This is in part due to the stochastic selection of precursorions, resulting in poor reproducibility as long as the number of peptides in the sample tested exceeds the available sequencing cycles on the mass spectrometer [103]. It is important to remember that since the lists of proteins are not comprehensive, the non-appearance of a protein does not necessarily mean that the protein is not present [20, 21]. In contrast,

absence of detection in SRM analysis means that the analyte is below detection limits [23].

The nature of the MS results are thus challenging, and the biological questions have to be answered by "data sets that are noisy, incomplete and contain large amounts of irrelevant data" [20]. To obtain meaningful biological data from proteomic experiments, such data must be at least semi-quantitative [21].

5.1.3 Quantitative proteomics

Applying SRM workflow on chosen protein candidates eliminates the stochastic component of discovery proteomics, enabling consistent quantification across multiple samples [103]. Nevertheless, in SRM-MS the interference from other peptides and small molecules in the sample matrix can be a challenge. Such interferences manifest through decreasing the ion current response of an analyte in an unpredictable manner, or the detection of other sample constituents that have both precursor and product ions with a *m/z* identical or nearly identical to the selected transitions [23]. Thus, SRM can be a strong method for precise measurement of relative changes in the target protein across samples, rather than to accurately determine the concentration in a sample [23].

In paper III we used label-free SRM (MIDAS) to quantify selected proteins. An MS/MS spectrum was acquired whenever a selected transition was detected, to ensure that the registered signal was in fact from the peptide of interest [102, 104]. We also compared the SRM results with a non-MS method, i.e. western blot, and obtained similar results, showing that the relative quantification done by MS was reflected by an antibody-specific technique.

5.1.4 Label-free quantification

Precise label-free quantification, such as spectral counting, on data sets obtained by discovery proteomics is challenging, since a peptide spiked into different backgrounds will result in different intensities depending on the sample and time of analysis. To control for these effects, normalization is performed, and this can correct for global shifts, but is depen-

dent on the analyzed samples being closely related in background and protein composition [102]. When working with tissue samples, the sample amount is usually correlated to tissue weight. This might distort the results when the tissue structures of the diseased and normal tissues have substantial differences [35, 102], as shown in our comparison of ECM components in healthy and malignant ovarian tissue in paper V. As the ECM had substantial differences in healthy and malignant tissue, the IF proteomes obtained and the comparison of these may be affected.

5.1.5 Western blot

The internal standard GAPDH was seen to vary between individual samples in paper III, although staining with Coomassie Blue was comparable in all wells, indicating that the variability in tumor composition may contribute substantially to variation in housekeeping proteins between tumor samples. Thus, total protein staining may be a better normalization control in western blot of samples from tumors in varying stages and representing different histological subtypes.

5.2 Importance of access to the tumor microenvironment in proteomic research

During the development of a malignant tumor the quantitative relationship of tissue specific proteins that are shed into the microenvironment is altered compared to in healthy tissue [33, 35]. As earlier hypothesized [35], and demonstrated to be true in paper IV, the concentration of such disease-related proteins are substantially higher in the tumor microenvironment (Figure 6).

Access to native interstitial fluid can enable the determination of important tumor parameters, as COP. The absolute quantification of signaling molecules and growth factors in the tumor microenvironment has been limited, due to the lack of techniques for the iso-

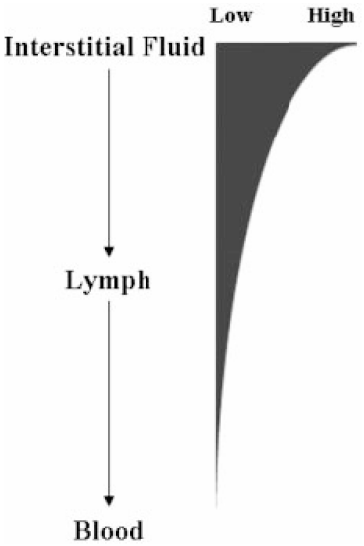

Figure 6: Relative concentration of tissue-specific proteins in TIF, lymph, and blood. The relative concentration of biomarkers is highest in TIF, which is in direct contact with cancer cells. TIF drains into lymph locally, which in turn merges with lymph from different tissues and finally drains into blood. The concentration of biomarkers from the local microenvironment of cancerous tissue is diluted approximately 1000 – 1500-fold during this process. From [35].

lation of undiluted IF. As shown for VEGF in the modeling study by Finley and Popel [111], the secretion rates of VEGF from tumors may predict response to anti-angiogenic treatment.

The later years have shown remarkable advances in proteomic technology, especially in quantitative proteomics [23, 103]. Although the resolution and speed of analysis improve, it is still imperative to understand the underlying biology and technical issues when analyzing body fluids or tumor tissue. Such knowledge is necessary to be able to find proteomic differences that are linked to tumor biology rather than the choice of sample and proteomic platform or sample preparation.

Proximal fluids that are found between the tumor and blood circulation, as ascites, have a concentration of tissue-specific proteins intermediate between plasma and IF, with a major influence of proteins from plasma, as we have shown by HPLC (Figure 4 in paper I). Ascites will contain proteins from not only tumor cells, but also liver and other tissues secreting proteins into the peritoneal cavity, and proteins with higher concentration than in plasma can originate from many sources, compared to proteins with high concentrations in the tumor interstitium [112]. Tumor interstitial fluid has the potential to meet this challenge,

having a higher concentration of locally produced and secreted proteins (Figure 6) than ascites or plasma, thus shifting the goal of proteomic analysis from the very low-abundance proteins in plasma, to the medium abundant proteins of TIF. In addition, we have demonstrated that the TIF-to-plasma ratio of locally produced proteins can relate to total tumor mass, and thus have prognostic value in itself (Paper IV).

5.3 Contribution of plasma proteins in TIF

Abundant plasma proteins are present in the interstitial fluid mainly from two sources. First, plasma proteins are transported across the capillaries to the interstitium by diffusion and convection. IF drains into lymph, and the lymphatic system returns fluid and proteins to the circulation [2]. The concentration of these plasma proteins in IF will be highly dependent on interstitial exclusion, as the ECM will restrict these large proteins from a substantial part of the interstitium. Thus, equilibrium is reached with lower total amounts of plasma proteins present than would be the case if proteins could distribute in the entire extracellular volume, as we have demonstrated in paper V.

Differential exclusion will affect the protein quantification, especially when comparing whole tissue homogenates that does not take extracellular volume and distribution into account. Care must be taken to exclude common plasma proteins that are shown as differentially expressed in proteomic studies, as these proteins are likely to originate not from the tumor, but from plasma. Differences in these proteins between healthy and malignant tissue can be caused by variable exclusion in different tissues.

The plasma residing in capillaries at the time of tissue sampling is the second source of plasma proteins in TIF. Studies on interstitial fluid in animal models have been able to quantify the extracellular space in the tissue by using ^{51}Cr-EDTA that was allowed to equilibrate until steady state was reached between the circulation and interstitium. Further, ^{125}I-HSA was infused in the last 5 minutes before cardiac arrest to label the vascular space. In trachea [113] and skin [114] the ratio between ^{125}I-HSA in IF and plasma was found to be

approximately 0.02, indicating a contribution from the vascular volume of 2 % in IF, while TIF had a ratio of 0.06 compared to plasma [114], thus suggesting that 6 % of the isolated fluid originating from the capillaries in the tissue. Thus, a part of the common plasma proteins in IF will originate directly from the vasculature in the excised tissue samples.

Plasma influences all extracellular body fluids, including IF, and it is important to understand the IF proteome in the context of plasma, as well as the structural elements in the tumor interstitium. Furthermore, the use of IF in biomarker discovery will reduce the concentration range between the abundant plasma proteins and locally produced proteins by increasing the concentration of the tissue-specific proteins for the disease that is studied, in addition to reducing the concentration and abundance of common plasma proteins.

5.4 Quantification of intracellular admixture

The main advantage of using TIF as a substrate for proteomics is the high concentrations of locally produced and secreted proteins. As these proteins are present in the extracellular microenvironment, they are more prone to enter the blood stream where non-invasive sampling and detection is possible [35, 37]. Therefore, an important question in TIF proteomics is whether the detected proteins are present extracellularly *in vivo*, or extracted from the cell during sample handling.

As discussed in paper II, and above, a limited number of proteomic studies on TIF validate the origin of the isolated sample as extracellular. In paper I, we used Na^+ and creatinine as extracellular endogenous markers. We compared the concentration in the interstitial fluid and plasma to quantify the admixture of intracellular fluid. Since these markers are in equilibrium between plasma and the interstitium in tumors, and are restricted from the cells, cell lysis *ex vivo* will result in a ratio between TIF and plasma below 1. Notably, *in vivo* cell rupture, e.g. caused by necrosis in the tissue, will affect the protein composition, but not the Na^+- or creatinine-ratios. Both molecules are fluid markers, and we assume that proteins are distributed in the intracellular fluid, and will be released from

the cells, when there is fluid admixture in IF. Vice versa, when the TIF-to-plasma ratios of Na^+ and creatinine are close to unity, neither fluid nor proteins are extracted from the intracellular compartment of the tumor due to sample handling.

Nevertheless, the TIF proteomes presented by us (Paper I, Paper III, Paper V), as well as TIF proteomes isolated by other isolation techniques [77, 115, 116, 117, 118, 119], only contain 16 to 41 % extracellular proteins based on the gene ontology (GO) classification system (Paper II). Similarly, the plasma proteome recently published by HUPO [120] contained 34 % extracellular proteins according to GO. Studies of lymph [121] and cultured media [122] show the same tendencies, with an overweight of proteins classified as intracellular, including proteins classified as nuclear and from other intracellular compartments. This raises the question of how many of the proteins classified as intracellular are actually secreted, and how to separate the proteins that are secreted from the proteins leaked from the cells due to sample handling.

In paper V, we measured Na^+ TIF-to-plasma ratios not significantly different from 1.0 for healthy and malignant ovarian tissue, while the endometrial tumor tissue yielded a much lower ratio, indicating intracellular admixture. Thus, the proteins present in the OC and OH proteomes most likely derive from the extracellular fluid phase. However, this measurement only determines fluid admixture, and as the protein concentration is much higher intracellularly than extracellularly [2, 123], a small admixture of fluid may influence the protein composition without changing the Na^+-ratio.

Abundant intracellular proteins have been suggested as indicators of cellular damage, e.g. as applied by Villarreal et al. [122]. They used ribosomal proteins as markers of cell lysis, and stress-related proteins as markers of initiated apoptosis. There were 73 ribosomal proteins detected in the Orbitrap-generated proteomes in paper V. The sum of all these ribosomal proteins, normalized on the total spectral counts in each sample, were significantly higher in endometrial carcinomas ($p = 0.004$, Mann-Whitney test) and ovarian carcinomas ($p = 0.03$, Mann-Whitney test) compared to healthy ovarian tissue, with the most promi-

nent differences being between EC and OH samples. The presence of ribosomal proteins can seem to be only partly related to the Na^+-ratio, and these proteins may leak into the interstitial fluid both *in vivo* (by cell necrosis) and *ex vivo* (during sample handling).

All but two of the stress-related proteins suggested by Villarreal et al. [122] were found in the Orbitrap-proteomes in Paper V. The sum of spectra for these proteins normalized on the total spectral counts for each sample was significantly higher in EC compared to both OC ($p = 0.04$) and OH ($p = 0.01$), and correlate well with the Na^+-ratios in Paper V.

5.5 Inflammation as a confounding factor

When proteins abundant in plasma and intracellular proteins not present extracellularly *in vivo* are disregarded, the task of finding proteins that are truly specific for the disease in question is still vast. In the biomarker studies published so far, there seem to be little overlap between proteomes (Paper II), and even in cell cultures, where specificity is high and the biological variability is minimal, the proteomic results deviate from each other [27].

In addition to the proteins that are not locally produced, as have been addressed in the previous sections, there is a large group of proteins related to inflammation that is up-regulated in cancer. This is a consequence of local as well as systemic inflammation responses in cancer patients, with tumors having many similarities to inflamed tissue [2, 38]. Such proteins have frequently been suggested as cancer biomarkers, but it is unlikely that they can be used to separate between different cancers as well as inflammatory conditions [38]. Proteins like the S100 calcium-binding protein family [124] and peroxiredoxins [125] are examples of such proteins, which have been shown up-regulated in numerous cancers (Paper II), but do not have the specificity required of a biomarker. Nevertheless, the study of these proteins may provide valuable information about cancer development and progression [124, 125].

5.6 Biomarkers in tumor interstitial fluid

An ideal screening biomarker has a sensitivity (proportion of cancer detected by a positive test), specificity (proportion of non-cancers identified with a negative test) and positive predictive value (proportion of tests that are true positives) of 100 %. The most limiting factor is the specificity, as most proteins suggested as biomarkers are tumor-associated rather than tumor-specific, and can be elevated in multiple cancers as well as benign conditions [30].

One challenge for identification of screening biomarkers is the fact that most biomarker studies utilize samples collected at diagnosis, and are more likely to increase sensitivity at time of diagnosis rather than contribute to early detection [30, 60]. Future studies searching for novel biomarkers for early detection should use samples collected up to three years before diagnosis [60].

In that perspective, the use of TIF proteomes are not optimal for the discovery of biomarkers for early detection. Rather, TIF as a proteomic substrate can contribute to the improvement of differential diagnosis, monitoring response to treatment, detecting recurrence and predicting prognosis. An example of this is the determination of the TIF-to-plasma ratio of CA-125 in paper IV, and the finding that this gradient is dependent on stage, or rather tumor mass. The determination of this gradient in the clinic may assist in a more accurate staging of disease.

New biomarkers for differential diagnosis may aid in the classification of type I (slow-growing, with good prognosis) and type II (aggressive, with poor prognosis) cancers for both ovarian and endometrial carcinomas. They should be contributing to the differentiation between patients that benefit from extensive surgery including lymphadenectomy and chemotherapy and patients that could be treated with surgery alone [79, 82]. Further, new biomarkers may be used to identify patients with drug resistant disease, for alternative treatments. Notably, the recent studies on the origin of ovarian carcinoma indicate that the distal fallopian tube is the starting point of type II serous ovarian carcinomas, and this must

be taken into account when choosing control tissues for the study of IF in ovarian cancer [30, 45].

The structure of the ECM in tumors will also have implications for treatment, and we have shown that proteomics can be used for the indirect determination of the structural elements of the tumor microenvironment in human samples. This provides us with knowledge transferable to the design of new therapeutic agents, to optimize penetration of the drug into the tumor tissue.

6 Conclusion

As the development of new platforms for proteomic studies move forward, the resulting lists of proteins get longer. Nevertheless, the challenges in biomarker discovery are multifaceted. First, the proteins identified in any one experiment will not be the complete proteome of the sample, but a subset of all the proteins present. Second, finding hundreds of proteins with an apparent change in expression, we lack the ability to choose the protein changes that are directly coupled to the condition that is being studied.

To answer the latter of these challenges, we have provided a sample collection strategy that limits the presence of contaminating proteins by using a proximal sample, i.e. the tumor interstitial fluid, which will be enriched with locally produced and secreted proteins. An additional advantage with our approach is the ability to assess the purity of the isolated IF before mass spectrometric analysis, enabling the selection of samples without detectable intracellular admixture before costly and time-consuming analysis.

We have demonstrated the advantage of using TIF for biomarker discovery, as the concentrations of tumor-related proteins, exemplified by CA-125, are much higher in TIF compared to plasma. Furthermore, analysis of undiluted and unfractionated IF with high-resolution MS techniques yields valuable proteomes with quantitative information as well as information on patient heterogeneity intact. TIF is a sample with an increased likelihood of detecting truly tumor-specific proteins. Using TIF, we have the ability to quantify the protein concentration in the tumor microenvironment that can contribute to improved differential diagnosis, better predicition of prognosis and the development of targeted agents in molecular imaging and therapy.

7 Future perspectives

In the work presented in this thesis, we have demonstrated how interstitial fluid can be isolated from healthy and malignant ovarian tissue by centrifugation. We envisage that the centrifugation approach can be validated for TIF isolation in other human tumor types, e.g. colon, breast and pancreatic cancer, to mention a few.

We have seen indications that the CA-125 ratio between TIF and plasma may add to differential diagnosis of ovarian cancer, and a larger study including more patients, especially with early stage disease, is warranted. In addition, a standardization of the centrifugation technique would be needed to include such testing in a clinical setting. Such standardization could make TIF sampling of clinical material in a biobank setting possible, thus facilitating future research.

We have attempted to highlight some of the advantages of using interstitial fluid in a proteomic approach, and produced extensive TIF proteomes of ovarian and endometrial carcinomas as well as healthy tissue. Substantial work remains in verifying and validating the findings in these proteomes further, as well as including additional controls, such as healthy endometrial and fallopian tube epithelial tissue. These may represent more valid control tissues, than can contribute to further in-depth analysis of the EC and OC proteomes.

In paper III, we found a substantial but variable increase of the actin-related protein WDR1 in ovarian cancer, and by measuring WDR1 in a larger patient material we can examine whether the WDR1 up-regulation is correlated to parameters as invasiveness and proliferation, or varies between histological subtypes.

In paper V we have published extensive proteomic data that may harbor a number of tumor-specific proteins that need further examination. A selection of proteins based on the abundance in TIF compared with plasma can give us an indication of which proteins are locally produced, aiding the selection for validation by MIDAS or SRM. By comparing OC

with OH and EC we can further identify proteins that are up-regulated in specific cancers, thereby avoiding selection of acute-phase proteins for further validation. By analyzing the proteomic data for a subgroup of patients, based on e.g. histological subgroup or type I and II cancers, we may identify proteins helpful in differential diagnosis.

The methods for determining available distribution volume in paper V can be applied to different types of tumors with varying stroma and extracellular matrix to search for differences in tumor parameters. We can hypothesize that the ECM structure may correlate to malignancy, invasiveness, or other important factors relevant for tumor growth or treatment of cancer, such as drug uptake. If so, such knowledge may aid the development of personalized medicine and determine course of treatment in cancer patients, e.g. through the identification of patients that are likely to develop drug resistance.

When applying proteomics to study TIF one should focus on the questions that can be answered by such a sample. In that aspect, early detection biomarkers would not be the main focus. Rather, the understanding of how up- or down-regulated proteins in TIF function in the tumor may contribute to improved differential diagnosis, prognosis and choice of treatment, and reveal possible targets for therapeutic intervention.

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