EXPLORING THE CLINICAL RELEVANCE OF CHONDROITIN SULFATE IN THE OVARIAN CANCER MICROENVIRONMENT

Sophie C.H.A. van der Steen
Exploring the clinical relevance of chondroitin sulfate in the ovarian cancer microenvironment

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Thesis, Radboud university medical center, Nijmegen, the Netherlands

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For reasons of consistency, terminology may be changed throughout this thesis when compared to the original publications.

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Chapter 1

Introduction and thesis outline

Based on Sulfated sugars in the extracellular matrix orchestrate ovarian cancer development: ‘when sweet turns sour’

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* Contributed equally
The extracellular matrix in ovarian carcinogenesis

Ovarian cancer is a major health threat in women, causing more deaths than all other gynecologic cancers combined. Worldwide, about 226,000 new patients and approximately 140,000 ovarian cancer related deaths are recorded each year.1 Due to absence of clear symptoms at an early stage and the lack of sufficient screening methods, over 70% of ovarian cancer patients are diagnosed with advanced stage (Fédération Internationale de Gynécologie et d’Obstétrique (FIGO) III and IV) of disease, and have a poor 5-year survival.2 First-line treatment, consisting of cytoreductive surgery and platinum-based chemotherapy, initially shows good response. Unfortunately, 70% of the patients with advanced disease quickly develop recurrent disease and eventually succumb.3

In the past, efforts in cancer research were predominantly focused on genetic changes in tumor cells that initiate cancer development. However, solely uncontrolled proliferation of tumor cells does not result in cancer. For tumor cells, in order to invade and disseminate, numerous hurdles in the surrounding micro-environment have to be taken. For example, preservation of normal tissue architecture surrounding tumor lesions prevents tumor cells from invading and metastasizing.4 As a consequence, tumor cells evolve and through proteolytic and glycosidic degradation of normal extracellular matrix (ECM) and de-novo-synthesis of ‘tumoral’ ECM they create a more protective environment. A large intratumoral ECM proportion has shown to correlate with poor prognosis in different types of cancer, including ovarian cancer.5

The ECM is made up of many different types of proteins and glycoproteins. Chondroitin sulfate (CS), a special class of glycosaminoglycans, is a major constituent of the ECM. Mainly positioned as side chains on the protein core of proteoglycans (PGs), they regulate many biological and pathological processes including cell differentiation, migration, adhesion and metastasis. An increase of CS and its different subfamilies has been noted in various types of cancer, including ovarian cancer (Table 1). Moreover, elevated levels of unique CS epitopes in ovarian cancer tissue and patient sera are associated with adverse prognostic factors and poor patient outcome.6 7 The functional significance of CS accumulation is largely unknown. Nevertheless, many mechanistic roles in the (tumoral) micro-environment have been indicated. A better understanding of the interplay between tumor cells and its surroundings could eventually lead to improved diagnostic and therapeutic modalities.

As the intricate play between core protein and glycosaminoglycan chains within proteoglycans is still unclear and core-mediated effects cannot always be separated from glycosaminoglycan-mediated effects, we have focused on CS proteoglycans as well.
Chondroitin sulfate in the extracellular matrix

A ‘sweet’ side to the extracellular micro-environment

The ECM is a highly organized three-dimensional mesh of fibrous proteins and glycoproteins. A well-operating ECM provides architectural support and molecular cues to the tissue. An ubiquitous component of the ECM are proteoglycans, highly versatile glycoproteins with a high degree of heterogeneity especially on the part of the glycosaminoglycan side chains. Glycosaminoglycans are linear polysaccharides that consist of a backbone of repeating disaccharide units composed of an amino sugar and an uronic acid. Four major families of glycosaminoglycans have been identified: chondroitin/dermatan sulfate, heparan sulfate/heparin, hyaluronan and keratan sulfate. To a large extent, the biological functions that proteoglycans possess mediate on the interactions of the glycosaminoglycan chains with various ligands. Moreover, the function of glycosaminoglycans is closely related to their spatial configuration and location. For instance, in the ECM they may form growth factor deposits, whereas at the cell surface they can function as (co)receptors regulating a wide range of cellular events. Through the binding of glycosaminoglycans with effector molecules such as cytokines, chemokines, and growth factors, glycosaminoglycans are able to regulate cell adhesion, proliferation, migration and angiogenesis.

The ECM is critical for everyday maintenance of all healthy organs. A “healthy” ECM surrounding cancer cells, can restrain or even overcome cancer progression, by retaining malignant tumors in an in situ situation. Conversely, the ECM also provides a scaffold to tumor cells and is therefore an essential intermediate player in tumor progression. Compared with non-neoplastic ECM, tumor associated ECM contains higher concentrations of various growth factors and high amounts of specific proteoglycans and glycosaminoglycans (Table 1). Moreover, tumor associated ECM is characterized by a predominant presence of (highly sulfated) CS, which outshines the other glycosaminoglycan families. Abnormal compilation of ECM facilitates tumor growth and cancer progression.

Chondroitin sulfate and its proteoglycans

The biosynthesis of glycosaminoglycan chains is a complex process and, unlike protein synthesis, not template driven. Instead, glycosaminoglycan formation relies on the activity of a group of specific glycosyltransferase, sulfotransferase and epimerase enzymes which are present in the Golgi apparatus. Only little is known about their regulation. The basic structure of the CS backbone is composed of a linear chain of repeating disaccharide units of glucuronic acid (GlcA) and N-acetylgalactosamine (GalNAc) (Figure 1A). Chain elongation occurs by sequential transfer of the appropriate monosaccharide by different glycosyltransferases (Figure 1B). Subsequent modifications involve CS-epimerization of GlcA to iduronic acid, thereby forming dermatan sulfate, and O-sulfation at C2 of GlcA/IdoA and/or C4 and/or C6 of GalNAc. The main structural categories of CS are illustrated in Figure 1A. Di-sulfation at C4 and C6 constitutes CS-E. Generally, CS is composed of various disaccharides. In addition, chains differ in length and special domains exist within one chain bearing high and low sulfated substitutions. The structural variation makes CS an information-dense molecule containing a large number of distinct oligosaccharide ‘motifs’. These domains are the key-elements on which growth factors and their receptors act, enabling them to
Table 1. Changes in chondroitin sulfate content and microstructure associated with various types of cancer.

<table>
<thead>
<tr>
<th>Type of cancer</th>
<th>CS/DS-related changes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Breast</strong></td>
<td>Increased CS-content</td>
<td>Alini et al., 1991; Suwanvat et al., 2004; Svensson et al., 2011; Vijayagopal et al., 1998; Weyers et al., 2012.</td>
</tr>
<tr>
<td></td>
<td>Decreased DS-content</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Increased C4S disaccharides</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Increased G6S disaccharides</td>
<td></td>
</tr>
<tr>
<td><strong>Pancreas</strong></td>
<td>Increased CS-content</td>
<td>Masuda et al., 2012; Theocharis et al., 2000.</td>
</tr>
<tr>
<td></td>
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<td></td>
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<tr>
<td></td>
<td>Increased C4S disaccharides</td>
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<tr>
<td></td>
<td>Increased C6S disaccharides</td>
<td></td>
</tr>
<tr>
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<td>Increased CS-content</td>
<td>Hatae et al., 1979; Horai et al., 1990; Masuda et al., 1987.</td>
</tr>
<tr>
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<td>Increased C4S disaccharides</td>
<td></td>
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<tr>
<td></td>
<td>Increased G6S disaccharides</td>
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</tr>
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<td><strong>Kidney</strong></td>
<td>Increased CS-content</td>
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<tr>
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<td></td>
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<tr>
<td></td>
<td>Increased C0S disaccharides</td>
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<tr>
<td><strong>Colorectum</strong></td>
<td>Increased CS-content</td>
<td>Theocharis et al., 2002; Tsara et al., 2002.</td>
</tr>
<tr>
<td></td>
<td>Increased DS-content</td>
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<tr>
<td></td>
<td>Increased G6S disaccharides</td>
<td></td>
</tr>
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<td><strong>Esophagus</strong></td>
<td>Increased CS-content</td>
<td>Theulin et al., 2012.</td>
</tr>
<tr>
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<tr>
<td></td>
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<td></td>
<td>Increased C4S disaccharides</td>
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</tr>
<tr>
<td></td>
<td>Decreased C6S disaccharides</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Decreased disulfated CS-content</td>
<td></td>
</tr>
<tr>
<td><strong>Stomach</strong></td>
<td>Increased CS-content</td>
<td>Sobue et al., 1980; Theocharis et al., 2003; Weyers et al., 2013.</td>
</tr>
<tr>
<td></td>
<td>Increased DS-content</td>
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<tr>
<td></td>
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<td></td>
<td>Increased C4S disaccharides</td>
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<tr>
<td></td>
<td>Increased G6S disaccharides</td>
<td></td>
</tr>
<tr>
<td><strong>Liver</strong></td>
<td>Increased CS-content</td>
<td>Jia et al., 2012; Kojima et al., 1975; Lapis et al., 1990; Lv et al., 2007</td>
</tr>
<tr>
<td></td>
<td>Increased C0S disaccharides</td>
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<tr>
<td></td>
<td>Increased C4S disaccharides</td>
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<tr>
<td></td>
<td>Increased C2S6S disaccharides</td>
<td></td>
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<tr>
<td></td>
<td>Increased C2S4S disaccharides</td>
<td></td>
</tr>
<tr>
<td><strong>Prostate</strong></td>
<td>Increased CS-content</td>
<td>De Klerk et al., 1984; Iida et al., 1997; Saikko et al., 2008; Ricciardelli et al., 1999; Teng et al., 2008.</td>
</tr>
<tr>
<td></td>
<td>Increased DS-content</td>
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<td></td>
<td>Increased C0S disaccharides</td>
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<td></td>
<td>Increased C4S disaccharides</td>
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<td></td>
<td>Increased G6S disaccharides</td>
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<tr>
<td><strong>Thyroid</strong></td>
<td>Increased CS-content</td>
<td>Emoto et al., 2007</td>
</tr>
<tr>
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<td></td>
<td>Increased C4S disaccharides</td>
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<td>Decreased C6S disaccharides</td>
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<tr>
<td></td>
<td>Decreased C2S6S disaccharides</td>
<td></td>
</tr>
<tr>
<td><strong>Ovary</strong></td>
<td>Increased C6S disaccharides</td>
<td>Potcharoen et al., 2006; Vallen et al., 2012.</td>
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<tr>
<td></td>
<td>Increased C4S6S disaccharides</td>
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<td></td>
<td>Increased C2S6S disaccharides</td>
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</table>

CS = chondroitin sulfate
DS = dermatan sulfate
C0S = non-sulfated chondroitin sulfate
C4S = monosulfated chondroitin sulfate at 4-O-position of N-acetylgalactosamine
C6S = monosulfated chondroitin sulfate at 6-O-position of N-acetylgalactosamine
C2S6S = disulfated chondroitin sulfate at 2-O position of uronic acid and at 6-O position of N-acetylgalactosamine
C4S6S = disulfated chondroitin sulfate at 4-O and at 6-O position of N-acetylgalactosamine
specifically promote various biological and pathological effects.\textsuperscript{12}

CS chains are bound to the core protein of a proteoglycan, which results in the formation of CS proteoglycans including; (part-time) cell surface CS proteoglycans, large hyalacticans and small leucine-rich proteoglycans (Figure 1C). In addition, proteoglycans generally carry more than one type of glycosaminoglycan, for instance aggrecan (one of the best studied proteoglycans) carries both chondroitin as well as keratan sulfate side chains.\textsuperscript{13}

**Figure 1.** Structure and diversity of chondroitin sulfate and associated proteoglycans.
Chondroitin sulfate in (ovarian) cancer

Cancer involves an intricate interplay between tumor cell proliferation, growth, angiogenesis, adhesion, migration, invasion, and survival. CS has shown to be involved in all these processes (Figure 2).

Altered expression of CS proteoglycans has been demonstrated in various types of cancer, including ovarian cancer. In addition, the type, i.e. sulfation pattern, of CS appears to be a critical factor in cancer progression. CS in normal, healthy tissue differs both in quantity and chain composition from CS present in tumors. In normal tissue monosulfated disaccharide units dominate the CS chains. By contrast, in cancerous tissue non-, di-, and trisulfated disaccharides are prominent (Table 1).

These structural alterations are brought about by the enzymes involved in CS biosynthesis (simplified overview; figure 1B). Therefore, it is not surprising that increased activity and altered

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Abbreviation</th>
<th>Reaction</th>
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<tbody>
<tr>
<td>Chondroitin sulfate GalNAc transferase (1/2)</td>
<td>GalNAc transfer II</td>
<td>Addition of GalNAc residues</td>
</tr>
<tr>
<td>Carbohydrate (chondroitin) synthase (1/2)</td>
<td>Chondroitin synthases</td>
<td>Chain elongation by alternating addition of GlcA/ GalNAc residues</td>
</tr>
<tr>
<td>Chondroitin-glucuronomate 5-epimerase</td>
<td>CS epimerase</td>
<td>Conversion of GlcA into IdoA</td>
</tr>
<tr>
<td>Chondroitin 4-O sulfo transferase (1/2/3)</td>
<td>C4ST</td>
<td>4-O-sulfation of GlcA/IdoA-GalNAc residue</td>
</tr>
<tr>
<td>Chondroitin 6-O sulfo transferase (1/2)</td>
<td>C6ST</td>
<td>6-O-sulfation of GlcA/IdoA-GalNAc residue</td>
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<tr>
<td>Chondroitin 4-sulfate 6-O sulfo transferase</td>
<td>C4S6ST</td>
<td>6-O-sulfation of GlcA/IdoA-GalNAc4S residue</td>
</tr>
<tr>
<td>Galactosaminyl uronyl 2-O sulfo transferase</td>
<td>UST</td>
<td>2-O-sulfation of GlcA/IdoA</td>
</tr>
</tbody>
</table>

A: Structure of the disaccharides variably present in chondroitin sulfates. Adapted with permission from Advances in Pharmacology, Volume 53 (Chondroitin Sulfate: Structure, Role and Pharmacological Activity), 2006, Page 35, Figure 1; B. Biosynthesis of chondroitin sulfate indicating the potential for structure heterogeneity; C. Various proteoglycans containing chondroitin sulfate side chains (artist’s impression).
function of these enzymes contributes to tumor progression as well. For example, increased expression of chondroitin-synthesizing enzymes such as chondroitin synthase I and III was seen in colorectal cancer. Whereas digestion of CS by chondroitinase AC had an adverse effect on proliferation and invasion of melanoma, endothelial, Lewis lung, breast and ovarian cancer cells.

**Figure 2.** Processes in ovarian carcinogenesis in which chondroitin sulfate participates as a critical player.

Legend

- chondroitin sulfate proteoglycan
- chondroitin sulfate chain
- effector molecule (e.g. growth factor)
- chondroitinase
- extracellular depot
- tumor cell
- proliferation
- adhesion
- invasion
- peritoneal lining
Ovarian cancer oncogenesis

The origin and pathogenesis of ovarian cancer has perplexed investigators for decades. Regarding the yet unexplained carcinogenesis and origin of ovarian cancer, CS is a molecule of interest.

Ovarian cancer is a heterogeneous disease composed of various epithelial subtypes with widely differing pathological features and clinical behavior. Currently, “ovarian cancer” is thought to comprise different diseases, wherein serous tubal intraepithelial carcinoma (STIC) in the tubal fimbriae has been identified as precursor lesion for the subgroup of high grade serous carcinoma.

Recently, a 2-way model of carcinogenesis was put forward stating that, among other things, divergent genetic mutations bring about two broad categories of ovarian carcinogenesis. Type I tumors represent low grade carcinomas which are genetically stable while type II tumors represent high grade, aggressive carcinomas which are genetically unstable. The TP53 gene is mutated in more than 80% of high grade serous carcinomas (type II) and p53 overexpression is significantly associated with important clinical prognosticators and worse survival in ovarian cancer patients.

The amount and sulfation pattern of CS has been correlated with the differentiation grade of several cancers. Poorly differentiated hepatocellular and prostate cancers display increased levels of CS with altered sulfation. In addition, the sulfation pattern alters during dedifferentiation, showing increased rates of non- and disulfated disaccharides and decreased levels of monosulfated disaccharides. Concerning the different ovarian cancer subtypes, serous ovarian carcinomas show an increased amount of CS-E compared to mucinous ovarian carcinomas. In conclusion, CS might represent an intermediate molecule in specific molecular pathways of ovarian cancer development.

Gene expression in cancer

Several genes are involved in the production of chondroitin and dermatan sulfate chains and changes in gene expression are associated with carcinogenesis. The position and degree of sulfation of chondroitin and dermatan sulfate chains involves the activity of a number of distinct carbohydrate sulfotransferases including; CHST3, CHST7, CHST11, CHST12, CHST13, CHST14, and CHST15. CHST11 and CHST13 are involved in the 4-O sulfation of CS, whereas CHST12 and CHST14 sulfate dermatan sulfate at the 4-O position. CHST3 and CHST7 share specificity for 6-O sulfation. Highly sulfated CS-E (4, 6-disulfated) disaccharides are produced when CHST15 transfers sulfate to a 4-O-sulfated monosaccharide. The literature on the regulation and underlying mechanisms involved in carbohydrate sulfation is still very limited. However, a number of studies have described some important alterations in cancer.

CHST15 is the sole sulfotransferase responsible for biosynthesis of highly sulfated CS-E. Altered transcription has been observed in various carcinomas including breast and ovarian cancer. Aggressive metastatic cell behavior has been correlated with high expression levels of CHST15 in Lewis lung carcinomas. Increased levels of CHST15 produced higher amounts of CS-E which, in turn, was accompanied by a decreased level of CS-A (the substrate for CS-E). Knockdown of CHST15 in Lewis lung carcinoma cells resulted in reduced proliferation and adhesiveness in vitro and inhibited pulmonary metastases in a mouse model. In astrocytic cancer, increased expression of CHST15 mRNA was correlated with poor patient prognosis. CHST11, which is primarily involved in the formation of CS-A, has also been correlated with metastatic potential of tumor cells. Overexpression of CHST11 was observed in breast cancer samples compared to normal tissue and a positive
correlation between the level of CHST11 expression and P-selectin binding to cells was observed suggesting the CHST11 gene as a player in the production of P-selectin ligands. TPS3 mutation is correlated with gene instability and is observed in various types of cancer including (type II) ovarian carcinoma. Among all different target genes of p53, CSPG2 (encoding versican) contains a putative p53 consensus binding site. It was shown that p53 binds specifically to the human versican regulatory element and over-expression of p53 showed a substantial induction of the versican gene. We speculate that various sulfotransferases might be involved in the TPS3 downstream pathway, and that they may be p53 target genes.

**Tumor growth**

All cancer cells share the same fundamental characteristic of uncontrolled growth, a result of disturbed regulation of cell proliferation and apoptosis. Ovarian cancer typically exhibits a very aggressive, rapidly growing nature, which causes patients to present with major tumor load throughout the abdomen. High proliferative activity in ovarian carcinoma has shown to associate with poor prognosis.

Growth factor binding to CS in the ECM is an important mechanism regulating growth factor availability, and thereby tumor growth. Similar to heparan sulfate, CS chains specifically interact with “heparin”-binding growth factors. Most growth factors require the presence of IdoA (DS-subunits) for binding, as was demonstrated by decreased cell proliferation of various cell lines after exogenous treatment with dermatan sulfate. However, highly sulfated CS, especially rich in CS-E subunits, has shown to serve as an important binding site as well. A large variety of growth factors bind to CS-E with strong affinity, including; fibroblast growth factor (FGF)2, FGF10, FGF16, FGF18, heparin-binding epidermal growth factor-like growth factor (HBEGF), hepatocyte growth factor (HGF), midkine (MDK), pleiotrophin (PTN), platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and transforming growth factor β (TGF-β). It was even shown that the binding affinity of CS-E for MDK, PTN and FGF18 (known as heparin-binding growth factors), was stronger for CS-E than their affinity for heparin. Furthermore, a synergistic effect of CS availability and FGF2 presence was evident, since a decrease in growth responsiveness to FGF2 was seen following chondroitinase treatment.

Versican, a large CS proteoglycan, is able to regulate tumor growth through binding of various growth factors with its CS side chains. Overexpression of versican on cancer cells (e.g. breast cancer) was associated with larger tumor masses in vivo and increased proliferation of SKOV-3 ovarian carcinoma cells and fibroblasts in vitro. Overexpression of human cationic antimicrobial protein 18 (hCAP-18)/pro inflammatory peptide (LL37) in the microenvironment of ovarian cancer cells contributes to increased cell proliferation and cancer progression through direct stimulation of tumor cells. Overexpression of versican, as shown for various ovarian cancer cell lines increased the release of LL-37 and hCAP18 by macrophages and multipotent mesenchymal stromal cells.

Decorin is a small proteoglycan which harbors only one glycosaminoglycan side chain. This side chain contains predominantly dermatan sulfate but can also hold CS. Decorin with dermatan attached to its core exhibits antiproliferative properties. When soluble decorin was added to the growth medium of SKOV-3 and MDAH2774 cells, proliferation was inhibited. Decorin can block tumor growth by binding growth factor TGF-β, thereby preventing it from interacting with its...
receptor complex. As a consequence, normal apoptosis can be restored. Binding of decorin to the epidermal growth factor (EGF) receptor induces an upregulation of cell cycle inhibitor p21 which in turn results in growth arrest of tumor cells. The binding of decorin to hepatocyte growth factor receptor (Met) results in impaired cellular proliferation and cell survival.

The glycosaminoglycan substitution of the decorin protein core changes during malignant transformation. In normal tissue, the ratio of chondroitin and dermatan sulfate side chains associated with decorin is balanced, however, in tumor tissue CS becomes the predominant side chain of decorin. This modification is reported for various carcinomas, including ovarian cancer. Depending on the glycosaminoglycan substitution of decorin, its functionality changes. Ovarian cancer patients suffering from advanced disease show high expression of decorin gene (DCN) and increased expression of decorin was associated with poor response to treatment and fast recurrence.

**Tumor angiogenesis**

Angiogenesis, the formation of new blood vessels from existing vasculature, is crucial for extensive tumor growth. Without sufficient delivery of oxygen and nutrients tumors cannot grow beyond a critical size. Ovarian carcinomas typically present as large, rapidly growing tumors which form large tumoral plaques in the abdomen. Several effector molecules of the angiogenic process have been reported, the most important being VEGF, FGF and PDGF. In ovarian cancer, the expression of VEGF is increased in primary tumor, ascites, and serum, and is associated with aggressive clinical behavior.

Tumor angiogenesis is at least partly depending on interaction with CS. Anti-CS antibody GD3G7, which reacts especially with CS-E rich epitopes, identified both VEGF-sensitive fenestrated and tumor blood vessels, which might implicate a role for CS-E in VEGF biology. CS-A has also been linked to VEGF induced angiogenesis. Human monocytic cell (THP-1) migration is normally accelerated by VEGF. However, when CS-A was exogenously added THP-1 cell migration was inhibited, eventually slowing down tumor angiogenesis and arresting tumor growth. Along with VEGF, PDGF has also shown to bind with CS-A. Exogenously added CS-A combined with PDGF showed a positive synergistic effect on the growth of fibrosarcoma cells, in vitro. It was put forward that this co-stimulatory effect of CS-A and PDGF was due to more efficient signaling. Clearly, the effect of CS on the binding and signaling effect of growth factors is complicated and likely dependent on the CS fine structure.

Versican is upregulated in the stromal compartment of various malignant tumors and is also seen around (pathological) blood vessels. The versican G3 domain has shown to stimulate the expression of fibronectin and VEGF, forming complexes with both molecules. These complexes enhance angiogenesis by modulating endothelial cell activity. The cleavage of versican splice variant V1 by several specific ADAMST (a disintegrin and metalloproteinase domain with thrombospondin type-1 motifs) metalloproteinases, has been proposed to play a role in angiogenesis through the release of pro-angiogenic particles into the tumoral extracellular environment. By contrast, decorin, which is also involved in the regulation of angiogenesis, blocks tumor cell mediated angiogenesis by downregulating VEGF production, Met activity and downstream pro-angiogenic pathways. Numerous studies have described a stimulating effect of various cleaving enzymes on
pathological angiogenesis including proteases, heparinases, and hyaluronidases. However, data for chondroitinases remains scarce.

**Tumor dissemination**
The high lethality of ovarian cancer is predominantly due to the advanced stage at time of diagnosis. The mechanism of dissemination of ovarian cancer is unique because it occurs mostly via intraperitoneal adhesion to the mesothelium and subsequent penetration of the sub-mesothelial matrix. Tumor cells are picked up by peritoneal fluid and are ‘seeded’ on to the peritoneal surface. In addition, the invasive character of ovarian cancer cells is limited, usually invading only the superficial layer of the peritoneum. Common sites of intraperitoneal seeding of ovarian carcinoma include the pelvic cavity, omentum, paracolic gutters, liver capsule, and diaphragm. The metastatic cascade involves detachment, adhesion and migration/invasion, and encompasses various interactions between tumor cells and components of the ECM.

**Detachment**
Before ovarian carcinoma cells detach they undergo epithelial-to-mesenchymal transition (EMT), which is in part regulated by the tumoral micro-environment. This change into a mesenchymal-like phenotype loosens the intercellular adhesions between cancer cells and between cancer cells and the ECM, thus facilitating cell separation and dissemination. An important characteristic of EMT in ovarian cancer cells is the “cadherin-switch”, involving the loss of E-cadherin and an upregulation of N-cadherin. When tumor cells arrive at the metastatic site, the micro-environment provokes mesenchymal-to-epithelial transition (MET), facilitating renewed cellular proliferation and upregulation of E-cadherin.

Versican has already been linked to MET. In fibroblasts, the versican V1 splice variant, which contains about half of the number of CS chains, has shown to influence the cadherin-switch from N- to E-cadherin. Versican overexpression caused expression of E-cadherin and thereby inactivation of N-cadherin, which was associated with dramatic alterations in membrane and cytoskeleton architecture. CS has also been directly linked to cadherin binding. In osteoblasts, highly sulfated CS-E promotes osteoblast differentiation by binding to both N-cadherin and cadherin-11. This effect was not seen for CS-A. The interaction between CS and N-cadherin suggests that the CS moiety on versican is an important player in binding cadherins in addition to the protein core.

**Adhesion**
Dissemination throughout the peritoneal cavity is greatly influenced by cell surface receptors that mediate the adhesion of ovarian cancer cells to the mesothelial lining of the peritoneum. It was demonstrated by Mizomoto et al. that down-regulation of CHST15, which produces the highly sulfated CS-E, resulted in reduced adhesiveness of Lewis lung carcinoma cells to ECM molecules and suppression of its metastatic capacity. Pretreatment of ovarian cancer cells with the CS cleaving enzyme chondroitinase ABC decreased cell adhesion to the ECM proteins fibronectin, type IV collagen and laminin and mesothelial cells.

Ovarian cancer cell adhesion involves cadherins, but is also depending on integrin-dependent mechanisms. Dimerization of integrins by the appropriate ligands leads to generation of intracellular
signaling and to physical changes in cell shape. Integrins bind directly to proteoglycans and their CS chains. The observation that proteoglycan binding sites are often expressed in close proximity to integrin binding domains suggests that cellular recognition of the ECM involves receptor clustering of both integrins and cell surface proteoglycans. Integrin receptor blockage could only partly inhibit ovarian cancer cell adhesion to collagen type I and III indicating a possible involvement of cell surface proteoglycans in adhesion. Antibody blockage of CD44 on SKOV-3 cells demonstrated that this CS proteoglycan is partly responsible for mesothelial attachment, likely through binding of hyaluronic acid.

**Migration and invasion**

Once attached to the metastatic site, ovarian cancer cells initiate the production of glycosidases and proteases. Although the invasive potential of ovarian cancer cells into the peritoneal surface is limited, the degradation of hostile host stroma is an important step for tumor cell invasion. In addition, the small liberated peptides may attract growth factors, further facilitating tumor progression. Metalloproteinases (MMPs) are an important group of matrix degrading enzymes contributing to cancer progression. MMP activation and regulation is influenced by the micro-environment including CS and its proteoglycans. Moreover, the sulfation pattern is again of crucial importance. For example, CS-A is crucial in the formation of a three-molecule complex consisting of CSPG4 (also known as melanoma-associated CSPG), membrane type 3 (MT3)-MMP and pro-MMP2. This complex leads to the activation of MMP2 via MMP16, enhancing tumor cell invasion. In addition to CS-A, CS-E is able to directly interact with various pro-MMPs, including pro-MMP7 and pro-MMP14, thereby promoting their activation and facilitating tumor cell metastasis. Membrane type 1 MMP (MT1-MMP/MMP14) plays a central role in tumor invasion. CD44, a cell surface proteoglycan containing CS chains, has shown to co-localize with MT1-MMP/MMP14. As a consequence, the newly formed complex increases MMP2 and MMP9 activity resulting in increased tumor cell invasion. Co-localization of CD44 with MT1-MMP/MMP14 facilitates cell migration by positioning MT1-MMP at the migration front of the cell. In addition, CD44 can dock MMP7 and MMP9 to the cell surface, attributing to cell adhesion properties.

Tumor cells exhibiting aggressive metastatic properties such as Lewis lung carcinoma cells have been characterized with marked upregulation of CS-E on the cell membrane inducing metastasis. Pretreatment with chondroitinase ABC of the tumor cells inhibited metastasis and blockage of CS-E with the single chain antibody GD3G7 restricted in vivo tumor cell dissemination. High expression of CS-E, seen immunohistochemically, surrounding primary ovarian tumor cells and in metastases could be involved in ovarian cancer progression. Enzymatic degradation of CS by chondroitinase AC has shown to inhibit melanoma cell proliferation and invasion. Conflicting data are available on the effect of enzymatic degradation of all available chondroitin and dermatan sulfate by intratumoral injections of chondroitinase ABC. In melanoma, chondroitinase ABC treatment resulted in the development of secondary tumors and increased lung metastases. However, in solid Ehrlich ascites tumors, growth was restricted after chondroitinase ABC injection. This suggests that targeting specific CSs, rather than all of them, is key to a potential anti-tumor strategy.
**Immunosilencing**

The immune system is a carefully regulated balance between immune-activating and immune-suppressing mechanisms and plays a major role in the clinical course of cancer. Ovarian cancer has the ability to escape from the immune system by creating a highly suppressive environment in the peritoneal cavity. Stroma has been shown to play a critical role in preventing or permitting immunological destruction of cancer cells in a tumor transplant. Also CS proteoglycans represent interesting components in the cancer-related immune response although their exact role has not yet been elucidated.

CSPGs are able to interact with the immune system through the binding of several chemokines and thereby prolonging their effect. This interaction is partly based on the overall negative charge of glycosaminoglycans and the density of sulfate and carboxylic groups on the GAG chains. Currently, our knowledge on specific interactions of CS with the immune system, whether influencing tumor immunogenicity in a negative or positive way, is limited. Said et al. reported that versican enhanced a tumor promoting inflammatory response in human bladder cancer by increasing the inflammatory cytokine chemokine ligand 2 (CCL2) and increasing the chemotactic effect on macrophages which directly facilitates metastasis. This increased tumor-promoting inflammatory response resulted in an increase of lung metastases in vivo.

TGF-β is a prominent immunosuppressive factor affecting proliferation, activation, and differentiation of immune cells and inhibits anti-tumor immune responses. Several CS proteoglycans including biglycan and decorin, bind to TGF-β suggesting that they may regulate TGF-β activity although precise interactions and effects have not yet be elucidated. Also interactions of CS with other chemokines have been described including TNF-α and interleukine-6 (IL-6), although usually in the context of inflammatory diseases.

Interestingly, CS can also directly influence T-helper (Th) cell differentiation into either a Th-1 or Th-2 subset. Th-1 cytokines, produced by Th-1 cells, stimulate the cellular immune response mainly through cytotoxic T-cells while Th-2 cytokines are associated with the humoral immune response. In cancer immunology, the Th-differentiation is shifted to a Th-2 cell response which is described as one of the escape mechanisms of ovarian cancer cells, abrogating a full blown cytotoxic T-cell response that is more potent to eradicate tumor cells. In addition, the Th-2 cell response may produce immune-suppressive cytokines, like IL-10. An in vitro experiment showed that splenocytes in the presence of CS affected Th-cell differentiation. The degree of sulfation was of significance for the immunological activity of CS and a larger effect for highly sulfated CS on Th-cell differentiation was observed.

Based on the limited research on the role of CS(PGs) in the cancer-immune response, we may conclude that the class of CS(PGs) is an interesting and potential critical player in the cancer inflammatory response.
Clinical relevance of chondroitin sulfate in ovarian cancer

Over the past decade, mortality rates for ovarian cancer have only been reduced by 4%.\textsuperscript{24} The poor outcome of ovarian cancer patients emphasizes the need to explore new therapeutic strategies improving (current) therapeutic efficacy. The underlying functionality of increased CSs in the tumoral microenvironment is still not completely understood. But our increased knowledge on the functional capacity of this dynamic molecule shows great potential for cancer therapy (Figure 3).

**Figure 3.** Various ways of interfering with chondroitin sulfate (proteoglycans) as a potential therapeutic strategy.
**Targeting chondroitin sulfate biosynthesis and degradation**

The rapid metastasizing character of ovarian cancer is the primary reason for poor patient outcome. One strategy to inhibit the effect of CS associate with the tumoral stroma is degradation of CS(proteoglycans) by enzymes. The growth of Ehrlich ascites tumors was inhibited after chondroitinase ABC treatment. Pre-incubation of Lewis lung carcinoma cells with anti-CS-E antibody GD3G7 and pretreatment with chondroitinase ABC strongly reduced secondary tumor growth. Moreover, eliminating endogenous CS by exogenously injected chondroitinase ABC was associated with inhibition of carcinogenic cellular activities including reduced proliferation, migration and invasion.

Besides interference with already formed CS chains, inhibition of CS proteoglycan biosynthesis may interfere with cancer progression, as demonstrated for the melanoma associated chondroitin sulfate proteoglycan (CSPG4). Intratumoral injection of a lentivirally encoded shRNA abrogated CSPG4 expression and function, thereby significantly affecting tumor cell proliferation and apoptosis, resulting in regression of primary tumors. Using modified monosaccharides (e.g. deoxy sugars), the biosynthesis of CS chains can also be blocked.

Interference with CS (proteoglycans) before or after its formation may be a feasible strategy to reduce the ovarian tumor cell growth and metastasis.

**Targeting the binding properties of chondroitin sulfate**

Monoclonal antibody-based therapy against CS proteoglycans in cancer is mainly focused on CSPG4 and versican. Cancer cells treated with CSPG4 specific antibodies showed diminished growth, adhesion and migration. After surgical removal of the primary tumor, prolonged disease-free and overall survival was demonstrated in cancer xenografts when treatment with anti-CSPG4 antibodies was continued.

One of the best studied therapeutic targets in the tumor microenvironment is VEGF, key player in tumor angiogenesis. Bevacizumab is a recombinant, humanized anti-VEGF monoclonal antibody inhibiting neovascularization, and has been recently approved in Europe as an additional treatment for ovarian cancer patients in a palliative setting. The addition of bevacizumab to standard chemotherapeutic regimens showed improved progression free survival. Its inhibiting effect on neovascularization primarily resulted in decreased tumor growth. In addition, patients showed a decrease in the amount of malignant ascites. This 'side-effect' may contribute substantially to an improved quality of life of end-stage ovarian cancer patients. The interaction of CS, specifically CS-E, with VEGF and FGF implicates that CSs are attractive targets for inhibiting tumor angiogenesis. Indeed histological evaluation of cancer xenografts treated with monoclonal antibodies against CS proteoglycans, with chondroitinase ABC or with lentivirally encoded CS proteoglycans shRNA showed a reduction of vascular density in the tumor micro-environment.

**Application of chondroitin sulfate as additional or direct therapy**

Besides therapeutic applications interfering with its expression, CS proteoglycans may also be used as an anti-cancer drug. Decorin has shown anti-tumoral properties in a number of different types of cancer, including ovarian cancer. In a breast carcinoma model, administration of decorin reduced primary tumor growth by 70% and eliminated metastasis; in addition, adenovirus-mediated decorin
delivery resulted in comparable effects.\textsuperscript{83} Cancer cells transfected with decorin, showed that ectopic decorin expression was able to reduce tumor outgrowth and angiogenesis.\textsuperscript{84} Combining carboplatin and decorin showed inhibiting synergistic effects on ovarian cancer cell growth \textit{in vitro}.\textsuperscript{58} Considering that platinum-based chemotherapy is still the leading strategy for ovarian cancer patients, this finding suggests it might be possible to lower the toxic dose of carboplatin and additionally address a novel secondary pathway for therapy.

A very promising novel therapeutic approach for ovarian cancer is combining currently used antitumor agents with agents targeting CS. Selective CS targeting therapy may provide a more efficient and safe way to deliver anticancer drugs than free anticancer agents. Cationic liposomes loaded with cisplatin bound to CS-E and showed selective and cytotoxic activities against tumors expressing large amounts of CS, \textit{in vivo}.\textsuperscript{85} These cationized liposomes were more potent in inhibiting liver metastases than either free cisplatin or plain liposomes loaded with cisplatin. Genetic fusion of a mAb specific for CSPG4 to TRAIL (Tumor Necrosis Factor Related Apoptosis Inducing Ligand), an immune cytokine with pronounced pro-apoptotic activity towards malignant cells, resulted in a bifunctional fusion protein designated anti-CSPG4:TRAIL.\textsuperscript{86} Treatment of malignant cells with anti-CSPG4:TRAIL resulted in a combined inhibition of carcinogenic CSPG4-signaling (focal adhesion kinase) and concordant activation of TRAIL-apoptotic signaling. Reduction of primary tumor size in cancer xenografts after anti-CSPG4:TRAIL administration was superior to combined treatment with TRAIL and anti-CSPG4. It was demonstrated by Wang et al. that anti-CSPG4 antibody inhibited lung metastasis and prolonged survival time in severe combined immunodeficient mice injected with a human melanoma cell line.\textsuperscript{87} Combining an anti-CSPG4 antibody to a cytotoxic agent may result in a tumor selective antidrug conjugate.

CS mimetics could also be used as anticancer agents. Such mimetics may compete with native CS with respect to interactions with e.g. growth factors and other effector molecules. Selectins and chemokines are sulfation dependent and oversulfated CS chains bind these molecules with stronger affinity.\textsuperscript{12} For instance, CS isolated from a sea cucumber, containing sulfated fucose branches (fucosylated CS (FucCS)), is a potent inhibitor of P- and L-selectin-mediated interactions resulting in reduced tumor cell-platelet association and reduced selectin-mediated lung metastasis in adenocarcinoma xenografts treated with FucCS.\textsuperscript{88}

Chemically (carbodiimide) modified CS chains showed to have strikingly different anticancer activities compared with native CS chains. Modified CS reduced cell viability of myeloma and breast cancer cells by inducing apoptosis \textit{in vitro}. In addition, tumor growth was abolished when these glycans were directly injected into breast tumors growing in mice.\textsuperscript{89}

In the future, CS mimetics might be of beneficial value when added to conventional therapy, either by having a synergistic effect on chemotherapy or as an additional way to attack tumor tissue. Still, much effort is needed to unravel the exact mechanisms by which CS (mimetics) may influence tumorigenesis.

For a long time the class of glycans (“sugars”) in the ECM has been neglected. Glycans were considered bystanders in protein and nucleic acid dominated physiological and pathophysiological processes. This especially applied for the class glycosaminoglycans, long anionic polysaccharides, which were (and actually are) difficult to study in part due to technical limitations associated
with their analysis. However, the last two decades have witnessed a number of studies showing the highly dynamic nature of these molecules, their physiological relevance, and their intrinsic involvement in the process of tumorigenesis. CS stands out as clear example of this.

Ovarian cancer is in definite need of novel avenues for diagnostic and therapeutic targeting and glycosaminoglycans, especially CS, may provide new leads in this respect. The qualitative and quantitative changes observed in these molecules provide novel handles for innovative strategies for early diagnosis and ways to interfere in processes as tumor growth and metastasis. Indeed, initial attempts have been made to inhibit the tumorigenic role of chondroitin(proteoglycans) including the use of (blocking) antibodies, CS mimetics, degrading enzymes, and other specific targeting strategies. The coming decade will see novel approaches to address tumor associated changes in CS. With the coming of age of the field of glycobiology, new prospects appear on the horizons in which modulation of glycans will contribute significantly to diagnostic and therapeutic strategies to fight ovarian cancer.

**Thesis outline**

This thesis addresses the ovarian cancer micro-environment by evaluating the involvement of extracellular chondroitin sulfates in the development and progression of ovarian cancer, and by investigating the potential of a clinical application of these matrix molecules (e.g. biomarker, therapy).

We evaluate the biomarker potential of highly sulfated chondroitin sulfate (CS-E) expressed in the extracellular matrix (ECM) using a large cohort of ovarian cancer patients, in chapter 2. We analyze the expression of CS-E in the different subtypes of ovarian cancer and healthy organs, and evaluate its prognostic value.

In chapter 3, we study the involvement of extracellular CS-E in the very early steps of carcinogenesis of the most prevalent and most clinically relevant subtype (high grade serous carcinoma) of ovarian cancer. We analyze the expression of CS-E in precursor lesions of the full spectrum of ovarian cancer development, and we address the immunological reactivity in the microenvironment. From here, we focus on infiltrating T cells involved in the immunological anti-cancer response in the remodeled micro-environment of ovarian cancer precursor lesions in chapter 4.

In chapter 5, we investigate the potential of an innovative approach for the treatment of ovarian cancer based on an ECM-targeting therapy. We create a drug delivery system that specifically targets highly sulfated chondroitin sulfates in the ovarian cancer ECM, and evaluate its functionality in vitro.

Finally we discuss relevant questions remaining from the abovementioned studies in chapter 6, and elaborate on the impact of our findings for future research.
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Chapter 2

Prognostic significance of highly sulfated chondroitin sulfates in ovarian cancer defined by the single chain antibody GD3A11

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**Abstract**

*Objective.* The extracellular matrix (ECM) of ovarian cancer may provide a number of potential biomarkers. Chondroitin sulfate (CS), a class of sulfated polysaccharides, is abundantly present in the ECM of ovarian cancer. Structural alterations of CS chains (i.e. sulfation pattern) have been demonstrated to play a role in cancer development and progression. In this study we investigate the potential of highly sulfated CS as a biomarker in ovarian cancer using the single chain antibody GD3A11 selected by the phage display technology.

*Methods.* The specificity of the antibody was determined by an indirect ELISA. GD3A11 epitope expression was assessed by immunohistochemistry in healthy organs, benign and malignant ovarian tumors ($N = 359$) and correlated to clinical parameters. The $CHST15$ gene, responsible for the biosynthesis of highly sulfated CS was evaluated for mutation and methylation status.

*Results.* The GD3A11 epitope was minimally expressed in normal organs. Intense expression was observed in the ECM of different ovarian cancer subtypes, in contrast to benign ovarian tumors. Expression was independent of tumor grade, FIGO stage, and the use chemotherapy. For the aggressive ovarian cancer phenotype, intense expression was identified as an independent predictor for poor prognosis. $CHST15$ gene analysis showed no mutations nor an altered methylation status.

*Conclusion.* Specific highly sulfated CS motifs expressed in the tumoral ECM hold biomarker potential in ovarian cancer patients. These matrix motifs constitute a novel class of biomarkers with prognostic significance and may be instrumental for innovative diagnostic and therapeutic applications (e.g. targeted therapy) in management of ovarian cancer.
Introduction

Epithelial ovarian cancer is the fifth leading cause of cancer death in women worldwide. The high mortality of this disease is related to the advanced stage of ovarian cancer (Fédération Internationale de Gynécologie et d’Obstétrique (FIGO) stage III-IV) in which most patients are diagnosed and the lack of effective therapies for this cancer. Although the majority of these patients initially show a good response on primary treatment, 70% will relapse within a few years and long term survival remains poor with a 5-year survival of less than 35%. Thus inventive clinical approaches are urgently needed to improve outcome of these patients.

Biomarkers in ovarian cancer are of value for the development of new diagnostic and therapeutic strategies. The search for new biomarkers should take into account that epithelial ovarian cancer is a heterogeneous disease comprising distinct subtypes with specific morphologic and molecular genetic characteristics. Recent knowledge may have implications for the development of novel clinical strategies.

In recent years, the tumoral extracellular matrix (ECM) has become a focus of research, with the understanding that alterations that occur in the ECM in a solid tumor might be relevant for prognosis and might generate new therapeutic targets. Modifications to the ECM composition are crucial for cancer development and progression. In ovarian cancer, a high intratumoral proportion of ECM has shown to correlate with poor prognosis. In the ECM, proteoglycans (PGs) are ubiquitous components and important players in the tumoral stromal reaction.

Proteoglycans are composed of a protein core to which glycosaminoglycan chains are covalently linked. Glycosaminoglycans are linear negatively charged polysaccharides built from repeating disaccharides generally consisting of units of N-acetylated hexosamine and uronic acid. Several major glycosaminoglycan families have been identified including chondroitin sulfate (CS)/dermatan sulfate, heparan sulfate/heparin, and hyaluronan. The degree and position of sulfation as well as the C-5 epimerization is highly variable in glycosaminoglycans and influence their biological properties. Through interacting with specific effector molecules (e.g. growth factors), glycosaminoglycans are able to regulate (cancer-associated) cell adhesion, proliferation, migration and angiogenesis.

Abnormal expression of the glycosaminoglycan chondroitin sulfate and chondroitin sulfate containing proteoglycans (e.g. versican) has been demonstrated in various types of cancer including ovarian cancer. Chondroitin sulfate consists of repeating disaccharide units composed of glucuronic acid (GlcA) and N-acetylgalactosamine (GalNAc), which are variously modified by O-sulfation. The monosulfated unit CS-A and CS-C consists of GlcA-GalNAc4S and GlcA-GalNAc6S disaccharide units, respectively, whereas the more rare disulfated unit CS-D or CS-E consists of GlcA2S-GalNAc6S or GlcA-GalNAc4S6S disaccharide units, respectively. In general, highly sulfated CS subtypes are rather rare and associated with pathologic circumstances including cancer. Increased expression of the highly sulfated CS subtype (CS-E) and the CSPG versican have been demonstrated in the ECM of ovarian cancer and have been correlated with poor prognosis.

Our recent studies demonstrated that tumor related alterations in CS structure in the ECM may represent interesting biomarkers for ovarian cancer. In this study, using the phage display technology, we selected the single chain variable fragment (ScFv) antibody GD3A11 which
strongly reacts with highly sulfated CS. In order to validate and expand previous results regarding the biomarker value of specific CS-like motifs upregulated in ovarian cancer, we investigated the expression of the GD3A11 epitope in a large cohort of ovarian carcinomas with respect to the distinct ovarian cancer subtypes. In addition, we studied the gene encoding the enzyme carbohydrate (N-acetylgalactosamine 4-sulfate 6-O) sulfotransferase 15 (CHST15) which is involved in the biosynthesis of highly sulfated CS.

Material and methods

Selection and production of scFv antibody GD3A11

The scFv GD3A11 antibody was selected using embryonic rat glycosaminoglycans as a source for carcinoembryonic antigens applying the phage display technology as previously described. For the selection of scFv antibodies, the human semisynthetic single chain variable fragment library was used, generously provided by Dr. G. Winter, Medical Research Council Molecular Biology, Cambridge, UK. The selection of phages displaying scFv antibodies and the production of the scFv antibody were performed as described previously.

Evaluation of specificity of the antibody GD3A11

Enzyme-linked immunosorbent assay (ELISA)

To determine the specificity of the scFv antibody GD3A11, an indirect ELISA was performed using various glycosaminoglycans including CS-A (from bovine trachea, Sigma-Aldrich), CS-B (dermatan sulfate) (from porcine intestinal mucosa, Celsus Laboratories Inc.), CS-C (from shark cartilage, Sigma-Aldrich), CS-D (from shark cartilage, Seikagaku), CS-E (from squid cartilage, Seikagaku), heparin (from porcine intestinal mucosa, Sigma-Aldrich), and heparan sulfate (from bovine kidney, Sigma-Aldrich) coated onto a 96-well microtiter plate. After blocking with phosphate buffered saline (PBS, pH 7.2) containing 1% Tween-20 (v/v) and 3% (w/v) bovine serum albumin (BSA, fraction V, Sigma-Aldrich), the plate was incubated successively with scFv GD3A11 (1:1), monoclonal mouse IgG anti-VSV antibody (clone P5D4, 1:10), and goat anti-mouse IgG alkaline phosphatase conjugated (Life Technologies, G-21060, 1:2000). After addition of the substrate (p-nitrophenyl phosphate in 1 M diethanolamine with MgCl₂, pH 9.8), absorbance was read at 405 nm after 90 min.

Immunofluorescence assay

Human ovarian cancer cryosections (5 µm) were pre-treated with 2 mM MgAc, in 25 mM Tris buffer (pH 8.0) with and without glycosaminoglycans-digesting enzymes including Chondroitinase-ABC, -AC, and -B (90 min, 37° C). Thereafter, sections were blocked with PBS containing 0.1% Tween-20 (PBS-T) and 1% BSA. Sections were incubated with the scFv antibody GD3A11 (1:5), a secondary mouse anti-VSV antibody and a tertiary goat antimouse IgG Alexa Fluor 488 (Life Technologies, A-11001, 1:500). Finally, cryosections were fixed in 100% ethanol and mounted in mowiol-488 (Calbiochem, La Jolla, CA, USA). Image processing was performed using ImageJ 1.48v (National Institutes of Health, USA). Brightness and contrast were adjusted similarly for all photos including the controls.
Patient cohort

Study approval was given by the Regional Committee for Medical Research Ethics and performed according to the Code for Proper Secondary Use of Human Tissue (Dutch Federation of Biomedical Scientific Societies, www.federa.org).

From patients diagnosed with epithelial ovarian cancer, tubal cancer, and peritoneal cancer between 1996 and 2014, paraffin embedded tissues of the primary tumor ($N=289$) were randomly collected from the department of Pathology at the Radboud University Medical Center. Only samples from patients who were initially treated according to the standard therapeutic regimen including surgery (and when indicated platin-based chemotherapy), were included ($N=278$). Both patients treated with adjuvant and neo-adjuvant chemotherapy were included. Hematoxylin and eosin stained ovarian tumor slides were revised by an experienced gynecologic pathologist (JB) and representative tumor sections were selected. Patients without evident tumor after the use of neo-adjuvant chemotherapy were excluded ($N=23$). The final study cohort included 255 ovarian carcinomas (168 serous, 22 mucinous, 25 endometrioid, 15 clear cell, and 25 undifferentiated), 62 benign ovarian tumors (27 serous, 24 mucinous, and 11 endometrioid), and 42 normal adnexa from patients with a benign gynaecologic abnormality (e.g. endometrial hyperplasia) as control group.

Patient clinical parameters were retrospectively retrieved from the medical records. Median age was 58 (range 37-83), 47 (range 22-86), and 61 (range 18-90) of patients with normal adnexa, benign tumors, and malignant tumors, respectively. 65%, 38%, and 78% of patients with respectively normal adnexa, benign tumors, and malignant tumors were postmenopausal at time of diagnosis. For analyses involving prognostic parameters, a homogeneous group of patients was composed consisting of only type II ovarian cancers based on morphology and grade of abnormality, and comprising high grade serous, high grade endometrioid, and undifferentiated adenocarcinomas ($N=197$). Median follow up was 22 months (range 0-160). Progression free survival (PFS) and overall survival (OS) were defined as the time-interval in months between completion of therapy and recurrent disease or death, respectively.

To assess the GD3A11 epitope expression in various normal human tissues, paraffin embedded tissues from the lung, liver, kidney, pancreas, adrenal gland, colon, breast, endometrium, and cervix were randomly collected from the department of Pathology at the Radboud University Medical Center. Hematoxylin and eosin stained sections of the tissues were reviewed by a pathologist and histology was assessed as normal.

Tissue GD3A11 epitope expression

The most representative section per case, as selected by an experienced gynecologic pathologist (JB), was immunohistochemically analyzed using the avidin-biotin complex method. In brief, 4 µm paraffin sections were de-waxed in xylene, rehydrated in graded ethanol and blocked for endogenous peroxidase activity by 0.3% $\text{H}_2\text{O}_2$ in methanol. After rinsing in PBS-T, sections were incubated with 10 mM sodium citrate buffer (pH 5.8) for 30 min and thereafter blocked with 2% BSA in PBS-T. Next, sections were incubated with the primary antibody GD3A11 (VSV-tagged), the secondary monoclonal mouse IgG anti-VSV antibody, and the tertiary biotinylated horse-anti-mouse IgG antibody (Vector Laboratories Inc., CA, USA, 1:200). After incubation with ABC reagent (Vectastain ABC anti-mouse-IgG kit, Vector Laboratories Inc.), bound antibodies were visualized
using 3-amino-9-ethyl-carbazole (AEC). Sections were counterstained with hematoxylin and mounted with Kaiser's glycerol gelatine (Merck, Darmstadt, Germany). As a negative control, the scFv GD3A11 was omitted, and sections with known strong staining intensity were included as positive control.

**Semi-quantitative assessment of GD3A11 staining**
GD3A11 staining was assessed by two independent observers (AvT and SvdS) without knowledge of clinicopathological parameters. Based on a commonly used semi-quantitative IHC scoring system, both the percentage of positively stained tumoral stroma and the maximum intensity of the staining were assessed. The percentage of positive tumor stroma was classified as: 0 = negative; 1 = 0-5%; 2 = 6-25%; 3 = 26-50%; 4 = 51-75% and 5 = 76-100% staining positive. The maximum intensity was scored as: 1 = weak, 2 = moderate and 3 = intense. The overall score was calculated by multiplying percentage and intensity (range 0-15 points). Two groups were formed: one group classified as no or mild staining (0-6 points) and a second group classified as intense staining (7-15 points). The cutoff point was based on its most discriminating character. In case of discordant scores, sections were re-evaluated by an independent observer (JB).

**CHST15 gene analysis**
Several genes are involved in the production of CS including sulfotransferases responsible for the position and degree of sulfation. Of these, the gene encoding the enzyme *N*-acetylgalactosamine 4-sulfate 6-O sulfotransferase (*CHST15*) is responsible for the biosynthesis of the GlcA-GalNAc4S6S residues (CS-E) by transferring a sulfate group to a GlcA-GalNAc4S (CS-A).\(^{20}\) To analyze the *CHST15* gene in ovarian cancer, its genomic mutation and methylation status was evaluated in ovarian cancer and normal tissue.

DNA from paraffin embedded tissues including 9 high grade serous carcinomas with intense expression of the GD3A11 epitope and 5 normal adnexa was isolated with TET-lysis buffer (10 mmol/l TrisHCl, pH 8.5; 1 mmol/l EDTA, pH 8.0; 0.1% Tween-20) containing 5% Chelex-100 (Bio-Rad, Hercules, CA). Protein digestion was performed by adding proteinase K and samples were incubated for 48 h at 56°C. Next, Proteinase K was inactivated at 95°C for 10 min and supernatant was collected after centrifuging at 24,000 g.

The sequences of the promoter region and 8 exons of the *CHST15* gene were analyzed for mutations by PCR amplification followed by sequencing the products (NCBI Reference Sequence: NG_032891.1). Methylation status of the CpG island in the *CHST15* promoter region was analyzed using the bisulfite conversion method converting unmethylated cytosines into uracil (EZ DNA Methylation-Gold\textsuperscript{TM} Kit, Zymo Research). DNA from 24 high grade serous carcinomas with intense expression of the GD3A11 epitope and 19 normal adnexa was used. The methylation profile of the bisulfite converted DNA was analyzed by PCR amplification with methylation-dependent primers and subsequently visualized on ethidium bromide 1% agarose gels. To quantify the amount of methylated and unmethylated PCR products, fragment analysis was performed on the bisulfite converted DNA PCR products using primers labeled with a fluorescent dye (6FAM/VIC).
Statistical analysis
Statistical analyses were performed using the software package SPSS 22.0 for Microsoft Windows (SPSS Inc., Chicago, IL). One way analysis of variance (ANOVA) was indicated to compare means of the measured absorbance at the ELISA approach. Inter-observer reliability for antibody expression was determined using Cohn’s Kappa. Chi-square test was used to assess correlations between GD3A11 epitope expression and clinicopathological factors (Fisher’s exact test was used when expected cell count was less than five). McNemar’s test was used to analyze the paired GD3A11 epitope expression. Survival estimates were plotted using the Kaplan Meier method and curves were compared using log-rank test. Cox’s proportion hazard model was used for univariate and multivariate analyses of prognostic values. All tests were two-sided and p-values < 0.05 were considered significant.

Results

Characterization of the antibody GD3A11
DNA sequence analysis revealed that the scFv antibody GD3A11 belongs to the V3 family, has a DP38 germline gene segment and contains the heavy chain complementarity determining region 3 (CDR3) amino acid sequence GTSiRD.

Specificity of the antibody GD3A11
To determine the specificity of the scFv antibody GD3A11 for various glycosaminoglycans an indirect ELISA approach was used (Figure 1A). The scFv antibody GD3A11 showed strong reactivity with the highly sulfated CS-E subtype rich in GalNAc4S6S disaccharides, while no reactivity was observed with other immobilized glycosaminoglycans including CS-A, CS-B (also known as dermatan sulfate), CS-C,CS-D, HS, and heparin (p < 0.001).

Cryosections of ovarian high grade serous carcinomas were pre-incubated with glycosaminoglycan degrading enzymes including chondroitinase-ABC, -AC, and -B and were subsequently stained with the scFv antibody GD3A11 (Figure 1B). Strong expression of the GD3A11 epitope was observed in the tumoral stroma of the sections without enzyme pre-incubation, whereas in the tumor sections pre-incubated with chondroitinase-ABC (digests CS and dermatan sulfate) and –AC (digests CS), staining was completely abolished. Sections pre-incubated with chondroitinase-B (digests dermatan sulfate) were not affected. From these data it can be concluded that the scFv antibody GD3A11 reacts with CS located in the extracellular matrix of ovarian cancer.

Distribution of the GD3A11 epitope in normal tissues
Normal human tissues including the lung, liver, kidney, pancreas, adrenal gland, colon, breast, endometrium, and cervix were analyzed for reactivity with the scFv GD3A11. A very restricting staining pattern was observed throughout the various organs (Figure 2A-K). The islets of Langerhans in the pancreas showed sometimes weak positive staining (Figure 2D).
The inter-observer reliability of scoring the GD3A11 epitope expression was assessed by using Cohen's kappa and estimated to be 0.81 (data not shown) which is considered to be good. All histological normal adnexa (including both the ovary and fallopian tube) showed no or very

Figure 1. Evaluation of the specificity of the scFv antibody GD3A11.

A. ELISA to determine the reactivity of the scFv antibody GD3A11 with glycosaminoglycans CS-A, CS-B (dermatan sulfate), CS-C, CS-D, CS-E, heparin, and heparan sulfate (HS). Bars represent mean with standard deviation (N=3). Absorbance was measured at 405 nm. B. H&E staining of ovarian cancer cryosection. Bar represents 100 µm. C. Effect of chondroitin sulfate degrading enzymes on staining with antibody GD3A11. Ovarian cancer cryosections were pre-incubated with chondroitinase-ABC (digests CS and dermatan sulfate), chondroitinase AC (digests only chondroitin sulfate) and chondroitinase-B (digests only dermatan sulfate). Bar represents 100 µm.

GD3A11 epitope expression in patients

The inter-observer reliability of scoring the GD3A11 epitope expression was assessed by using Cohen's kappa and estimated to be 0.81 (data not shown) which is considered to be good. All histological normal adnexa (including both the ovary and fallopian tube) showed no or very
Prognostic significance of highly sulfated chondroitin sulfates in ovarian cancer defined by the single chain antibody GD3A11

minimal expression of the GD3A11 epitope in the stroma and in basement membrane zone underlying the epithelial cell layer (Figure 2J-K). Most benign ovarian tumors (81%) showed no or mild GD3A11 epitope expression while in 19% intense expression was observed in the area directly underlying the epithelial cell layer (Figure 2L). The majority (86%) of malignant ovarian tumors showed intense GD3A11 epitope expression in the stroma and basement membrane zone surrounding the epithelial cancer cells (Figure 2M-O). Additionally, in most ovarian cancer samples intratumoral capillaries stained positive for the GD3A11 antibody.

Comparison of the GD3A11 epitope expression showed that ovarian carcinomas have a significantly stronger expression compared to normal ovaries (p < 0.001) and compared to benign ovarian tumors (p < 0.001) (Figure 2P). In addition, benign ovarian tumors have a significantly stronger GD3A11 epitope expression compared to normal adnexa (p = 0.002).

**GD3A11 epitope expression in ovarian cancer subtypes**

The expression of the GD3A11 epitope was evaluated for the different ovarian cancer subtypes (Table 1). Most ovarian cancer subtypes including low grade serous, low grade endometrioid, clear cell, high grade serous, and undifferentiated adenocarcinomas were associated with intense expression in >85% of the samples. Mucinous ovarian carcinomas showed intense GD3A11 epitope expression in 55% of the samples. The GD3A11 epitope expression profiles of low grade serous compared to high grade serous carcinomas, and low grade endometrioid compared to high grade endometrioid carcinomas were not significantly different (p = 1.00 and p = 0.364, respectively). Of the type II ovarian carcinomas, 69% of high grade endometrioid carcinomas showed an intense GD3A11 epitope expression compared to 90% high grade serous and 96% undifferentiated carcinomas (p = 0.024 and p = 0.026, respectively).

**Table 1.** GD3A11 epitope expression correlates with ovarian cancer subtypes. Chi squared test and Fisher’s exact test were indicated to calculate p-values.

<table>
<thead>
<tr>
<th>Type I carcinomas</th>
<th>GD3A11 epitope expression</th>
<th>Type II carcinomas</th>
<th>GD3A11 epitope expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mild</td>
<td>Intense</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>Serous low grade</td>
<td>1</td>
<td>8.3</td>
<td>11</td>
</tr>
<tr>
<td>Endometrioid low grade</td>
<td>1</td>
<td>11.1</td>
<td>8</td>
</tr>
<tr>
<td>Mucinous*</td>
<td>10</td>
<td>45.5</td>
<td>12</td>
</tr>
<tr>
<td>Clear cell</td>
<td>3</td>
<td>20</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serous high grade</td>
<td>15</td>
<td>9.6</td>
<td>141</td>
</tr>
<tr>
<td>Endometrioid high grade</td>
<td>5</td>
<td>31.2</td>
<td>11</td>
</tr>
<tr>
<td>Undifferentiated</td>
<td>1</td>
<td>4</td>
<td>24</td>
</tr>
</tbody>
</table>

* p < 0.001 for mucinous carcinomas compared to type II carcinomas.

| p < 0.05 for high grade endometrioid carcinomas compared to high grade serous carcinomas and undifferentiated carcinomas.

**Relation of the GD3A11 epitope expression with prognostic parameters**

Analysis of prognostic parameters and the GD3A11 epitope expression included only patients with type II ovarian carcinomas (N = 197, Table 2). Intense GD3A11 epitope expression was observed in early stage (FIGO stage I-II) as well as in advanced stage (FIGO stage III-IV) ovarian carcinomas; 81% and 90.3% respectively (p = 0.251). In patients with advanced stage ovarian cancer, no correlation
Figure 2. Immunohistochemical evaluation of the highly sulfated CS distribution in normal human tissues and ovarian tumors using the scFv antibody GD3A11 (bar represents 100 µm).
Prognostic significance of highly sulfated chondroitin sulfates in ovarian cancer defined by the single chain antibody GD3A11

Figure 3 shows the Kaplan-Meier curves for GD3A11 epitope expression with respect to OS and PFS in ovarian cancer type II patients. Patients with ovarian cancer and intense expression of the GD3A11 epitope demonstrated a significantly shorter overall survival compared to patients with mild expression (p = 0.013 for FIGO stage I-IV; p = 0.013 for FIGO stage III-IV ovarian cancer patients). No significant correlation for PFS and GD3A11 epitope expression was observed (p = 0.264 for FIGO stage I-IV; p = 0.641 for FIGO stage III-IV ovarian cancer patients).

Table 3 shows the analysis of prognostic parameters for OS using the Cox proportional hazard model. Univariate analysis showed a significant prognostic value for GD3A11 epitope expression in OS for FIGO stage I-IV ovarian cancer patients (p = 0.016). Also the FIGO stage and CA-125 serum level were identified as significant prognostic factors. Significant parameters were entered in a multivariate analysis and an independent prognostic significance for GD3A11 epitope expression and FIGO stage were demonstrated for OS, respectively p = 0.019 and p = 0.001.
Table 2. Correlation of the GD3A11 epitope expression with clinicopathological parameters of patients with type II ovarian carcinomas. Chi squared test and Fisher’s exact test were indicated to calculate p-values.

<table>
<thead>
<tr>
<th>GD3A11 epitope expression</th>
<th>Mild</th>
<th>Intense</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>%</td>
<td>N</td>
</tr>
<tr>
<td>Type II tumors (N = 197)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FIGO stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early (I-II)</td>
<td>4</td>
<td>19</td>
<td>17</td>
</tr>
<tr>
<td>Advanced (III-IV)</td>
<td>17</td>
<td>9.7</td>
<td>159</td>
</tr>
<tr>
<td>Ca-125</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 35 U/ml</td>
<td>2</td>
<td>16.7</td>
<td>10</td>
</tr>
<tr>
<td>&gt; 35 U/ml</td>
<td>19</td>
<td>10.6</td>
<td>161</td>
</tr>
<tr>
<td>Type II tumors, FIGO III-IV (N = 176)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary surgery</td>
<td>9</td>
<td>11.8</td>
<td>67</td>
</tr>
<tr>
<td>Intervention surgery</td>
<td>8</td>
<td>8</td>
<td>92</td>
</tr>
<tr>
<td>Outcome debulking</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Optimal (≤ 1 cm)</td>
<td>11</td>
<td>9.8</td>
<td>101</td>
</tr>
<tr>
<td>Incomplete (&gt; 1 cm)</td>
<td>5</td>
<td>8.1</td>
<td>57</td>
</tr>
<tr>
<td>Response primary treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complete/partial</td>
<td>16</td>
<td>11.4</td>
<td>124</td>
</tr>
<tr>
<td>Progression</td>
<td>0</td>
<td>0</td>
<td>24</td>
</tr>
<tr>
<td>Progression/recurrence*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 6 months</td>
<td>3</td>
<td>4.8</td>
<td>60</td>
</tr>
<tr>
<td>&gt; 6 months</td>
<td>12</td>
<td>12.9</td>
<td>81</td>
</tr>
</tbody>
</table>

* included only patients with a follow up of > 6 months (N = 156).
Table 3. Univariate and multivariate Cox regression analysis of clinicopathological parameters in all stage (FIGO I-IV) and advanced stage (FIGO III-IV) type II ovarian cancer patients with respect to OS.

<table>
<thead>
<tr>
<th>Type II tumors (N = 197)</th>
<th>Univariate</th>
<th>Multivariate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>p-value</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;70</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>≥70</td>
<td>1.41 (0.95-2.10)</td>
<td>0.091</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-serous</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Serous</td>
<td>1.51 (0.99-2.30)</td>
<td>0.055</td>
</tr>
<tr>
<td>FIGO stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-II</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>III-IV</td>
<td>6.36 (2.77-14.62)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>CA 125</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 35 kU/L</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>&gt;35 kU/L</td>
<td>3.77 (1.53-9.27)</td>
<td>0.004</td>
</tr>
<tr>
<td>GD3A11 expression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mild</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Intense</td>
<td>2.14 (1.15-3.98)</td>
<td>0.016</td>
</tr>
<tr>
<td>Type II tumors, FIGO III-IV (N = 176)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td>0.039</td>
</tr>
<tr>
<td>&lt;70</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>≥70</td>
<td>1.55 (1.02-2.358)</td>
<td>0.262</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-serous</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Serous</td>
<td>1.29 (0.83-2.01)</td>
<td>0.213</td>
</tr>
<tr>
<td>FIGO stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>1.33 (0.85-2.10)</td>
<td>0.070</td>
</tr>
<tr>
<td>CA 125</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 35 kU/L</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>&gt;35 kU/L</td>
<td>3.64 (0.90-14.71)</td>
<td>0.009</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary debulking</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Intervention debulking</td>
<td>1.61 (1.13-2.30)</td>
<td>0.004</td>
</tr>
<tr>
<td>Outcome debulking</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Optimal (≤ 1 cm)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Incomplete (&gt; 1 cm)</td>
<td>1.66 (1.17-2.36)</td>
<td>0.018</td>
</tr>
<tr>
<td>GD3A11 expression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mild</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Intense</td>
<td>2.39 (1.16-4.90)</td>
<td>0.018</td>
</tr>
</tbody>
</table>

Abbreviations: CI, confidence interval; HR, hazard ratio.
For advanced stage ovarian cancer patients (FIGO stage III-IV), univariate analysis showed a significant prognostic value for GD3A11 epitope expression in OS (p = 0.018). Also age, type of treatment, and outcome of debulking were identified as prognostic factors for OS. Multivariate analysis demonstrated an independent prognostic significance for the GD3A11 epitope expression for OS in patients with advanced stage ovarian cancer (p = 0.019). Age, type of treatment, and outcome of debulking were also identified as independent prognostic factors for OS, p = 0.049, p = 0.005, and p < 0.001 respectively.

**Figure 3.** Kaplan-Meier curves for GD3A11 epitope expression with respect to OS and PFS in patients with type II ovarian cancer. Log-rank p-values are indicated.

**A.** Overall survival curve for all stage ovarian cancer type II patients. **B.** Progression free survival curve for all stage ovarian cancer type II patients. **C.** Overall survival curve for stage III-IV ovarian cancer type II patients. **D.** Progression free survival curve for stage III-IV ovarian cancer type II patients.
**GD3A11 epitope expression before and after chemotherapy**

To analyze the effect of platin-based chemotherapy on the GD3A11 epitope expression in the ECM of ovarian carcinomas, samples of representative vital ovarian carcinomas before and after chemotherapy from the same patient were compared ($N = 17$). 94% of the cases demonstrated to have a similar GD3A11 epitope expression before and after chemotherapy; in one case both mild expression and in 15 cases both intense expression. In one case an intense GD3A11 epitope expression before chemotherapy (biopsy) was seen while the expression was scored as mild after chemotherapy since (<25% of the tumoral stroma showed an intense expression). McNemar’s test demonstrated equal GD3A11 staining in vital ovarian cancer tissues before and after chemotherapy ($p = 1.00$). Also, the GD3A11 epitope expression in ovarian carcinomas was not correlated to the use or non-use of chemotherapy before debulking surgery ($p = 0.393$) (Table 2). Evaluation of ovarian cancer samples including necrosis as effect of chemotherapy showed that the ECM of yet vital tumor adjacent to necrotic areas was still strongly positive for the GD3A11 antibody (Figure 2O).

**Mutation/methylation analysis of CHST15 gene**

Sequence analysis of the CHST15 gene including the promoter region and 8 exons did not reveal relevant mutations in high grade serous cancer samples compared to normal controls (data not shown). The methylation profile of the CpG islands in the CHST15 promoter region were evaluated using DNA bisulfite conversion. PCR amplification with methylation-dependent primers showed detectable PCR products for all unmethylated-specific primers, while no PCR products of the methylated-specific primers were detected in both high grade serous carcinomas and normal adnexa (data not shown). Also, fragment analysis showed that the mean percentage of methylated DNA was similar for both groups: 17.82% in the high grade serous carcinoma group compared to 17.27% in the control group control ($p = 0.609$).

**Discussion**

The identification of new biomarkers and treatment targets for ovarian cancer has typically focused on molecules expressed by malignant cells, while molecules mainly produced by stromal cell remain relatively unexplored. The glycosaminoglycans family stands out with their unique ability to regulate a diverse range of molecular activities through highly dynamic interactions in the tumoral ECM. In this study, we focused on ovarian cancer-associated matrix alterations of specific chondroitin sulfates by the use of the scFv antibody GD3A11 reactive with GlcA-GalNAc4S6S residues, and we demonstrated the prognostic biomarker potential of this class of matrix molecules.

Chondroitin sulfates are a major component of a healthy ECM and fine structural alterations of CS motifs are associated with cancer development and progression. The highly sulfated CS-E subtype, rich GlcA-GalNAc4S6S disaccharides, was found to be abundantly expressed in ovarian carcinomas while no or minimal expression was seen in most benign ovarian tumors, and expression was hardly present in the stroma of normal fallopian tubes and ovaries. In addition, expression of the GD3A11 epitope was very limited in normal human organs, making the specific CS motif an attractive target. From the same phage display antibody library the antibody was
selected previously and reactivity with the high molecular weight melanoma associated antigen (HMW-MAA), also known as chondroitin sulfate proteoglycan 4 (CSPG4) was observed. CSPG4 is an integral membrane proteoglycan which contains chondroitin sulfate side chains and is expressed by several carcinomas including melanoma and breast cancer.

The understanding of the origin and development of ovarian cancer has dramatically changed in recent years. Several ovarian cancer subtypes have been identified, with a specific molecular genetic pathway of carcinogenesis. In this respect, our data demonstrated that intense stromal upregulation of the GD3A11 epitope was seen in different ovarian cancer subtypes including serous, endometrioid, clear cell and undifferentiatedadenocarcinomas, and independent of the tumor grade. In the mucinous ovarian cancer subtype, intense stromal upregulation was less frequently observed. Apparently, highly sulfated CS seems to represent a molecule influencing the process of carcinogenesis in a more general context since it is involved in many distinct specific molecular pathways of the ovarian cancer development, e.g. low grade and high grade serous cancer. In addition, the GD3A11 epitope expression in vital ovarian cancer ECM is demonstrated to be independent from the use or non-use of chemotherapy suggesting that this class of molecules is a potential useful biomarker for a large group of ovarian cancer patients during a wide course of their disease.

Sulfotransferases are involved in the biosynthesis of CS and dictate the position and degree of sulfation. The gene encoding the enzyme CHST15 transfers a sulfate group to a GlcA-GalNAc4S (CS-A) thereby forming a highly sulfated GlcA-GalNAc4S6S residue (CS-E). Increased CHST15 transcription has been observed in various carcinomas including ovarian cancer, and correlated to more aggressive tumors and poor outcome. Genetic or epigenetic alterations in the CHST15 gene might be involved in this process, but our analysis showed no mutations in the CHST15 gene in high grade serous carcinomas nor an altered methylation status of the CpG island in the CHST15 promoter region. At the same time, the cell type(s) responsible for the increased CS-E production in ovarian carcinomas is not known. Based on the stromal location of the CS-E, it seems to be plausible that a substantial part of CS-E is produced by cancer-associated fibroblasts. The molecular pathway underlying the increase in GlcA-GalNAc4S6S expression, however, remains to be elucidated.

Newly identified prognostic biomarkers might be of value to aid clinical decision making in ovarian cancer management. We analyzed prognostic parameters for a large and homogeneous cohort of aggressive phenotype ovarian cancer patients. Because poorly differentiated ovarian carcinomas are difficult to classify into morphological categories and both high grade serous carcinomas and high grade endometrioid carcinomas display similar gene expression profiles, we included high grade serous, high grade endometrioid, and undifferentiated adenocarcinomas (also known as type II ovarian carcinomas). Our data demonstrated a significant correlation between intense GD3A11 epitope expression and a poor overall survival for patients suffering from ovarian type II cancer. Furthermore, intense expression of the GD3A11 epitope was identified as an independent prognostic factor for overall survival. Recently, gene expression profiling has proposed several subtypes of type II ovarian carcinomas with specific molecular characteristics and distinct biology. A poor prognosis subtype (called C1 or mesenchymal) was defined by a reactive stroma gene expression signature correlating with extensive desmoplasia. An enhanced stroma response has a significant effect on tumor behavior and clinical outcome. The class of chondroitin
sulfates seems to be involved in this cancer-associated stroma activation and its associated poor outcome.

In contrast to the correlation of intense stromal GD3A11 epitope expression with poor overall survival, intense GD3A11 epitope expression was not correlated with a shorter progression free survival. Well known prognostic factors including age and outcome of debulking for advanced stage ovarian cancer patients showed their significant prognostic value in our cohort indicating that this patient cohort is indeed a representative reflection.\textsuperscript{32} For patients with no or mild tumoral GD3A11 epitope expression, early recurrence of disease was not associated with short survival. The tumor biology of these mild stromal expression type II ovarian carcinomas might differ from ovarian carcinomas with intense stromal expression resulting in a variable progression of disease from the moment of first recurrence.

Compared to previous data,\textsuperscript{14, 15} our study confirmed the prognostic biomarker value of specific upregulated CS motifs in the ECM of ovarian carcinomas. However, in contrast to these studies we found no correlation with tumor grade, FIGO stage, and progression free survival. One of the reasons for this apparent discrepancy might be the relatively small cohort of patients used in both previous studies. In addition to the sample size, the composition of the patient cohorts in both previous studies differed significantly from our study. In previous studies all ovarian cancer subtypes were included representing a more heterogeneous patient group also including mucinous carcinomas associated with mild stromal expression while prognostic analyses in our study included only type II ovarian carcinomas. The scFv antibodies GD3G7, GD3A10, and GD3A11 used in the different studies all belong to V\textsubscript{\mu}3 family and have a DP38 germline gene segment however they each have their unique heavy chain CDR3 amino acid sequence. All these scFv antibodies demonstrated to react with a CS-like epitope overexpressed in ovarian cancer however the precise epitopes composition (i.e. the exact saccharide motif) have not been established. The presence of (subtle) differences in epitope structure is unclear.

The class of glycosaminoglycans comprises a group of matrix molecules very eligible for the use as a diagnostic tumor marker since these glycosaminoglycans are usually secreted in bodily fluids including urine and blood. The majority (81\%) of the early stage (FIGO stage I-II) aggressive type II ovarian carcinomas demonstrated to have intense expression of the GD3A11 epitope indicating that matrix alterations with respect to GlcA-GalNAc4S6S motifs occur in an early stage of ovarian cancer progression. Previously, Pothacharoen et al. have demonstrated the potential diagnostic biomarker value of serum CS epitopes for early stage ovarian cancer including all different histological subtypes.\textsuperscript{33} Also a specific serum N-glycan profile was observed in patients with both early and late stage serous ovarian cancer.\textsuperscript{34, 35} Elevated and/or altered glycosaminoglycans including specific CS epitopes may provide a promising diagnostic target for novel screening strategies of disease in patients with ovarian cancer.

As a result of the development of platinum-resistance over time, most patients with ovarian cancer eventually succumb to the disease. Thus, the identification of novel treatment targets is important to improve clinical outcome. Tumor-associated ECM may provide numerous targets for therapy because of being an active player in cancer progression. A multifaceted regulatory role of CS and CS-conjugated proteins (proteoglycans) is described in e.g. tumor associated proliferation, invasion, and metastasis pointing out that this class of molecules may represent an attractive
target for cancer therapy.\textsuperscript{8, 10, 36} Preclinical studies focused on interfering with the biosynthesis or degradation of CS as well as targeting the binding properties of CS show that CS-targeted treatments have anti-tumor effects.\textsuperscript{26, 37, 38} Another promising approach is using upregulated stromal CS motifs as a micro-environmental catch for targeted anti-cancer therapy.\textsuperscript{39} Delivering antitumor drugs to the tumoral stroma has already been shown to be successful in destroying tumor cells and their micro-environment in \textit{in vivo} studies.\textsuperscript{40, 41}

\textbf{Concluding remarks}

Extracellular matrix chondroitin sulfates are unique and multifaceted molecules functioning as key regulators in multiple cancer-associated processes thereby being an attractive class of molecules for the development of novel ovarian cancer management approaches. In this study we demonstrated the biomarker potential of specific highly sulfated CS-E rich in GlcA-GalNAc4S6S units in several ovarian cancer subtypes. The prognostic significance of upregulated stromal highly sulfated CS in type II ovarian carcinomas may be a useful tool in clinical decision making. In addition, these molecules may serve as a potential diagnostic or therapeutic target. In the future, ovarian cancer patients with stromal upregulation of highly sulfated CS might benefit from promising glycosaminoglycan-targeted therapies. In conclusion, our findings suggest an important biomarker role for highly sulfated chondroitin sulfate in the aggressive ovarian cancer phenotype.
References

Chapter 3

Changes in the extracellular matrix are associated with the development of serous intraepithelial carcinoma (STIC) into high grade serous carcinoma

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Johan Bulten
Koen. K. Van de Vijver
Toin H. van Kuppevelt
Leon F.A.G. Massuger
Abstract

Objective. The identification of a marker for early progression of pre-invasive lesions into invasive pelvic high grade serous carcinoma (HGSC) may provide novel handles for innovative screening and prevention strategies. The interplay between cancer cells and the extracellular matrix (ECM) is one of the main principles in cancer development and growth, but has been largely neglected in pre-invasive lesions. This is the first study addressing the involvement of the extracellular matrix (ECM) in the ‘step-by-step’ transition of normal fallopian tube epithelium into pre-invasive lesions, and eventually the progression of pre-invasive lesions into invasive HGSC.

Methods. The expression of highly sulfated chondroitin sulfate (CS-E), a characteristic glycosaminoglycan of the cancer-associated ECM, was assessed by immunohistochemistry in a large cohort of precursor lesions of the full spectrum of HGSC development including 97 serous tubal intraepithelial carcinomas (STICs), 27 serous tubal intraepithelial lesions (STILs), and 24 p53 signatures. Additionally, the immunological reactivity in the microenvironment was evaluated.

Results. Increased stromal expression of highly sulfated CS-E was observed in 3.7%, 57.7%, and 90.6% of STILs, STICs, and invasive HGSCs respectively (p < 0.001). No or limited expression was found in p53 signatures and normal tubal epithelium (compared to STIC; p < 0.001). A gradual increase in the amount of CS-E expression between STIC and paired HGSC was demonstrated. Intense stromal CS-E expression in STICs was significantly associated with an immune infiltrate (p < 0.001).

Conclusion. Our study showed that increased stromal CS-E expression is related to the degree of the tubal epithelium abnormality. Specific alterations in the ECM (i.e. CS-E expression) occur early in pelvic HGSC development and may represent a novel biomarker of early cancer progression, useful for the identification of novel clinical strategies.
Introduction

Epithelial ovarian cancer is the fifth leading cause of female death worldwide of which high grade serous carcinoma (HGSC) represents the majority of all subtypes. HGSCs are genetically highly unstable and associated with clinically aggressive behavior. TP53 mutations occur in more than 95% of these carcinomas and numerous DNA amplifications and deletions have been described. Recent histological and molecular genetic studies have provided accumulating evidence that the fallopian tube epithelium may be the site of origin of most pelvic HGSCs. Putative precursor lesions in the distal fallopian tube include serous tubal intraepithelial carcinoma (STIC), a focal non-invasive cytological appearance of HGSC, and ‘p53 signature’, normal appearing secretory cells that overexpress p53. The detection of identical TP53 mutations in STICs and their paired HGSCs indicate a clonal relationship and support the tubal origin of HGSC. In addition to STIC and p53 signature, another group of lesions displaying atypia that falls short of a STIC has been identified. These lesions, usually termed as serous tubal intraepithelial lesion (STIL), may be an intermediate between STIC and p53 signature.

Molecular characterization of precursor lesions is elementary in elucidating the, still poorly understood, pathogenesis of HGSC and might carry relevant implications for diagnosis and prevention. While most research focuses on (genetic) cellular changes, the initial steps of cancer development, i.e. abnormal cellular proliferation and survival, depend substantially on the largely neglected tumor microenvironment: drastic remodeling of the extracellular matrix (ECM) occurs thereby affecting cancer-associated cell processes including adhesion, invasion, and migration. The ECM represents a highly organized three-dimensional network of fibrous proteins and proteoglycans interacting with each other and with surrounding cells. To a large extent, proteoglycans function through their glycosaminoglycan side chains, linear negatively charged polysaccharides which can be sulfated in variable positions and quantities.

The cancer-associated ECM is characterized by a predominant presence of chondroitin sulfate (CS), a class of glycosaminoglycans which outshines other glycosaminoglycan subtypes. The 4,6-sulfated chondroitin sulfate subtype (CS-E) is overexpressed in more than 90% of HGSCs, in both early and advanced FIGO stages, while rarely expressed in normal organs. Compared to the less sulfated CS subtypes present in healthy tissues (i.e. CS-A and CS-C), the highly sulfated motif of CS-E chains enables specific interactions with various molecules such as growth factors (e.g. vascular endothelial growth factor, VEGF) and adhesion molecules. By facilitating various cancer-associated cell processes including proliferation, adhesion, and angiogenesis, CS-E motifs expressed in the ECM may contribute to ovarian cancer development and progression.

In this study, we aim to broaden the understanding of HGSC development by studying specific changes, the highly sulfated CS-E expression, in the ECM of HGSC precursor lesions. Additionally, the immunological reactivity in the microenvironment was evaluated. Our study cohort included the largest series of precursor lesions of HGSC (i.e. p53 signatures, STILs, and STICs) until now. This is the first study addressing the involvement of ECM molecules in the early development of pelvic HGSC.
Materials and methods

Case selection
Study approval was given by the Regional Committee for Medical Research Ethics and performed according to the Code for Proper Secondary Use of Human Tissue (Dutch Federation of Biomedical Scientific Societies, www.federa.org). Precursor lesions and associated HGSCs were collected from the Radboud university medical center, the Netherlands Cancer Institute, the Elisabeth-TweeSteden hospital, and the Canisius-Wilhelmina hospital in the Netherlands using the nationwide network and registry of histopathology and cytopathology (PALGA) as the primary source (range 1997-2015). Pathology reports were screened and relevant cases (N = 114) were used for histological analysis. Fallopian tubes from patients with a BRCA mutation (N = 10) and from patients with a benign gynecologic abnormality (N = 10) were included as a control group.

Categorizing HGSC precursor lesions
All sections were independently assessed by two experienced gynaecological pathologists (JB and KVdV) for morphology, p53, Ki-67, and CS-E immunoreactivity and subsequently categorized according to the algorithm for diagnosis of serous tubal intraepithelial carcinoma. In case of no consensus, sections were reevaluated and discussed to reach consensus. In 70 of the 114 cases one or more HGSC precursor lesions were identified, 148 precursor lesions in total. Cohen’s kappa coefficient for interobserver reliability was 0.71. In 49 of the 70 cases (70%), fallopian tubes had been sectioned and embedded in total (e.g. SEE-FIM protocol: Sectioning and Extensively Examining the Fimbriated end).

Histological parameters of precursor lesions
Several histological aspects of the precursor lesions were evaluated using H&E staining. The location of the precursor lesion was determined as located in either the fimbriated end or the ampulla/infundibulum. The position of the precursor lesion to the corresponding HGSC was classified as isolated from the tumor when no HGSC was present, or as adjacent to the tumor when HGSC was present in the section of the localized precursor lesion. Further, the presence of an immune infiltrate in H&E stained precursor lesions was evaluated using a semi-quantitative analysis. Twenty normal fallopian tubes were used as a reference and precursor lesions were scored to have either a low (mononuclear cell density comparable to controls) or high (increased mononuclear cell density) immune cell infiltration.

Immunohistochemistry
Paraffin-embedded sections (4 µm) were deparaffinized in xylene, hydrated, and stained with hematoxylin and eosin (H&E) or processed for immunohistochemical analysis. HGSCs with known strong primary antibody staining intensity were used as positive controls and primary antibodies were omitted for negative controls.

Chondroitin sulfate E
As previously described, the avidin-biotin complex method was used for immunohistochemical
Changes in the extracellular matrix are associated with the development of STIC into high grade serous carcinoma. In brief, sections were incubated with the single chain antibody GD3G7 (VSV-tagged, 1:5) specific for CS-E, the monoclonal mouse IgG anti-VSV antibody, and the biotinylated horse-anti-mouse IgG antibody (Vector Laboratories Inc., CA, USA, 1:200). After incubation with ABC reagent (Vectastain ABC anti-mouse-IgG kit, Vector Laboratories Inc.), bound antibodies were visualised using 3-amino-9-ethyl-carbazole (AEC).

**P53 and Ki-67**

Heat induced epitope retrieval was performed using 10 mM citrate buffer (pH 6.0, ScyTek Laboratories) for 15 min and endogenous peroxidase activity was blocked with 3% hydrogen peroxide in phosphate buffered saline (PBS). Subsequently, the sections were incubated overnight at 4°C with mouse anti-human p53 tumor suppressor protein (clone DO7, ImmunoLogic, 1:400) or monoclonal mouse anti-human Ki-67 antigen (clone MIB-1, Dako, 1:100) diluted in normal antibody diluent (ImmunoLogic). Thereafter, sections were incubated for 60 min with poly-HRP-anti-mouse/rabbit/rat-IgG (ImmunoLogic). Bound antibodies were visualized with 3,3-diaminobenzidine (ImmunoLogic). Sections were counterstained with hematoxylin, dehydrated, and mounted with Quick-D mounting medium (Klinipath).

**Scoring CS-E, p53, and Ki-67 expression**

Immunoreactivity of CS-E, p53, and Ki-67 was scored for the proportion (range 0-100%) and intensity (either negative, weak, moderate, or intense) of positive cells or positive ECM underlying the epithelial layer. The percentage of CS-E expression in the stroma was classified as: 0 = negative; 1 = 0-5%; 2 = 6-25%; 3 = 26-50%; 4 = 51-75% and 5 = 76-100%, and the most intense area was scored as: 1 = weak, 2 = moderate, and 3 = intense. Based on the sum of the percentage and intensity (range 0-8), two groups were formed: 1) no or mild CS-E expression (0-4) and 2) intense CS-E expression (5-8) [15]. The expression pattern of p53 was classified as wild type or mutated. A mutation pattern includes either a diffuse moderate to strong nuclear staining of >75% of epithelial cells or a completely negative nuclear staining. The Ki-67 proliferation index was determined as low or high depending on whether nuclear expression was observed as <10% or ≥10%, respectively.

**Statistics**

To evaluate the association between CS-E expression and the histological tissue subtype, and between having any CS-E positive STIC and the presence of associated HGSC, chi-square tests were used. (Fisher’s exact test when expected cell count was less than five.) To assess the mean difference in CS-E expression as continuous variable (range 0-8) between STIC and its paired HGSC within patients, paired T-tests were performed. In case of more than one STIC per patient, the highest value of CS-E expression in STIC was included in the test. To assess the association between CS-E expression and histopathological characteristics of precursor lesions, logistic regression analyses (using a Generalized Estimating Equations model) with correction for within subject correlation were performed. All tests were two-sided and p-values < 0.05 were considered significant. Statistical analyses were performed using the software package SPSS 22.0 for Microsoft Windows (SPSS Inc., Chicago, IL).
Results

Clinicopathological characteristics precursor lesions
A total of 148 lesions from 70 patients were defined as HGSC precursor lesion according to the STIC algorithm and classified as either p53 signature ($N=24$), STIL ($N=27$), or STIC ($N=97$). Most patients ($N=54, 77.1\%$) were diagnosed with having pelvic HGSC while 16 (22.9\%) patients only had a precursor lesion. In 42 patients (60.9\%) more than one precursor lesion was identified. Table 1 shows baseline patient characteristics.

Table 1. Baseline clinical characteristics of the patient cohort ($N=70$).

<table>
<thead>
<tr>
<th></th>
<th>$N$</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age (range)</td>
<td>63</td>
<td>(33-82)</td>
</tr>
<tr>
<td>Germline BRCA mutation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No BRCA mutation</td>
<td>11</td>
<td>15.7</td>
</tr>
<tr>
<td>BRCA1 mutation</td>
<td>9</td>
<td>12.9</td>
</tr>
<tr>
<td>BRCA2 mutation</td>
<td>8</td>
<td>11.4</td>
</tr>
<tr>
<td>Unknown</td>
<td>42</td>
<td>60</td>
</tr>
<tr>
<td>Pelvic HGSC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No HGSC</td>
<td>16</td>
<td>22.9</td>
</tr>
<tr>
<td>FIGO stage I</td>
<td>6</td>
<td>8.6</td>
</tr>
<tr>
<td>FIGO stage II</td>
<td>8</td>
<td>11.4</td>
</tr>
<tr>
<td>FIGO stage III</td>
<td>32</td>
<td>45.7</td>
</tr>
<tr>
<td>FIGO stage IV</td>
<td>7</td>
<td>10</td>
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<tr>
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<td>1</td>
<td>1.4</td>
</tr>
<tr>
<td>Surgical intervention</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Risk reducing salpingo-oophorectomy</td>
<td>10</td>
<td>14.3</td>
</tr>
<tr>
<td>Complete staging</td>
<td>11</td>
<td>15.7</td>
</tr>
<tr>
<td>Primary debulking</td>
<td>18</td>
<td>25.7</td>
</tr>
<tr>
<td>Interval debulking</td>
<td>25</td>
<td>35.7</td>
</tr>
<tr>
<td>Other*</td>
<td>6</td>
<td>8.6</td>
</tr>
</tbody>
</table>

* Other surgical interventions included extirpation of the adnexa because of a benign ovarian cyst ($N=4$) and extirpation of the uterus and adnexa because of either fibroids ($N=1$) or endometria hyperplasia ($N=1$).

Histological and immunohistochemical characteristics of the precursor lesions are summarized in Table 2. All types of precursor lesions were predominantly located at the fimbriated end. Concerning the localization of precursor lesions in relation to the associated HGSC, 86.7\% of p53 signatures, 45\% of STILs, and 46.1\% of STICs were found to be isolated from the HGSC. All associated HGSCs showed similar p53 immunoreactivity as observed in the precursor lesion except for two cases (3.8\%) in which both the STIC and HGSC showed a completely negative p53 immunoreactivity, while the additional p53 signature(s) showed p53 overexpression.

An evident immune infiltrate in the microenvironment was observed in 1 (3.7\%) STIL and 51 (52.7\%) STICs, while no infiltrate was observed in normal fallopian tube samples ($N=20$) and p53 signatures ($N=24$) ($p<0.001$). Immune cells were morphologically characterized as predominantly stromal infiltrating lymphocytes located in the ECM rather than intraepithelial lymphocytes.
Changes in the extracellular matrix are associated with the development STIC into high grade serous carcinoma

Table 2. Overview of histological and immunohistochemical characteristics of the defined precursor lesions including p53 signature, STIL and STIC (N = 148).

<table>
<thead>
<tr>
<th></th>
<th>PS signature N = 24</th>
<th>STIL N = 27</th>
<th>STIC N = 97</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>%</td>
<td>N</td>
</tr>
<tr>
<td><strong>CS-E expression</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No or mild</td>
<td>24</td>
<td>100</td>
<td>26</td>
</tr>
<tr>
<td>Intense</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><strong>Pelvic HGSC present</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>9</td>
<td>37.5</td>
<td>7</td>
</tr>
<tr>
<td>Yes</td>
<td>15</td>
<td>62.5</td>
<td>20</td>
</tr>
<tr>
<td><strong>Location</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ampulla/infundibulum</td>
<td>5</td>
<td>20.8</td>
<td>7</td>
</tr>
<tr>
<td>Fimbriated end</td>
<td>19</td>
<td>79.2</td>
<td>20</td>
</tr>
<tr>
<td><strong>PS3</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overexpression</td>
<td>24</td>
<td>100</td>
<td>26</td>
</tr>
<tr>
<td>No expression</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Wild type</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><strong>Ki-67 proliferation index</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;10%</td>
<td>24</td>
<td>100</td>
<td>21</td>
</tr>
<tr>
<td>10-50%</td>
<td>0</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>&gt;50%</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Immune infiltrate</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No infiltrate</td>
<td>24</td>
<td>100</td>
<td>26</td>
</tr>
<tr>
<td>Evident infiltrate</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><strong>Position to HGSC</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isolated from HGSC</td>
<td>13</td>
<td>86.7</td>
<td>9</td>
</tr>
<tr>
<td>Adjacent to HGSC</td>
<td>2</td>
<td>13.3</td>
<td>11</td>
</tr>
</tbody>
</table>

*Includes only precursor lesions (N = 124) from patients with having pelvic HGSC.

CS-E expression in precursor lesions and associated HGSC

The expression of CS-E was evaluated in precursor lesions, associated HGSCs, and controls. Expression of CS-E was only observed in the ECM underlying the epithelial layer and no expression was observed in epithelial cells. Representative examples of CS-E expression in normal fallopian tube epithelium, p53 signature, STIL, STIC, and HGSC are presented in Figure 1A, including H&E, p53, and Ki-67 stainings. Intense CS-E expression in the ECM was observed in 56 (57.7%) STICs and in one STIL (3.7%) while all p53 signatures (N = 24) showed no or mild CS-E expression (p < 0.001 for CS-E expression in STIC compared to either STIL or p53 signature) (Figure 1B). Most associated pelvic HGSCs (90.6%) showed intense stromal CS-E expression (p < 0.001 for CS-E expression in STIC compared to HGSC). All normal fallopian tube epithelium samples (N = 20) showed no or mild expression (p < 0.001 for CS-E expression in STIC compared to normal epithelium). Accordingly, the percentage of CS-E expressing lesions was gradually increased with the degree of the serous tubal epithelium abnormality: between either normal epithelium, p53 signature or STIL and STIC, and between STIC and invasive HGSC (p < 0.001).

In addition, the mean difference in CS-E expression as continuous variable (range 0-8) between STIC and paired HGSC within patients was analyzed. In case of multiple STICs, the highest value of CS-E expression in STIC was used in paired T-testing. One paired HGSC was not assessed for CS-E immunoreactivity due to its absence in additional stainings and thus not included in this analysis.
**Figure 1.** Immunohistochemical CS-E staining in normal fallopian tube epithelium, precursor lesions, and HGSC.

<table>
<thead>
<tr>
<th></th>
<th>H&amp;E staining</th>
<th>p53 staining</th>
<th>Ki-67 staining</th>
<th>CS-E staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td><img src="image1" alt="Normal" /></td>
<td><img src="image2" alt="Normal" /></td>
<td><img src="image3" alt="Normal" /></td>
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<td><img src="image7" alt="p53 signature" /></td>
<td><img src="image8" alt="p53 signature" /></td>
</tr>
<tr>
<td>STIL</td>
<td><img src="image9" alt="STIL" /></td>
<td><img src="image10" alt="STIL" /></td>
<td><img src="image11" alt="STIL" /></td>
<td><img src="image12" alt="STIL" /></td>
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<tr>
<td>STIC</td>
<td><img src="image13" alt="STIC" /></td>
<td><img src="image14" alt="STIC" /></td>
<td><img src="image15" alt="STIC" /></td>
<td><img src="image16" alt="STIC" /></td>
</tr>
<tr>
<td>HGSC</td>
<td><img src="image17" alt="HGSC" /></td>
<td><img src="image18" alt="HGSC" /></td>
<td><img src="image19" alt="HGSC" /></td>
<td><img src="image20" alt="HGSC" /></td>
</tr>
</tbody>
</table>
Changes in the extracellular matrix are associated with the development STIC into high grade serous carcinoma.

The expression of CS-E as continuous variable was significantly lower in STIC compared to the expression in paired invasive HGSC (mean difference 2.0, p < 0.001) (Figure 1C).

**CS-E expression in STIC associated with clinicopathological factors**

Associations between CS-E expression in STIC and several histopathological parameters were evaluated by univariate analysis. CS-E expression was neither associated with the position of STIC towards the corresponding HGSC, either isolated or adjacent, nor with its localization, either fimbriated end or ampulla/infundibulum (p = 0.647 and p = 0.078, respectively). Intense CS-E expression was significantly associated with a complete negative p53 immunoreactivity while no significant association with the Ki-67 proliferation index (either 10-50% or >50%) was observed (p = 0.005 and p = 0.237, respectively) (Table 3). A significant association between intense CS-expression and the presence of an immune infiltrate was observed, p < 0.001 (Figure 2). Both a negative p53 immunoreactivity and immune infiltrate were by multivariate analysis identified as independent factors associated with intense stromal CS-E expression in STIC (p = 0.003 and p < 0.001, respectively).

Finally, the association between STIC with intense CS-E expression and having invasive HGSC was evaluated. Of the patients having HGSC and STIC (N = 52), 34 (65.4%) had at least one STIC with intense CS-E expression while 18 (34.6%) had no STIC with intense CS-E expression. When compared to patients without having HGSC (N = 7), five (71.4%) patients had one or more STIC with intense CS-E expression while two (28.6%) patients had no STIC with intense CS-E expression (p = 1.00). In 23/34 (67.6%) patients having HGSC and having one or more CS-E positive STIC, and in all patients (5/5) not having HGSC and having one or more CS-E positive STIC, also an immune
**Figure 2.** Examples of STICs with intense CS-E expression in the underlying extracellular matrix and the association with an invading immune infiltrate. Bar represents 100 µm.

A-B. STIC with intense CS-E expression and no evident immune infiltrate present.  
C-D. STIC with intense CS-E expression and the presence of an invading immune infiltrate of predominantly lymphocytes, mainly localized in the extracellular matrix.
Changes in the extracellular matrix are associated with the development of STIC into high-grade serous carcinoma.

Table 3. Association of CS-E expression in the extracellular matrix of STIC \((N = 97)\) to (immuno)histological parameters of the STIC lesion. Univariate and multivariate logistic regression analysis with correction for within subject variables was used to assess correlations.

<table>
<thead>
<tr>
<th>CS-E expression</th>
<th>No or mild</th>
<th>Intense</th>
<th>Univariate</th>
<th>Multivariate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(N)</td>
<td>(n)</td>
<td>(%)</td>
<td>OR (95% CI)</td>
</tr>
<tr>
<td>P53</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overexpression</td>
<td>67</td>
<td>35</td>
<td>52.2</td>
<td>32</td>
</tr>
<tr>
<td>No expression</td>
<td>30</td>
<td>6</td>
<td>20</td>
<td>24</td>
</tr>
<tr>
<td>Ki-67 proliferation</td>
<td>77</td>
<td>35</td>
<td>45.5</td>
<td>42</td>
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<td>10-50%</td>
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<td>30</td>
<td>14</td>
</tr>
<tr>
<td>&gt;50%</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
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<tr>
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<td>31</td>
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<tr>
<td>Evident infiltrate</td>
<td>51</td>
<td>10</td>
<td>19.6</td>
<td>41</td>
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Abbreviations: CI, confidence interval; OR, odds ratio.

Discussion

There is increasing evidence that most pelvic HGSCs may arise from the serous fallopian tube epithelium and STIC is defined as the non-invasive precursor lesion. Additionally, several lesions including STIL and p53 signatures have been proposed as precursors of STIC. The identification of precursor lesions offers new prospects in understanding the (molecular) pathway of HGSC development that subsequently will have impact on the design of novel strategies for early detection and prevention of HGSC. In this respect, we have studied the largest cohort of HGSC precursor lesions, from p53 signature to HGSC, until now.

While the interplay between cancer cells and the ECM has been recognized as one of the main principles in cancer development and progression, the involvement of ECM molecules in pre-invasive lesions has been largely neglected.\(^{10, 12, 13}\) To our knowledge, we are the first group addressing the involvement of the ECM in the early steps of HGSC development. Our data show that in addition to intrinsic cellular changes (e.g. \(TP53\) mutation), specific alterations in the ECM (i.e. glycosaminoglycan expression) occur in the pre-invasive stage.

The altered functional expression of glycosaminoglycans has been marked as a major characteristic of the remodeled cancer-associated ECM. Upregulation of highly sulfated CS-E is strongly associated with invasive HGSC and participates in tumor growth and metastasis by influencing several cell processes.\(^{14-21}\) In this study, we observed a gradually increased percentage of CS-E expressing lesions with the degree of tubal epithelium abnormality in the spectrum of
HGSC development; between normal epithelium, p53 signature, STIL and STIC, and between STIC and HGSC. Furthermore, a gradual increase in the amount of expressed CS-E between STIC and its paired HGSC was demonstrated, mainly due to a more restricted percentage of stromal CS-E in STIC rather than restricted intensity. The altered CS expression in STIC marks the involvement of these matrix molecules in the early steps of HGSC development from a certain degree of (genetically) mutated epithelial cells (i.e. STICs) that strengthens in further malignant progression (i.e. invasive HGSC) (Figure 3).

Although a STIC is not invading the basal membrane and underlying stroma, our results indicate that these mutated epithelial cells are able to influence the neighboring ECM (i.e. by inducing CS-E expression). Enzymatic degradation of the ECM including loss of the basement membrane has been observed to occur already in pre-invasive lesions of cervical and breast cancer. A modified ECM may be created facilitating progression of the non-invasive STIC into an invasive tumor. The upregulation of highly sulfated CS-E in the ECM provides a range of different binding sites for growth factors as CS-E interacts with various 'heparin binding' growth factors including VEGF, fibroblast growth factor-2 (FGF-2), FGF-10, FGF-16, FGF-18, midkine (MK), pleiotrophin (PTN), and heparin-binding epidermal growth factor-like growth factor (HB-EGF). These growth factors are all well known mediators of various biological processes during tumor growth and metastasis and their storage and release by CS-E chains localized in the ECM may promote their signaling pathways. In this context we suggest that increased stromal CS-E expression in STICs is an early marker of progression into an invasive carcinoma since CS-E is an ubiquitous component of the cancer-associated ECM while absent in the stroma underlying normal fallopian tube epithelium as well as absent in a wide range of other normal organs. Consequently, we hypothesize that STICs with intense CS-E expression are more likely to progress into invasive HGSC than STICs without CS-E expression (Figure 3).

In the search for molecular biomarkers and targets of HGSC precursor lesions, highly sulfated chondroitin sulfates in the ECM represent a novel class, useful for the development of screening and prevention purposes. Today clinicians are puzzled about the most appropriate management of patients diagnosed with a HGSC precursor lesion, especially STIC, in the absence of HGSC (termed as isolated STIC); proposed strategies vary from surgical staging to adjuvant chemotherapy to an expectative approach. As most STICs affect the fimbriated end of the fallopian tube, shed epithelial cells have direct access to the peritoneal cavity and it is thought that dissemination can occur without stromal invasion at the site of origin. The development of pelvic HGSC after diagnosis of an isolated STIC was reported to be 4.5% for high risk patients (BRCA mutation carriers) without adjuvant chemotherapeutic treatment. Our cohort included one case with an isolated STIC who had developed pelvic HGSC several years later; this STIC was observed with intense CS-E expression. In this respect, highly sulfated CS motifs hold biomarker potential for the development of novel strategies to aid clinical decision making in patients with an isolated STIC. Also, the class of glycosaminoglycans represents a promising diagnostic target for screening purposes as these molecules are secreted in bodily fluids.

In the spectrum of HGSC precursor lesions, an immune infiltrate was observed in the majority of STICs. Infiltrating lymphocytes were mainly located in the ECM and strongly associated with increased CS-E expression. In the tumor microenvironment, the immunological anti-cancer
Changes in the extracellular matrix are associated with the development of STIC into high grade serous carcinoma. The presence of tumor infiltrating cytotoxic T-cells is associated with prolonged survival and in contrast, lower ratios of cytotoxic T-cells/ regulatory T-cells are associated with poor survival as regulatory T-cells suppress the anti-cancer immune response. Knowledge about the immunological response in the early steps of HGSC development is limited. Only one study evaluated immune infiltrates in a small cohort of STICs and observed lymphocytes and macrophages to be present. We hypothesize that the presence of CS-E molecules in the remodeled ECM of precursor lesions, necessary to enable cell invasion, may be recognized by lymphocytes as pathogenic and activate the immune response. On the other hand, upregulated CS-E motifs may play a more active role in the initiation of the cancer inflammatory response as highly sulfated and oversulfated CS are known modulators of immunological responses through specifically interacting with chemokines, e.g. stromal derived factor-1 (SDF-1). In addition, these glycosaminoglycan chains are able to interfere with T-cell processes including chemotaxis and Th-cell differentiation. Further research should elucidate whether the created microenvironment in STICs is immune-activating or immune-suppressing regarding the anti-cancer response, and determine the exact role of CS-E in this context.

**Figure 3.** Location of chondroitin sulfate E expression in the extracellular matrix during the development of normal fallopian tube epithelium into pelvic HGSC.
This study addresses the extracellular matrix in pre-invasive lesions of the pelvic high grade serous carcinoma. Our findings show that increased expression of highly sulfated chondroitin sulfate (CS-E), a characteristic glycosaminoglycan in the cancer-associated matrix, was related to the degree of the tubal epithelium abnormality. Increased CS-E expression was observed in the majority of serous tubal intraepithelium carcinomas (STICs) and was associated with an immune infiltrate. Specific alterations in the extracellular matrix (i.e. CS-E expression) occur at an early stage of pelvic high grade serous cancer development and may represent a novel class of biomarkers for early cancer progression, useful for the identification of novel screening and prevention strategies.
References

27. Nasr M, Ayyad SB, El-Lamie IK, Mikhail MY. Expression of matrix metalloproteinase-2 in preinvasive and invasive carcinoma


Chapter 4

Infiltrating T cells within the serous tubal intra-epithelial carcinoma (STIC) microenvironment

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In preparation.
Abstract

The host immunological anti-cancer response comprises a complex and dynamic interplay between various immune factors, and may either negatively or positively affect high grade serous ovarian cancer progression. Until now, it is unknown whether the immune response is involved in the pre-invasive stage during carcinogenesis. In this study, we evaluated T cells in serous tubal intraepithelial carcinoma (STIC), the precursor lesion of pelvic high grade serous carcinoma (HGSC) and evaluated associations with several clinicopathological parameters. We applied a novel and powerful technique of multiplex immunofluorescent staining followed by quantitative and automated whole-slide analysis by an optimized pathology imaging system on a large cohort of STICs, associated carcinomas and normal fallopian tubes as control (N = 59). Compared to normal fallopian tubes, STICs were observed to have a significantly increased density of CD3+ T cells (p < 0.001), including increased densities of helper, cytotoxic, and regulatory T-cell subsets (p < 0.001, p = 0.01, and p < 0.001, respectively). Within subjects, an additional increase of infiltrating cytotoxic and regulatory T cells was observed in invasive HGSC compared to the paired STIC (p < 0.001 and p = 0.01, respectively). The increase of CD3+ T cells in STIC and HGSC was more significant in the stromal compartment rather than intraepithelial. Recently, a specific change in the stromal compartment of STIC (i.e. the upregulation of stromal chondroitin sulfate E) has been linked to malignant progression, and increased numbers of infiltrating helper, cytotoxic, and regulatory T cells were significantly associated with the presence of this stromal change in STIC (p = 0.039, p < 0.001, and p = 0.001, respectively). Finally, CD3+ T-cell densities in STIC were not associated with the presence or absence of paired invasive HGSC. We conclude that the host immune response occurs in a pre-invasive stage of pelvic HGSC development as is associated with stromal changes. These findings may serve as a starting point in our understanding of cancer immunity in STIC and the potential effect on malignant progression.
Introduction

High grade serous carcinoma (HGSC) is the most common subtype of epithelial ovarian cancer, characterized by clinically aggressive behavior and a poor overall survival. In recent years, major advances in the molecular understanding of ovarian cancer as a heterogeneous disease have emerged new prospects for improved screening, prevention, and therapy.

Pelvic HGSCs are marked by high genetic instability with a TP53 mutation being present in more than 95%. There is compelling evidence from clinical and molecular studies that most pelvic HGSCs arise from the serous fallopian tube epithelium. The serous tubal intraepithelial carcinoma (STIC), a focal non-invasive cytological appearance of HGSC typically located in the distal fallopian tube, has been identified as putative precursor lesion of pelvic HGSC. STIC is composed of secretory cells, the non-ciliated population of the fallopian tube epithelium, and exhibit features as variable stratification, increased proliferation and loss of nuclear polarity. Genetic analysis has revealed that STICs harbor identical TP53 mutations as their associated invasive HGSCs, supporting the fallopian tube as site of origin for pelvic HGSC. The immune system plays an important role in (ovarian) cancer and is considered to have a significant influence on its clinical outcome. In ovarian carcinomas, high numbers of tumor infiltrating cytotoxic T cells (CD8+, CD8+ CD103+, CD103+ PD-1+) are associated with prolonged patient survival. In contrast, infiltration of regulatory T cells (CD4+ CD25+ Foxp3+) and lower ratios of CD8+ regulatory T cells have an unfavorable effect on prognosis as regulatory T cells are able to suppress the ovarian cancer immune response. It has been suggested that in advanced stage ovarian cancer, an immune suppressive microenvironment dominates within the abdominal cavity, which may enable the tumor to escape the immune system. Based on the current knowledge, several promising anti-cancer immunotherapeutic strategies have nowadays been developed in order to boost the anti-tumor immune response.

Information about the immunological response in the early (pre-invasive) steps of HGSC development is limited and may be helpful to determine its role in the progression of pre-invasive STIC into a full blown HGSC. We hypothesize that differences in the immunogenicity of STIC might reflect its potency of progressing into an invasive carcinoma. STIC may be recognized by the immune system as pathogenic and may be cleared or at least controlled, thus preventing tumor development. On the other hand, precursor lesions might escape immune surveillance or attract cells that suppress the local immune system thereby creating a microenvironment that promotes tumor development. In a substantial subset of STICs, the presence of an immune infiltrate has been observed. In addition, George et al. analyzed immune cells in a small cohort of STICs and detected increased numbers of macrophages (CD68+) while numbers of lymphocytes (CD3+) were found to be comparable to healthy controls. Still, information on the role of different immune cells within the STIC is lacking.

Recently, the presence of an immune infiltrate in STIC has been associated with stromal upregulation of chondroitin sulfate E. Chondroitin sulfate E is a highly sulfated glycosaminoglycan that is abundantly expressed in the ovarian cancer microenvironment, while practically not expressed in healthy stroma. In addition, the upregulation of stromal chondroitin sulfate E has been linked to the progression of STIC into HGSC. This class of multifaceted molecules is known
to participate in various biological responses including immune signaling pathways, through the interaction with effector molecules (e.g. chemokines and selectins).\textsuperscript{26, 27} Stromal upregulation of chondroitin sulfate E may have an effect on the immune microenvironment within STIC, hence we address this association in the current study.

In order to characterize the immune micro-environment in precursor lesions of high grade serous ovarian cancer, we analyzed the presence of T cells in STIC. A recently developed method of multiplex immunofluorescent staining was used, followed by high-resolution whole-slide imaging, and quantification of cells by an automated image analysis software, providing us with the opportunity to perform an automated and objective measurement of different T-cell subsets within tissues. We applied this technology to a large cohort of STIC, paired HGSC, and normal fallopian tubes, and evaluated T cells associated with STIC. In addition, we evaluated associations between T-cell densities and stromal changes in STIC.

**Materials and methods**

**Case selection**
This study was approved by the Regional Committee for Medical Research Ethics and performed according to the Code for Proper Secondary Use of Human Tissue (Dutch Federation of Biomedical Scientific Societies, www.federa.org). By using the nationwide network and registry of histopathology and cytopathology (PALGA) as the primary source, STICs and associated HGSCs were collected from the Radboud university medical center, the Netherlands Cancer Institute, the Canisius-Wilhelmina hospital, and the Elisabeth-TweeSteden hospital in the Netherlands (range 1997-2016).\textsuperscript{28} After reviewing pathology reports, 114 relevant cases were selected for histological analysis. As a control group, fallopian tubes from patients with a BRCA mutation (\(N = 5\)) and from patients with a benign gynecologic abnormality (\(N = 5\)) were included.

**Identification of STICs**
For the identification of STIC, sections were independently assessed by two experienced gynecological pathologists (JB and KVdV). Lesions were categorized according to the algorithm for diagnosis of serous tubal intraepithelial carcinoma based on morphology, p53 and Ki-67 immunoreactivity.\textsuperscript{29, 30} In case of no consensus, sections were reevaluated to reach consensus. One or more STICs were identified in 59 cases, Cohen’s kappa coefficient for inter-observer reliability of the characterization of the tubal lesions (i.e. STIC or no STIC) was determined to be 0.81. In 40 cases (67.8%), fallopian tubes were sectioned and embedded in total (e.g. SEE-FIM protocol).\textsuperscript{31}

**Immunohistochemistry**
Paraffin-embedded sections (4 \(\mu m\)) were deparaffinized in xylene, hydrated in graded ethanol. Heat-induced antigen retrieval was performed in 10 mM citrate buffer (pH 6.0, ScyTek Laboratories, 15 min).
**Immunofluorescent staining**

After antigen retrieval, sections were blocked with 1% (w/v) bovine serum albumin (BSA, fraction V, Sigma-Aldrich) in Tris-buffered saline 0.05% (v/v) Tween 20 (TBST) for 10 minutes. Samples were stained by Opal TSA Plus multiplex tissue staining kits (PerkinElmer) according to the manufacturer’s protocol. All slides were stained by following panel of antibodies (antibodies were added in order as mentioned): 1) anti-CD45RO (1:12640, clone UCHL-1, cat MS-112, Thermo Scientific) labeled with Opal 620 fluorophore, anti-CD8 (1:1600, clone C8/144B, cat M7103, Dako) labeled with Opal 570 fluorophore, anti-CD3 (1:400, clone sp7, cat RM-9107, Thermo Scientific) labeled with Opal 520 fluorophore, anti-Foxp3 (1:300, clone 236A/E7, cat 14-4777, eBioscience Affymetrix) labeled with Opal 540 fluorophore. To identify epithelial cells, all sections were stained with anti CK8.18 (1:100, clone cam5.2, cat 563613, BD) labeled with Opal 650 fluorophore. Poly-HRP-anti-mouse/rabbit/rat-IgG (ImmunoLogic) was used as secondary antibody. Finally, slides were counterstained with DAPI and mounted in Fluoromount-G (Southern Biotechnology Associates, Inc).

Multispectral scanning was performed using the Vectra slide scanner (version 2.0.7; PerkinElmer). Multispectral images were unmixed using spectral libraries build from images of single stained tissues for each reagent and unstained tissue using inForm (version 2.1.1; PerkinElmer). A selection of 30 to 40 representative original multispectral images of ovarian carcinomas and controls were used to train inForm for quantitative image analysis; segmentation of epithelial (tumor) cell/stromal tissue based on Opal 650, cell segmentation based on DAPI and membrane stain signals, and finally phenotyping of different cell types). After optimizing the analysis software, phenotyping and epithelial/stromal segmentation were performed and depicted in a computer-generated overview. The automated quantification and segmentation were manually checked and were found to be adequately performed.

All settings applied to the training images were saved within an algorithm and used for batch analysis. Vectra Review (Version 2.0.8, PerkinElmer Inc.) was used to select the areas for analysis; epithelial cells and underlying stroma of STIC, HGSC, and normal fallopian tube as control. Additionally, STICs were precisely marked by applying regions of interest using ImageJ 1.48v (National Institutes of Health, USA).

**P53 and Ki-67 staining**

After antigen retrieval, peroxidase activity was blocked with 3% hydrogen peroxide in phosphate buffered saline (PBS). Sections were then incubated overnight at 4°C with mouse anti-human p53 tumor suppressor protein (1:400, clone DO7, ImmunoLogic) or monoclonal mouse anti-human Ki-67 antigen (1:100, clone MIB-1, Dako) diluted in normal antibody diluent (ImmunoLogic). Next, sections were incubated with poly-HRP-anti-mouse/rabbit/rat-IgG (ImmunoLogic, 60 min) and bound antibodies were visualized with 3,3-diaminobenzidine (ImmunoLogic). Sections were counterstained with hematoxylin, dehydrated, and mounted with Quick-D mounting medium (Klinipath).

Immunoreactivity of p53 and Ki-67 were scored for the intensity (weak, moderate, or intense) and proportion (range 0-100%) of positive cells. The expression pattern of p53 was classified as mutated (diffuse moderate to strong nuclear staining of >75% of epithelial cells or a completely negative nuclear staining), or as wild type. The Ki-67 proliferation index was determined as low when nuclear expression was observed as <10% or high when expression was ≥10%.
**Chondroitin sulfate E staining**

The expression of chondroitin sulfate E was immunohistochemically evaluated by using the avidin-biotin complex method. In brief, slides were incubated with the GD3G7 antibody specific for chondroitin sulfate E (1:5), followed by a mouse anti-VSV antibody (clone P5D4, 1:10), biotinylated horse-anti-mouse IgG antibody (Vector Laboratories Inc., CA, USA, 1:200), and ABC reagent (Vectastain ABC anti-mouse-IgG kit, Vector Laboratories Inc.). Immunoreactivity of chondroitin sulfate E was scored for the proportion and intensity of positive stroma and classified as either mild or intense.

**Statistics**

Logarithmic transformation was applied in order to achieve normal distribution of T cell density in the subgroups (logarithmic transformation \((x + 1)\) was indicated if zeros were included). In case of multifocal STIC, all STIC regions were added up and T-cell densities of the total area were calculated and used in statistical analysis. T-tests were performed to evaluate the difference in mean T-cell density between subgroups. Paired t-tests were used to compare the mean difference in T-cell density between STIC and invasive carcinoma within patients. To assess the association between T-cell density and the expression of chondroitin sulfate E, logistic regression analyses (using a Generalized Estimating Equations model) with correction for within subject correlation were performed. All tests were two-sided and p-values < 0.05 were considered significant. T-cell densities that are presented in graphics comprise the non-logarithmic transformed values. Statistical analyses were performed using Graphpad Prism version 5.03 (Graphpad software, La Jolla, CA, USA).

**Table 1.** Clinicopathological characteristics of the patient cohort \((N = 59)\).

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Results

Clinicopathological features of STICs
Using the validated STIC algorithm, STIC was identified in 59 patients and included 96 focal STIC lesions in total. Clinical and histological features of the cohort of cases with STIC are detailed in Table 1. The median patient age was 63 years (range 34-82). Most patients (N = 52, 88%) were diagnosed with STIC and associated invasive HGSC and seven patients (12%) had STIC only. Twenty-five patients (42%) received neoadjuvant chemotherapy prior to surgery while 34 (56%) did not. Multifocal STIC was detected in 19 cases (32%). One of the patients diagnosed with STIC only, had developed pelvic HGSC two years after initial diagnosis.

Automatic quantification of T cell subsets
Tissue sections were stained with CD3, CD8, CD45RO, Foxp3, CK8.18, and DAPI using 6-plex fluorescent immunohistochemistry with tyramide signal amplification. DAPI visualizes the nucleus, whereas CK8.18 was used to stain epithelial cells, in both healthy and cancerous tissues. CD3, CD8, CD45RO and Foxp3 were used to phenotypically distinguish several T-cell subsets: CD3+ CD8- CD45RO- Foxp3- (representing CD4+ effector T cells), CD3+ CD8- CD45RO+ Foxp3- (representing CD4+ memory T cells), CD3+ CD8+ CD45RO- Foxp3- (representing CD8+ effector T cells), CD3+ CD8+ CD45RO+ Foxp3- (representing CD8+ memory T cells), CD3+ CD8- CD45RO Foxp3+ (representing regulatory T cells), and CD3+ CD8- CD45RO Foxp3+ (representing regulatory memory T cells). To measure the presence of T-cell subsets within tissues, we performed a quantitative and automated whole-slide analysis using an optimized pathology imaging system. Figure 1 shows examples of opal 6-color immunohistochemistry in tissues, automated T-cell subset quantification, and a computer-generated overview.

Presence of T cells in STIC
In order to explore the presence of lymphocytes in STIC, we first focused on the presence of CD3+ T cells. The total T-cell density (cells/mm²) was calculated and included both the stromal and epithelial compartment. The CD3+ T-cell density in STIC was significantly increased compared to the density in normal fallopian tubes (mean difference 340 cells/mm², p < 0.001) (Figure 2A). By applying epithelial cell/stromal tissue segmentation, we compared CD3+ T-cell densities between these compartments in STIC and normal controls. In STIC, there was no difference in CD3+ T-cell density between the epithelial and stromal compartment (mean difference 80 cells/mm², p = 0.23) (Figure 2B). In normal fallopian tubes, the intraepithelial CD3+ T-cell density was significantly higher compared to the density in the stromal compartment (mean difference 161 cells/mm², p < 0.001).

Presence of T cells within paired STIC and HGSC from single patients
The immunological response in the (pre)cancer microenvironment may substantially vary between and within subjects. By analyzing the presence and localization of T cells in STIC and the paired invasive HGSC, we might get insight into the intrinsic patient-related variation. Due to our unique collection of paired STIC and HGSC we were able to analyze intralesional variation in 51 cases. One paired carcinoma was not assessed for T-cell presence due to its absence in additional staining.
**Figure 1.** Multiplex fluorescent staining and phenotyping of T cells in normal fallopian tubes, STIC and invasive HGSC.

**A.** Examples of multiplex opal 6-color immunofluorescent staining of normal fallopian tube epithelium, STIC, and invasive HGSC (20x zoom).

**B.** InForm software was applied for automatic, quantitative image analysis including segmentation of epithelial and stromal tissue and phenotyping of different cell types (20x zoom).

**C.** Results of quantitative analysis were presented in a computer-generated overview. Please note that a region of interest (marked by the light red area) indicates STIC.

**Legend**

**A.** Multispectral imaging

- CD3+
- CD8+
- CD45RO+
- Foxp3+
- CK8.18
- DAPI

**B.** Phenotyping

- CD3+ CD8- CD45RO- Foxp3-
- CD3+ CD8- CD45RO+ Foxp3-
- CD3+ CD8+ CD45RO- Foxp3-
- CD3+ CD8+ CD45RO+ Foxp3-
- CD3+ CD8- CD45RO- Foxp3+
- CD3+ CD8- CD45RO+ Foxp3+
- CD3+ CD8- CD45RO- Foxp3+
- CD3+ CD8- CD45RO+ Foxp3+
- CD3+ CD8- CD45RO- Foxp3+
- CD3+ CD8- CD45RO+ Foxp3+

**C.** Overview:

- CD3+ CD8- CD45RO- Foxp3-
- CD3+ CD8- CD45RO+ Foxp3-
- CD3+ CD8+ CD45RO- Foxp3-
- CD3+ CD8+ CD45RO+ Foxp3-
- CD3+ CD8+ CD45RO+ Foxp3-
- CD3+ CD8+ CD45RO- Foxp3+
- CD3+ CD8+ CD45RO+ Foxp3+

- 1-10 cells
- 11-50 cells
- 51-500 cells

**A.** Examples of multiplex opal 6-color immunofluorescent staining of normal fallopian tube epithelium, STIC, and invasive HGSC (20x zoom). **B.** InForm software was applied for automatic, quantitative image analysis including segmentation of epithelial and stromal tissue and phenotyping of different cell types (20x zoom). **C.** Results of quantitative analysis were presented in a computer-generated overview. Please note that a region of interest (marked by the light red area) indicates STIC.
Infiltrating T cells within the serous tubal intra-epithelial carcinoma (STIC) microenvironment

**Figure 2.** Density (cells/mm$^2$) of T cells associated with STIC.

A. CD3$^+$ T-cell density in STIC, HGSC, and normal controls; B. Stromal and intra-epithelial CD3$^+$ T-cell density in STIC, HGSC, and normal controls; C. CD3$^+$ T-cell density in multifocal STIC derived from individual patients; D. Distribution of subsets of helper, cytotoxic, and regulatory T cells in STIC; E. Distribution of subsets of helper, cytotoxic, and regulatory T cells in normal controls; F. Distribution of subsets of helper, cytotoxic, and regulatory T cells in invasive HGSC; Red horizontal line represents mean. ** indicates a statistical difference with $p < 0.01$, and *** indicates $p < 0.001$. 
Overall, the density of CD3+ T cells in STIC was significantly lower than the density of CD3+ T cells in paired invasive HGSC (mean difference 239 cells/mm², p = 0.035) (Supplementary data, Table S1). Additionally, we compared T-cell densities for each compartment (i.e. epithelial or stromal) between the paired lesions. The intraepithelial density of CD3+ T cells was not different between STIC and paired HGSC (mean difference 31 cells/mm², p = 0.91), while the density of CD3+ T cells in the stromal compartment in STIC was significantly lower than the density in paired HGSC (mean difference 314 cells/mm², p = 0.012) (data not shown).

**Presence of T cells in multifocal STIC**
Within a single patient multiple focal lesions of STIC can be detected. Our unique collection of multiple STIC per patient made it possible to analyze whether there are (subtle) differences between these focal lesions. A total number of 19 patients with a median of 2 STICs per patient were analyzed, mostly including unilaterally located STIC except for patient 45 and 51 with bilaterally located STIC. The density of CD3+ T cells was assessed for up to 10 focal lesions derived from single patients. As shown in figure 2C, the density of CD3+ T cells was comparable in paired multifocal lesions in a majority of patients (e.g. patient 4, 11, 23, 38, 51, and 58), while a few cases showed large differences in T-cell densities between focal lesions (e.g. patient 49 and 54).

**Presence of T-cell subsets in STIC**
We evaluated the immunological response in STIC more precisely by determining different T-cell subsets including helper T cells (CD3+ CD8- Foxp3-), cytotoxic T cells (CD3+ CD8+ Foxp3-), and regulatory T cells (CD3+ CD8 Foxp3+), examples of fluorescent staining are shown in Figure 3. Numbers of helper, cytotoxic, and regulatory T-cell subsets were significantly increased in STIC compared to numbers in normal fallopian tubes (mean difference 137 cells/mm², p < 0.001; mean difference 160 cells/mm², p = 0.01; and mean difference 43 cells/mm², p < 0.001; respectively) (Table 2).

In STIC, the density of cytotoxic T cells was significantly higher than the density of helper and regulatory T cells (mean difference 104 cells/mm², p < 0.01 and mean difference 201 cells/mm², p < 0.001 respectively) (Figure 2D). In addition, numbers of helper T cells were higher than regulatory T-cell numbers (mean difference 97 cells/mm², p < 0.001) in STIC. A comparable distribution profile of T cells (cytotoxic T cell > helper T cell > regulatory T cell) was observed in normal fallopian tubes (Figure 2E).

Next, we focused on T-cell subset densities in STIC and paired invasive HGSC within subjects. The density of helper T cells was comparable in STIC and paired HGSC (mean difference 33 cells/mm², p = 0.79) while numbers of cytotoxic and regulatory T cells were significantly lower in STIC compared to the associated HGSC (mean difference 163 cells/mm², p = 0.031 and mean difference 43 cells/mm², p < 0.001; respectively) (Table 2). In the immune microenvironment of HGSC, the cytotoxic T-cell density was significantly higher than the density of helper and regulatory T cells, while densities of these last two subsets were not significantly different (Figure 2F).
Infiltrating T cells within the serous tubal intra-epithelial carcinoma (STIC) microenvironment

**Figure 3.** Examples of fluorescent staining of T cell subsets in normal fallopian tube, STIC, and invasive HGSC (zoom 20x).

- **A.** Normal fallopian tube epithelium;
- **B.** STIC (case without associated HGSC);
- **C.** STIC (case with associated HGSC);
- **D.** The paired HGSC of STIC C;
- **E.** Overview of STIC (case with associated HGSC), STIC is marked by the dotted line.

**Legend**

- CD3+ CD8- Foxp3- (helper T cell)
- CD3+ CD8+ Foxp3- (cytotoxic T cell)
- CD3+ CD8- Foxp3+ (regulatory T cell)
- CK8.18 (epithelial cell)
- DAPI (cell nucleus)

*A. Normal fallopian tube epithelium; B. STIC (case without associated HGSC); C. STIC (case with associated HGSC); D. The paired HGSC of STIC C; E. Overview of STIC (case with associated HGSC), STIC is marked by the dotted line.*
Table 2. Mean T-cell densities (cells/mm²) of subsets of helper, cytotoxic, and regulatory T cells in normal fallopian tubes, STIC, and paired invasive HGSC (data included T cells located in the stromal and epithelial compartment).

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Presence of T cells in STIC associated with clinicopathological factors

To gain more insight into the presence of T cells in STIC without the existence of invasive HGSC, we compared the T-cell density in patients diagnosed with STIC only and patients diagnosed with STIC and associated HGSC (Figure 4A). There was no difference in CD3⁺ T-cell density between these two groups (mean difference 281 cells/mm², p = 0.21). Regarding the different subsets of T-cells, numbers of helper T cells were significantly higher in STIC only compared to STIC with associated HGSC (mean difference 136 cells/mm², p = 0.049), while numbers of cytotoxic and regulatory T cells were comparable (mean difference 138 cells/mm², p = 0.41 and mean difference 7 cells/mm², p = 0.08; respectively) (Figure 4B).

Furthermore, treatment might have an influence on the presence of T cells in STIC and HGSC. We therefore compared T-cell densities in patients that were and were not treated with chemotherapy before tissue collection. No significant difference in the density of CD3⁺ T cells in STIC of patients that did or did not receive platin-based chemotherapy was observed (mean difference 101 cells/mm², p = 0.27), neither the distribution of subpopulations of T-cells (i.e. helper, cytotoxic, and regulatory T-cell) was different (Figure 4C, D).

Presence of T cells in STIC associated with stromal changes

Changes in the microenvironment (e.g. stroma) during malignant epithelial progression into STIC might affect the influx of immune cells. Recently, the stromal upregulation of chondroitin sulfate E has been associated with the presence of an immune infiltrate in STIC. We evaluated the association between quantified T-cell densities and the stromal expression of chondroitin sulfate E in focal STIC lesions with correction for within subject correlations. The density of CD3⁺ T-cells was significantly increased in STIC with intense chondroitin sulfate E expression (mean difference 97 cells/mm², p < 0.001) compared to STIC with mild expression (Figure 4E). Also densities of helper, cytotoxic, and regulatory T-cell subsets were significantly higher in STIC with intense chondroitin sulfate E expression (mean difference 8 cells/mm², p = 0.039; mean difference 64 cells/mm², p < 0.001; and mean difference 24 cells/mm², p = 0.001; respectively) (Figure 4F).
Figure 4. Density (cells/mm²) of T cells in STIC associated with clinicopathological parameters.

A. CD3⁺ T-cell density in STIC cases without and with associated invasive HGSC. B. Subsets of helper, cytotoxic, and regulatory T cells in STIC cases without and with associated invasive HGSC. C. CD3⁺ T-cell density in STIC and HGSC before and after the use of platin-based chemotherapy. D. Subsets of helper, cytotoxic, and regulatory T cells in STIC before and after the use of platin-based chemotherapy. E. CD3⁺ T-cell density in STIC with mild and intense chondroitin sulfate E expression. F. Subsets of helper, cytotoxic, and regulatory T cells in STIC with mild and intense chondroitin sulfate E expression. Red horizontal line represents mean. Abbreviation: CS-E, chondroitin sulfate E. *Graphics includes T-cell densities of focal STIC lesions. * indicates a statistical difference with p < 0.05, ** indicates p < 0.01, and *** indicates p < 0.001.
**Discussion**

The presence of tumor infiltrating lymphocytes within the (pre-)cancer microenvironment is considered to reflect the immunological response against cancer antigens. In the present study, we evaluate the presence of T cells in serous tubal intraepithelial carcinoma (STIC), the precursor lesion of pelvic high grade serous cancer (HGSC), by applying a comprehensive and powerful method of multiplex immunofluorescent staining followed by high-resolution whole-slide imaging, and quantification of cells by automated image analysis software. In this study, we focused on the CD3$^+$ T-cell population including its helper, cytotoxic, and regulatory T-cell subsets.

Herein, we report that STICs have a significantly increased density of CD3$^+$ T cells, compared to normal fallopian tube tissue. T-cell densities between STIC and paired invasive carcinoma within subjects were observed to be more elevated in the associated HGSC than in STIC. The gradual increase in CD3$^+$ lymphocytes between normal epithelium and STIC, and between STIC and paired HGSC indicates an immune response that is associated with the hypothesized malignant progression from normal epithelium to STIC to invasive HGSC.

Most studies investigating immune responses towards (high grade serous) ovarian cancer have focused on invasive primary carcinomas with little attention to the immune response associated with its pre-invasive stage located in the fallopian tube. In this study, an immune response was observed in the vast majority of STICs marked by an elevated CD3$^+$ T-cell density compared to normal fallopian tubes. In addition, the density of helper, cytotoxic, and regulatory T-cells was found to be increased in STIC. Our results are in contrast with the study of George et al., which showed similar levels of CD3$^+$ T cells in STIC, HGSC, and normal controls.$^{23}$ Their small sample size ($N = 15$) may explain this contradicting finding, since we studied a large cohort of 59 patients with STIC. Recently, increased numbers of T cells (CD3$^+$, CD4$^+$, CD8$^+$, and regulatory T cells) have been identified in the pre-invasive stage of breast cancer (ductal carcinoma in situ, DCIS) supporting our findings.$^{32-34}$

From studies focused on ovarian carcinomas, several effects of tumor infiltrating T cells on the clinical outcome are known. Subsets of CD8$^+$, CD8$^+$ CD103$^+$, and CD103$^+$ PD-1$^+$ tumor infiltrating lymphocytes correlate most strongly with favorable patient outcome.$^{10-15}$ On the other hand, relatively high levels of regulatory T cells have a negative effect on patient outcome, which is consistent with their proposed immunosuppressive role.$^{10, 16, 17}$ Eventually, the balance between immune promoting and immune inhibiting cells may determine the effect on patient prognosis. The anti-cancer immunity in advanced stage ovarian cancer has been proposed to be counterbalanced by an immune suppressive microenvironment.$^{18, 19}$ In STIC, a significant influx of both immune activating and immune inhibiting T cells was observed, however, the increase of regulatory T-cell numbers was relatively higher than the increase of cytotoxic T-cell numbers. These data may cautiously suggest that a significant immunosuppressive response exists within the microenvironment of STIC. A strong increase of regulatory T-cell numbers has also been associated with the progression of normal epithelium into DCIS.$^{32}$ Nevertheless, Morita et al. reported that CD8$^+$ T cells might be involved in the healing of DCIS.$^{33}$ Finally, additional characterization of immune parameters is needed to expand our understanding of cancer immunity in pre-invasive HGSC.

Little is known about the evolution of the immunological response against aberrant cells within
Infiltrating T cells within the serous tubal intra-epithelial carcinoma (STIC) microenvironment

the microenvironment along the continuum from pre-invasive lesions into invasive carcinoma. Our results show that the density of CD3+ lymphocytes was significantly lower in STIC compared to associated invasive carcinoma within patients. It seems likely that the immune microenvironment in STIC and paired invasive HGSC may be largely comparable, marked by comparable numbers of helper T cells and a relatively comparable increase of cytotoxic and regulatory T cells between STIC and paired HGSC. Further characterization of the evolving immune microenvironment in malignant epithelial progression is necessary to assess whether immune-based therapies are a treatment option for pre-invasive lesions of the pelvic HGSC.

The prognostic relevance of T-cell localization within the cancer microenvironment, i.e. stromal or intra-epithelial, is not entirely clear. The presence of lymphocytes in the stromal compartment has been less significantly associated with prolonged survival rather than intra-epithelial localization. On the other hand, it was recently reported that stromal CD8+ T cells have a positive effect on the prognosis of ovarian cancer patients by abrogating stromal mediated chemo resistance. Here, we observed a high intra-epithelial/stromal T-cell ratio in normal fallopian tubes that shifted into a more or less equal ratio in STIC, while an additional increase in numbers of stromal CD3+ cells was observed in paired HGSC. It seems that the influx of T cells in the stroma is associated with malignant epithelial progression into STIC and HGSC. We suggest that stromal changes associated with malignant progression might have an effect on the infiltration of T cells in STIC. Recently, the presence of an immune infiltrate in STIC has been associated with intense stromal expression of chondroitin sulfate E. This highly sulfated glycosaminoglycan has been identified as a characteristic component of the extracellular matrix in (high grade serous) ovarian cancer and is almost not present in healthy stroma. Through interactions with biologically active molecules (e.g. growth factors), chondroitin sulfate E is involved in several cancer-promoting signaling pathways. Further, chondroitin sulfate E can interfere with immunological responses via its binding affinity for chemokines (i.e. stromal derived factor-1, secondary lymphoid tissue chemokine, and interferon gamma-induced protein-10) and selectins. Our study confirmed the association between intense chondroitin sulfate E expression and the infiltration of T cells in STIC. Increased numbers of helper, cytotoxic, and regulatory T-cell subsets were associated with stromal upregulation of chondroitin sulfate E in STIC. Although both microenvironmental changes have been linked to malignant epithelial progression, their exact interaction remains unclear. Stromal chondroitin sulfate E might play a role in orchestrating immune signaling pathway by chemokine binding and sequestering. However, the association between the infiltration of all different T-cell subsets and intense chondroitin sulfate E expression in STIC does not point in direction of a particular immune signaling pathway. Alternatively, stromal chondroitin sulfate E might be considered as a bystander in the immune microenvironment and may only evoke a general influx of immune cells. Nevertheless, the microenvironment of STIC characterized by intense chondroitin sulfate E expression and high numbers of infiltrating T cells, resembles closely that of the invasive HGSC microenvironment, in contrast to STIC with mild chondroitin sulfate E expression and low numbers of infiltrating T cells. This finding may support the involvement of microenvironmental changes associated with the progression of STIC into invasive HGSC.

The presence of multifocal STIC in the fallopian tube is not uncommon, but little is known about this phenomenon. Multifocal (unilateral) STICs might originally belong to a single largely
outstretched STIC lesion (i.e. before sectioning and embedding), however, subtle differences between the multifocal lesions regarding their potency for further progression may exist. The density of infiltrating T cells in paired multifocal lesions was generally found to be in a similar range, only in a few cases substantial differences were seen. In addition, a relatively high inter-patient variability of infiltrating T-cell density was observed when compared to the intrinsic patient-related variability (even when STIC was bilaterally located), as also noticed in DCIS.32

Currently, there is no data about the immune response in STIC without the presence of associated invasive carcinoma, and the risk for development of pelvic HGSC afterwards. It is proposed that epithelial cells shed from STIC, have direct access to the peritoneal cavity and may disseminate and give rise to invasive disease.37 The risk for development of pelvic HGSC after STIC is largely unknown, yet reported to be at least 4.5%.38 Although the number of patients diagnosed with STIC only are small and results should be interpreted with caution, increased numbers of infiltrating T cells were identified, including the patient who developed invasive HGSC two years after diagnosis of STIC. The distribution of T-cell subsets within the immune infiltrate was largely comparable to STIC with associated HGSC, although marginally significant increased numbers of helper T cells were observed in STIC only. Necessarily, these data should be validated in a larger study cohort.

Chemotherapeutics including platin-based agents are considered to have an effect on the immune microenvironment in carcinomas. In this respect, both negative and positive regulations on the immune response have been described.39-41 We observed no different T-cell density in STIC cases that were treated with chemotherapy, neither any difference in the distribution of T-cell subsets. Still, from these data we cannot exclude an effect on the immune response as the intrinsic patient-related effect was not studied.

In addition to analyzing T cells in STIC, we studied the presence of T cells in the normal (non-cancer) microenvironment. Histologically normal fallopian tubes from patients with a benign gynecologic diagnosis or BRCA-mutation were analyzed for the presence of T cells. We observed relatively low amounts CD3$^{+}$ T cells in healthy fallopian tubes and T cells were found to be predominantly located in the intra-epithelial, rather than in the stromal compartment. Furthermore, the subpopulation of cytotoxic T-cells was most abundantly present, while levels of helper T cells, and especially levels of regulatory T cells, were significantly lower. The substantial population of intraepithelial cytotoxic T cells in normal fallopian tubes may represent tissue-resident CD8$^{+}$ memory T cells, non-recirculating memory T cells that persist long-term in epithelial barrier tissues like the reproductive tract.42, 43 Indeed, we observed the expression of CD45RO in a large subpopulation of intraepithelial cytotoxic T cells, which indicates a memory T cell. A common feature of tissue-resident memory T cells is their expression of CD103.30, 32, 33 As high levels of intratumoral CD8$^{+}$ CD103$^{+}$ T cells have been associated with favorable prognosis in ovarian cancer patients, tissue-resident memory T cells located in the fallopian tube may be involved in the immunological response against STIC and progression into HGSC.11, 12

In conclusion, we demonstrate increased levels of T cells in the microenvironment of serous tubal intraepithelial carcinoma (STIC), the putative precursor lesion of invasive high grade serous ovarian cancer (HGSC). The infiltration of T cells is associated with stromal changes in STIC and reflects the existence of a host anti-cancer response in an early, pre-invasive stage of pelvic HGSC development. Our findings may serve as a starting point in our understanding of cancer immunity in STIC.
References

17. van der Steen SC, van der Vlijver K, van Kuppevelt TH, Massuger LFAG. Changes in the extracellular matrix are associated with the development of serous intraepithelial carcinoma (STIC) into high grade serous carcinoma. Int J Gynecol Cancer 2017; in press.


Supplementary data

Table S1. Density of T cells in STIC and paired HGSC (N = 52).

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Chapter 5

Targeting the extracellular matrix of ovarian cancer using functionalized, drug loaded lyophilisomes

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Abstract

Epithelial ovarian cancer is characterized by a high mortality rate and is in need for novel therapeutic avenues to improve patient outcome. The tumor’s extracellular matrix ("stroma") offers new possibilities for targeted drug-delivery. Recently we identified highly sulfated chondroitin sulfate (CS-E) as a component abundantly present in the ovarian cancer extracellular matrix, and as a novel target for anti-cancer therapy. Here, we report on the functionalization of drug-loaded lyophilisomes (albumin-based biocapsules) to specifically target the stroma of ovarian carcinomas with the potential to eliminate cancer cells. To achieve specific targeting, we conjugated single chain antibodies reactive with CS-E to lyophilisomes using a two-step approach comprising sortase-mediated ligation and bioorthogonal click chemistry. Antibody-functionalized lyophilisomes specifically targeted the ovarian cancer stroma through CS-E. In a CS-E rich micro-environment in vitro lyophilisomes induced cell death by extracellular release of doxorubicin which localized to the nucleus. Immunohistochemistry identified CS-E rich stroma in a variety of solid tumors other than ovarian cancer, including breast, lung and colon cancer indicating the potential versatility of matrix therapy and the use of highly sulfated chondroitin sulfates in cancer stroma as a micro-environmental hook for targeted drug delivery.
**Introduction**

Epithelial ovarian cancer is the fifth leading cause of cancer-related death in women worldwide.\(^1\) Most patients are diagnosed with an advanced stage of disease (Fédération Internationale de Gynécologie et d’Obstétrique (FIGO) stage III-IV) and suffer from extensive abdominal metastaeses.\(^2,3\) Aggressive surgical cytoreduction and chemotherapy are used as primary treatment, but nevertheless up to 70% of these patients will develop recurrent disease and eventually succumb. Long term survival is poor with a 5-year survival of less than 35%.\(^2,3\) Overall survival statistics have not significantly improved over the last decades and new avenues for better treatment are clearly warranted.\(^4\)

Conventional chemotherapeutics affect proliferating cancer cells as well as normal cells, resulting in systemic adverse events that greatly affect quality of life. As a consequence, the dose administered has to be limited resulting in a suboptimal treatment that negatively affects prognosis of cancer patients. The use of drug delivery systems may be helpful to overcome these problems by improving biodistribution, resulting in high local drug concentrations at the tumor site while minimizing exposure to healthy cells.\(^5\) Beneficial effects of drug delivery systems such as liposomal doxorubicin (Caelyx/Doxil) and albumin bound paclitaxel (nab-paclitaxel), have been reported in several (pre)-clinical studies.\(^6-9\) Previously we described a novel class of drug delivery vehicles, lyophilisomes, which are spherical nano- to microsized biocapsules that can be prepared from various proteins (e.g. albumin, collagen, and elastin).\(^10,11\) Albumin-based lyophilisomes can be efficiently loaded with doxorubicin and are able to eliminate ovarian cancer cells in vitro.\(^10\) In addition, the albumin wall of lyophilisomes offers opportunities for functional modification, e.g. by the incorporation or conjugation of components in and/or on the wall. Antibody-conjugated lyophilisomes have been shown to specifically bind to cancer cells expressing the corresponding antigen, thus enabling active cancer-targeting.\(^12\)

Although it is hypothesized that active targeting of cancer cells by drug delivery systems using specific antibodies or ligands has the potential to broaden the therapeutic index of anti-cancer drugs, the favorable effect of tumor-cell targeting over non-targeting systems was reported to be disappointing.\(^5,6,13\) As most of these studies have focused on targeting cancer cells, other approaches such as targeting the cancer extracellular matrix (ECM) may offer valuable alternatives.\(^14\)

The ECM represents a network of proteins and proteoglycans that is abundantly remodeled during cancer development and actively contributes to cancer progression.\(^15,16\) A large amount of intratumoral matrix correlates with poor prognosis in cancer, including ovarian cancer.\(^17\) Major components of the ECM are collagen, laminin and proteoglycans. Proteoglycans function to a large extent through their glycosaminoglycan side chains; linear negatively charged polysaccharides built from repeating disaccharides.\(^18\) Highly 4,6-sulfated chondroitin sulfate (CS-E), a specific class of glycosaminoglycans, is found to be abundantly expressed in the ovarian cancer stroma while being absent or present in only very small amounts in healthy stroma, thus representing an attractive target for anti-cancer therapy.\(^19,20\) Interestingly, the amount of these targets expressed in the stroma is relatively high when compared to targets expressed at the cancer cell surface.

In this study, we present an innovative concept of an anti-cancer strategy aiming at forming a depot of chemotherapeutic-loaded lyophilisomes in the ovarian cancer ECM. Targeting the
cancer ECM rather than cancer cells might be helpful to overcome hurdles observed in cell-targeting therapies as the cancer ECM is a relatively stable structure, unlike cancer cells that are characteristically genetically instable.\textsuperscript{21} Due to intratumoral heterogeneity, cell-targeting therapies may only affect subpopulations of cancer cells, and leave other cancer cells and cancer-promoting cells (e.g. cancer-associated fibroblasts, endothelial cells, and macrophages) unaffected.\textsuperscript{22} Release of chemotherapeutics from a depot of drug-loaded lyophilisomes in the cancer ECM may affect all cells in its vicinity including cancer cells, cancer stem cells and cancer-associated stromal cells. Collagens have been used as micro-environmental anchors for targeted anti-cancer therapy,\textsuperscript{23, 24} but collagen is also abundantly present in normal tissues. Therefore, in this study we focus on CS-E as a much more cancer-specific molecular target. We describe the construction and evaluation of a lyophilisome-based drug delivery system specifically targeting highly sulfated CS-E in the ovarian cancer stroma.

Materials and methods

Patient material
Study approval was given by the Regional Committee for Medical Research Ethics and performed according to the Code for Proper Secondary Use of Human Tissue (Dutch Federation of Biomedical Scientific Societies, www.federa.org). Cryosections (5 µm) of advanced stage high grade serous ovarian cancer were used for immunofluorescent analysis of antibody-functionalized lyophilisome specificity. Paraffin embedded sections (4 µm) of lung, cervical, breast, renal cell, endometrial, and colon cancer were used for immunohistochemical analysis of CS-E expression.

Production of antibody-functionalized lyophilisomes

\textbf{Modification of GD3G7 antibodies for sortase-mediated conjugation}

The single chain antibody GD3G7 was previously selected against embryonic glycosaminoglycans and showed specificity for CS-E.\textsuperscript{19} For site-selective conjugation of GD3G7 at the carboxy terminus, leaving the antigen-binding parts of this antibody intact, the LPETG sortase A-recognition motif was introduced. To this end the GD3G7 reading frame was cloned in plasmid pHENIX-LPETG-His-VSV to yield pHENIX-GD3G7-LPETG-His-VSV. Expression of the fusion protein in \textit{E.coli} strain ER2566 was induced with isopropyl β-D-thiogalactoside (IPTG) as described previously.\textsuperscript{25} GD3G7-LPETG-His-VSV was released from the periplasmic space via osmotic lysis using 200 mM Tris-HCl, pH 8.0, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 20% (w/v) sucrose containing protease inhibitors. Purification by nickel-NTA affinity chromatography was performed as described (NTA-Ni sepharose*, IBA Life sciences).\textsuperscript{26}

\textbf{Introduction of DBCO functionality to GD3G7 by sortagging}

pGBMCS-SortA, a gift from Dr. Fuyuhiko Inagaki\textsuperscript{27} was transfected into \textit{E. coli} ER2566 for standard protein expression. Bacterial expression was performed as described and IPTG-induced cells were lysed by sonication at 4°C using a Bandelin Sonopuls HD2070 sonicator. His-tagged sortase was purified with NTA-Ni Sepharose as described above.
To equip the GD3G7 antibody with a bio-orthogonal chemical click handle (Figure 1), 16 µM GD3G7-LPETG-His-VSV was incubated overnight at room temperature with 4 mM amino-PEG$_4$-DBCO (Click Chemistry Tools, Scottsdale, USA) in the presence of 40 µM sortase A in 50 mM Tris, 150 mM NaCl, 10 mM CaCl$_2$, pH 7.5. Reaction product was cleared from unreacted GD3G7-LPETG-His-VSV, cleaved G-His-VSV tags and sortase A by depletion on nickel-NTA beads. Free amino-PEG$_4$-DBCO was removed by filtration in PBS over a 10 kDa centrifugal filter device (Amicon® Ultra-4, Merck Millipore) using standard protocols. Routinely, filters were washed five times to obtain highly purified product.

**Figure 1.** Construction of cancer targeting lyophilisomes. Schematic overview of the conjugation between albumin-based lyophilisomes and GD3G7 antibodies reactive with CS-E, by applying a two-step approach comprising sortase mediated ligation and click chemistry.

**A.** Modification of antibodies for click chemistry

**B.** Modification of lyophilisomes for click chemistry

**C.** Generation of GD3G7-lyophilisomes using click chemistry

**A.** LPETG-His-VSV tagged single chain GD3G7 antibodies were modified for click chemistry by introducing amino-PEG$_4$-DBCO through a reaction mediated by Sortase A. **B.** Lyophilisomes were functionalized for click chemistry by conjugating PEG$_4$-azide to the primary amine groups of lyophilisomes mediated by N-hydroxysuccinimide (NHS). **C.** Antibody-functionalized lyophilisomes were generated by a click reaction between azido-conjugated lyophilisomes and GD3G7-PEG$_4$-DBCO antibodies. Abbreviations: CS-E, chondroitin sulfate E; DBCO, dibenzyclooctyne; $\gamma_L$, light chain variable domain; $\gamma_H$, heavy chain variable domain; SrtA, Sortase A.
The sortase mediated reaction was evaluated by applying bioorthogonal click chemistry between DBCO and azide. The sortagged product was incubated with azido-cyanine-7.5 (Lumiprobe GmbH, Hannover, Germany) for 1 h at 4 °C, followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 10% gels and gel imaging at 800 nm (Odyssey® CLx imaging system). Thereafter, gels were stained for presence of proteins with 0.1% (w/v) Coomassie Brilliant Blue R-250 solution (MP Biomedicals, Santa Anna, CA) in 50% (v/v) methanol and 10% (v/v) acetic acid in water.

Preparation of lyophilisomes and introduction of azide functionality
Lyophilisomes were prepared from bovine serum albumin (BSA; PAA Laboratories, Linz, Austria) as described previously. Briefly, droplets of 20 µl 2.5 mg/ml BSA (containing 10% FITC-labeled BSA (Sigma-Aldrich, St. Louis, MO, USA)) in 0.01 M acetic acid were snap frozen in liquid nitrogen. Capsules were formed using an annealing and lyophilization regimen. Large structures were removed by centrifugation (60 x g).

Lyophilisomes were prepared for click chemistry by introducing azide groups to the surface of lyophilisomes. Lyophilisomes were suspended in PBS containing 0.1% tween20 (v/v) (PBST; pH 8.0), sonicated (Cycle 0.5; Amplitude 20; 10 cycles) with a Sartorius labsonic P sonicator (Göttingen, Germany), mixed with 100 times molar excess NHS-PEG₄-azide (Jena Bioscience, Jena, Germany) and incubated under rotation at room temperature overnight. Next, lyophilisomes were washed three times with PBST and centrifuged at 17,000 x g for 5 min to remove free NHS-PEG₄-azide, and stored in PBST at 4°C.

Modification of lyophilisomes with PEG₄-azide was analyzed using flow cytometry. Lyophilisomes (2.5 µg) with or without PEG₄-azide were incubated with 1 µg/ml DBCO-IR dye 680RD (LI-COR Biotechnology, Bad Homburg, Germany), which binds only azido modified lyophilisomes, in PBST under rotation at room temperature for 1 h. Control samples were incubated in PBST only. Lyophilisomes were three times washed with PBST and centrifuged at 17,000 x g for 5 min, re-suspended in PBST and analyzed for their 680RD signal with a BD FACSCalibur flow cytometer (BD Biosciences, Breda, the Netherlands). Data were analyzed using FlowJo software (Version 10, Treestar, Ashland, OR).

Loading of lyophilisomes with doxorubicin
Loading of lyophilisomes with doxorubicin was performed as described previously. In short, 200 µg of lyophilisomes with or without GD3G7 antibody were washed twice with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.4; Promega, Madison, WI, USA) and centrifugated at 17000 x g for 5 min at 4°C. Next, lyophilisomes were incubated overnight with 500 µl 0.5 mg/ml doxorubicin (Accord Healthcare, the Netherlands) in 10 mM HEPES buffer v/v at room temperature. Non-entrapped doxorubicin was removed by centrifuging at 17 000 x g, 5 min, 4°C and collecting the supernatant. Subsequently, the lyophilisomes were washed three times with 10 mM HEPES buffer (17 000 x g, 5 min, 4°C) and the supernatants were collected. The doxorubicin concentration in 10 mM HEPES buffer of the three collected supernatants was quantified spectrophotometrically at 490 nm (Synergy BioTek 2 Plate reader, BioTek, Winooski, VT, USA). The entrapment efficiency was calculated using the following formula:
Antibody functionalization of lyophilisomes

In order to achieve specific targeting of lyophilisomes to CS-E, GD3G7 antibodies were conjugated to lyophilisomes through bioorthogonal click chemistry. A 1.25x molar excess GD3G7-LPET-PEG-DBCO was reacted to azido-conjugated lyophilisomes in PBST via the scheme depicted in Figure 1C. Reaction was allowed to proceed overnight at room temperature. In a control reaction, azide-conjugated lyophilisomes were incubated with unmodified GD3G7 antibodies. Free antibodies were removed through three washing steps with PBST by centrifugation at 17,000 x g for 5 min.

Conjugation was evaluated using horseradish peroxidase-conjugated Protein A that binds to the Vγ3 domain of the antibody. Antibody-functionalized lyophilisomes and non-functionalized lyophilisomes (2.5 µg) were incubated with 0.1 ug/ml peroxidase-Protein A (Merck Millipore, Darmstadt, Germany) in PBST for 1 h under rotation at room temperature. Afterwards, lyophilisomes were washed three times with PBST, centrifuged at 17,000 x g for 5 min and peroxidase activity in the pellets was measured by reaction in 0.0243 M citric acid, 0.0514 M K2HPO4, 0.012% H2O2 (v/v) and 0.04% ortho-phenylenediamine (w/v). After 30 min at room temperature, 12.5% H2SO4 (v/v) was added to stop the reaction. Subsequently, lyophilisomes were centrifuged and the absorbance of the supernatant was measured at 492 nm using a Synergy BioTek 2 Plate reader (BioTek, Winooski, VT, USA).

Evaluation of the targeting potential of antibody functionalized lyophilisomes

Binding to ovarian cancer stroma

Human ovarian cancer cryosections (5 µm) were pre-treated with 2 mM MgAc2 in 25 mM TrisHCl buffer (pH 8.0) with and without the chondroitin sulfate digesting enzyme chondroitinase-AC (30 mU/ml, 1 h, 37° C). After blocking with 2% BSA in PBS (w/v), sections were incubated with either antibody-functionalized lyophilisomes or non-functionalized lyophilisomes (0.1 mg/ml in 2% BSA in PBS) for 1 h. Nuclei were visualized by incubation with 10 µg/ml 4',6-diamidino-2-phenylindole (DAPI; Merck, Darmstadt, Germany) and CS-E was visualized by immunofluorescence, sections were mounted with cover glasses in mowiol-488.

Binding to peri-cellular chondroitin sulfate E in vitro

Although CS-E in ovarian carcinomas is predominantly localized in the cancer stroma and not (peri)cellularly, we used cell lines which do or do not produce CS-E in vitro as a model for analyzing the binding properties of the delivery system. Cell lines SKOV3 (ATCC, #HTB 77) and SKOV3-F7 (overexpressing CS-E) showed strong (peri)cellular CS-E expression while cell line HFF1 (ATCC, #SCR-1041) showed no CS-E expression (Supplementary data, Figure S1). Cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM)-glutamax (Gibco, ThermoFisher scientific, Waltham, MA, USA) containing 10% fetal bovine serum (PAN Biotech, Aidenback, Germany) (v/v) and 100 I.U/ml penicillin and 100 µg/ml streptomycin (Amresco, Solon, OH, USA), at 37° C in a humidified incubator in a 5% CO2 atmosphere. When 80% confluency was reached, cells were dissociated using 0.05% trypsin (w/v) in 0.53 mM EDTA in Hank’s balanced salt solution (Corning Mediatech,
Tweksbury, MA, USA) and maintained as proliferating cultures. Cells were tested for mycoplasma contamination every four months using a MycoAlert™ mycoplasma detection kit (Lonza, Basel, Switzerland). After thawing, cells remained in culture for a maximum of six months.

Cells were cultured in 10-well glass slides in 75 µl medium to ≥90% confluency. Next, cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) in PBS (v/v) for 20 min, blocked with 2% BSA in PBS (w/v) and subsequently incubated for 1 h with 0.1 mg/ml antibody-functionalized lyophilisomes or non-functionalized lyophilisomes. Cells were incubated with 10 µg/ml DAPI for nuclear staining and mounted in Mowiol-488.

**Binding to immobilized chondroitin sulfate E**

To evaluate the specificity of the antibody-functionalized lyophilisomes for CS-E, the following glycosaminoglycans were coated onto a 10-well glass slide (Thermo Fisher Scientific, Waltham, USA) all at 0.1 mg/ml: CS-A (from bovine trachea, Sigma-Aldrich), CS-B (dermatan sulfate, from porcine intestinal mucosa, Celsus Laboratories Inc.), CS-C (from shark cartilage, Sigma-Aldrich), CS-D (from shark cartilage, Seikagaku), CS-E (from squid cartilage, Seikagaku), heparin (from porcine intestinal mucosa, Sigma-Aldrich), and heparan sulfate (from bovine kidney, Sigma-Aldrich). After blocking with 2% BSA in PBS (w/v), glass slides were incubated for 1 h at room temperature with either antibody-functionalized lyophilisomes or non-functionalized lyophilisomes (0.1 mg/ml), or GD3G7 antibodies as control. Thereafter, glass slides were rinsed in PBS and mounted in Mowiol-488 (Calbiochem, La Jolla, CA, USA). Lyophilisomes were visualized using their FITC label.

Analyses were performed using a Leica DM6000B fluorescent microscope. Image processing was performed using ImageJ 1.48v (National Institutes of Health, USA). For visualization purposes, brightness and contrast were adjusted similarly for all images including the controls.

**Visualization of chondroitin sulfate E**

The expression of CS-E was visualized by immunofluorescence or by applying the avidin-biotin complex method.20 In brief, slides were incubated with the GD3G7 antibody (1:5) after blocking, followed by a mouse anti-VSV antibody (clone P5D4, 1:10) and either a goat anti-mouse IgG Alexa Fluor 488 (Life Technologies, 1:500) or biotinylated horse-anti-mouse IgG antibody (Vector Laboratories Inc., CA, USA, 1:200) and ABC reagent (Vectastain ABC anti-mouse-IgG kit, Vector Laboratories Inc.).

**Evaluation of cytotoxic potential of antibody-functionalized lyophilisomes**

**Cytotoxicity analysis in vitro**

The cell eliminating potential of doxorubicin loaded, antibody-functionalized lyophilisomes was investigated using a cell viability assay. To mimic a CS-E rich extracellular matrix, 96-wells cell culture plates (Corning Costar, NY, USA) were coated overnight with CS-E (0.1 mg/ml). The next day, non-immobilized CS-E was removed by washing with PBS and wells were incubated with either: (1) culture medium, (2) Caelyx (PEGylated liposomal doxorubicin, Janssen-Cilag B.V., Tilburg, the Netherlands), (3) free doxorubicin (Accord Healthcare, Utrecht, the Netherlands), (4) empty non-functionalized lyophilisomes, (5) empty antibody-functionalized lyophilisomes, (6) doxorubicin loaded non-functionalized lyophilisomes and (7) doxorubicin loaded, antibody-functionalized
lyophilisomes, all preparations were diluted in culture medium. Doxorubicin concentration was 20 µM and the amount of lyophilisomes with or without doxorubicin were equal. After incubation for 1 h at 37 °C, wells were washed with culture medium. Subsequently, 5000 SKOV3-F7 cells were seeded in 100 µl medium and cell viability was measured after 5 days. Wells were washed with medium and 100 µl medium containing 10% (v/v) Alamar blue reagents (ThermoFisher Scientific) was added with an incubation time of 4 h at 37 °C. Cell viability was measured by fluorescence (excitation at 570 nm, emission at 585 nm) using a Synergy BioTek 2 Plate reader (BioTek, Winooski, VT, USA).

**In vitro doxorubicin release**

To determine whether lyophilisomes can eliminate cells by releasing doxorubicin, but without entering cells, an in vitro experiment using 2 kDa cut-off membranes (Slide-A-Lyzer®MINI dialysis units 2,000 MWCO, Thermo Scientific) was conducted. Free doxorubicin will pass the membrane, but doxorubicin in lyophilisomes or liposomes will not. 5000 SKOV3-F7 cells were seeded in the upper compartment of the membrane, while in the lower compartment the following conditions were added: (1) culture medium, (2) 5 or 20 µM Caelyx (liposomal doxorubicin), (3) 5 or 20 µM free doxorubicin, (4) lyophilisomes, (5) 5 or 20 µM doxorubicin in lyophilisomes, (6) PBS and (7) 5 µM doxorubicin in lyophilisomes in PBS. The amount of doxorubicin loaded and empty lyophilisomes was equal. After 48 h, 30 µl medium containing 30% (v/v) Alamar blue was added to the cells, with an incubation time of 4 h at 37 °C. Cell viability was measured as described.

**Visualization of cellular doxorubicin uptake**

To visualize uptake of doxorubicin from lyophilisomes into cells (nuclei), 5000 SKOV3F7 cells were cultured on a glass slide overnight and subsequently incubated with either free doxorubicin, Caelyx (liposomal doxorubicin) or lyophilisomes with or without 5 µM doxorubicin for 24 h. Finally, cells were washed once with PBS and fixed with 4% paraformaldehyde (Sigma-Aldrich) in PBS (v/v) for 10 min. Nuclei were stained with 10 µg/ml DAPI in PBS (w/v) for 10 min and cells were mounted in Mowiol-488. Analyses were performed using a Leica DM6000B fluorescent microscope.

**Statistical analysis**

Statistical analyses were tested by one-way analysis of variance (ANOVA) with posthoc Bonferroni’s Multiple Comparison Test using Graphpad Prism version 5.03 (Graphpad software, La Jolla, CA, USA). All tests were two-sided and p-values < 0.05 were considered significant. All experiments were performed at least three times independently.

**Results**

**Generation and evaluation of antibody-functionalized lyophilisomes**

Because random conjugation of single chain antibodies to albumin lyophilisomes using classical 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/N-Hydroxysuccinimide (EDC/NHS) chemistry carries a risk of modifying amino acids that are crucially involved in antigen binding, we chose to
develop a method of controlled and site-specific conjugation using sortagging (Figure 1). GD3G7 antibodies harboring the LPETG sortase consensus motif (molecular weight of ~28.5 kDa) could be readily produced and purified and were labeled with PEG-DACO (Figure 2A). The product was analyzed by a click reaction between DBOC and azido-cyanine 7.5, and in line with expectation, was only seen for GD3G7-PEG-DACO as was demonstrated by SDS-PAGE analysis (lane 3 in Figure 2A; note that the molecular weight of the GD3G7-DACO-N\textsubscript{3}-cyanine 7.5 is slightly lower than the unreacted GD3G7-LPETG-His-VSV in lane 2, due to loss of the G-His-VSV tag during the sortase reaction).

**Figure 2.** Evaluation of antibody-functionalized lyophilisomes.

A. SDS-PAGE evaluation of sortase mediated ligation of amino-PEG4-DACO to the GD3G7 antibody harboring a LPETG motif, using azido-cyanine 7.5 labeling. Coomassie brilliant blue staining and visualization in the 800 nm channel of GD3G7-PEG4-DACO and unmodified GD3G7 antibodies after azido-cyanine 7.5 labeling. **B.** Median fluorescent intensity (MFI) of azide-conjugated lyophilisomes and non-conjugated lyophilisomes after DBOC-IR dye 680RD labeling assessed by flow cytometry. **C.** Absorbance of DBOC-modified GD3G7 antibodies conjugated to azide-conjugated lyophilisomes detected by the binding of peroxidase-conjugated protein A. Unmodified GD3G7 antibodies and non-conjugated lyophilisomes were included as controls; Bars represent mean ± standard deviation (N = 3). *** indicates a statistical significant difference with p < 0.001.
The conjugation of amino-PEG₄-azide to the surface of lyophilisomes was evaluated using flow cytometry. After incubation with DBCO-IR dye 680RD, which only binds azide modified lyophilisomes, the median fluorescent intensity of azide-conjugated lyophilisomes was 631 (95% confidence interval [CI]: 578-684) compared to 26 (95% CI: 21-30) for non-conjugated lyophilisomes (p < 0.001) (Figure 2B). Conjugation was further evaluated by using peroxidase-conjugated Protein A, which binds to single chain antibodies of the V₃ class, as is antibody GD3G7. High levels of Protein A bound to antibody-functionalized lyophilisomes compared to non-functionalized lyophilisomes [0.71 (95% CI: 0.53-0.89) vs 0.19 (95% CI: 0.11-0.27), p<0.001] (Figure 2C).

Loading of lyophilisomes with doxorubicin resulted in a mean entrapment efficiency of 28% (95% CI: 18-38%), which corresponds with a mean drug loading of 0.35 mg doxorubicin / mg lyophilisomes (95% CI: 0.23-0.48).

**Specificity of antibody-functionalized lyophilisomes**

Targeting properties of antibody-functionalized lyophilisomes were analyzed in patient derived ovarian cancer tissues (Figure 3A-B). Cryosections of high grade serous ovarian carcinomas were incubated with antibody-functionalized lyophilisomes and non-functionalized lyophilisomes. An abundancy of antibody-functionalized lyophilisomes associated with the cancer stroma (containing CS-E), whereas almost no binding to ovarian cancer epithelial cells was observed. Non-functionalized lyophilisomes showed background reactivity with both the ovarian cancer stroma and epithelial cells (Figure 3A). Specificity of binding was assayed by pretreatment of ovarian cancer sections with the chondroitin sulfate degrading enzyme chondroitinase-AC. Enzymatic treatment abolished the reactivity of antibody-functionalized lyophilisomes with the ovarian cancer stroma (Figure 3A).

The specificity of antibody-functionalized lyophilisomes was further analyzed in vitro using two human ovarian cancer cell lines producing CS-E (SKOV3 and SKOV3F7) and a cell line not producing CS-E (HFF1 cells, human foreskin fibroblasts) (Figure 3B). The antibody-functionalized lyophilisomes showed strong reactivity with the CS-E producing cell lines (SKOV3 and SKOV3F7) compared to the CS-E-negative cell line HFF1. The non-functionalized lyophilisomes showed limited reactivity with any type of cell line.

Finally, the specificity of the antibody-functionalized lyophilisomes for various glycosaminoglycans was determined. Antibody-functionalized lyophilisomes showed strong reactivity with the highly sulfated CS-E subtype (Figure 3C), while only background signal was observed with other immobilized glycosaminoglycans including CS-A, CS-B (also known as dermatan sulfate), CS-C, CS-D, heparan sulfate and heparin. The non-functionalized lyophilisomes showed no significant reactivity with any type of glycosaminoglycan (Supplementary data, Figure S2).
**Figure 3.** Specificity of antibody-functionalized lyophilisomes for highly sulfated chondroitin sulfate E (CS-E).

A

- ChAC-, Ab-functionalized lyophilisomes
- ChAC+, Ab-functionalized lyophilisomes
- H&E staining ovarian carcinoma
- ChAC-, non-functionalized lyophilisomes
- ChAC+, non-functionalized lyophilisomes

B

- SKOV3F7, Ab-functionalized lyophilisomes
- SKOV3, Ab-functionalized lyophilisomes
- HFF1, Ab-functionalized lyophilisomes
- SKOV3F7, non-functionalized lyophilisomes
- SKOV3, non-functionalized lyophilisomes
- HFF1, non-functionalized lyophilisomes
Targeting the extracellular matrix of ovarian cancer using functionalized, drug loaded lyophilisomes

Cytotoxic potential of drug loaded, antibody-functionalized, lyophilisomes in vitro

A CS-E rich environment was created to assess the effect of doxorubicin loaded antibody-functionalized lyophilisomes on ovarian cancer cells. As shown in Figure 4A, viability of cells cultured in CS-E coated wells that were pre-incubated with doxorubicin loaded antibody-functionalized lyophilisomes was decreased to 30% (95% CI: 22-39%), and was significantly lower compared to the cell viability after pre-incubation with doxorubicin loaded non-functionalized lyophilisomes, free doxorubicin, and Caelyx (liposomal doxorubicin) of 77% (95% CI: 65 – 89%), 70% (95% CI: 65 – 75%), and 103% (95% CI:97 – 109%), respectively (p < 0.001). Empty lyophilisomal control conditions (functionalized or non-functionalized) did not affect cell viability.

Mode of doxorubicin release and nuclear localization

To study whether cell death occurs by extracellular release of doxorubicin from lyophilisomes or by cellular uptake of doxorubicin loaded lyophilisomes, an in vitro study using 2 kDa cut-off membranes was performed. 2 kDa membranes allow free doxorubicin (544 Da) to pass, but not larger components such as lyophilisomes-associated doxorubicin, Caelyx (liposomal doxorubicin), cells, and enzymes. Free doxorubicin and doxorubicin loaded lyophilisomes in culture medium containing 10% FBS decreased cell viability in the upper compartment to 46.7% (95% CI: 39.4 – 54.0%, p<0.001) and 39.5% (95% CI: 25.9 – 53.1%, p<0.001), respectively (Figure 4B). In contrast, equal doses of Caelyx and empty lyophilisomes did not significantly affect cell viability, 97.9% (95% CI: 83.3 – 112.4%) and 93.9% (95% CI: 74.6 – 113.2%), respectively. Moreover, a lower dose of 5 µM of free doxorubicin and doxorubicin in lyophilisomes significantly affected cell viability, whereas the
Figure 4. Cell viability and doxorubicin release \textit{in vitro}.

\textbf{A}. A chondroitin sulfate E (CS-E) rich environment was pretreated with either doxorubicin loaded lyophilisomes with/without antibody functionalization, free doxorubicin or Caelyx (liposomal doxorubicin), all preparations containing 20 µM doxorubicin. After washings, ovarian tumor cells (SKOV3F7) were cultured and viability was assayed. Antibody-functionalized lyophilisomes with doxorubicin were most effective in eliminating ovarian cancer cells.

\textbf{B}. Viability of SKOV3F7 cells cultured on a 2kDa dialysis membrane separating them from a lower compartment containing various conditions. Doxorubicin loaded lyophilisomes and free doxorubicin were equally effective, whereas Caelyx (liposomal doxorubicin) did not affect the cell viability.

\textbf{C}. Nuclear localization of lyophilisomal derived doxorubicin \textit{in vitro}. SKOV3F7 cells were incubated for 24 h either with free doxorubicin, Caelyx (liposomal doxorubicin), empty lyophilisomes (depicted in green) or doxorubicin loaded lyophilisomes (depicted in red). Nuclei were stained blue with DAPI. Free doxorubicin and released doxorubicin from lyophilisomes localized to the nuclei (colored red). *** indicates a statistical significant difference with non-asteriks marked conditions with p < 0.001. Bars represent mean ± standard deviation (N = 3). Scale bar represents 100 µm.
same concentration of Caelyx did not; interestingly, doxorubicin loaded lyophilisomes in PBS did not significantly affect cell viability (Supplementary data, Figure S3).

After incubation of cells with lyophilisomes loaded with doxorubicin, the drug localized to the nuclei, the site of action (Figure 4C). This was also observed with free doxorubicin, but not with Caelyx under the same conditions (24 h of incubation).

**Generality of CS-E expression in the stroma of solid cancers**

In order to explore the stromal CS-E expression associated with various solid cancers other than ovarian, we immunohistochemically analyzed five samples of lung, cervical, breast, renal cell, endometrial, and colon cancer for the expression of CS-E. In the vast majority of each cancer type, stromal overexpression of CS-E was observed (Figure 5).

**Figure 5.** Stromal chondroitin sulfate E (CS-E) expression (in red) in various solid cancers including lung cancer, cervical cancer, breast cancer, renal cell cancer, endometrial cancer, and colon cancer. Scale bar represents 100 µm.

**Discussion**

In the current study, we have evaluated the feasibility of a novel concept of therapeutic tumor targeting based on an ECM-targeting drug delivery system. Largely neglected, the cancer ECM provides potential targets for therapy and may have advantages over targeting cancer cells. Due to intra-tumoral heterogeneity, targeted therapies against cancer cell specific targets may act only on a subpopulation of cancer cells whilst other subpopulations not expressing the target, and cancer-associated stromal cells (i.e. fibroblasts and endothelial cells), are left unaffected.\(^{21}\) Cancer-associated stromal cells have been identified as significant contributors to cancer growth and dissemination, and the additional elimination of these cells may benefit clinical outcome.\(^{30, 31}\) Furthermore, expression of cancer cell specific targets may change over time resulting in resistance to the applied targeted therapy.\(^{13}\) Targeting anti-cancer drugs to the more stable ECM may be
helpful to overcome these hurdles.

The potency of ECM-targeting therapies is supported by recent studies which have demonstrated that delivering anti-cancer drugs to the tumor stroma can successfully eliminate tumor cells and their micro-environment in vivo. However, the approaches that were used in these studies were of limited translational value because these were not specific for the cancer stroma. Because the antibody-functionalized lyophilisomes in our study target a unique cancer-specific stromal antigen, this may result in a more specific tumor targeting with concomitant less exposure to healthy surrounding tissues.

Here we report that antibody-functionalized lyophilisomes, generated by a stable and specific bioorthogonal click reaction between GD3G7-PEG₄-DBCO and azido-functionalized lyophilisomes, have specific binding properties for the ovarian cancer stroma rich in CS-E motifs. In addition and focusing on targeting characteristics, ovarian cancer cells in a CS-E rich micro-environment were efficiently eliminated by antibody-functionalized lyophilisomes loaded with doxorubicin, in contrast to non-functionalized lyophilisomes and Caelyx (liposomal doxorubicin), the latter being a commonly used second line chemotherapeutic for the treatment of ovarian cancer. These results indicate the potential of the ECM-targeting drug delivery system as a novel class of targeted therapy for the treatment of ovarian cancer.

To achieve specific stroma-targeting of the drug delivery system, we applied a two-step approach comprising sortase mediated ligation and bioorthogonal click chemistry. First, we equipped single chain GD3G7 antibodies with a click chemistry handle using the recently published sortagging approach. Sortase A, a transpeptidase from Staphylococcus aureus, recognizes a LPXTG motif and catalyses cleavage between threonine and glycine residues forming an intermediate complex. The complex is then resolved by a nucleophilic attack of the N-terminal protein thus forming a covalent bond. Recently, it has been demonstrated that amine groups of other compound (e.g. amino-PEG) can be substrate for the LPETG-sortase intermediate thus offering a versatile toolbox for sortase A catalyzed ligations in protein engineering. By positioning the LPETG sequence upstream of purification tags, these tags can be replaced with handles for click chemistry, while allowing convenient and rapid purification of the reaction product from unreacted proteins and by-products of the reaction. Using this system we generated GD3G7-PEG₄-DBCO with high purity. Of note, because the click handle is positioned at the extreme carboxyterminal end, there is a minimal risk of affecting antibody affinity, unlike the widely used approach of EDC/NHS-mediated conjugation.

Lyophilisomes were used here as biocapsules for doxorubicin, and represent an attractive class of drug delivery systems for ECM-targeting since they can be prepared in a wide range of size (100 nm - 10 µm), thereby being eligible for systemic as well as intraperitoneal administration. Ovarian cancer has a unique tumor biology and metastatic spread pattern since it is usually confined to the peritoneal cavity. Standard treatment regimes include debulking surgery and systemic chemotherapy. As these patients especially suffer from extensive abdominal metastases, delivering adjuvant chemotherapeutics into the peritoneal cavity in addition to systemically administered chemotherapy, has been shown to significantly improve survival rates of ovarian cancer patients and is currently the standard of care for a selected group of patients. Lyophilisomes can be prepared with a diameter as small as 100-200 nm allowing them to extravasate from leaky intratumoral vessels and accumulate in the cancer interstitium (enhanced permeability and
Targeting the extracellular matrix of ovarian cancer using functionalized, drug loaded lyophilisomes

Intense stromal CS-E expression has been associated with various ovarian cancer subtypes including low grade and high grade serous, clear cell, and low grade and high grade endometrioid cancer. In this study we showed that CS-E was highly upregulated in the stroma of a variety of solid tumors including breast cancer, endometrial cancer, cervical cancer, lung cancer, colon cancer, and renal cell cancer. Accordingly, delivery systems targeting CS-E may be applicable to a large and diverse group of cancers.

Release of drugs from stroma-targeting lyophilisomes is essential to eliminate cells. We demonstrated that doxorubicin loaded lyophilisomes in fetal bovine serum enriched culture medium release a substantial part of their drug resulting in nuclear localization of the drug (the site of action) and ovarian cancer cell death, in contrast to (Caelyx) liposomal doxorubicin that did not affect cell viability. This release cannot be explained by simple diffusion as the release in a neutral buffer (PBS) was minimal and did not result in cell death. Albumin represents an important transport protein and is known for its non-covalent reversible ligand-binding capacity. Moreover, it has several binding sites for hydrophobic components which may explain the affinity of albumin for doxorubicin, maintaining the drug within lyophilisomes and which may contribute to the high drug loading capacity compared to Caelyx (0.35 mg doxorubicin / mg lyophilisomes vs 0.125 mg doxorubicin / mg liposomes). We hypothesize that hydrophobic components (e.g. fatty acids) or free albumin in serum-enriched medium may compete with the albumin-doxorubicin binding and lead to drug release. In addition, proteolytic enzymes in the cancer stroma may contribute to the degradation of the albumin lyophilisomes thus enabling release of its payload. In order to enhance drug release, antibody-functionalized lyophilisomes hold potential for additional functionalization, e.g. by the incorporation of substrates for proteolytic enzymes upregulated in the ovarian cancer ECM, into the albumin wall.

**Conclusion**

In this study, we constructed and evaluated a drug delivery system targeting the cancer-associated stroma, based on albumin lyophilisomes loaded with doxorubicin and functionalized with antibodies to highly sulfated chondroitin sulfates. The delivery system may contribute to a novel class of therapy, based on addressing specific components in the extracellular matrix of tumors.
References

44. Martin F. Food and Drug Administration. 2016.
**Supplementary data**

**Figure S1.** *In vitro* expression of CS-E, defined by the GD3G7 antibody.

Cell lines SKOV3F7 and SKOV3 showed strong (peri)cellular expression of the epitope while the cell line HFF1 showed no expression. Scale bar represents 100 μm.
**Figure S2.** Reactivity of antibody-functionalized lyophilisomes and non-functionalized lyophilisomes with various immobilized glycosaminoglycans.

<table>
<thead>
<tr>
<th>GD3G7-antibodies</th>
<th>Ab-functionalized lyophilisomes</th>
<th>Non-functionalized lyophilisomes</th>
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<tr>
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<td>CS-B</td>
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<tr>
<td>CS-E</td>
<td><img src="image9" alt="Image" /></td>
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**Figure S2.** Continued

Antibody-functionalized lyophilisomes showed strong reactivity with CS-E while no reactivity was observed with other glycosaminoglycans including CS-A, CS-B (also known as dermatan sulfate), CS-C, CS-D, heparan sulfate (HS), and heparin. No reactivity with glycosaminoglycans was observed for the non-functionalized lyophilisomes. GD3G7 antibody staining was performed as control and showed strong reactivity with CS-E while no reactivity with other glycosaminoglycans was observed. Abbreviations: Ab, antibody. Scale bar represents 100 µm.

**Figure S3.** *In vitro* doxorubicin release and cytotoxicity analysis.

Cell viability of SKOV3F7 cells cultured on a 2kDa dialysis membrane containing different conditions (equivalent to 5 µM doxorubicin) in the lower compartment. Cells cultured with free doxorubicin that was able to diffuse through showed the lowest cell viability (21.9% [95%CI: 9.2 – 34.6%]). Moreover, doxorubicin loaded lyophilisomes in medium significantly affected cell viability (51.7% [95%CI: 51.7 – 79.4%]), while non-loaded lyophilisomes in medium or doxorubicin loaded lyophilisomes in PBS did not affect the cell viability. **p < 0.01. *** p < 0.001. Bars represent mean ± standard deviation (N = 3).
Chapter 6

General discussion and future outlook

Sophieke C.H.A. van der Steen
The extracellular matrix in ovarian cancer

Epithelial ovarian cancer is characterized by a high mortality rate which has not substantially decreased over the last decades.\textsuperscript{1,2} Often, ovarian cancer is described as an elusive disease: the molecular pathway of ovarian cancer development is largely unexplained, symptoms of ovarian cancer manifest only in an advanced stage of disease, and therapeutic strategies are generally ineffective to completely eradicate the disease. A better understanding of molecules and mechanisms involved in the (early) carcinogenesis is fundamental in identifying targets useful for the development of novel avenues regarding prevention, diagnosis, and treatment. The cancer-associated microenvironment, especially glycosaminoglycans (e.g. chondroitin sulfates) located in the extracellular matrix (ECM), may provide new leads in this respect.

Chondroitin sulfates orchestrate ovarian cancer development

Chondroitin sulfates are molecules with a highly dynamic nature and play an active role in various signaling pathways involved in physiological and pathological processes. The highly sulfated chondroitin sulfate (CS-E) has been identified as a characteristic component of the ECM in solid carcinomas including ovarian cancer while almost not expressed in healthy tissues. We suggest that the upregulation of CS-E is involved in a common carcinogenic mechanism since stromal overexpression of CS-E has been associated with various distinct molecular pathways of cancer development and progression, e.g. low grade and high grade serous ovarian cancer, breast cancer, and lung cancer.

Because of their involvement in cancer growth and dissemination, chondroitin sulfates (especially highly sulfated chondroitin sulfates) have gained interest as potential targets for anticancer therapy. Nonetheless, the exact role of chondroitin sulfates in cancer remains a matter of debate. Although various studies have provided evidence that CS-E plays a promoting role in (ovarian) cancer progression, it could also be suggested that the stromal upregulation of CS-E may represent a host reaction against cancer growth. Solid evidence for a fundamental role of CS-E in the (ovarian) carcinogenesis may be critical to determine its potential therapeutic value. Recently, it was demonstrated that silencing the gene responsible for the biosynthesis of CS-E (chondroitin sulfate sulfotransferase 15, \textit{CHST15}) by small interfering RNA (siRNA) resulted in strongly reduced pancreatic cancer growth \textit{in vivo}.\textsuperscript{3} Additional evidence for the intrinsic carcinogenic role of CS-E may be obtained by studying cancer growth in \textit{CHST15} knockout mice that completely lack the biosynthesis of CS-E.\textsuperscript{4} The evaluation of cancer growth after the intraperitoneal administration of ovarian cancer cells may eventually determine whether highly sulfated chondroitin sulfate in the ECM is indeed of importance for cancer growth, and whether its absence may limit (or even prevent) ovarian cancer development. If so, a wide range of patients with (ovarian) cancer might benefit from CS-E targeting therapies in the future. Beside, \textit{CHST15} knockout mouse mice were observed to function normally, except for a decreased activity of bone marrow-derived mast cells.\textsuperscript{4} These
findings may suggest a low probability for severe adverse effects associated with CS-E targeting strategies, that supports its feasibility for a clinical application.

CS-E characterizes the remodeled matrix in pre-invasive ovarian cancer

The interplay between cancer cells and the ECM has been recognized as a major determinant in cancer development, but its involvement in pre-invasive (carcinoma in situ) lesions is largely unknown.5-7 Our findings demonstrate the involvement of ECM molecules (i.e. highly sulfated chondroitin sulfate) in the progression of pre-invasive serous tubal intraepithelial carcinoma (STIC) into invasive high grade serous carcinoma (HGSC). The de-novo-synthesis of other cancer-promoting ECM molecules versican and tenascin has been previously observed in precursor lesions of breast cancer (ductal carcinoma in situ), and supports the significance of ECM-changes associated with early (pre-invasive) cancer development.8-11

Since increased stromal CS-E expression in STIC may reflect progression into invasive pelvic HGSC, we address in this thesis CS-E as a novel class of molecular biomarkers and targets of HGSC precursor lesions, useful for screening and prevention purposes. Today, the most appropriate clinical management (e.g. surgical staging, adjuvant chemotherapy, or expectative) of patients diagnosed with isolated STIC remains uncertain, since there is a significant risk for the development of pelvic HGSC afterwards.12 In addition, there are currently no prognostic handles available to aid clinical decision making in patients with isolated STIC. Staining STIC for CS-E expression might be helpful to obtain more information about the level of progression, nevertheless, the biomarker potential of CS-E for early cancer progression and the feasibility of this epitope for the use in clinical practice should be determined in future studies. In this perspective, a large cohort of patients diagnosed with an isolated STIC should be analyzed for the expression of CS-E to evaluate the association between stromal CS-E expression in STIC and the development of invasive HSGC afterwards. Since the incidence of an isolated STIC has been reported to be very low, strong (international) collaborations are required to succeed.

Regarding the development of novel screening purposes for the identification of early (pre-invasive) stages of ovarian cancer, the class of glycosaminoglycans is eligible for the use as a diagnostic target. Chondroitin sulfates are usually secreted in bodily fluids and may be detected in blood, urine, cervix smear, or uterine lavage.13, 14 Nonetheless, we expect that levels of CS-E may be marginally elevated in an early (pre-invasive) stage, thus being difficult to trace. Considering the state of the art of the molecular technology, the identification of genetic mutations (i.e. TP53) for screening purposes seems to have a greater potential due to the powerful technique of DNA amplification.15 In contrast to protein- and nucleic acid-related research, the study of glycosaminoglycans is complex and challenging in part due to technical limitations. At this moment, defining the glycosaminoglycan fine structure (i.e. exact saccharide motif) or amplifying these molecules are beyond the bounds of possibility. Future advances in the molecular glycosaminoglycan-related technology may emerge novel perspectives for clinical applications of chondroitin sulfates.
In addition, the potential of highly sulfated chondroitin sulfate as diagnostic (or therapeutic) target should be further investigated by analyzing the association between CS-E expression and other conditions than cancer. Although we identified restricted expression of CS-E in a wide range of healthy organs including the fallopian tube and the ovary, we cannot absolutely exclude that stromal CS-E upregulation is not involved in other processes associated with tissue remodeling (e.g. wound healing). It is known that the class of chondroitin sulfates have multifaceted functions including tissue regeneration and a role in inflammatory diseases.\textsuperscript{16, 17} In our studies, we incidentally noticed increased stromal CS-E expression in an area of chronic inflammation of the fallopian tube, and in breast tissue wound healing after a fine needle puncture. Exploring the specificity of CS-E expression for various pathological processes associated with ECM remodeling, may further determine the value of CS-E as a target for anti-cancer strategies.

The matrix and immune system, interactions within the pre-invasive micro-environment

The (pre-) cancer microenvironment represents a complex and dynamic network that comprises various players and interactions. In addition to the ECM, the immune system is considered to play a substantial role in the (ovarian) carcinogenesis, marked by infiltrating T cells in the microenvironment of STIC, especially in the stromal compartment.

In the remodeled microenvironment of STIC, CS-E might be involved in the stromal influx of T cells through the binding and sequestering of chemokines. Although our knowledge is limited, CS-E does bind a number of chemokines (i.e. stromal derived factor-1, secondary lymphoid tissue \textit{chemokine, and} interferon gamma-induced protein-10) and selectins (i.e. P- and L-selectin), and is implicated in T-cell differentiation.\textsuperscript{18, 19} Probably, more (still unidentified) interactions do exist. Intense stromal CS-E expression in ovarian cancer has been associated with progression of disease and is considered to have a promoting effect on cancer growth rather than an inhibiting effect. It remains unclear whether the suggested interaction between stromal CS-E and the host anti-cancer response in the (pre)-invasive microenvironment may be in favor of high grade serous cancer growth. A strong infiltration of immune inhibiting regulatory T cells in STIC is associated with CS-E overexpression, in addition to the infiltration of immune promoting helper and cytotoxic T-cell subsets. Future in-depth studies are needed to determine relevant interactions between CS-E and immunological agents and to evaluate its potential effect on T cell signaling pathways related to the immunological anti-cancer response.

Understanding the precise effects of CS-E on tumor infiltrating immune cells may broaden our understanding of interplays within the cancer microenvironment, and may be instrumental for the development of immunological anti-cancer therapies. This promising class of anti-cancer treatments aims to boost the host immune response, e.g. by interfering with immune checkpoint proteins. Immune checkpoint proteins such as cytotoxic T-lymphocyte antigen 4 (CTLA-4) and programmed death receptor 1 (PD-1) are negative regulators on T-cell immune function.\textsuperscript{20} New insights into cancer immunology have recently lead to the development of cancer immune-
therapeutics that inhibit immune checkpoints and subsequently boost T cells to attack cancer cells.\textsuperscript{21, 22} In addition, natural killer cell-based strategies for the treatment of solid carcinomas have gained interest during recent years and may represent another potential class of immunotherapy of cancer.\textsuperscript{23-25} Nevertheless, the anti-cancer activity of natural killer cells in solid carcinomas is often disappointing compared to its effect in hematopoietic cancers, possibly due to a negative impact of the cancer microenvironment on natural killer cell efficacy. Hence, it may be of significant relevance to find out the extent to which stromal changes in (pre)invasive ovarian carcinomas may interfere with the immune microenvironment. If stromal CS-E has a role in specific immune pathways (e.g. recruitment of cytotoxic, regulatory T cells, or natural killer cells), we hypothesize that an additional glycosaminoglycan-interfering therapy might be applied to favorably modulate the effect of immunotherapy in cancer.

**Extracellular chondroitin sulfates as target for anti-cancer therapy**

While significant advances in the development of targeted anti-cancer therapies have been made during the last decades, the area of targeted drug delivery to solid tumors still needs substantial improvement. In this thesis we address an alternative, potential, approach for the treatment of ovarian cancer by targeting the cancer-associated ECM. Targeting the cancer stroma might be helpful to overcome hurdles observed in cancer cell-targeting strategies including intra-tumoral heterogeneity and the intra-tumoral stroma as a barrier against effective tissue penetration of therapeutic agents.\textsuperscript{26-31}

The concept of ECM-targeting lyophilisomes that we designed holds anti-cancer potential, and may serve as a starting point in the development of ECM-targeting strategies for the treatment of ovarian cancer. In addition to the use of lyophilisomes, also other carrier systems may be of interest as its size, shape and surface properties, may significantly affect the biodistribution and immunogenicity, and ultimately influence its efficacy and toxicity. Also, ECM-targeting drug delivery systems might be loaded with other compounds than doxorubicin thus offering versatile possibilities for anti-cancer therapies. For example, the targeted delivery of siRNA silencing oncogene expression, molecules targeting signaling pathways, or a combination of different (synergistic) anti-cancer agents, could possibly be facilitated by ECM-targeting drug delivery systems.\textsuperscript{32} Additional (in vivo) exploration of ECM-targeting strategies is needed to determine future directions regarding the engineering of a highly functional ECM-targeting drug delivery system for the treatment of ovarian cancer.

Nevertheless, even more possibilities for the development of innovative ECM-targeting constructs appear on the horizon, e.g. the generation of antibody drug conjugates by applying a sortase mediated ligation for the conjugation of cytotoxic agents directly to single chain antibodies. It is well known that small particles have slower removal from the circulation and have more effective tissue penetration thus illustrating the potency of such a drug-antibody-conjugate. Release of the conjugated drug after binding to the ECM could be enabled by incorporating a cleavage site for
enzymes located in the cancer-associated ECM, e.g. metalloproteinase 2/9 for ovarian carcinomas.  

In addition to the use of the cancer-associated ECM as a hook for drug delivery, selectively interfering with chondroitin sulfate-protein interactions may be an alternative approach for the treatment of ovarian cancer. A series of preclinical studies indicate that reducing endogenous chondroitin sulfates (i.e. by blocking its biosynthesis or by enzymatic degradation) and interfering with its biological active domain structures (i.e. by blocking antibodies or by chondroitin sulfate mimetics) inhibits the carcinogenic role of chondroitin sulfate by affecting extracellular signaling pathways. At the same time, accumulating evidence from clinical studies suggests that the glycosaminoglycan heparin, principally used as anti-thrombotic therapy in cancer patients, significantly improves survival by directly affecting cancer growth and dissemination. Hence, the identified heparin-induced anti-cancer activity in cancer patients signifies the potency of extracellular glycosaminoglycans as target for anti-cancer therapy. To determine the anti-cancer potency of CS-E interfering therapies, a relatively simple in vivo study may bring us relevant knowledge, e.g. by studying the effect of exogenous CS-E on ovarian cancer growth.

To end, we would like to stress that in the future a large and diverse group of cancer patients may benefit from chondroitin sulfate-targeting therapies since various stromal-rich solid tumors have been associated with overexpression of (highly sulfated) chondroitin sulfate including ovarian and breast cancer. We should realize that this class of therapy may be of insufficient therapeutic value as single anti-cancer agent but may be particularly useful when combined with other treatment modalities (e.g. immune therapy, signaling pathway-targeted therapy, or gene therapy) since synergistic effects have been described.
References


General discussion and future outlook

Chapter 7

Summary / Samenvatting

Sophieke C.H.A. van der Steen
Summary

Epithelial ovarian cancer is the fifth leading cause of cancer death in women worldwide. The high mortality of this disease is related to the usually advanced stage of ovarian cancer at diagnosis and the lack of effective therapies to completely cure this disease.

The development and progression of (ovarian) cancer depend for a major part on the largely neglected cancer microenvironment. Considerable remodeling of the extracellular matrix (ECM) occurs and affects cancer-associated processes such as cell proliferation. The ECM represents a highly organized three-dimensional network of fibrous proteins and proteoglycans interacting with each other and with the surrounding cells. Proteoglycans function to a large extent through their glycosaminoglycan side chains which are linear negatively charged polysaccharides built from repeating disaccharides.

This thesis focuses on chondroitin sulfate (CS), a specific class of glycosaminoglycans abundantly present in the ECM of various types of cancer including ovarian cancer. For a long period, chondroitin sulfates were considered as bystanders in physiological and pathological processes including cancer. However, two decades of research has shown the highly dynamic nature of these molecules and their intrinsic involvement in cancer development and progression. In this thesis, we evaluated the involvement and the potential clinical application of chondroitin sulfates in epithelial ovarian cancer.

In chapter 1, we comprehensively reviewed literature on chondroitin sulfate (proteoglycans) in the (ovarian) cancer micro-environment and discussed its potential clinical relevance in the management of ovarian cancer. In various solid carcinomas, qualitative and quantitative changes in chondroitin sulfate expression in the ECM have been observed. The highly sulfated chondroitin sulfate subtype (CS-E) is associated with the remodeled ECM in carcinomas and rarely expressed in normal organs, while the less sulfated chondroitin sulfate subtypes are usually present in healthy tissues. By the interaction with effector molecules (e.g. growth factors and adhesion molecules), chondroitin sulfates are able to interfere with cell signaling pathways involved in proliferation, adhesion, invasion, migration, angiogenesis, and immnosilencing. Changes in the expression of chondroitin sulfate and its carcinogenic role may provide novel handles for innovative methods for diagnostic and therapeutic targeting. Initial attempts have been made to inhibit the carcinogenic role of chondroitin sulfates or to use these molecules as a micro-environmental hook for targeted therapy.

The identification of biomarkers for epithelial ovarian cancer may be instrumental for the development of new diagnostic and therapeutic modalities. In addition to intrinsic cancer cell characteristics, the ovarian cancer-associated ECM may provide potential biomarkers.

In chapter 2, we investigated the biomarker potential of highly sulfated chondroitin sulfate (CS-E) in ovarian cancer using a single chain antibody selected by phage display technology. The single chain antibody showed specificity for the glycosaminoglycan CS-E as determined by ELISA, the epitope being minimally expressed in a wide range of normal human organs. Intense CS-E expression in the ECM was observed in the majority of ovarian carcinomas including all subtypes,
while restricted expression of CS-E was observed in normal adnexa and benign ovarian tumors. In the most prevalent and most aggressive ovarian cancer subtype (type II carcinomas), the expression of CS-E was found to be independent of FIGO stage and the use of chemotherapy. Further, intense CS-E expression was identified as a significant independent predictor for poor prognosis. Analysis of the CHST15 gene, responsible for the biosynthesis of CS-E, showed no mutations in ovarian carcinomas nor an altered methylation status. To conclude, specific highly sulfated CS motifs expressed in the epithelial ovarian cancer ECM hold biomarker potential, and may contribute to the identification of new clinical strategies.

The origin and pathogenesis of epithelial ovarian cancer has been unexplained for decades. Today, major advances in the understanding of early steps in the ovarian cancer development have emerged, and new prospects for improved screening, prevention, and therapy are arising. The interplay between cancer cells and the ECM, however, has been largely neglected in pre-invasive lesions while this is characterized as a hallmark in cancer development. In chapter 3, we analyzed the expression of CS-E in precursor lesions (i.e. p53 signature, serous tubal intraepithelial lesion (STIL), and serous tubal intraepithelial carcinoma (STIC) – mentioned in order of progression into cancer) of pelvic high grade serous carcinoma (HGSC), a type II ovarian carcinoma. Additionally, we addressed the immunological reactivity in the micro-environment. We found that the expression of stromal CS-E was related to the degree of the tubal epithelium abnormality: intense CS-E expression was observed in the ECM in 3.7%, 57.7%, and 90.6% of STILs, STICs, and invasive HGSCs respectively, while all normal tissues and p53 signatures showed no CS-E expression. Further, intense CS-E expression was significantly associated with an invading immune infiltrate consisting of lymphocytes. In conclusion, specific alterations in the ECM (i.e. CS-E expression) occur early in the development of the pelvic high grade serous carcinoma and may represent a new biomarker of early cancer progression.

The host immunological anti-cancer response in the microenvironment is considered to play a substantial role in ovarian cancer progression, but it is largely unknown whether the immune response is involved in pre-invasive stages of pelvic HGSC. In chapter 4, we evaluated the presence of T cells in STIC, by applying a comprehensive and powerful method of multiplex immunofluorescent staining, high-resolution whole-slide imaging, and quantification of cell subsets by automated image analysis software, and we analyzed associations with clinicopathological parameters. Compared to normal fallopian tubes, STICs were observed to have a significantly increased density of CD3+ T cells, including helper, cytotoxic, and regulatory T-cell subsets. Within subjects, an additional increase of infiltrating cytotoxic and regulatory T cells was observed in invasive HGSC compared to the paired STIC. We observed that the increase of T cells in STIC and HGSC was more significant in the stromal compartment rather than intraepithelial. In addition, increased levels of infiltrating helper, cytotoxic, and regulatory T cells in STIC were significantly associated with the stromal upregulation of CS-E. We concluded that the host immune response occurs in a pre-invasive stage of pelvic HGSC development, and is associated with stromal changes. Our findings may serve as a starting point in our understanding of cancer immunity in STIC and its potential effect on malignant progression.
Considering the poor long-term prognosis of ovarian cancer patients, there is an urgent need for novel therapeutic strategies. In chapter 5, we evaluated an innovative approach for the treatment of ovarian cancer based on an ECM-targeting drug delivery system. We identified CS-E as an attractive molecular target for anti-cancer therapy, since this glycosaminoglycan is abundantly expressed in (ovarian) carcinoma stroma while not expressed in healthy tissues. To achieve specific targeting, we generated an ECM-targeting drug delivery system using a two-step approach. By applying a sortase-mediated ligation and click chemistry, single chain antibodies specific for CS-E were conjugated to lyophilisomes, spherical albumin based biocapsules that can be loaded with doxorubicin. Specificity of the ECM-targeting lyophilisomes was observed for the glycosaminoglycan CS-E. Additionally, ECM-targeting lyophilisomes were observed to bind specifically to the CS-E rich stroma of ovarian carcinoma tissues and binding was abrogated after incubation with a chondroitin sulfate degrading enzyme. In vitro, doxorubicin loaded ECM-targeting lyophilisomes were able to efficiently destroy ovarian carcinoma cells in a CS-E rich micro-environment by release of the doxorubicin which localized to the nucleus. To conclude, the use of extracellular chondroitin sulfates as an anchor for anti-cancer therapy shows potential therapeutic value and may provide new leads in the development of innovative cancer therapies.

In chapter 6, we discussed relevant questions remaining from the abovementioned studies and we elaborated on perspectives for future research.
Samenvatting

Wereldwijd is het ovariumcarcinoom (eierstokkanker) de vijfde oorzaak van sterfte door kanker bij vrouwen. De hoge mortaliteit is gerelateerd aan het meestal vergevorderde stadium van de ziekte ten tijde van de diagnose, en de beperkte mogelijkheden van huidige therapieën om vergevorderde ziekte volledig te genezen.

De ontwikkeling en progressie van het ovariumcarcinoom wordt voor een groot deel beïnvloed door de omgeving van de tumor. De extracellulaire matrix is een belangrijk onderdeel van deze omgeving en is aanzienlijk veranderd in kanker in vergelijking met de matrix in gezond weefsel. Veranderingen in de matrix kunnen verschillende met kanker geassocieerde celprocessen beïnvloeden, zoals proliferatie en adhesie. De extracellulaire matrix is een goed georganiseerd driedimensionaal netwerk van (fibreuze) eiwitten en proteoglycanen, welke met elkaar en met omliggende cellen communiceren. Proteoglycanen functioneren voor een groot deel door middel van hun zijketens, glycosaminoglycanen. Dit zijn lineaire negatief geladen polysacchariden (suikers) bestaande uit zich herhalende disacchariden.

Dit proefschrift richt zich op chondroïtinesulfaat, een specifieke klasse van glycosaminoglycanen welke overvloedig aanwezig is in de extracellulaire matrix van verschillende soorten kanker zoals het ovariumcarcinoom. Lange tijd werd gedacht dat de invloed van chondroïtinesulfaat in verschillende fysiologische en pathologische processen (bijv. kanker) beperkt was. Echter, de laatste twee decennia hebben laten zien dat deze moleculen dynamische eigenschappen bezitten en intrinsiek betrokken zijn bij de ontwikkeling en progressie van kanker. In dit proefschrift onderzochten we de betrokkenheid en mogelijke klinische toepassingen van chondroïtinesulfaat in het ovariumcarcinoom.

In hoofdstuk 1 beschreven we op basis van een uitvoerige literatuurstudie de mogelijke relevantie van extracellulaire chondroïtinesulfaten (en hun proteoglycanen) in de klinische benadering van het ovariumcarcinoom. In verschillende vormen van kanker zijn kwalitatieve en kwantitatieve veranderingen in de expressie van chondroïtinesulfaat in de extracellulaire matrix waargenomen. Het hooggesulfateerde chondroïtinesulfaat (CS-E) is geassocieerd met de veranderde matrix in kanker en wordt nauwelijks gezien in gezonde organen. Daarentegen zijn de minder gesulfateerde chondroïtinesulfaten over het algemeen wel aanwezig in gezonde weefsels. Door een interactie met verschillende moleculen (bijv. groeifactoren), zijn chondroïtinesulfaten in staat invloed uit te oefenen op verschillende met kanker geassocieerde processen zoals proliferatie, adhesie, invasie, migratie, angiogenese en immunologische respons. Veranderingen in de expressie van chondroïtinesulfaat en hun rol in de carcinogenese kunnen nieuwe uitgangspunten vormen voor de ontwikkeling van innovatieve diagnostische en therapeutische benaderingen. Zo zijn recentelijk pogingen gedaan om de carcinogene eigenschappen van chondroïtinesulfaat te remmen of te blokkeren, en om deze moleculen in de omgeving van de tumor te gebruiken als target voor medicijnen gericht tegen kanker.

Het identificeren van biomarkers voor het ovariumcarcinoom is van belang voor het ontwikkelen van nieuwe diagnostische en therapeutische toepassingen. Naast eigenschappen van de kankercel...
zelf, kan ook de extracellulaire matrix van de tumor als biomarker fungeren. In hoofdstuk 2 hebben we onderzoek gedaan naar biomarkerwaarde van hoog gesulfateerd chondroitinesulfaat (CS-E) voor het ovariumcarcinoom. We hebben hiervoor gebruik gemaakt van een antilichaam met slechts één enkele keten, welke geselecteerd werd door de faagdisplay techniek. Dit antilichaam toonde specificiteit voor het glycosaminoglycaan CS-E dat we vastgesteld hebben middels ELISA. Het epitoot dat door het antilichaam wordt herkend, wordt nauwelijks tot expressie gebracht in verschillende normale organen. In de matrix van goedaardige ovariumtumoren werd beperkte CS-E expressie gezien terwijl in de meerderheid van verschillende soorten ovariumcarcinoom sterke CS-E expressie werd gezien. In het meest voorkomende en meest agressieve subtype ovariumcarcinoom (type II), was de expressie van CS-E onafhankelijk van het FIGO stadium en het gebruik van chemotherapie. Sterke CS-E expressie in het type II ovariumcarcinoom was een onafhankelijke voorspeller van een slechte prognose. De analyse van het CHST15 gen, dat verantwoordelijk is voor de biosynthese van CS-E in ovariumcarcinomen, toonde geen mutaties noch een veranderde methylering. Concluderend, hoog gesulfateerd chondroitinesulfaat in de extracellulaire matrix vormt een biomarker voor het ovariumcarcinoom en kan bijdragen aan de ontwikkeling van nieuwe klinische strategieën voor deze ziekte.

De ontstaanswijze van het ovariumcarcinoom was decennia lang een raadsel, maar inmiddels zijn er grote stappen vooruit gezet in de inzichten over de vroege ontwikkeling van het ovariumcarcinoom hetgeen nieuwe perspectieven biedt voor screening, preventie en therapie. De interactie tussen kankercellen en de extracellulaire matrix is nog nauwelijks onderzocht in een pre-invasief stadium ovariumcarcinoom, terwijl deze interactie als fundamenteel wordt beschouwd in de progressie van kanker. In hoofdstuk 3 analyseerden we de expressie van CS-E in de matrix van verschillende voorstadia van het hooggradig sereus ovariumcarcinoom (type II), te weten p53 signature, sereus tubair intra-epitheliale laesie (STIL) en sereus tubair intraepitheliaal carcinoom (STIC) – genoemd in volgorde van toenemende atypie. Daarnaast bestudeerden we de immunologische reactiviteit in de omgeving van de voorloper laesies. We zagen dat de expressie van CS-E in de matrix was gerelateerd aan de mate van atypie van het epitheel in de tuba Fallopii: sterke CS-E expressie werd waargenomen in 3,7% van STILs, in 57,7% van STICs en 90,6% van invasieve hooggradig sereuze carcinomen, terwijl alle in normale weefsels en p53 signatures nauwelijks CS-E expressie werd waargenomen. Daarnaast was er een significant verband tussen sterke expressie van CS-E in de matrix en de aanwezigheid een lymfocyteninfiltraat. Specifieke veranderingen in de extracellulaire matrix (expressie van CS-E) treden op in een vroeg stadium van ontwikkeling van het hooggradig sereus ovariumcarcinoom, en vertegenwoordigen mogelijk een nieuwe klasse van biomarkers voor progressie naar invasie.

Er wordt verondersteld dat de immunologische respons een belangrijke rol speelt in de progressie van het ovariumcarcinoom, het is echter onbekend of de immunologische respons ook betrokken is in een pre-invasief stadium van het hooggradig sereus ovariumcarcinoom. In hoofdstuk 4 onderzochten we de infiltratie van T cellen in STIC door middel van een krachtige methode waarbij we gebruik maakten van zowel multiplex fluorescente kleuringen, als hoge resolutie afbeeldingen van de gehele coupe, als het kwantificeren van de verschillende fenotypes van cellen door
middel van automatische analysesoftware. In STIC werd een hogere dichtheid van CD3⁺ T cellen waargenomen dan in normale tubae Fallopii, waaronder een toename van helper, cytotoxische en regulatoire T cellen. Het aantal cytotoxische en regulatoire T cellen was nog meer toegenomen in het invasieve carcinoom wanneer vergeleken met de geassocieerde STIC van dezelfde patiënt. De infiltratie van T cellen was groter in het stromale compartiment dan in het epitheliale compartiment. Daarnaast was de infiltratie van helper, cytotoxische en regulatoire T cellen in STIC geassocieerd met sterke expressie van CS-E in de extracellulaire matrix. We concludeerden dat de immunologische respons al in een pre-invasief stadium van het hooggradig sereus ovariumcarcinoom optreedt en is geassocieerd met stromale veranderingen in STIC. Onze bevindingen vormen een uitgangspunt voor het beter begrijpen van de immunologische response in pre-invasieve lesies van het ovariumcarcinoom en het effect hiervan op verdere progressie.

Gezien de slechte prognose voor patiënten met ovariumcarcinoom is er behoefte aan nieuwe en meer effectieve therapeutische strategieën. In hoofdstuk 5 evalueerden we een innovatieve benadering voor de behandeling van het ovariumcarcinoom. De basis van deze nieuwe strategie omvat een geneesmiddelafgiftesysteem dat doelgericht naar de extracellulaire matrix wordt gebracht. We identificeerden CS-E als een aantrekkelijk target voor kankertherapie, omdat deze moleculen overvloedig voorkomen in de matrix van het ovariumcarcinoom en nauwelijks in gezonde weefsels. Om specifieke targeting te bereiken, ontwikkelden we een geneesmiddelafgiftesysteem met specificiteit voor CS-E. Door middel van een door sortase-gemedieerde ligatie en vervolgens klikchemie, werden antilichamen met één enkele keten gericht tegen CS-E aan lyophilisomen geconjugeerd. Lyophilisomen zijn biologisch afbreekbare biocapsules van albumine en kunnen worden opgeladen met cytostatica. Matrix-targeting lyophilisomen vertoonden specificiteit voor CS-E en toonden geen reactiviteit met andere glycosaminoglycanen. Ook werd sterke reactiviteit waargenomen met de CS-E rijke extracellulaire matrix van ovariumcarcinoomweefsel, deze reactiviteit was nauwelijks meer aanwezig nadat het weefsel met een chondroitinesulfaatafbrekend enzym was behandeld. Matrix-targeting lyophilisomen in een CS-E rijke micro-omgeving waren in staat ovariumcarcinoomcellen efficiënt te vernietigen door middel van het vrijkomen van doxorubicine. Extracellulair chondroitinesulfaat toont een potentiële therapeutische waarde als een target voor antikankertherapie en draagt mogelijk bij aan de ontwikkeling van innovatieve kankertherapieën.

In hoofdstuk 6 bediscussieerden we verschillende nog openstaande vragen vanuit de bovengenoemde studies en we bespraken we relevante perspectieven voor verder onderzoek.
Appendix

Curriculum Vitae
List of publications
Portfolio
Dankwoord
van der Steen SC, Bulten J, Van de Vijver KK, van Kuppevelt TH, Massuger LFAG. Changes in the extracellular matrix are associated with the development of serous intraepithelial carcinoma (STIC) into high grade serous carcinoma. Int J Gynecol Cancer 2017; in press.


van der Steen SC, de Nieuwenhof HP, Massuger L, Bulten J, de Hullu JA. New FIGO staging system of vulvar cancer indeed provides a better reflection of prognosis. Gynecol Oncol 2010;119:520-5.
# Portfolio

**Graduate School Radboud Institute for Molecular Life Sciences (RIMLS)**

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<th><strong>Courses &amp; Workshops</strong></th>
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* ECTS, European Credit Transfer System  
** Post Academic Education
Dankwoord

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