Glycosaminoglycans in cancer treatment
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ABSTRACT
Studies aimed at the identification of biomarkers and treatment targets of cancer have focused on mRNAs, miRNAs, and proteins expressed by malignant cells, while glycoproteins mainly produced by stromal cells remain relatively unexplored. Glycans lack a given template for their biosynthesis that involves the concerted action of several, sometimes >15 different enzymes. This fact complicates the analysis at the genomic level of the role of glycoproteins in clinical oncology. The glycosaminoglycans (GAGs) stand out as highly polyanionic components at the surface of malignant and stromal tumor cells as well as their surrounding matrix. Published data thus describe a multifaceted regulatory role of GAGs and GAG-conjugated proteins, proteoglycans, in e.g. tumor associated angiogenesis, coagulation, invasion, and metastasis. Relatively small, randomized clinical trials suggest that heparin, an over-sulfated variant of the GAG heparan sulfate, may have direct, anti-tumor effects. Several ongoing trials aim at establishing whether heparin and its derivatives should be added to standard treatment of cancer patients or not, based on progression free- and overall survival end-point data. Given the potential bleeding complications with this treatment, other strategies to block GAG function should provide interesting alternatives. In the emerging era of personalized medicine, one can foresee the development of predictive biomarkers to select patients that may benefit from GAG-targeted treatments, aiming at individualized prevention of thromboembolic complications as well as inhibition of tumor development and progression. Here, the role of GAGs as targets and vehicles of cancer treatment is discussed with special emphasis on angiogenesis and coagulation associated mechanisms.

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INTRODUCTION
The initial steps of cancer development, i.e. genetic alterations resulting in the abnormal proliferation and survival of malignant cells, are highly dependent on contextual factors of the pericellular microenvironment [1], such as the glycocalyx [2]. Thus, normal cells acquire a number of capabilities characteristic of most, if not all, tumors through intercellular cross-talk involving diffusible growth factors, cytokines, and exosomes provided by adjacent cells or transmitted through contact with the extracellular matrix (ECM). An early and decisive event during tumor development is insufficient oxygen supply, hypoxia that triggers a metabolic shift and induces processes such as coagulation, angiogenesis, and ECM remodeling [3,4]. A vicious cycle of hypoxia, abnormal angiogenesis and tumor intravascular thrombosis drives local tissue invasion and systemic spread of malignant cells that is responsible for the vast majority of cancer related deaths. This requires altered functional expression of macromolecules involved in cell-cell and cell-matrix interactions as well as ECM remodeling. Here, the glycosaminoglycans (GAGs), take centre stage with their unique ability to regulate a diverse range of molecular activities through highly dynamic interactions in the ECM, and at the surface of stromal as well as malignant tumor cells [5-9]. In addition to these molecular interactions, GAGs of the glycocalyx may play important roles in the mechanotransduction pathways involved in flow regulated tumor invasion and metastasis [2]. This review will discuss some of these activities and interactions, and put emphasis on the still controversial utility of GAGs and related substances as anti-cancer therapeutics.

Glycosaminoglycan biosynthesis and structure
GAG biosynthesis is a well-studied process that takes place in a sequential manner by membrane spanning enzymes residing in the endoplasmic reticulum and the Golgi apparatus. Heparan sulfate (HS), which is the major GAG discussed herein, consists of a -glucuronic acid (GlcA)-galactose-galactose-xylose- linker region that is further elongated by the addition of N-acetyl glucosamine (GlcNAc), followed by the sequential addition of GlcA-GlcNAc disaccharide repeats. As the chain polymerizes, HS can be enzymatically modified at various positions through an ordered manner with the product of one modification serving as substrate for the next. The initial modification enzyme, N-deacetylase/N-sulfotransferase (NDST),
substitutes the N-acetyl group with a sulfate group in between clusters of GlcNAc, leaving regions of the chain unmodified. Further modifications are epimerization of GlcA into iduronic acid (IdoA), 2-O-sulfation of IdoA and more seldom GlcA, and subsequently sulfation at position C6 and, rarely, at C3 of GlcNS. As the HS modification reactions are clustered around the GlcNS-containing disaccharides, a characteristic HS domain structure of highly sulfated stretches (NS domains) interspersed between largely unmodified regions (NA domains) will evolve [9]. Altogether, this biosynthetic machinery results in tremendous diversity. In general, ligand binding depends on the arrangements of NS and NA domains and on the modified residues within the NS domains. Regions consisting of relatively common disaccharides arranged in a specific pattern, as in the case of fibroblast growth factor-2 (FGF-2) and its signaling pathway inhibitor-1 and its signaling receptor, can provide specific interactions. Also, relatively rare modifications can be recognized, as exemplified by the specific, antithrombin binding HS pentasaccharide containing an internal 3-O-sulfation. The binding motifs for the negatively charged HS chains are usually polybasic protein domains containing a stretch of basic amino acids (Table 1). The abundance of such domains and peptide stretches in naturally occurring proteins is consistent with HS binding promiscuity [10]. HS-binding ligands have been clustered into functional interaction networks responsible for the regulation of complex biological processes such as angiogenesis, morphogenesis, ECM assembly, and regulation of the coagulation system. These data support the view of HS-SPGs as key mediators of the assembly of molecular complexes at the cell surface and in the pericellular environment [10].

### Proteoglycans - proteins conjugated with glycosaminoglycans

Sulfated GAGs are normally conjugated with a diverse family of proteins, proteoglycans (PGs), at specific serine-glycine acceptor sites. In fact, compared with the high number of proteins substituted with N-linked or O-linked oligosaccharides, relatively few proteins are classified as PG core proteins. However, all cells investigated, including erythrocytes, appear to contain HS PGs [9]. GAG-conjugated PGs can be found both intracellularly, at the cell surface, and in various niches of the ECM. Basement membrane PGs include perlecan, agrin and collagen XVIII, which are primarily substituted with HS GAGs. Other ECM PGs are mostly substituted with the GAGs chondroitin sulfate (CS), dermatan sulfate (DS) and/or keratan sulfate [11]. The dominating cell surface PGs are the HS substituted families of the membrane spanning syndecans (SDCs) and the glycosyl-phosphatidylinositol (GPI)-anchored glypicans (GPs) [12,13]. There are four SDCs (SDC 1–4) and six GPs (GPC 1–6) identified in mammals. Notably, the distribution of HS chains on cell surface core proteins differs; whereas GPC HS chains are attached to the core protein close to the plasma membrane, SDC HS chains are located at more peripheral sites. Shedding of HS-SPGs from the cell surface represents another level of regulation of HSPG distribution; whereas SDCs are enzymatically released by a variety of matrix proteinases, GPs are shed by GPI-specific lipases [12,13]. HSPG shedding is expected to down regulate HSPG functions at the cell surface; however, shed HSPG may act as an extracellular chaperone that transfers bound ligands to signaling receptors on neighbouring cells [14]. Further, regulation of HS structure also occurs post synthetically by the action of extracellular sulfatases (SULFs) and heparanase, which have been shown to regulate HSPG-dependent signaling by releasing tumor promoting ligands, including FGF-2, and vascular endothelial growth factor (VEGF) [15,16]. In this manner, extracellular enzymes are involved in shaping the fine structure of HS chains with implications in cancer.

<table>
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<tr>
<th>Table 1</th>
<th>Examples of HSPG ligands and their binding motifs</th>
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<tr>
<td>Ligand</td>
<td>Main Function</td>
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<tr>
<td>Apolipoprotein E</td>
<td>Lipid metabolism</td>
</tr>
<tr>
<td>FGF-2</td>
<td>Cell-growth/Wound healing</td>
</tr>
<tr>
<td>HIV-TAT</td>
<td>Viral transcriptional activator</td>
</tr>
<tr>
<td>Tissue factor pathway inhibitor-1</td>
<td>Coagulation inhibitor</td>
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<tr>
<td>VEGF</td>
<td>Cell-growth/angiogenesis</td>
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### Targets of glycosaminoglycan associated cancer treatments

#### Angiogenesis

The progression from occult cancer into manifest tumor disease requires the recruitment of blood vessels, angiogenesis, which is a multi-step process dependent on endothelial cell activation, the recruitment of mural cells, and coordinated differentiation into a functional vessel. HPSG has long been implicated in the control of angiogenesis, an area that was initiated by pioneering work by Folkman and co-workers who described an inhibitory effect of mast cell-derived heparin on tumor angiogenesis [17]. Since then, HPSG has evolved as an important co-receptor for e.g. FGFs, VEGF, heparin-binding EGF like GF (HB-EGF) and platelet derived growth factor (PDGF) activity in endothelial cells, smooth muscle cells and fibroblasts [8,10,18]. Binding of a majority of known angiogenic factors thus underlines the importance of HSPGs in angiogenesis. Several of these growth factors and cytokines are regulated by hypoxia during early steps of tumor expansion. As cell surface HS-SPGs act as intimate co-receptors and modulators of these factors, one might speculate that HSPGs themselves are regulated by hypoxia. In support of this notion, it was shown that hypoxia regulates the expression of regulatory enzymes responsible for HS chain synthesis, resulting in increased responsiveness of hypoxic endothelial cells to FGF-2 [19]. Indirect genetic evidence, implicating a central role for HSPGs in developmental and tumor angiogenesis comes from transgenic mouse studies where specific deletions of the HS-binding isoforms of VEGF or PDGF resulted in severely impaired vascular patterning and pericyte recruitment to tumor vasculature, respectively [20,21]. More direct evidence comes from studies with endothelial cell Ndst1 knockout mice, which displayed decreased FGF and VEGF signalling in tumors, and reduced tumor angiogenesis [18]. Developmental angiogenesis was not affected in these mice, suggesting that HSPGs of the tumor vasculature could be specifically targeted. Along the same line, microRNA-24 dependent targeting of NDST1 was shown to reduce HS sulfation, resulting in reduced VEGF-HS binding and endothelial cell migration [22]. In ovarian cancer, increased HS 6-O-sulfotransferase activity in tumor-associated endothelial cells was shown to promote VEGF and FGF signalling [23]. Moreover, it was recently shown that targeted knockdown of sulf1 results in abnormal vasculature formation due to insufficient VEGF signalling activity [24].

As for core protein specific functions, it was shown that GPC-1, a major cell surface HSPG in the macrovascular system, modulates the biological activity of FGF [25]. GPC-1 was found highly expressed in glioma tumor vasculature, while absent in normal brain endothelial cells; over-expression of GPC-1 in normal endothelial cells resulted in increased FGF-mediated proliferation [26]. It was further demonstrated that GPC-1 could restore the biological activity of oxidized VEGF, suggesting a more general role of GPC-1 in angiogenesis [27]. Notably, GPC-1...
Interestingly, synstatin, ternary receptor complex in angiogenic endothelial cells [30]. Integrins and insulin-like growth factor-1 receptor, forming a domain has been specifically implicated in the assembly of disease of the pancreas and lungs belong to the groups with a compared with individuals without malignancy. Tumor increased approx. 7-fold in patients with any malignancy [4,39,40]. The overall risk of VTE has been estimated to be associated mortality and is commonly associated with a poor prognosis and morbidity in patients with malignant disease [13,36]. In vivo models of cancer progression indicate a link between TF and the aggressiveness of tumor disease; in patient tumors, the malignant potential of tumor cells has been found to correlate with their TF expression level. More recent, experimental studies indicated that TF can promote tumor progression independently of blood clot formation via cleavage-activation of a unique class of G protein-coupled protease activated receptors (PARs), in particular PAR-2, and down-stream phosphorylation of the cytoplasmic domain of TF (pTF) [4]. In support of this idea, we have demonstrated the existence of pTF in experimental as well as in clinical breast cancer, and that pTF was associated with poor patient outcome [42]. It may thus be hypothesized that TF-dependent PAR signaling is a key determinant for the documented negative effects of hypercoagulative activity on tumor progression. A remaining key question is what triggers TF-dependent coagulation in the tumor microenvironment. It has been suggested that hypoxia induces the expression of TF and FVIIa in malignant cells that together with leakage of plasma coagulation factors through dysfunctional tumor vessels may trigger procoagulant signaling in the tumor [43]. Recent data suggested that MVs provide cancer cells with a powerful signalling mechanism in hypoxia-driven modulation of stromal cells. More specifically, hypoxic glioma cells were shown to release MVs loaded with the TF/FVIIa coagulation initiation protease complex that in a paracrine manner could activate PAR-2 of endothelial cells [41].

**Invasion and metastasis**

Altered expression of PG core proteins and HS modifying enzymes have been described in many tumor types and are often associated with changes in their invasive and metastatic activities [5]. Heparanase represents one of the well-studied examples, and its overexpression has been closely correlated with an invasive phenotype in experimental models. Moreover, heparanase overexpression has been linked to worse outcome of cancer patients [15]. Mechanistically, these effects have been related to degradation of HSPGs in basement membranes, resulting in enhanced escape of malignant cells over the vascular wall. In addition, released HS fragments can stabilize and activate pro-invasive factors of the tumor microenvironment. Human heparanase is primarily found within endolysosomes, but also at the cell surface and within the nuclear compartment. Notably, heparanase has been ascribed some non-enzymatic activities, independent of its involvement in ECM degradation and remodeling of the extracellular microenvironment. For example, cell surface expression of heparanase elicits firm cell adhesion, reflecting an involvement in cell–ECM interaction. SDC-1 may negatively regulate invasion and metastasis through maintenance of cell-cell and cell-ECM adhesions. Transforming cells may express less HS as compared with non-malignant cells, which is further supported by high metastatic activity of epithelial tumors with reduced expression of SDC-1. It may be
concluded that SDC-1 on malignant cells can acts as a suppressor of the metastatic phenotype. It has been suggested that this activity is coordinate regulated with E-cadherin, i.e. a known inhibitor of mesenchymal transition and metastasis [44,45]. Notably, the role of SDC-1 in cancer cell dissemination appears to be dependent on tumor type, i.e. in myeloma SDC-1 was shown to promote metastasis [46].

Importantly, not only HS-substituted PGs have been implicated in the regulation of tumor development and progression. A tumor suppressor role of a CS/DSPG, i.e. decorin, has been well established [47]. Initially, decorin showed neutralization of TGF-β mediated signaling [48]. Later studies demonstrated a pleiotropic role of decorin as a negative regulator of additional membrane receptors, including EGFR, Met, and the insulin-like growth factor-1 receptor, all of which have been implicated in invasion and metastasis. Consistently, decorin gene delivery resulted in growth suppression and reduced metastasis in a variety of experimental tumor models, and reduced expression of decorin was associated with a worse prognosis of breast and prostate cancer. As an apparent contradiction, a large body of evidence indicates that CS/DS may support the malignant behaviour of cancer cells [49]. It was reported that CS derivatives may potently reduce the viability of breast cancer cells, and inhibit tumor growth as well as metastasis in experimental models [50]. Other studies revealed that high CS expression correlates with worse clinical outcome in primary breast and prostate cancer [51,52]. Versican, i.e. another CS/DSPG of the ECM, seems to facilitate growth and invasion of several tumor types. Accordingly, versican overexpression has been associated with worse outcome in patients with breast, prostate, and many other tumor types [53,54]. In further support of a role of CS/DS GAGs in the metastatic process, removal of CS/DS chains was shown to inhibit invasion of melanoma cells [55], and highly sulfated CS structures on the cell surface were important for the dissemination of lung carcinoma and osteosarcoma cells [56,57]. Mechanistically, CS/DS chains may bind e.g. FGF and hepatocyte growth factor (HGF) that are major players in cancer migration and invasion. DS-epimerase-1 is responsible for the conversion of GlcA into IdoA, and thus the formation of DS. Because of its overexpression in most cancers it has been suggested to be a cancer-associated antigen. It was recently shown that DS-epimerase-1 is overexpressed in esophageal cancer cells together with increased enzyme activity in cancer tissue as compared with normal tissue. Interestingly, DS-associated IdoA appeared to co-localize with HGF, and there was a strong dependence on intact IdoA formation for efficient HGF-mediated cancer cell migration and invasion [58].

**Glycosaminoglycans as anti-cancer therapeutics**

Most experimental studies have been focused on heparin and CS, their derivatives, and other polyanionic compounds, such as dextran sulfate and suramin [59,60]. Other strategies to interfere with PG function include false substrates for HS biosynthesis, e.g. xylosides [61], inhibitors of heparanase [62,63], and proteins or peptides with polybasic domains that act as competitive inhibitors of growth factor binding to HS chains [64]. Recently, a chemically sulfated polysaccharide PI-88 and inhibitor of SULFs [65] was given as adjuvant therapy to hepatocellular carcinoma patients in a randomized Phase II safety trial. Notably, this inhibitor was shown to exhibit severe adverse effects, including immune induced thrombocytopenia [66]. Interestingly, van Kuppevelt and co-workers have characterized several phase display derived anti-HS single chain fragment antibodies directed at specific HS epitopes in various tissues [67,68]. The effect of these and other anti-HS antibodies on in vivo tumor development remains to be investigated.

Heparin and other sulfated GAGs are structurally highly complex molecules that, owing to their polyanionic nature, interact with and regulate the activity of a multitude of ligands. It is not surprising but rather to be expected that a relatively high concentration of GAGs inhibits tumor metastasis in the experimental setting, i.e. where they are co-administered during systemic tumor cell circulation. Given the role of the coagulation system in tumor development and metastasis (see above), the negative regulation by heparin of Fxa’s and thrombin’s proteolytic activities as well as platelet activation may partly explain its inhibitory effect. As a consequence, an obvious drawback is the relative unspecific mode of targeting, and significant risks of bleeding complications. As described above, the important role of PGs as co-receptors of several key molecules in angiogenesis, invasion and metastasis presents an attractive target for competitive inhibition by GAGs, resulting in down-regulation of e.g. tyrosine kinase receptor activation. In addition, experimental studies support anti-metastatic effects of GAGs by interference with lectin- and integrin-dependent adherence of metastatic tumor cells to platelets and the endothelium at the metastatic site [69,70]. However, still very little is known about how heparin and related derivatives are processed in the tumor microenvironment, e.g. what is the steady state concentration in the pericellular milieu and how are these substances distributed within the ECM as well as in the intracellular compartment? In other words, it is very difficult to foresee the net effect of GAG-based therapeutics in the clinical setting.

**Clinical trials**

Novel therapies targeted at specific drivers of tumor disease have been introduced over the last 15 years, which has significantly contributed to a better prognosis for several tumor groups, e.g. breast cancer patients. However, the overall mortality remains virtually constant due to a steady increase of total cancer incidence and a dismal prognosis for major patient groups, most importantly lung cancer patients. A meta-analysis of eleven clinical trials testing the efficacy of low molecular weight heparin (LMWH) vs. unfractionated heparin (UFH) as initial treatment of VTE showed a reduced mortality with LMWH among patients with a cancer diagnosis, which was not due to a reduction of fatal VTE [71]. In a retrospective analysis within the CLOT-trial, comparing LMWH with coumarin as treatment for deep vein thrombosis, a significant reduced 1-year mortality with LMWH was seen in cancer patients (n=602, mixed diagnoses) without metastatic disease [72]. In the MALT-study, 302 patients with various advanced malignancies randomly received LMWH treatment or placebo for six weeks. The survival analysis showed a significantly reduced hazard ratio of mortality in the LMWH group as whole, with the largest effect seen in the a priori defined group with a life expectancy of more than 6 months [73]. In the FAMOUS-trial, 385 patients with different malignancies randomly received a low dose of daily LMWH (deltaparin) or placebo for one year or until death. No significant benefit in 1-year-survival was observed but a better outcome in 2- and 3-year survival was seen in a post-study defined group of patients with a favorable prognosis [74].

Lung cancer is one of the most common malignancies in the world with an increasing trend in women. The majority of patients present with advanced disease, and only 15-20% are amenable to curative treatments, of which surgical resection remains the most important. The 5-year-survival rate in all patients is approx. 15% with modern treatment. Small cell lung cancer (SCLC) makes up about 20% of all lung cancers and is
generally characterized by a typical histological picture, fast growth and early metastases. The standard treatment of SCLC consists of combination chemotherapy (e.g. cis- or carboplatin plus etoposide), and in some cases thoracic radiotherapy, and prophylactic cranial irradiation. Even though the majority of patients have partial or complete tumor remissions, the overall prognosis is poor. With standard treatment, the 5-year survival rate is only 1% in extensive disease and 10% in limited disease. The treatment of SCLC thus remains a challenge, despite the initial sensitivity to combination chemotherapy and radiation therapy. Novel and “targeted” therapies have been tested but have failed. Two randomized clinical trials of chemotherapy ± heparin in prophylactic dosages in SCLC have shown positive results. In a study by Lebeau et al. (n=277), UFH was given as an adjunct to chemotherapy for five weeks, which resulted in an increased median survival of 317 days compared with 261 days in patients receiving chemotherapy only [75]. In another study, published by Altinbas et al. (n=84), LMWH was used during the entire 18 week period of chemotherapy. The results showed an impressive median survival of 13 months in the LMWH group compared with 8 months in the control group [76]. Notably, these studies were either small or did not use modern chemotherapy. More recently, a retrospective, single-center analysis of the clinical and therapeutic characteristics of SCLC patients treated during 1990-2002 was reported [77]. The 2 year survival rates were 27 and 12% among patients treated with the PE (cisplatin or carboplatin + etoposide) regimen with and without UFH, respectively, supporting the added value of UFH in combination with standard chemotherapy. Further, a Cochrane report concluded that the effect of anticoagulation (with either UFH or LMWH) on mortality of patients with cancer who have no therapeutic or prophylactic indication for anticoagulation was statistically and clinically significant at 24 months but not at 12 months. Subgroup analysis for mortality at 12 months suggested survival benefit specifically in patients with SCLC but not in patients with other types of cancer [78]. A very recent meta-analysis by Zhang et al., which included nine different studies with 2185 participants in total, came to the very same conclusion, i.e. that anticoagulation showed a survival benefit specifically in SCLC patients [79]. According to the ClinicalTrials.gov database, using the search terms “heparin and cancer”, several studies currently investigate the utility of LMWH as treatment of lung cancer, but also of pancreas and colorectal cancer (Table 2). The RASTEN trial provides an internationally unique study population of SCLC patients that will clarify the tumor-inhibiting effect of LMWH (enoxaparin) in a well-defined cancer patient subgroup. Two studies (TILT and SYRINGES) investigate the effect of LMWH on overall survival of non-SCLC patients. Importantly, there is still no published, properly designed study that can either validate or disprove positive effects of heparin or its derivatives on the survival of cancer patients directly related to their tumor-inhibiting effects.

Future perspectives

At present, the general recommendations for the use of GAGs in the management of cancer patients is limited to LMWHs for primary prophylaxis of patients at high risk of developing VTE, patients with confirmed VTE, and secondary prophylaxis of patients with a history of VTE. So far, there are no established biomarkers to guide clinicians in tailoring individualised LMWH treatment, and to motivate a more general use of this class of drugs also in a health economics perspective. The identification of cancer patients who would benefit from LMWH therapy by improved survival or prevention of VTE-associated morbidity thus remains a challenge of high clinical relevance. The complexity of HSPG structure and function calls for caution in the future development of more potent, PG-targeting drugs. PG-mediated interactions are essential during development and adult tissue homoeostasis, i.e. animals deficient in HSPG core proteins or HS biosynthetic enzymes display a wide variety of phenotypes, including early embryonic lethality to no apparent signs of anomalies. Loss of HS chains or major perturbation of HS structure by interference with polymer modification is generally associated with severe phenotypes. HSPG core protein knockout animals in most cases display relatively less severe phenotypes, conceivably due to functional redundancy between different PG core protein families. Interesting challenges clearly lie ahead to decipher how GAGs should be utilized as targets and treatments of cancer patients, and how such strategies may be combined with existing therapies.

### Abbreviations

Chondroitin sulfate: CS; dermatan sulfate: DS; fibroblast growth factor-2: FGF-2; glucuronic acid: GlcA; glycosaminoglycan: GAG; glycosyl-phosphatidylinositol: GPI; glypicanc:GPC; heparan sulfate: HS; heparin-binding EGF like GF: HB-EGF; hepatocyte growth factor: HGF; Iduronic acid: IdoA; low molecular weight heparin: LMWH; microvesicles: MVs; N-acetyl glucosamine: GlcNAc; N-deacetylase/N-sulfotransferase: NDST; platelet derived growth factor: PDGF; protease activated receptors: PARs; proteoglycan: PG; small cell lung cancer: SCLC; sulfatases: SULFs; syndecan: SDC; tissue factor: TF; unfractionated heparin: UFH; vascular endothelial growth factor: VEGF; venous thromboembolism: VTE.

### Acknowledgements

I apologize to all those authors whose important work could not be cited in this overview due to space restrictions. This work was supported by grants from the Swedish Cancer Fund; the Swedish Research Council; the Gunnar Nilsson, Anna Lisa and Sven Eric Lundgren, and Kamprad Foundations; the Skåne University Hospital donation funds; and the Governmental funding of clinical research within the national health services (ALF).
Conflict of interest statement

No conflict of interest

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