MALIGNANT PLEURAL MESOTHELIOMA:
NOVEL BIOMARKERS AND RELATED PATHWAYS

Filip Mundt

Stockholm 2013
Front cover: a part of John Rocque’s Map of London from 1746 (section C:2). The curvature of the Thames resembles the pleural cavity and the various ships (biomarkers) carry different cargo (biological information). Reprinted with permission from Motco©.

Back cover: a pathway identified using Ingenuity Pathway Analyser in paper II.

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“One of the advantages of being disorderly is that one is constantly making exciting discoveries.”

– A. A. Milne (author of Winnie-the-Pooh)

To my parents
ABSTRACT

Malignant mesothelioma is an asbestos induced cancer that is difficult to diagnose. Several studies have combined biomarkers to improve mesothelioma diagnosis, but with moderate success, and there is a need for new mesothelioma biomarkers. The tumour is often resistant to treatment and most patients will survive less than a year. An indicator of patient survival is the tumour’s growth pattern, which in turn is influenced by expressed proteoglycans.

In this thesis work, we aim to improve the possibilities to diagnose malignant mesothelioma by combining biomarkers and by identifying new ones. We also investigate tumour driving mechanisms with focus on one of these suggested biomarkers, the cell-bound proteoglycan syndecan-1.

We were able to construct a diagnostic two-step model based on biomarkers in patient material. By implementing a cut-off level and thereafter focusing on unresolved patients we combined hyaluronan and N-ERC/mesothelin (paper I), which significantly increased the diagnostic accuracy for malignant mesothelioma. To further improve diagnosis, we used mass spectrometry to find new biomarkers. We identified and validated galectin-1, which was excellent in discriminating mesotheliomas from adenocarcinomas (paper II). In the same study, we were also the first to describe aldo-keto reductase 1B10 as a novel prognostic mesothelioma biomarker.

Syndecan-1 has been indicated as a marker for carcinomas. In paper I we describe how higher levels of syndecan-1 indicate the presence of a carcinoma over a mesothelioma. This was verified in paper II when syndecan-1 was identified as downregulated in fluids from mesothelioma patients compared to lung cancer patients. Paper III and paper IV focus on this proteoglycan.

Malignant cell lines transfected with syndecan-1 and various truncated forms of syndecan-1 affected adhesion and migration, which are key features of cancer invasion (paper III). The results showed a domain- and cell type specific effect on the cells’ motility. Regulating syndecan-1 levels and analysing the global gene expression of mesothelioma cells made it evident that this proteoglycan has a strong influence on transforming growth factor β signalling and several growth factor pathways (paper IV). Links to cell migration and proliferation were furthermore identified, along with glycosaminoglycan modifying enzymes. These results can shed light on the complex role of syndecan-1 in invasion and growth of malignant mesenchymal cells.

Taken together, this thesis work describes a complement to conventional mesothelioma diagnosis and identifies novel biomarkers. Furthermore, the potential biomarker syndecan-1 was shown to have an effect on cell motility and proliferation. These results increase our understanding of this aggressive malignancy.
LIST OF PUBLICATIONS

This thesis is based on the following publications, which will be referred to in the text by their Roman numerals:


II. **Filip Mundt**, Henrik Johansson, Jenny Forshed, Sertaç Arslan, Muzaffer Metintas, Katalin Dobra, Janne Lehtiö and Anders Hjerpe. Proteome screening identifies galectin-1 as a negative predictor for malignant mesothelioma in pleural effusions. *Manuscript*.


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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AGS</td>
<td>Altered gene set</td>
</tr>
<tr>
<td>AP1</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under curve</td>
</tr>
<tr>
<td>BAP1</td>
<td>BRCA1-associated protein 1</td>
</tr>
<tr>
<td>CA125/MUC16</td>
<td>Cancer antigen 125</td>
</tr>
<tr>
<td>CEA</td>
<td>Cancer embryonic antigen</td>
</tr>
<tr>
<td>CID</td>
<td>Collision induced dissociation</td>
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<tr>
<td>CS</td>
<td>Chondroitin</td>
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<tr>
<td>CV</td>
<td>Cross-validation</td>
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<tr>
<td>Da</td>
<td>Daltons</td>
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<tr>
<td>DE</td>
<td>Differentially expressed</td>
</tr>
<tr>
<td>DS</td>
<td>Dermatan sulphate</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>EGF receptor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immune-sorbent assay</td>
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<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>EMA</td>
<td>Epithelial membrane antigen</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-mesenchymal-transition</td>
</tr>
<tr>
<td>Erc</td>
<td>Expressed in renal carcinoma</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>Extracellular signal-regulated kinases 1 and 2</td>
</tr>
<tr>
<td>FDR</td>
<td>False discovery rate</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FGS</td>
<td>Functional gene set</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>GalNAc</td>
<td>N-acetylgalactosamine</td>
</tr>
<tr>
<td>GF</td>
<td>Growth factors</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-acetylglucosamine</td>
</tr>
<tr>
<td>GlcUA</td>
<td>Glucuronic acid</td>
</tr>
<tr>
<td>GO</td>
<td>Gene ontology</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosyl-phosphatidylinositol</td>
</tr>
<tr>
<td>GSEA</td>
<td>Gene set enrichment analysis</td>
</tr>
<tr>
<td>HAS</td>
<td>Hyaluronan synthase</td>
</tr>
<tr>
<td>HCD</td>
<td>Higher-energy collision dissociation</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor/scatter factor</td>
</tr>
<tr>
<td>HPCE</td>
<td>High performance capillary electrophoresis</td>
</tr>
<tr>
<td>HPLC</td>
<td>High pressure liquid chromatography</td>
</tr>
<tr>
<td>HS</td>
<td>Heparan sulphate</td>
</tr>
<tr>
<td>IEF</td>
<td>Isoelectric focusing</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukins</td>
</tr>
<tr>
<td>IPA</td>
<td>Ingenuity pathway analyser</td>
</tr>
<tr>
<td>IPG</td>
<td>Immobilized pH gradient</td>
</tr>
<tr>
<td>ITRAQ</td>
<td>Isobaric tag for relative and absolute quantification</td>
</tr>
<tr>
<td>MARS</td>
<td>Multi removal affinity system column</td>
</tr>
<tr>
<td>MET</td>
<td>Mesenchymal-epithelial-transition</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>NCAM</td>
<td>Neural cell adhesion molecule</td>
</tr>
<tr>
<td>NEA</td>
<td>Network enrichment analysis</td>
</tr>
<tr>
<td>NF2</td>
<td>Neurofibromin 2</td>
</tr>
<tr>
<td>nLC</td>
<td>Nano-liquid chromatograph</td>
</tr>
<tr>
<td>OPLS-DA</td>
<td>Orthogonal partial least squares discriminant analysis</td>
</tr>
<tr>
<td>PCA</td>
<td>Principal component analysis</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>ROC</td>
<td>Receiver operating characteristic</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SAM</td>
<td>Significant analysis of microarrays</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>TNFR</td>
<td>TNFα receptor</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
</tbody>
</table>
1 BACKGROUND

1.1 THE HALLMARKS OF CANCER AND TUMOUR HETEROGENEITY

Cancer is a collective term for around 200 different diagnoses. In 2008 there were 12.7 million new cancers diagnosed globally, and 7.6 million people would die due to their cancer in the same year\(^5\). A recent review\(^6\) summarised the growing framework of the disease – a conceptualisation that is a work in progress and describing cancer as an intricate society with a limited set of underlying hallmarks.

Even when a cancer arises from mutations in a single cell, the resulting tumour is a mixture of several different malignant cell populations\(^7\). The resulting heterogeneity is illustrated in a set of studies that sequenced the genome of several cancer types and identified a large amount of mutations\(^8\)-\(^10\). Surprisingly, the majority of the mutations do not overlap with another cancer from the same origin and between tumour types the overlap is virtually non-existing. So how is it that different proliferative malignant diseases can be described using only a handful of common hallmarks\(^6\) if so few of the mutations are shared? It seems that even though the overlap of mutated genes between tumours is very small, the pathways they influence are very similar\(^10\). In other words, the driving mutations might vary between tumours, but the effects these mutations have will overlap. This high degree of heterogeneity, still with adherence to characteristic pathways is also encountered in malignant mesothelioma and will affect our interpretation of the disease when aiming for diagnoses, therapeutic measures and prognosis.

1.2 MALIGNANT MESOTHELIOMA

Malignant mesothelioma is a rare but highly aggressive tumour. The mortality is one of the highest associated with cancers\(^11\). Since the mesothelial tissues arise from mesoderm and commonly express neural cell adhesion molecule (NCAM), this malignancy is classified as a sarcoma, however, often with epithelial resemblance. There are two major localizations of malignant mesothelioma: the pleura and peritoneum\(^12\). Malignant mesothelioma may also arise in the pericardium\(^12\) or tunica vaginalis testis\(^13\).

This thesis regards the pleural form of malignant mesothelioma.

1.2.1 The pleura

Each lung is covered by a thin mesenchymal tissue, the pleura, which also covers the chest wall. The pleural tissue covering the lungs is the visceral pleura and the layer lining the chest is the parietal pleura. The cells in this tissue originate from the mesoderm, but resemble epithelium by covering a surface in an epithelial-like manner, hence the word mesothelium.\(^14\) The entire pleural tissue consists of a single layer mesothelium resting on a basal membrane with underlying connective tissue. The mesothelial cells have a flattened shape with a distinct epithelioid phenotype and often a multitude of apical microvilli, which are a distinct attribute\(^14,15\). The connective tissue is intersected with lymphatic vessels, blood vessels, immune cells and fibroblast-like cells. It has been
shown that fibroblast-like cells can differentiate and replace the damaged mesothelium following injury\textsuperscript{14,16}.

In the pleural cavity between the two layers there is a small amount of fluid for lubrication that minimises friction between the visceral and parietal layers during breathing. Usually the serous fluid is less than a few millilitres in an average sized body.\textsuperscript{14} This fluid is continuously circulating, from the intercostal arteries, partly produced by the mesothelial cells and then drained into the lymphatic circulation\textsuperscript{17}.

Apart from facilitating relatively frictionless movement between the chest wall and the lungs, the pleural mesothelium also controls additional tissue functions. These include trans-membrane material flux, regulation of fibrinolysis, maintenance of serosal integrity by producing growth factors (GF) and extracellular matrix (ECM) components. The pleura also aid leukocyte migration to sites of inflammation, synthesis of cytokines and inflammatory mediators as well as contributing to antigen presentation. Among the most common growth factors found in the pleural space are transforming growth factor β (TGFβ), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF; scatter factor) and heparin-binding epidermal growth factor-like growth factor. Common cytokines and chemokines include interleukins (IL) 1, 6, 8 and 15; prostaglandins and SDF-1.\textsuperscript{14}

\subsection*{1.2.2 Pathological conditions of the pleura}

The pleura encounter many carcinogens and pathogens that cause various cellular injuries. Infections and inflammation are common and pose challenges in diagnosis and treatment.\textsuperscript{18} Long-term inhalation of several industrial and natural fibres causes chronic inflammation and scarring of the pleura. Inflammation and injury result in cellular responses both from the immune system and the mesothelium itself.

Cancers in the pleural cavity are most often metastatic adenocarcinomas from other organs\textsuperscript{19}, but there are also tumours arising from the pleural tissue. Solitary fibrous tumours of the pleura are mostly benign mesenchymal tumours without the typical mesothelial immunophenotype, and if diagnosed early total resection results in a 97 per cent 5-year survival. Prognosis becomes quickly poor if re-resection is necessary or malignant transformation has occurred. This tumour can be difficult to distinguish from the most common primary malignancy of pleural origin, the malignant pleural mesothelioma.\textsuperscript{20}

\subsection*{1.2.3 Malignant pleural mesothelioma}

The pleural form of malignant mesothelioma is the most common type accounting for more than 70 per cent of all mesothelioma cases\textsuperscript{12,21}.

\subsubsection*{1.2.3.1 Epidemiology}

\textit{Incidence, latency, survival and mortality}

The best known causative agent for mesothelioma is asbestos (see section \textit{Asbestos}, under 1.2.3.2) and even though the relationship between asbestos and mesothelioma has been known for over half a century and asbestos usage is banned in a large part of
the world, the malignant mesothelioma incidence is still increasing globally\textsuperscript{22}. In 2010, Australia was reported to have the world’s highest national incidence of malignant mesothelioma at circa 35 cases per million inhabitants\textsuperscript{23}. In the United States the incidence ranges between 20 and 25 per million for men and around 2 per million for women. The incidence might already have peaked in the United States\textsuperscript{24}, but in the United Kingdom the incidence is expected to reach its peak in 2015\textsuperscript{25}.

Sweden has seen a steady increase of mesothelioma cases per year since the 1970s (figure 1). A large part of this increase can be linked to improved diagnosis, which is probably true for all reported estimates. The incidence in Sweden is approx. 10 per million for men and 1 to 2 per million for women (data extracted from the Swedish National Cancer Registry). The difference between the sexes is due to the work-related exposure to asbestos. There is, however, also a non-asbestos induced malignant mesothelioma representing a constant background prevalence of the disease\textsuperscript{26}.

The latency period between asbestos encounter and first clinical symptoms is usually longer than 20 years\textsuperscript{12}, not uncommonly up to 40 years or longer\textsuperscript{27}. This characteristically long time is a contributing factor to the continued rise in the incidence of the disease – even in countries that have long restricted usage of asbestos. The median age of diagnosis has been reported to be between 72 and 74 years\textsuperscript{12,21} which is in line with work-related exposure to asbestos and a long latency period. The lifetime risk of malignant mesothelioma persists, even after exposure has ceased\textsuperscript{26,29}. This is the opposite of individuals who stop smoking and decrease their lung cancer risk over the following years.

Malignant mesothelioma is a fatal disease and patients have an overall median survival of between 9 and 12 months\textsuperscript{22}. Tumour stage does not seem to be a good predictor of survival and survival itself has not improved significantly over the last few decades\textsuperscript{21}. Five-year survival for malignant mesothelioma is a gloomy 9 per cent\textsuperscript{30}, although some individuals may survive for 10 years or more.

Malignant mesothelioma has been called a \textit{man-made} disease and its slow but widespread increase in occurrence has been described as an epidemic. Without doubt, malignant mesothelioma has a tremendous socio-economic and political effect on society. The economic impact of asbestos related lung cancers and malignant mesothelioma is estimated to be three times higher than the revenue of asbestos trade\textsuperscript{31}. Company litigations alone are estimated to cost a staggering 300 billion USD in the western world in the next few decades\textsuperscript{12}. Additional costs of asbestos removal and
associated administration adds to malignant mesothelioma being regarded as one of the world’s worst industrial accidents. In the 1970s the USA Occupational Safety and Health Administration initiated a screening program to identify individuals in need of treatment. The program used chest X-ray, health history and spirometry, but showed poor ability to predict a malignant mesothelioma\(^{33}\).

1.2.3.2 Aetiology

Asbestos

Asbestos is by far the most common causative agent for malignant mesothelioma\(^{34-36}\), with 80 per cent of mesothelioma patients reporting asbestos exposure\(^{27}\). As well, mesothelioma is the primary malignancy resulting from asbestos exposure\(^{30}\) with 5 to 10 per cent of heavily asbestos exposed individuals developing the disease\(^{11}\). The relationship between asbestos and malignant mesothelioma was described for the first time in South African miners by Wagner et al. in 1960\(^{37}\). This relationship is one of the clearest between a carcinogen and its associated cancer.

Asbestos is a group of hydrated fibrous silicate minerals that occur in nature and comes in two major groups: serpentines, including chrysotile (white asbestos; figure 2a) and the amphiboles, including crocidolite (blue asbestos; figure 2a) and amosite (brown asbestos)\(^{36}\). While white asbestos comprises 90 per cent of the world’s entire asbestos consumption\(^{38}\), blue asbestos is the most carcinogenic form\(^{39}\). There has been a long standing debate whether the white form leads to malignant mesothelioma or not\(^{39}\). However, exposure to white asbestos predisposes individuals to smoking-induced bronchogenic lung cancers\(^{38}\) and there is evidence showing an increased overall cancer incidence\(^{34}\) and mortality in exposed cohorts\(^{40}\). It should also be cautioned that white asbestos can be contaminated with blue asbestos.

The word asbestos is Greek and means unquenchable; it is naturally formed over millennia under high pressure and in the right chemical milieu\(^{41}\). The properties of asbestos have been known for thousands of years. Due to its remarkable heat-resistant capacities asbestos has been called a miracle-fibre and been extensively used in industry\(^{42}\) (it has even been thought to defeat superheroes: see box 1). Hence, asbestos has been widely used as insulation and fire-protection in plumbing, cars and houses (figure 2b). The people most exposed to this carcinogen are therefore asbestos miners, shipyard workers, plumbers, electricians, construction workers and people in similar professions\(^{21,22,43,44}\). The commercial use of asbestos peaked between 1930 and 1960\(^{43}\), but asbestos has been banned, or strongly restricted, in several countries since then\(^{45}\). Still, due to its low cost – at least in the short term – many countries import and consume asbestos even today\(^{46,47}\). Restrictions of asbestos in the western world has not deterred an increase in its sale and usage in developing countries\(^{48}\), possibly foretelling of yet another mesothelioma epidemic to come.

There are also some natural deposits of asbestos and asbestos-like minerals, around the world that can lead to unprovoked asbestos exposure. A Japanese study analysed
In the Cappadocia region of Turkey there is an asbestos-like mineral occurring naturally. This fibre, named erionite, is believed to be even more carcinogenic than asbestos and is strongly linked to mesothelioma\textsuperscript{50}. Erionite containing rocks are used to build houses in certain villages and the mineral itself produces a white powder that is a popular component of indoor paint. This environmental and domestic exposure has led to a much higher incidence of mesothelioma in these settings compared to for example Sweden\textsuperscript{51}. The incidence is not only drastically higher but with the different exposure in the domestic environment the risk is also differentially distributed between the sexes. Reports are not uncommon with erionite exposure where more than half the studied mesothelioma patients were women\textsuperscript{52}. Studies in Sweden, the Netherlands and USA report a low incidence in women\textsuperscript{44,53}. In some of these Turkish villages erionite has caused mesothelioma in half the population, also affecting younger people than would be expected\textsuperscript{26,50}. Extremely high incidence rates are fortunately very rare, but have also been reported in the Australian mining community of Wittenoom\textsuperscript{29}, one of the world’s few blue asbestos mining communities. Wittenoom’s mine closed in 1966 due to unprofitability and growing health concerns, and the entire town was officially abolished by the Australian government in 2007.

Asbestos fibres of a certain length and width are inhaled all the way out to the alveoli where they lodge in the lung tissue. Over time, these fibres migrate out to both layers of the pleura. Fibre presence is evident from tissue samples containing encapsulated asbestos fibres\textsuperscript{54}. These fibres are like thin spears that can penetrate plasma membranes without killing the cells. During mitosis the asbestos fibres can cause aberrant karyotypes with possible malignant outcome. Furthermore, upon attempted
ingestion by macrophages and other cells the asbestos fibres become covered by iron deposit and iron-rich proteins\textsuperscript{49}. These \textit{ferruginous bodies} (figure 2c) may lead to increased formation of reactive oxygen species (ROS). ROS lead in their turn to cellular damage, especially DNA mutations\textsuperscript{55}, and have been linked to tumour progression\textsuperscript{56}. Macrophages that try to phagocytose the asbestos fibres fail, but in the process produce more ROS and cytokines\textsuperscript{57} (see section below concerning IL1β, TNFα and HMGB1). This so called \textit{frustrated phagocytosis} is part of a chronic inflammation in the lung and pleura. The clastogenic properties of the asbestos fibres, the increased ROS production along with a never-ending inflammation are thought to be some of the main causes of malignant mesotheliomas\textsuperscript{58} and a dose-dependent effect has been described\textsuperscript{59}. For details on the molecular pathogenesis of malignant mesothelioma see section 1.2.3.3.

\textbf{Box 1: Asbestos the \textit{“miracle fibre”}}

Asbestos was held in high regards during parts of the 20\textsuperscript{th} century. Its heat resistant capacity was even regarded potent enough to stop superheroes. Or at least it was tried by two super-villains: \textit{the Asbestos Lady} and later the \textit{Asbestos Man}. They both fell short. Only \textit{the Asbestos Lady} reappeared, this time with a cancer diagnosis. Images are reprinted with permission from \textit{Marvel Comics©}.

\textbf{Simian Virus 40}

Simian virus 40 (SV40) is a DNA virus known to infect monkeys. It seems to have been introduced to the human population by contaminated polio-vaccines in the 1950s and 1960s\textsuperscript{60}, but can possibly transmit horizontally as well. The question of whether SV40 has a causal role in malignant mesothelioma development has been discussed for many years.

Studies show that blue asbestos together with SV40 cause mesothelioma in a synergistic fashion in hamsters\textsuperscript{61}. Molecular investigations imply that the large T-antigen of the virus is able to inhibit several tumour suppressor genes (including p53\textsuperscript{62} and pRB\textsuperscript{63}). However, other studies warn for false positive detection of SV40\textsuperscript{60}. For an overview on the SV40 controversy, please see references 64 and 65.

\textbf{Smoking}

Remarkably, there is no connection between smoking and malignant mesothelioma\textsuperscript{12,66} as there is for a multitude of other human malignancies\textsuperscript{67}. On the other hand, smoking and asbestos induce lung cancer in a synergistic manner\textsuperscript{68}.

\textbf{1.2.3.3 Molecular pathology of malignant pleural mesothelioma}

Only 5 to 10 per cent of individuals that are heavily exposed to asbestos develop malignant mesothelioma\textsuperscript{11}. This may indicate an underlying factor in these persons that make them susceptible to the fibre; perhaps a contribution of genetic predisposition
and complex relationship to environmental influences (e.g. duration of asbestos exposure or SV40 infection).
As with all cancers, malignant mesothelioma is caused by a series of genetic changes and aberrations, a few are well known and well described. Increasing knowledge of these molecular changes might allow screening of risk groups for early detection and designing novel therapeutic alternatives. Studies in animals and humans have shown extensive chromosomal rearrangements and losses, particularly of chromosome 22, but also 1p, 3p, 6q and 9p. Several of the affected loci contain important tumour suppressor genes, such as neurofibromin 2 (22q12), p16 and p14 (both located in the CDKN2A locus at 9p21) and BRCA1-associated protein 1 (3p21).

**Neurofibromin 2**
Heterozygous loss of neurofibromin 2 (NF2) has been reported in up to 100 per cent of mesothelioma cell lines and in approx. half of all mesothelioma patients. The product of the NF2 gene is merlin (moesin-ezrin-radixin-like protein), a membrane-cytoskeleton scaffolding protein, linking actin filaments to cell membrane or membrane proteoglycans. Merlin asserts tumour suppressor properties through contact-mediated growth inhibition via the hippo and mTOR pathways, and via transcriptional control of integrins and receptor tyrosine kinases. At least 75 per cent of mesothelioma patients show inactivation of merlin, SAV1 or LATS2 (two proteins downstream in the hippo pathway).

**The CDKN2A locus**
The CDKN2A locus is an important genetic segment containing the tumour suppressor genes p16\textsuperscript{INK4A} and p14\textsuperscript{ARF}. These tumour suppressors are the product of exon splicing. p16 prevents phosphorylation of pRB by binding CDK4/6, which in turn prevents the cell going through the G1 and S phases. The alternative splice variant p14 is involved in MDM2 sequestering and/or degradation, facilitating both pRB and p53 stabilisation and thereby arresting cells in G1 and G2 cell cycle phases. So even though p53 itself is rarely deleted or mutated in malignant mesothelioma, it is indirectly silenced through the loss of p14. Disruption of the CDKN2A genes are linked to the development of several cancers and the loss of p16 expression is considered an essential event in the development of malignant mesothelioma.

**BRCA1-associated protein 1**
The BRCA1-associated protein 1 (BAP1) gene product is a deubiquitinase that translocates to the nucleus and interacts with the BRCA1 protein. Current studies have reported an association with BAP1 deletion or mutation and malignant mesothelioma. In one study, the analysis of the genome revealed inactivating mutations in 12 of 53 mesothelioma patients, while the well-known tumour suppressors PTEN and p53 had few or no mutations. It has also been shown that individuals with germline mutations of BAP1 are predisposed to develop malignant mesothelioma. The inactivation of BAP1 in mesothelioma seems to be more specific for the epithelioid phenotype, with 81 per cent of these patients having mutations compared to 14 per cent of non-epithelioid cases.
Asbestos activated pathways
As already touched upon, several pathways can be activated in normal and malignant mesothelial cells by the asbestos fibres themselves, their involvement in ROS generation or their incomplete phagocytosis. The first study to show that asbestos fibres could interfere with the molecular machinery of cells, reported a dose-dependent induction of c-fos and c-jun mRNA as a response to blue asbestos in pleural mesothelial cells. The two proteins c-fos and c-jun heterodimerize to form activator protein 1 (AP1) and in response to extracellular stimuli this transcription factor binds DNA and regulates the expression of several proteins involved in proliferation, differentiation and apoptosis. It has also been shown that the asbestos fibre itself can directly interfere with and activate the epidermal growth factor receptor (EGFR) in mesothelial cells. This receptor normally responds to various ligands (e.g. EGF, transforming growth factor α and heparin-binding EGF) and can be the oncogenic addiction of many cancers.

Blue asbestos fibres can dimerize EGFRs leading to autophosphorylation and lead to induction of extracellular signal-regulated kinases (ERK) 1 and 2. ERK1/2 stabilization of c-fos is regulated by fra1 in mesothelial cells. Fra1 is needed for AP1-dependent mesothelial cell growth, migration and transformation. Further studies elaborate on a link between ERK1/2 and fra1 expression of c-met and CD44 (hyaluronan receptor), two proteins involved in cancer initiation and progression. Fra-1, c-met and CD44 may be partly responsible for mesothelioma invasion. Asbestos fibres have also been shown to elicit a NFkB response in mesothelial cells in vitro. NFkB is a transcription factor linked to proliferation, apoptosis and chemokine/cytokine production.

IL1β is a chemokine produced by macrophages and can be induced as a response to asbestos mediated killing of mesothelial cells. When asbestos induced cell damage occurs in the mesothelium, HMGB1 (a DNA binding protein and a cytokine for inflammation contained in the nucleus) is released and in response macrophages secrete IL1β and TNFα – leading to inflammation in the tissue. The mesothelial cells can express receptors for both IL1β and TNFα (TNFR), initiating a paracrine loop for maintained survival and proliferation; a survival that might partly be NFkB and IL1/AP1-dependent. Some studies also show TNFR activation through asbestos fibres and/or ROS. This might lead to the survival of damaged mesothelial cells with increased transformation potential.

Furthermore, malignant mesothelioma is frequently associated with certain growth factors and cytokines, including increased levels of secreted PDGF, HGF, FGF, VEGF; IL6, IL8 and TGFβ. Some can be produced by mesothelial cells due to inflammation; possibly acting in autocrine manner to aid survival, and/or affecting surrounding stroma to facilitate angiogenesis and invasion. Various forms of PDGF have long been known to have an effect on malignant mesothelioma cell proliferation and chemotaxis. Human mesothelial and malignant mesothelioma cell lines secrete TGFβ and inhibition of TGFβ receptors reduce the proliferative potential of murine mesothelioma cells. It is known that there is a cross-talk between TGFβ and the hippo pathway in mesothelioma cells linking the pivotal NF2 deletions with the plasticity of malignant
mesothelioma. For two comprehensive reviews on asbestos affected pathways in lung epithelia and pleural mesothelium, see references 86 and 94.

1.2.3.4 Mesothelioma phenotypes and epithelial-mesenchymal transition
Malignant mesothelioma originates in a mesenchymal tissue. The histology of the cancer is represented clinically by three different growth patterns: epithelioid, sarcomatoid and a mixture of these two, called biphatic. Estimates of the different forms vary, but the epithelioid is the most common finding (50 to over 70 per cent), usually followed by the biphasic (10 to 40 per cent), while the sarcomatoid only constitutes 10 to 15 per cent of all mesothelioma cases12,103,104. However, these fractions will be influenced by the diagnostic technique and size and number of examined biopsies – it might be that the biphasic phenotype is more prevalent if mesothelioma tumours were even more carefully examined105. The phenotypes are closely linked to patient survival, the median survival time has been reported to fall from 12 months for epithelioid mesotheliomas to only 4 months for sarcomatoid mesotheliomas106.

The mesothelial cells of the pleura have the ability to transdifferentiate between an epithelioid and a fibroblast-like phenotype107,108. This observation is confirmed in embryological and cell culture experiments which demonstrate that mesothelial cells may display fibroblast-like characteristics under certain conditions14,109.

Some differences have been shown between the two phenotypes. In a qPCR based in vitro experiment it was reported that epithelioid mesothelioma cells produced more membrane bound proteoglycans (syndecan-2 and syndecan-4) as well as hyaluronan synthase, while sarcomatoid mesothelioma cells overexpressed extracellular proteoglycans (versican, decorin and biglycan). Furthermore, silencing of syndecan-1 by antisense targeting, morphed an epithelioid cell shapes into more a fibroblast-like.110 Moreover, mesothelin is expressed in greater quantities from epithelioid mesotheliomas111 and to some extent so is hyaluronan112.

When a cell changes from an epithelial to a fibroblast-like phenotype, this is called epithelial-mesenchymal-transition (EMT). EMT is a feature of invasive carcinomas and intertwined with the hallmark of cancer – invasion and metastasis – that presents one of the greatest challenges in cancer treatment today. The change in morphology enables a proliferative cancer in situ to start migrating and invade surrounding tissue, eventually leading to the seeding of daughter cells.113 EMT will lead to loss of cell-to-cell contacts and initiation of a migratory (invasive) programme. Some of the factors that seem to be responsible for this transformation include a loss of: E-cadherin, syndecan-1, MUC1 and cytokeratin to a gain of: N-cadherin, αSMA, β-catenin, vimentin, desmin and α5β1 integrins.113-115 However, this transition should best be viewed as a tissue specific and graded continuum. The induction and aiding of EMT in carcinomas has been linked to signals emanating from the tumour-associated stroma (notably, HGF, EGF, PDGF, metalloproteases and TGFβ) and as an intrinsic drive from the tumour itself.113,116
Interestingly, studies acknowledge the resemblance between malignant mesothelioma and EMT, arguing that the three mesothelioma phenotypes (epithelioid, sarcomatoid and biphasic) could be actual snap-shots of the EMT axis\textsuperscript{117,118}. When analysing epithelioid to sarcomatoid mesothelioma tissues, a switch from epithelial to mesenchymal markers were seen in the direction of EMT (\textit{e.g.} loss of E-cadherin with an increase in N-cadherin and vimentin). Moreover, inhibition of two mesenchymal markers (ZEB1 and ZEB2) led to increased E-cadherin expression and reduced migration and invasion of mesothelioma cells \textit{in vitro}\textsuperscript{118}. Another study evaluating the role of EMT in mesothelioma analysed 29 genes involved in EMT or stemness and found that they distinguished mesothelioma histology from normal pleura and lung tissue\textsuperscript{117}.

However, other studies show that even though mesothelioma can express both E-cadherin and N-cadherin, the mesothelial marker NCAM is predominant in both phenotypes\textsuperscript{119} and a large proportion of epithelioid mesotheliomas express vimentin\textsuperscript{120}. It should furthermore, not be forgotten that it can be difficult to distinguish EMT from the reversal \textit{mesenchymal-epithelial-transition} (MET) which is needed for secondary tumour formation at a new location\textsuperscript{121}. Still, evidence for an EMT-like description of malignant mesothelioma is intriguing and might carry validity.

\subsection*{1.2.4 Diagnosis}
Whenever a patient presents with dyspnoea due to a pleural effusion and chest wall pain the probability of a malignant mesothelioma must always be considered\textsuperscript{22}. The diagnosis is difficult and often a lengthy process. The main differential diagnoses to a malignant mesothelioma are metastatic adenocarcinomas (most often from the breast, adjacent lung or ovary) and benign mesothelial hyperplasia\textsuperscript{122}.

The clinical procedure in short: imaging with chest x-ray can show the effusion and the tumour or pleural thickening. Computed tomography can show a pleural mass and invasion (\textbf{figure 2d}). More advanced imaging techniques, such as magnetic resonance imaging and positron emission tomography can be helpful in evaluating tumour likelihood, invasiveness and staging. Staging is of little importance for medical management, however it is useful in planning surgical management of malignant mesothelioma\textsuperscript{22}.

\subsection*{1.2.4.1 Morphological evaluation}
It has been recommended that the mesothelioma diagnosis should be based on histological assessment of biopsy material\textsuperscript{123}. To obtain sufficient material for the diagnosis, the biopsy should preferably be taken during thoracoscopy or thoracotomy. Coarse-needle biopsies are used less today due to problems with the diagnostic yield and high complication rate\textsuperscript{124}. A specific diagnosis can also be obtained based on effusions that often are sampled early in the process. Cytology together with ancillary analyses will provide an early diagnosis in a large proportion of cases\textsuperscript{22,125-127}. 
Histology and immunohistochemistry
Haematoxylin-eosin staining of tissue sections for light-microscopy, together with immunohistochemistry will allow for classification of a large proportion of the patients. The recommendation for tissue markers are at least two positive and two negative markers with no less than 80 per cent sensitivity or specificity. For example, calretinin will stain most cells of mesothelial origin, and NCAM, epithelial membrane antigen (EMA) with cell membrane accentuation and Wilms’ tumour 1 are indicative of a mesothelioma, while CD15, B72.3 and Ber-EP4 are often negative in malignant mesothelioma. Desmin and the glucose transporter 1 can be used to separate a malignancy from a benign reaction, so can necrotic areas in some instances – less useful are mitotic index and cytological atypia.

Cytology and immunocytochemistry
Sixty to eighty per cent of pleural mesothelioma patients present with an effusion at their first clinical examination, but as many as 95 per cent develop an effusion during the course of the disease. Thoracentesis is required for these patients to relieve the discomfort of effusion. With minimal invasion, effusions and exfoliated cells will hence be available for analysis and it has been estimated that 92 per cent of thoracocenteses can provide clinically useful information. A pleural effusion is however, a sign that the disease has already progressed and most mesotheliomas are diagnosed at stage IV.

Cells extracted from various bodily fluids are examined in the diagnostic routine for several human diseases. However, cytology has had historical difficulties to establish itself as a diagnostic alternative for malignant mesothelioma. Still, when a serous cavity is affected by disease, an exudate with exfoliated cells is often the first symptom. By incrementing the procedure with specific antibodies (similar to those for immunohistochemistry) and ancillary measures such as fluorescence in situ hybridization, electron microscopy (EM; figure 3) and chemical analyses of biomarkers in the effusion supernatant, cytology should be recognised as a minimal-invasive and quick arm of mesothelioma diagnosis.

1.2.4.2 Soluble biomarkers
The measurement of disease specific markers in patient material has a long history in the clinical routine and taking the step to an personalized patient care in the future will greatly rely on accurate biomarker evaluation.

The quantification of soluble markers in pleural effusions, serum and plasma can be invaluable complements to morphological examination of malignant mesothelioma. These measurements
could speed up diagnosis, increase accuracy, predict treatment outcomes, survival and be used to follow treatment efficacy. Additionally, blood based tests could be used to monitor risk groups for mesothelioma (i.e. individuals with known asbestos exposure and/or known gene alterations) and detect signs of early disease. However, today there are few options of soluble biomarkers with high enough accuracy for a malignant mesothelioma\textsuperscript{135,136}.

**Mesothelin**

In humans, the *MSLN* gene coding for mesothelin is found on the 16p13.3 locus. Its glycosylated product is one of the most evaluated soluble mesothelioma biomarkers today. Mesothelin was first described by Chang et al. in 1992 as being expressed by ovarian carcinomas and mesothelial tissues\textsuperscript{137}; the name even refers to its strong mesothelial expression. Mesothelin has been characterised as a membrane-bound protein anchored by a glycosyl-phosphatidylinositol (GPI) link, which is evident by its release after phosphatidylinositol phospholipase C treatment\textsuperscript{138}. Mesothelin has 4 isoforms: mesothelin 1 to mesothelin 4. Isoform 1 is considered the canonical sequence, isoform 2 has a 24 base pair insertion and isoform 3 has a 82 base pair C-terminal insertion, while isoform 4 differs only by lacking a glutamic acid at position 44\textsuperscript{139}. Isoform 3’s C-terminal insertion predicts a soluble form and was assumed to be the main isoform detected in fluids from mesothelioma patients. However, additional research has shown that this is rarely the case; the primary found isoform is mesothelin 1. This protein is occasionally named as pre-pro-megakaryocyte potentiating factor. Post-translational cleavage by trypsin-like, or furin-like proteases, creates a C-terminally peptide (~40kDa) as well as an N-terminal soluble peptide (~30kDa)\textsuperscript{140} (figure 4, upper part). The GPI-bound C-terminal protein is informally referred to as mesothelin or C-ERC/mesothelin while the solubilized N-terminal 30kDa part is referred to as megakaryocyte potentiating factor or N-ERC/mesothelin. ERC is derived from studies on the Erc (Expressed in renal carcinoma) gene, which is a functional orthologue to mesothelin in the Eker rat model of hereditary renal carcinoma\textsuperscript{141}. C-ERC/mesothelin has been shown to bind cancer antigen 125 (CA125 or MUC16). This interaction affects cell adhesion and migration and could be involved in the seeding of ovarian carcinoma cells in the abdominal cavity\textsuperscript{142}. N-ERC/mesothelin is involved in the fate of megakaryocytes\textsuperscript{142,143}. Otherwise mesothelin’s physiological roles are largely unknown.

The diagnostic utility of C-ERC/mesothelin was first evaluated in sera from patients with ovarian carcinoma, where it was significantly upregulated compared to a control group\textsuperscript{144}. The enzyme linked immune-sorbent assay (ELISA) established in this study was based on the monoclonal OV569 and 4H3 antibodies and was soon used to evaluate mesothelioma patients. The first study reported an encouraging 84 per cent sensitivity at 98 per cent specificity in serum\textsuperscript{111}. Several studies have followed with similar results\textsuperscript{145-149}. The OV569 and 4H3 based assay has since been marketed under the name MesoMark™ as a blood based test to aid mesothelioma diagnosis. This particular ELISA-kit is the most readily used for this purpose in clinics as well as research today.
MesoMark™ has also been evaluated on pleural effusions from malignant mesothelioma patients\textsuperscript{147,148,150-155} where it also adds diagnostic information.

The proteolytically generated N-ERC/mesothelin fragment of mesothelin was investigated later as a mesothelioma marker. N-ERC/mesothelin was first found in a pancreatic cell line\textsuperscript{143} but further assessment showed a significant increase in sera from mesothelioma patients\textsuperscript{156,157}; though, with less discriminatory capacity than C-ERC/mesothelin\textsuperscript{158}. Shortly after, a new N-ERC/mesothelin ELISA was constructed changing the detection antibody from a polyclonal to a monoclonal, thereby increasing accuracy\textsuperscript{159}. This new ELISA has a diagnostic performance comparable with the MesoMark™-kit in sera from mesothelioma patients and controls\textsuperscript{160}.

Levels of C-ERC/mesothelin and N-ERC/mesothelin reflect tumour burden after administration of chemostatic drugs or surgical resection: both serum markers decrease initially and then increase almost to baseline levels before the recurring tumour is clinically measurable again. This indicates that both mesothelin fragments can be analysed longitudinally as surrogate endpoints. However, neither C-ERC/mesothelin nor N-ERC/mesothelin seem to be prognostic\textsuperscript{161}.

Elevated levels of mesothelin in a patient with suspected mesothelioma clearly warrant further clinical investigation\textsuperscript{162,163}. However, low sensitivity diminishes the possibility of using this biomarker as a screening marker in risk populations\textsuperscript{163}.

There are a few important pitfalls to keep in mind when measuring mesothelin related proteins in fluids: \textit{i}) decreased kidney function might lead to increased serum levels of mesothelin\textsuperscript{164-166}, \textit{ii}) mesothelin levels increase with age\textsuperscript{159}, \textit{iii}) in addition to mesothelioma, mesothelin is produced and secreted by both ovarian and pancreatic carcinomas\textsuperscript{144,167} and \textit{iv}) mesothelin is only produced by epithelial mesothelioma cells\textsuperscript{111,159}. Taking into account these confounding factors, glomerular filtration rate should be monitored whenever assessing soluble mesothelin proteins and low levels of mesothelin never exclude a sarcomatoid mesothelioma.

Regarding kidney status, a recent study described a higher diagnostic performance of pleural C-ERC/mesothelin over serum C-ERC/mesothelin\textsuperscript{155}. The explanation was that serum is more susceptible to changes in kidney clearance, as compared to pleural effusions. Furthermore, cytostatic treatment with platinum based drugs is known to cause nephrotoxicity\textsuperscript{168} and can thereby raise mesothelin values even in the absence of mesothelioma recurrence.

\textbf{Hyaluronan}

Hyaluronan is a linear glycosaminoglycan (GAG), formally known as hyaluronic acid or hyaluronate. Its structure consists of disaccharide repeats of glucuronic acid (GlcUA) and N-acetylglucosamine (GlcNAc) connected by β-linkages (GlcAβ(1→3)GlcNAcβ(1→4))\textsuperscript{169}. The biosynthesis of hyaluronan is performed by hyaluronan synthase (HAS). In humans there exist at least three homologues: HAS1, HAS2 and HAS3. The HAS enzyme is imbedded in the plasma membrane of the cells and synthesises the growing hyaluronan chain out into the extracellular space (figure 4, middle).\textsuperscript{170} One molecule of hyaluronan
can exceed 30,000 disaccharide repeats; amounting to several millions of Daltons (Da) in molecular weight\textsuperscript{171}. The architecture is simple but also very peculiar in character since it is the only known GAG that is not linked to a protein, or sulphated\textsuperscript{172}. Degradation occurs by hyaluronidases and is rapid: hyaluronan is degraded when passing the liver and its half-life in blood is only a few minutes\textsuperscript{173}.

Hyaluronan was described for the first time in isolates from the vitreous humour (hyaloid in Greek) of a cow’s eye\textsuperscript{174}, but seems to be omnipresent in vertebra tissue\textsuperscript{171}. Hyaluronan is involved in several physiological processes and can for example activate CD44 (hyaluronan receptor) in various cell types. This interaction leads to induction of VEGF synthesis and cellular proliferation in endothelial cells\textsuperscript{175}. Recent evidence indicates that hyaluronan-CD44 interactions help to regulate fibrocyte differentiation following tissue injury\textsuperscript{176}. The CD44 receptor is known to participate in cell adhesion, proliferation, cellular growth and survival, motility, invasion and differentiation\textsuperscript{177-179}.

Hyaluronan is involved in angiogenesis\textsuperscript{180} inflammation and cytokine induction\textsuperscript{181}, and rapid cell proliferation\textsuperscript{182,183}. Conditioned media from mesothelioma cells induce hyaluronan expression in fibroblasts\textsuperscript{184} and hyaluronan is also known to be expressed in tumour stroma of breast, ovarian and prostate cancers, which correlates with a poor prognosis for the patient\textsuperscript{185}. Hyaluronan asserts a multitude of specific cellular functions dependent on its molecular weight, temporospatial expression and interactions with hyaladherins (hyaluronan binding proteins).

Six years after its discovery, hyaluronan was shown to be increased in effusions from mesothelioma patients\textsuperscript{186}. Hyaluronan can be demonstrated using high pressure liquid chromatography (HPLC)\textsuperscript{187}, high performance capillary electrophoresis (HPCE)\textsuperscript{188} or electrophoresis\textsuperscript{189} as well as ELISA based techniques. Hyaluronan detecting ELISA methods utilize various hyaladherins as capturing entities\textsuperscript{190}. Elevated concentrations of hyaluronan in mesothelioma effusions have since been demonstrated by several authors\textsuperscript{153,186,191-199}. This linear GAG is a soluble mesothelioma biomarker with high specificity, but limited sensitivity for a malignant mesothelioma. At 100 per cent specificity the sensitivity ranges from circa 50 to 60 per cent\textsuperscript{153,196,197}. Hyaluronan expression seems to be indicative for the epithelioid phenotype\textsuperscript{198,199}. Additionally, hyaluronan might carry prognostic information\textsuperscript{200}. 
FIGURE 4. Established biomarkers for mesothelioma diagnosis and the syndecan family.

Upper part: Mesothelin is linked to the cell membrane with a glycosyl-phosphatidylinositol (GPI) link. Proteolytic cleavage generates a membrane bound (C-ERC/mesothelin) and a solubilised (N-ERC/mesothelin) fragment. Middle: Hyaluronan synthase (HAS) is a family of enzymes responsible for hyaluronan synthesis. This glycosaminoglycan is made up of repeating glucuronic acid (GlcA) and N-acetylglucosamine (GlcNAc) disaccharides. UDP = uridine diphosphate. Lower part: The syndecan family has four members. Their extracellular ectodomain (grey) differ between members. The transmembrane domain (dark grey) and the two conserved regions in the cytoplasm (black) show high sequence homology between members. The cytoplasmic variable region (white) exerts different functions for the syndecans. Black triangles indicate heparin sulphate attachment sites. Grey triangles indicate attachment sites for chondroitin sulphate.
There are some caveats that might affect hyaluronan sensitivity and specificity:  

1) Due to the rapid turnover of hyaluronan in blood, this marker is best suited for detection in other body fluids, such as pleural effusions.  

2) Since degradation takes place in the liver, severe liver disease might lead to increased hyaluronan levels in serum and result in false positives.  

3) Moderate levels of hyaluronan are also measured in empyema, bacterial infections (where the bacteria synthesize hyaluronan), metastatic disease and in serum of patients with rheumatoid arthritis.

**Osteopontin**

Osteopontin is a heavily post-translationally modified matricellular protein produced by a smorgasbord of cell types. Osteopontin isoforms range in size from 41 to 75 kDa and are expressed as secreted glycoprophosphoproteins with several functional domains. Osteopontin has been linked to many cellular functions including immune modulation, wound healing, angiogenesis, bone remodelling, tumour progression and metastasis. Osteopontin also works as a chemoattractant for macrophages and monocytes. Furthermore, osteopontin promotes cell survival, chemotaxis and adhesion through interactions with CD44 and various integrins and also potentiates invasion (for a review see reference 205). The CD44 interaction, and metastasis formation, can be out-competed by hyaluronan. Osteopontin is expressed in several human cancers, including melanoma, lung, head and neck, colorectal, cervical, breast, ovarian, gastric and prostate carcinomas.

A transcriptomic profiling linked osteopontin to mesothelioma and a validation study showed strong reactivity for the protein in tissue sections as well as increased levels in serum from mesothelioma patients. This first study reported a promising diagnostic ability of osteopontin. However, validation studies from other groups never reached the same diagnostic performance, either in sera, plasma or pleural effusions and osteopontin’s role as a diagnostic mesothelioma biomarker seems to be limited.

Higher levels of osteopontin in pleural effusions seem to predict shorter patient survival. Osteopontin levels also appear to mirror the effect of chemotherapy as well as the response to surgical resection of the tumour. However, there are conflicting data and the changes are not as clear as for C-ERC/mesothelin and N-ERC/mesothelin.

To be aware of when measuring osteopontin for diagnostic purpose:  

1) The general problem with osteopontin is low specificity in most settings, which is due to osteopontin’s involvement in a multitude of cancers and other conditions.  

2) The osteopontin protein has several internal cleavage sites for proteolytic degradation by thrombin and matrix metalloproteases. This means that the protein is easily degraded and lost for immunogenic analyses that target the native form. It is preferable to measure osteopontin in plasma since it seems more stable.  

3) As with mesothelin, osteopontin is also negatively correlated to the glomerular filtration rate.  

4) Additionally, the different isoforms of osteopontin seem to have different properties in malignant mesothelioma and different splice variants of the protein might be more...
accurate than others for diagnostic measures. v) Lastly, the analysis of osteopontin is strictly dependent on which ELSIA-kit is used\textsuperscript{226}, perhaps reflecting isoform diversity.

**Novel diagnostic biomarkers and biomarker panels**

In a set of meta-analyses the usage of minimal-invasive laboratory tests to assess biomarkers were deemed of limited help when diagnosing a malignant mesothelioma\textsuperscript{135,136,162,163}. Hence, novel biomarkers, or a combination of biomarkers to improve the diagnostic accuracy of this disease are needed.

It is improbable that a single biomarker would uphold both excellent sensitivity and specificity. The idea of combining biomarkers is to utilize their incremental value. For example one biomarker may correctly classify the mesothelioma patients (high sensitivity), while another correctly excludes all non-mesothelioma patients (high specificity). On the other hand, two biomarkers might both classify mesothelioma patients, but different ones, and together have high sensitivity. A different approach to aid the diagnosis of mesotheliomas by soluble compounds is the search for specific anti-tumour immune responses\textsuperscript{227}.

**Thioredoxin**

Thioredoxin-1 is a 12kDa redox enzyme involved in the cellular defence against oxidative stress. It has been shown to increase proliferation\textsuperscript{228} and inhibit apoptosis\textsuperscript{229}. Upregulation of thioredoxin has been reported in some cancers\textsuperscript{229} and DNA binding of NF-kB and AP-1 may be thioredoxin-1-dependent\textsuperscript{230}. Mesothelioma cells often have upregulated levels of both thioredoxin-1 and thioredoxin reductase\textsuperscript{231,232}.

The clinical role of thioredoxin-1 has been evaluated recently, in serum from both malignant pleural\textsuperscript{233} and peritoneal\textsuperscript{234} mesothelioma patients. Thioredoxin-1 could distinguish pleural mesothelioma patients from benign and lung cancer patients and possibly had prognostic value\textsuperscript{233}. Thioredoxin also seemed to aid the diagnosis of peritoneal mesothelioma patients compared to asbestos exposed control patients\textsuperscript{234}. These studies were rather small and need validation with more representative control groups. Still, thioredoxin seems to play a role in malignant mesothelioma pathogenesis and possibly in its diagnosis.

**Apolipoprotein-CI**

Apolipoprotein-CI achieved similar accuracy as C-ERC/mesothelin when using MALDI-ToF to screen pleural effusions from mesothelioma patients\textsuperscript{235}. When comparing 41 mesotheliomas to 48 effusions from other cancers and benign disease, apolipoprotein-CI outperformed osteopontin, CYFRA21-1 and human epididymis protein 4\textsuperscript{235}, which has earlier been described as upregulated in mesotheliomas compared to benign conditions\textsuperscript{236}. Hence, apolipoprotein-CI is a candidate that warrants further investigation.

**Fibulin-3**

A recent study verified and validated fibulin-3 as a potent new soluble biomarker for malignant mesothelioma\textsuperscript{237}. The study was based on a former gene expression analysis where fibulin-3 was described as being upregulated in diffuse malignant peritoneal
Fibulin-3 is a member of the extracellular glycoprotein fibulin family. Its expression is sparse in normal adult tissues where fibulin-3 is involved in cell-to-cell and cell-to-matrix communication, suppressing proliferation and having angiogenic effects. Suppression of fibulin-3 has been reported in several cancers. However, the protein is also upregulated in metastatic adenocarcinoma of the pancreas.

Fibulin-3 levels were measured in plasma, pleural effusions as well as serum from pleural mesothelioma patients and controls (including other cancers and asbestos exposed individuals). Initial findings showed an excellent diagnostic accuracy. However, in one blinded validation it failed to diagnose malignant mesothelioma. It was speculated that this could be due to proteolytic degradation since there are at least two reported thrombin cleavage sites in the protein. In a second blinded validation study it showed significant diagnostic value. Furthermore, pleural effusion levels of fibulin-3 increased with the stage of the disease, and were associated with shorter survival. After cytoreductive surgery fibulin-3 plasma levels dropped significantly, possibly enabling its use as a surrogate endpoint. The staining intensity and distribution of fibulin-3 were similar in epithelioid, biphasic and sarcomatoid mesothelioma as evident by immunohistochemistry. This was however, not evident for fluid levels. Fibulin-3 is a highly interesting novel biomarker for malignant mesothelioma. It needs to be compared to mesothelin and hyaluronan levels and corrected for kidney function before it can be used in clinical diagnosis, possibly in a panel of biomarkers.

**Combination of established markers**

Combinations of the most established markers seem to be difficult. An effort to join hyaluronan and C-ERC/mesothelin was considered of no clinical use. The same was concluded when trying to combine osteopontin and C-ERC/mesothelin, C-ERC/mesothelin and N-ERC/mesothelin, or all three of them (C-ERC/mesothelin, N-ERC/mesothelin and osteopontin). In paper I, however, such a model is possible to establish.

**Combining C-C motif chemokine 2, galectin-3, and C-ERC/mesothelin**

Based on a previous genome-wide gene expression analysis C-C motif chemokine 2, galectin-3, and C-ERC/mesothelin combination improves the diagnosis of mesothelioma in pleural effusions. In this study the markers were combined in a logistic regression model and assessed on pleural effusions. The model increased the diagnostic accuracy of a malignant mesothelioma over C-ERC/mesothelin alone. However, this panel needs a proper external validation.

**Genome and proteome screenings**

Oomics screenings have combined large arrays of markers to enhance diagnostic accuracy for malignant mesothelioma. The omics approach is probably the ultimate combinatorial effort. However, results including tens or hundreds, even thousands, of variables are hard to evaluate and there is often a considerable decrease in the diagnostic performance at validation (if validated at all). At present it might furthermore be technically difficult to implement such a measure in the routine clinic; a situation that will hopefully change in the future. Oomics studies are on the other hand
excellent ways of discovering novel biomarkers associated with a certain
disease.\textsuperscript{235,237,238,243}

A new technique used slow off-rate modified aptamers (SOMAmers\textsuperscript{TM}), which are
oligonucleotides with affinity for proteins (or any molecule). In a sequential manner
these SOMAmers can quantify thousands of proteins with reportedly high sensitivity
and specificity\textsuperscript{243}. This rather novel multiplexing technique was used to evaluate serum
from 117 malignant pleural mesothelioma patients and 142 asbestos-exposed controls.
Measuring approx. a thousand proteins, a model composed of 13 proteins showed
excellent diagnostic capability in training, as well as in an independent blinded
verification and validation. The model however lacks sensitivity for the diagnosis of
malignant mesothelioma at high specificity.\textsuperscript{244}

1.2.5 Proteoglycans

1.2.5.1 Structure
Proteoglycans are expressed on all nucleated cells in humans and regulate many cellular
processes\textsuperscript{245}. As the name suggests proteoglycans are proteins with covalently attached
glycosaminoglycan (GAG) chains at specific locations. These GAG chains are linear
polysaccharides with negative charge. They are repeats of disaccharides composed of a
hexuronic acid and a hexosamine making up four classes of GAGs: heparan sulphate
(HS), chondroitin and dermatan sulphate (CS and DS, respectively), keratan sulphate and
hyaluronan. HS, CS and DS are the three major GAGs found on proteoglycans, while
hyaluronan is the only GAG not connected to a core protein\textsuperscript{246,247}. The syntheses of
GAGs are non-template driven and lack proof-reading. All GAGs except hyaluronan are
synthesised and attached to their core protein in the Golgi apparatus by a set of
enzymes in a specific sequential order (sometimes referred to as the GAGosome). These
enzymes are expressed in isoform and tissue specific patterns\textsuperscript{248} and the final GAG
chains can make up the majority of the proteoglycans final weight\textsuperscript{249}. The core protein,
number and length of GAGs as well as GAG modifications (\textit{e.g.} sulphation) will to a great
extent determine the biological function of each proteoglycan.\textsuperscript{247}

1.2.5.2 Location of proteoglycans
Depending on their cellular localisation the proteoglycans are classified further into
three groups: tethered to the plasma membrane, secreted out into the ECM or retained
in the cytoplasm.

The two main families of membrane bound proteoglycans, with four and six
members respectively, are syndecans and glypicans, but CD44 and a few others have
been characterised\textsuperscript{247,250}. The syndecans are intercalated in the membrane via a
hydrophobic transmembrane segment in their core proteins, while the glypicans
are attached with a GPI-anchor. These proteoglycans are considered to be co-receptors for
heparin-binding mitogenic growth factors.

The extracellular proteoglycans include large aggregating proteoglycans called
hyalectans and small leucine-rich proteoglycans (SLRPs). Versican belongs to the
hyalectans while decorin and biglycan are both members of the SLRP family. Hyalectans
have the ability to bind hyaluronan through their N-terminal globular domain. Biglycan is thought to be the result of decorin gene duplication.

Serglycin is the only known intracellular proteoglycan and is found in haematopoietic and endothelial cells. Serglycin interacts with histamine and TNFα and is important for the storage of these inflammatory mediators in secretory vesicles.\textsuperscript{247}

1.2.5.3 Functions of proteoglycans
Tethered to the membrane or secreted into the ECM, proteoglycans are involved in a myriad of cellular activities including cell-to-cell communication, adhesion, cell survival and differentiation. On a tissue level, proteoglycans are involved in angiogenesis, axonal-growth, tissue architecture and integrity. However, proteoglycans are seldom the primary receptors for various growth factors and cytokines, but rather act as co-receptors to each ligand's prototypic receptor.\textsuperscript{250}

Versican, which is shown to be expressed by sarcomatoid mesothelioma cells\textsuperscript{110}, has been shown to bind hyaluronan, CD44, EGFR as well as integrins and toll-like receptors. These interactions are HS and DS-dependent while others are mediated through the core protein. These interactions regulate cell adhesion, proliferation, apoptosis, migration, invasion and ECM assembly and are highly involved in cancer motility and progression. Furthermore, TGFβ has been shown to regulate versican expression in several sarcomas\textsuperscript{247}. Decorin, also expressed by sarcomatoid mesothelioma cells\textsuperscript{110}, is substituted with both CS and DS and has been described as an inhibitor of tumour growth\textsuperscript{251}. It is understood that decorin inhibits tumour growth by preventing EGFR phosphorylation\textsuperscript{252}. However, the opposite has also been shown\textsuperscript{247}, underlining the importance of tissue and co-factor context for proteoglycan functions.

1.2.5.4 Syndecans
Expression
As one of the major families of membrane bound proteoglycans, the four syndecans (syndecan-1 to syndecan-4) are expressed on the majority of human cells. They are evolutionary related and have orthologues in most species\textsuperscript{253}. In humans, syndecan-1, often referred to as CD138, is expressed on epithelial cells and is a marker for plasma cells, syndecan-2 is synthesised by mesothelium, while syndecan-3 is common in neuronal tissue and syndecan-4 is expressed on most tissue\textsuperscript{254,255}. For extensive reviews see the following references: 247, 256 and 257.

Syndecan-1 expression has been shown to be negatively regulated by TNFα, but only in the endothelium, while TGFβ induced syndecan-1 surface expression occurs on epithelial cells in a post-translational fashion\textsuperscript{257}. Moreover, PDGF and TGFβ induce syndecan-1 expression in human fibroblasts and osteoblasts\textsuperscript{258}. These findings indicate tissue specific regulation.

The changes in syndecan levels can be controlled post-transcriptionally. This is evident for syndecan-1 protein levels that can increase drastically without changes in syndecan-1 mRNA level. The cell surface increase of syndecan-1 could be dependent on cyclic AMP, at least in macrophages\textsuperscript{259} and syndecan-1 can be stored in cytoplasmic vesicles\textsuperscript{260}.
In contrast, high amount of syndecan-3 mRNA can be found in heart tissue where the protein is barely detectable. In addition, syndecan-1 expression is decreased in several tumours following malignant transformation, even though the mRNA levels stay unchanged. The underlying mechanisms are often unknown. Furthermore, the syndecan family members seem to be able to regulate each other's expression, possibly in a compensatory fashion.

**Syndecan structure**
The syndecans are membrane proteoglycans with covalently bound HS. Additionally, syndecan-1 and syndecan-3 are substituted with CS. HS and CS chains are attached to a serin residue in a serin-glycin motif, and starts with a xylose-galactose-galactose-glucuronic acid tetrasaccharide, elongated with unbranched saccharides into a mature GAG chain. The core proteins vary in length from 198 to 442 amino acids and in the number of attachment sites for GAGs. Syndecan-1 and syndecan-3 show the highest sequence homology within the family, while syndecan-1 and syndecan-4 exhibit functional similarity. Each individual syndecan core protein has long stretches of individual sequence, but also shares motifs. For a schematic representation of the four syndecans see **figure 4, lower part**; for a more detailed depiction of syndecan-1 see **figure 5**.

The extracellular ectodomains vary between the four syndecans, but close to the membrane they share common cleavage sites for various proteases. The HS chains are attached close to the N-terminus on the ectodomain on all syndecans, while the CS chains have attachment sites close to the plasma membrane of syndecan-1 and syndecan-3. However, it has recently been postulated that CS can replace HS at some N-terminal sites in certain settings. Below the HS chains are amino acid sequences thought to be involved in protein-protein interaction for syndecan-1 and syndecan-4.

The transmembrane domain of hydrophobic amino acids traverses the membrane once and anchors the syndecans. Also, the transmembrane domain contains a GXXXG motif important for syndecan dimerization. The syndecans dimerize to create homodimers and heterodimers that are essential for a majority of functions.

There is a short cytoplasmic C-terminal sequence which is composed of three motifs. Nearest the plasma membrane is a conserved region (C1), followed by a varied region (V) and ends with another conserved region (C2). The conserved regions are shared between syndecans, while the variable sequence is not. The C1 domain is thought to facilitate linkage to cytoskeletal filaments. This conserved domain starts with the consensus sequence: RMKKK, which has been proposed to be a novel nuclear localisation sequence for syndecan-1. The intersectional V domain regulates cell spreading through actin and fascin bundling, and in syndecan-4 the V domain has been shown to mediate binding and regulation of PKCα and PIP2. The C2 domain is also highly conserved and comprises an EFYA motif, responsible for binding PDZ-containing intracellular proteins such as ezrin.
and syntenin. EFYA mediated bindings facilitates syndecan recycling, clustering, connection to the cytoskeleton and regulates several cellular functions\textsuperscript{250,256,269,270}.

**Ligand affinity**

The syndecans bind various ligands in the ECM and can by doing so increase the concentration of growth factors and cytokines proximal to the plasma membrane, and also present ligands to their primary receptor\textsuperscript{250}. Ligands the syndecans bind include: ECM components (e.g. fibronectin and laminins), growth factors (e.g. FGF2, VEGF and TGFβ) and several integrins. Binding is frequently mediated via the GAG chains or sometimes through specific parts of the core protein\textsuperscript{256}. GAG binding is described as being mostly low in specificity, increasing the number of possible ligands\textsuperscript{271}. Nonetheless, antithrombin has been shown to bind a highly specific HS sequence, emphasizing the importance of details\textsuperscript{272}. The significance of intact GAG chains has been shown by the decreased effect of growth factors and ECM ligands after adding soluble competitors or disrupting GAG synthesis\textsuperscript{250}.

FGF-2, which is a heparin binding factor with strong mitogenic activity has been shown to have association with all syndecans\textsuperscript{267,273,274}. For example, it has been shown that syndecan-4 mediate FGF-2 signalling dependent on both HS and its cytoplasmic domain\textsuperscript{275}.

**Cellular compartmentalization**

"Location, location, location!"

\textsuperscript{– K. Dobra}

Traditionally syndecan-1 is thought of as an exclusively membrane bound proteoglycan. However, there is increasing evidence showing a more complex cellular distribution with major effects on function. Translocation to the nucleus of syndecan-1\textsuperscript{276} together with FGF-2 might regulate proliferation\textsuperscript{267} in a heparanase-dependent manner\textsuperscript{277}. Nuclear localization has also been shown for other HS proteoglycans\textsuperscript{278}. Syndecan-1 is also associated with tubulin\textsuperscript{276} and stored in intracellular vesicles\textsuperscript{260}. Specific localisation of syndecans might be key for regulation and function.

**Shedding of the syndecan ectodomain**

Shedding of the syndecan ectodomain is a process with great impact on syndecan function. All syndecans have protease cleavage sites in their ectodomains close to the plasma membrane and the ectodomain is readily cleaved off and retrievable in cell culture media\textsuperscript{255} and body fluids\textsuperscript{256}.

The shed ectodomain can bind the same ligands as when tethered to the cell surface. This means that solubilized syndecan ectodomain can sequester growth factors and cytokines, competing with cell bound receptors. In this fashion, soluble syndecan can assert opposite function to when cell-bound. Shed ectodomain can also bind and facilitate gradients of growth factors and chemokines/cytokines that cells can then migrate along\textsuperscript{247}. The proteases facilitating ectodomain shedding of syndecan-1 are not all known, but several metalloproteases have been described\textsuperscript{279,280}. Shedding can be accelerated by growth factors, chemokines, cellular stress\textsuperscript{281} and heparanase\textsuperscript{282}. Furthermore, Rab5 regulates shedding via syndecan-1’s cytosolic domain\textsuperscript{283}. 
**Physiological and pathophysiological roles**

The syndecans affinity for such a plethora of ligands is mirrored by the vast quantity of cellular functions they affect. There is a well-established link to wound healing *in vitro* and *in vivo*\(^{250,256,257}\). The syndecans, furthermore, seem to play various roles in neuronal development, axonal growth and possibly regulating food consumption and weight gain\(^{256}\).

The syndecans are involved in both adhesion and migration; two linked processes. The migratory cell response needs an optimal, intermediate adhesion phenotype, where the cell is not too loose, or too adherent\(^{284}\). Moreover, the initiated migration can be either random or directionally persistent. The latter can be caused by several external or intrinsic stimuli, as is referred to as chemotaxis\(^ {285}\). Both syndecan-1 and syndecan-4 are involved in fibroblast adhesion and migration\(^ {250,286}\). Syndecan-1 and \(\alpha_\beta_3\) integrins mediated cell spreading of a breast cancer cell line\(^ {287}\). Overexpression of syndecan-1 leads to increased invasion of fibrosarcoma cells\(^ {288}\), while syndecan-1 depletion increased adhesion, migration, and resistance to irradiation of breast cancer cells\(^ {289}\).

The syndecans are also involved in aspects of vascular physiology. Syndecan-1, syndecan-2 and syndecan-4 have been implicated in vascular development and organization. Their angiogenic effect might in part be explained by syndecan specific HS interactions with thrombospondin-1 and thrombospondin-2. These two matricellular glycoproteins are well-known to inhibit angiogenesis in normal and malignant conditions, but also possess proangiogenic activity. Furthermore, syndecan-1’s V region seems important for thrombospondin-1 mediated adhesion of endothelial cells\(^ {256,257}\).

Syndecan-1 furthermore regulates angiogenesis through core protein interaction and activation of \(\alpha_\beta_3\) and \(\alpha_\beta_5\) integrins in human mammary carcinoma cells. The peptide synstatin (amino acid 82 to 130 in the ectodomain of syndecan-1; *figure 5*) was found responsible and identified to have strong antiangiogenic properties in a mouse model\(^ {290}\). It has since been shown that synstatin selectively inhibits a syndecan-1 coupled IGF1R-\(\alpha_\beta_3\) integrin complex in angiogenesis and tumourigenesis, and its role as a novel therapeutic strategy is discussed\(^ {291}\).

The syndecans partake in many different pathological conditions, including cancer, viral infections (HIV can in some cases be syndecan-1-dependent), obesity and myocardial infarctions (as reviewed in reference 256).

The syndecans have been implicated in the regulation of many aspects of cancerous growths. Syndecan-1 has been connected to diagnosis, patient survival, differentiation and tumour stage; proliferation, invasion and angiogenesis of many carcinomas\(^ {290,292-295}\).

Overall, the published research points to a strong link between syndecan-1 and cancer. Again it should be noted that the effects asserted by syndecan-1 seem to be cell type and context dependent.

The importance of cellular distribution, including ectodomain shedding, was illustrated in a study where mammary adenocarcinoma cells were transfected with different
constructs of syndecan-1. One construct expressed a membrane bound syndecan-1 and one translated to a constitutively shed syndecan-1. In this model, membrane bound syndecan-1 was associated with proliferation, but decreased invasiveness, while cells constitutively shedding syndecan-1 proliferated less but were more invasive\textsuperscript{296}. In another study, a shed polypeptide of syndecan-1 promoted invasion in an epithelial cell line\textsuperscript{297}.

These findings stand in stark contrast to the effects of shed syndecan-1 in myeloma cells \textit{in vivo} where transfection of the syndecan-1 ectodomain increased myeloma cells proliferation\textsuperscript{298}. These seemingly conflicting results are probably due to cell type specific effect of syndecan-1. There are additionally studies on GAG chain and their modifications relating to disease states. In malignant myeloma it has been shown that syndecan-1 HS has a greater amount of sulphated motifs than benign plasma cells\textsuperscript{299}. This could increase HS affinity for mitogens and angiogenic factors and thereby promote cancer progression.

Since syndecan-1 is predominantly expressed on epithelial cells\textsuperscript{300} and syndecan-2 is abundant in mesenchymal tissue\textsuperscript{301}, they have been proposed as a tool for distinguishing between adenocarcinomas and malignant mesothelioma\textsuperscript{302,303}. In mesothelioma, syndecan-1 correlates to cell differentiation\textsuperscript{304} and overexpression of syndecan-1 has been correlated to induction of epithelial morphology and inhibition of proliferation\textsuperscript{261}. Additionally, loss of syndecan-1 in epithelial cells leads to an epithelial to mesenchymal transformation\textsuperscript{305}. However, only a handful of studies have addressed syndecan-1’s role in malignant mesothelioma.
FIGURE 5. Schematic view of syndecan-1 and its glycosaminoglycans (GAG). The core protein is comprised of an extracellular ectodomain (ED), a transmembrane domain (TM) and a cytoplasmic domain (C1, V and C2). Heparan sulphate (HS) and chondroitin sulphate (CS) attach to serine residues in a serine-glycine motif (-SG-). HS and CS start with a tetrasaccharide of xylose-galactose-galactose (-xyl-gal-gal-) and a glucuronic acid (GlcA). HS is synthesised by alternate adding of N-acetylglucosamine (GlcNAc) and GlcA saccharides. Most GlcA is then epimerised to iduronic acid. CS constitutes of N-acetylgalactosamine (GalNAc) and iduronic acid. GAG initiation and elongation occurs in the Golgi apparatus by the GAGosome. Sulphation patterns are modified on the mature GAG at the cell surface by specific enzymes.

Sequences: synstatin (amino acids 82 to 130) have been shown to activate integrins during angiogenesis, GL is a cleavages site for ectodomain shedding, DRKE is proposed to affect oligomerisation and so is GXXXG. The RMKKK motif has been proposed as a nuclear localization signal and the EFYA motif facilitates PDZ-dependent bindings.
2 THE PRESENT STUDY

Malignant mesothelioma is notoriously hard to diagnose and the prognosis is poor. The ancillary use of soluble biomarkers with conventional morphological examination could add accuracy and shorten time to final diagnosis. One possible biomarker, syndecan-1, is involved in the drive and progression of many cancers, but information about its effects and role in malignant mesothelioma is sparse. A better understanding of this proteoglycan in malignant mesothelioma might lead to increased options for treatment in the future.

2.1 AIMS

The overall aim of this thesis focuses on the diagnostic accuracy of soluble biomarkers and the molecular machinery governed by syndecan-1. This thesis is based on four studies with specific aims as follows:

- **Aim paper I**: to combine established and novel biomarkers in an interpretation algorithm to increase the accuracy of mesothelioma diagnosis.
- **Aim paper II**: to discover new soluble diagnostic biomarkers for malignant mesothelioma.
- **Aim paper III**: to characterise mesenchymal tumours cell adhesion, migration and motility in regard to specific syndecan-1 domains.
- **Aim paper IV**: to investigate the global gene change in a mesothelioma cell line after syndecan-1 overexpression and silencing.
3 REMARKS ON METHODOLOGY

The following is a short discussion on methods and statistics involved in all the four papers. Methods exclusive for paper I and paper II are described under DIAGNOSTICS, while paper III and paper IV methods are described under SYNDICAN-1. For in-depth details please see the individual papers.

3.1 DIAGNOSTICS

3.1.1 Patient material and study designs

Large enough patient cohorts, representative control groups and external validation are crucial elements in the design of biomarker studies. Pleural effusions collected from patients by thoracocentesis were used in the translational part of this thesis work. The internal material in paper I was collected at the Department of Pathology and Cytology, Karolinska University Hospital in Huddinge, Sweden, and used for model generation. The external material was collected at the Medical Faculty of Eskisehir, Department of Chest Diseases, Turkey and was used for validation. Both the internal and the external material were collected in a prospective and consecutive way. This leads to control groups that are more representative of a clinical setting. The inclusion criteria for the internal material were strict, including only definite diagnoses and patients with long follow-up. This is important so that the model building is not undermined due to erroneously classified patients. However, the internal material was enriched for mesothelioma patients and hyaluronan had in some cases been included in the diagnostic work-up (although all diagnoses were verified with at least one independent method such as immunohistochemistry and/or EM). Therefore the internal material could have introduced biases. However, external validation on a large representative material safeguards final conclusions.

As evident from an extensive immunohistochemistry verification of a subgroup of patients from the external material it needs to be stated that the initial diagnoses were not 100 per cent accurate (see supplementary table S2, in paper I) – something that cannot be expected of any material, but needs to be kept in mind.

In paper II all material, both for initial proteome screening and validation, was from the Turkish sample collection. The validation sample in paper II were composed by randomly selecting similar numbers of mesothelioma patients and control patients resulting in a heterogeneous control group with the main differential diagnoses represented.

3.1.2 Integrity of the analyte

There are important discussions about which type of sample to analyse and how to acquire and store the material before analysis. When whole blood is collected the first step is often to eliminate erythrocytes and other cells. The acellular plasma is left to coagulate and a fibrin rich precipitate can be eliminated through a simple centrifugation step; what remains is serum. This loss of material (the coagulate) has been shown to compromise the analysis of some biomarkers while not effecting others. In our diagnostic studies (paper I and paper II) we use pleural effusions, which are thought to
resemble serum in their protein composition, and furthermore display a similar coagulative precipitate. The effusion is normally not treated to prevent coagulation and possible losses through fibrin precipitation cannot be excluded. Samples were frozen at -20°C or -80°C and care was taken to keep the number of freeze-thaw cycles to a minimum. No degradation of measured molecules was evident over time when plotting year of collection and biomarker expression values (data not shown).

A pleural effusion is often the first symptom of a malignant mesothelioma and needs to be drained. This results in samples available for analysis early in the process. Additionally, some of the effusion content will have been produced by the mesothelioma cells. There is a close proximity to the pleural tumours and a higher concentration (often ten to a hundred times higher) of measured molecules compared to serum meaning that rare proteins are more likely to be measurable. The closed pleural space is also less affected by elimination via organs outside of that circulation (e.g. hyaluronan degradation by the liver or mesothelin elimination via the kidneys) and mixing of fluids from other body compartments.

3.1.3 Measurement of biomarker levels and related biostatistics

3.1.3.1 Enzyme linked immune-sorbent assay

The main method to measure protein and hyaluronan levels in paper I, as well as validating biomarkers candidates in paper II, was enzyme linked immune-sorbent assay (ELISA) based. ELISA is a rather inexpensive technique with semi-high-throughput.

Since ELISA techniques utilises antibodies (in the case of hyaluronan it was a hyaluronan-binding protein), it also inherits all the caveats of the antibody field. Due to antibody differences there can be a large variance between different ELISA-kits using different antibody clones. This is exemplified by the development of the N-ERC/mesothelin ELISA, but the importance of choosing a suitable ELISA-kit is also evident for other proteins.

Hyaluronan is historically often measured by ion suppression HPLC, a technique that separates molecules due to differences in dissociation constant between anionic compounds. This technique is more exact but also more time consuming compared to an ELISA based assay.

3.1.3.2 Western blot

When determining established biomarker levels by ELISA in paper I, osteopontin did not discriminate a mesothelioma in our hands. To estimate osteopontin degradation we analysed a few mesothelioma patient effusions by Western blot. With correct antibodies, this technique shows the entire amount of a specific protein with possibility to distinguish eventual degradation.

3.1.3.3 Statistical measures

Logistic regression

Logistic regression is a multivariate regression analysis that measures the relationship between a categorical dependent variable (e.g. disease group) and one or several continuous independent variables (e.g. protein levels). It will give a probability that one
or several proteins are associated with a certain patient group. This analysis was performed in paper I and paper II.

**Internal validation**

Internal validation refers to testing the validity of a generated model in the same sample from which the model was generated. Permutation methods such as bootstrapping and cross-validation may be used. These methods can thus be performed on data at hand, without further enrolment of patients or analysis of samples. However, it is not as informative on the generalizability of the model as if an external validation is conducted. Bootstrapping is used in paper I, and 7-fold cross validation is used in paper II (see section Multivariate analyses (paper II), under 3.1.3.4).

The bootstrapping technique creates a new dataset by randomly selecting patients from an existing dataset with replacement. Patients can thus be selected more than once. The created dataset is yet again used to assess a biomarker or model and the results might vary from the first assessment. It is common to repeat this procedure at least a thousand times. The average model is then reported.

**Receiver operating characteristic (paper I and paper II)**

One way to describe the diagnostic capability of a biomarker is to plot its true positive rate (sensitivity) on the y-axis against its false positives rate (100 per cent minus specificity) on the x-axis. This results in a receiver operating characteristic (ROC) curve, and the area under this curve (AUC) is a measure of the biomarker’s overall accuracy. The highest possible AUC is 1, which corresponds to 100 per cent sensitivity and 100 per cent specificity. An AUC of 0 would be the perfect negative predictor. In contrast, an AUC of 0.5 indicates that the biomarker has no diagnostic value.

ROC curves are good to visualize a biomarker’s predictability and in most cases AUC values can be used to compare biomarkers. However, often the ROC does not tell the entire story and it will be especially difficult if the two curves cross. Therefore, novel measures have been suggested when comparing diagnostic predictability of biomarkers.

**Novel measures (paper I)**

Calibration slopes: It is important to evaluate the correlation between the observed risk and the predicted risk. If a new biomarker or model was perfect, then these risks would be the same and the calibration slope would overlap completely with the ideal slope. Deviations of calibration from the ideal can suggest which risks a model classifies better. For example, it can be discerned that a model is better at classifying individuals with high risk of having a disease compared to those with a low risk. For an example see figure 5c and figure 5d in paper I.

Net reclassification improvement: Diagnostic improvement by a biomarker or a model does not necessarily translate into significant changes in AUC. Net reclassification improvement tests a biomarker on how well it classifies the patients with and without the diagnosis separately. If adding a biomarker leads to more patients being classified correctly, then this is an improvement in reclassification. If fewer patients are classified
correctly with the novel measure, then that indicates worse reclassification. The sum of these changes is the net reclassification improvement.

Integrated discrimination improvement and discrimination slope: The integrated discrimination improvement is a continuation of the net reclassification improvement, where net reclassification is measured over all possible cut-offs. This is equivalent to the change in discrimination slopes. The discrimination slopes are calculated as the difference in the means from the disease group and the control group (see figure 6 in paper I).

These novel measures have been proposed as a complement and to give more diagnostic information than conventional measures of performance\textsuperscript{312,313}.

3.1.3.4 Proteomics

Identification and quantification of proteins

In a discovery study, such as the one described in paper II, mass spectrometry (MS) is a powerful tool to identify proteins. MS based proteomics was basically used to weigh peptides for identification and from them deduce information about proteins. By inducing a charge (proton) to the peptide, they can be moved by electric fields, separated by mass and detected. Using this information, the peptide mass can be measured and by including a peptide fragmentation step, the peptide sequence can be identified. From the identified peptides, proteins can be inferred — all this without using antibodies.

By measuring patient material, MS has long been thought to identify biomarkers for all conceivable diseases, but has not yet delivered to the hoped extent\textsuperscript{314}. This is partly due to the high level of complexity of biological material as well as biological and technical variation resulting in a small overlap of identified proteins between analysed samples. Furthermore, one of the most studied materials — serum — is particularly hard to analyse due to its dynamic range of proteins. In serum, one protein can be present in femtomolar while another is present in micromolar. The dynamic range of human serum is believed to stretch over more than 14 magnitudes\textsuperscript{315}. Even experienced MS laboratories have difficulties to span this dynamic range. The problem is that highly abundant proteins will mask the presence of low abundant proteins, which will be lost for analysis. Pleural effusions, as mentioned, are a similar to serum in composition and therefore offer the same challenge.

Sample pre-treatment for proteomics

In the discovery phase of paper II we selected homogenous patient groups with regards to age and phenotype of mesothelioma and controls (only lung adenocarcinomas and pleurisy). We furthermore excluded patients with known systemic diseases (such as diabetes mellitus and rheumatoid arthritis) or collision tumours. These steps were performed to reduce inter-patient variance. All pleural effusions were passed through a multi removal affinity system column (MARS-14). The MARS-14 column has antibodies directed against the human sera 14 most abundant proteins. These 14 proteins correspond to more than 90 per cent of the entire protein content of human serum\textsuperscript{316}. 
Affinity removal reduces the dynamic range, hopefully enabling the detection of low abundant disease-specific proteins. There is however, a risk that this step removes a possible mesothelioma biomarker if it shares binding site with any of the antibodies or is sequestered by one of the removed proteins.

Samples were digested using trypsin, so the resulting samples contained peptides rather than proteins. To analyse peptides for protein identifications on a mass spectrometer is called shotgun proteomics, or bottom-up proteomics. Some of the advantages of working with peptides are that they more often result in clearer spectra. Also, even though some proteins are highly hydrophobic, they will still result in some hydrophilic peptides – this is important for sequential steps in our workflow. One of the drawbacks with peptide-centric shotgun proteomics is that several isoforms can contribute with indistinguishable peptides, making it impossible to resolve some protein isoforms (protein inference). Only unique/proteotypic peptides in the data set were used for quantification of a protein group in paper II.

We used a relative quantification method called isobaric tag for relative and absolute quantification (iTRAQ). The iTRAQ mass tags label primary amines in peptides and proteins. The isobaric in iTRAQ means that all the different iTRAQ-labels have the same mass due to a variable region of the molecule. So a tagged peptide from one sample still have the same mass as the same peptide from a different sample tagged with another iTRAQ-label. At dissociation in MS/MS each iTRAQ-label produces a different reporter ion with a unique mass, giving relative quantifiable data from each individual sample. Labelling samples and pooling them reduce technical variance in following steps. In paper II we used two 8-plex iTRAQ-labels (i.e. we could label and pool 14 samples when one mass tag from each plex was used to create an internal standard to link the two resulting pools). This means that the number of samples is low; however the number of identified peptides that are common between samples will increase since 7 samples are quantified from a single peptide.

There are similar ways to label samples for pooling, with comparable pros and cons. Another option could be to screen samples label-free. Such measures of precursor ion intensity in the first MS spectra has been described as a sensitive method for estimating low abundant biomarkers. However, the number of identified proteins would decrease.

Another obstacle to overcome when screening the proteome is the complexity of biological samples. In paper II we were able to reduce the complexity by several fractionation steps. The most rigorous fractionation was achieved by ultra-narrow isoelectric focusing using an immobilized pH gradient (IEF-IPG). In brief, the samples are run on a gel-strip which has a pH gradient stretching from 4.0 to 4.25 (the range used in paper II). The peptides will migrate until they reach their isoelectric point where the net charge of the peptide is zero. Peptides with an isoelectric point outside this gradient will be lost to analysis and thereby the complexity is reduced. Here is another advantage of working with peptides, not only are they soluble at this step, but even in
this narrow pH range are the majority of all proteins are represented by at least one peptide. So, after reducing complexity, this method still retains the possibility to identify most proteins. Prior to the IEF run, we had performed a theoretical in silico digestion of known mesothelioma biomarkers so that we knew that the selected pH range would contain peptides of interest. The gel-strip is then physically fractionated into 72 parts; peptides from each are extracted and further fractionated on a nano-liquid chromatograph (nLC) for increased proteome coverage.

**Mass spectrometry**

For paper II we used the LTQ-Orbitrap Velos mass spectrometer, which is a hybrid instrument composed of a linear ion-trap and an Orbitrap. The peptides were charged by electron spray ionisation. Only peptides with charge $Z \geq +2$ were considered for fragmentation since trypsin cleaves after arginine and lysine residues, which creates one positive charge at the N-terminus in addition to arginine and lysine on the C-terminus. Five precursors were selected for collision induced dissociation (CID) and higher-energy collisional dissociation (HCD) and then analysed in the linear ion-trap quadrupole and Orbitrap, sequentially. With its hybrid system the LTQ-Orbitrap has quickly established itself as the gold standard for proteome research today, allowing for a broader dynamic range and highly accurate mass measurements. Furthermore, the LTQ-Orbitrap is suitable for iTRAQ-ion dissociation and quantification since it is possible to fragment precursors by HCD and then analyse low mass range fragments in the Orbitrap.

**Data mining**

For each analysis, there is an inverted relationship between data size and ambiguity of the results. This means that when the gigabytes of acquired MS/MS spectra are boiled down to a manageable list of identified proteins the uncertainty has increased due to inherent uncertainties in every step along the way.

The scan speed of the mass spectrometer decides the amount of spectra actually acquired and algorithms match these spectra to amino acid sequences in a database. There are different algorithms for this purpose, with some leeway allowing specific amino acid modifications and missed cleavage sites. A database is preferably a non-redundant, curated and frequently updated database. This means that there are no multiple entries per gene/protein, which make the search more efficient. Obsolete and false sequences/entries are deleted and it is up to date with high quality sequences. Non-existing sequences in the database will not be mapped. The UniProtKB/Swiss-Prot database was used in paper II, which is a non-redundant, manual annotation, high-quality (i.e. frequently reviewed and curated) database.

False discovery rate (FDR) was evaluated in paper II by the SEQUEST and Percolator algorithms using a target decoy database approach. A decoy database contains nonsense sequences (usually reversed protein sequences) that the spectra are searched against. All hits in the decoy database are considered false positives and the amount is used to estimate the FDR for that search in the target database. In paper II FDR was not
allowed to be more than 5 per cent (i.e. with those settings not more than 5 out of 100 peptides identifications would be false discoveries).

**Multivariate analyses (paper II)**
Principal component analysis (PCA) was performed to see sample distributions related to protein expression in a multivariate unsupervised way. By using all analysed protein levels, a PCA show similarities and dissimilarities between samples. In this way PCA can give an overview of the data, showing possible clusters and outliers. Values were unit variance scaled and mean centred to assume the same importance of each protein irrespective of relative abundance and variance between samples. Furthermore, log$_2$-transformation was performed to give equal weight to up and down regulation of proteins.

Orthogonal partial least squares discriminant analysis (OPLS-DA) is a supervised linear multivariate model, in which multiple proteins can be joined in a model to differentiate between sample groups. OPLS-DA gives a linear model that can predict new samples and identify related/dependent variables (proteins). The proteins that best explain the variance between selected groups are interpreted from the model.

Seven-fold cross-validation (CV) was performed on each OPLS-DA model. With this internal validation method, 1/7 of the samples is excluded from the model and predicted by a model created from the remaining 6/7. The cross-validation is repeated until all samples had been left out once. In this manner it is possible to calculate the significance of the built model by an ANOVA on the predicted scores from the CV.

**Univariate analysis (paper I)**
A normal t-test will quickly run into problems when applied to too many comparisons at once. This is becoming increasingly common in today’s medical research when the number of simultaneous analyses increase. The conventional level of $p < 0.05$, when applied to thousands of tests will inevitably lead to an increasing number of false positives (i.e. falsely rejecting the null-hypothesis). In such a situation it is needed to estimate the FDR. In paper II significant analysis of microarrays (SAM) was used for differentially expressed proteins between groups. In this method, the data is repeatedly permuted and used to assess if a protein is significantly regulated in the disease group. While performing multiple non-parametric t-tests, SAM also calculates and reports the FDR as $q$-values (corrected $p$-values).

### 3.2 SYNDECAN-1

#### 3.2.1 Cell cultures
In paper III and paper IV, we used the STAV-AB malignant mesothelioma cell line, which grows with an epithelioid phenotype. The B6FS fibrosarcoma cell line used in paper III displays a more fibroblast-like morphology. These cell lines are suitable for syndecan-1 induction studies since their endogenous level of syndecan-1 is low. Clones with stable syndecan-1 transfection were established from both cell lines by geneticin selection. Transfection rate was lower in the STAV-AB cells compared to the B6FS cell line. Additionally, it was only possible to establish sub-clones with high or low syndecan-1
expression from the B6FS cells. These sub-clones retained their respective syndecan-1 levels in later passages.

3.2.2 Syndecan-1 constructs and syndecan-1 silencing
In paper III, several constructs of syndecan-1 were used to evaluate the effects of different core protein segments on cell adhesion and motility. Cells were transfected with three truncated syndecan-1 versions either mimicking syndecan-1 shedding by i) lacking the entire ectodomain (construct 77), ii) lacking most of the ectodomain with the exception for the DRKE sequence (construct 78), or alternatively iii) only containing the penta-peptide RMKKK that is close to the plasma membrane on the cytoplasmic side. The 77 and 78 constructs resembles remaining syndecan-1 after shedding, and the DRKE sequence is thought to be important for oligomerisation. The RMKKK oligopeptide, on the other hand, is a proposed nuclear localization signal. An additional construct contained the entire, full-length syndecan-1. All four constructs were EGFP tagged and the control construct carried an EGFP protein only. Overexpression of proteoglycans might lead to aberrant GAG synthesis due to exhaustion of the GAGosome, which will have to be kept in mind during result interpretation.

STAV-AB cells stably overexpressing syndecan-1, together with STAV-AB cells silenced for syndecan-1 were analysed for their total mRNA content in paper IV. Silencing was transiently achieved by a cocktail of three siRNA constructs. A scrambled siRNA sequence was used as a negative control. Successful overexpression and silencing of syndecan-1 was evaluated both on mRNA and protein level using qPCR and fluorescent cell activated sorting (FACS), respectively.

3.2.3 Adhesion and motility (paper III)
3.2.3.1 Cell adhesion assay
To evaluate the rate of cell adhesion in paper III, the cells were allowed to adhere to the bottom of a 96-well plate for 5 minutes. After washing, adherent cells were lysed and their number was indirectly measured by staining the released DNA with CyQUANT® dye and reading as fluorescence. The acquired measure focuses on early adhesion responses.

3.2.3.2 Random movement assay
Sub-confluent cells were grown on glass bottom culture plates and time-laps imaging was conducted with an interval of 15 minutes for 16 hours. Cell movements were classified in two ways: total distance and final displacement. The first indicates the length the cells have moved away from the start position, while the latter indicates the total distance the cells have traversed regardless of direction.

3.2.3.3 Wound healing assay
Migration was evaluated by the ability of cells to close an artificial wound in their confluent population. The wound was afflicted by scratching the cell layer with a sterile
pipette tip and healing was measured with image processing software. The distance between the leading edges of the wound was measured at six random points at three, 6 and 12 hours.

3.2.3.4 Chemotaxis assay

Chemotactic migration was assessed using a 24-Transwell plate, where cells were seeded in serum-free medium in the upper compartment and allowed to migrate down through micropores towards serum supplemented medium. The number of migrated cells was then measured by collecting the cells that had migrated through and measuring their DNA content with CyQUANT® dye.

3.2.4 Transcriptomics

The Affymetrix GeneChip® Human Gene 1.0 ST microarray was used to evaluate the global gene expression of STAV-AB cells in paper III and paper IV. This gene chip contain probes mapping the entire length of each gene analysed. In this manner splice variants can be discerned, and in combination with random primers for cDNA synthesis, mRNA 3’ degradation bias is partly avoided.

To focus the global gene analysis in paper III, we used the gene ontology (GO) annotations adhesion, migration and chemotaxis. These identified genes associated with these cellular functions. There are however, misannotations and positive literature bias (see following section) that might cloud the interpretation of the results.

3.2.4.1 Gene set enrichment, network enrichment and pathway analysis (paper IV)

Characterizing differentially expressed (DE) genes as whole groups, sub-groups, or individually can be difficult. Generally, genes with altered expression (altered gene sets; AGS) are matched to known functional groups (e.g. GO terms; i.e. functional gene sets; FGS) and in classical gene set enrichment analysis (GSEA) a specific function will be ascribed to the AGS if it shares an enriched fraction of genes with a specific FGS (i.e. contains more than can be assumed by random chance alone). This analysis, however, only regards DE genes that are assigned to any FGS, while disregarding known functional links within the analysed AGS or between the AGS and FGS.

Pathway analysis (ingenuity pathway analyser; IPA), also reveals associations between sets of altered genes and known pathways or other functional sets, but the analysis is applied to so called networks. They are compact clusters or modules of genes tightly interconnected in the global network and enriched in DE genes. Then these networks are characterized as separate units. Thus, IPA analysis employs only such DE genes that can be grouped in modules, which might not always be the case.

The novel network enrichment analysis (NEA) works in similar manner to conventional GSEA, with the difference that NEA considers also links between genes of AGS and FGS in the global network. NEA was shown to increase the sensitivity (statistical power) of associating AGS with FGS compared to GSEA\textsuperscript{321}. In distinction from IPA, NEA does not
expect any ready modules in the network and uses all AGS genes that have direct network links to genes of known FGS.

GSEA, IPA and NEA employ network links derived from literature searches and high-throughput data analysis for each gene/protein, both inside and outside of canonical pathways and GO terms. This information might be too focused on published interactions that are proven to exist, while negative experimental results largely remain unpublished (the positive literature bias). This is a generic feature of all the three methods.

3.2.4.2 Verification and validation of transcriptomic findings
In paper IV, a set of identified DE genes were verified on mRNA level using qPCR or validated on protein level using a Western blot based proteome profiler assay. Verification at the mRNA level indicates the integrity of the microarray results, while validation at the protein level will strengthen the conclusions about syndecan-1’s postulated effect on cell behaviour.

3.2.5 Functional assays (paper IV)
The effect of syndecan-1 silencing on cell proliferation was evaluated by the viability WST-1 assay. WST-1 is a tetrazolium salt that acts as a substrate for many mitochondrial enzymes. The generated products colour the cell media red, and absorption is measured at 450 nanometre as an indirect correlate to cell number.

Effect of syndecan-1 silencing on mesothelioma cell proliferation was further assessed by analysing cell cycle distribution. Syndecan-1 silenced cells and controls were fixed and partly permeabilized using cold ethanol. Total nucleic acid content was stained with propidium iodide (PI) after elimination of RNA by RNase pre-treatment. Cells were analysed by FACS. Cells in G0/G1 have a certain intensity of PI, visible as a peak and corresponding to their amount of DNA. Cells with double intensity of PI are presumed to be in G2/M phase and all cells in-between these two peaks should then correspond to cells in S-phase.

To assess apoptosis, non-fixed syndecan-1 silenced cells were stained with PI and FITC conjugated Annexin-V. PI enters cells with severely damaged plasma membrane (necrotic). Annexin-V binds phosphatidylserine, a phospholipid that normally faces the cytoplasm, but in early apoptosis becomes visible on the surface of the cell. By FACS analysis we could thereby describe cell populations in early apoptosis as well as distinguish the apoptotic and necrotic fractions. However, late stages of cell death are not distinguishable using this method. Furthermore, cell stress can give reversible Annexin-V reactivity, possibly resulting in false positives.¹²²
4 RESULTS AND DISCUSSION

4.1 PAPER I: PLEURAL EFFUSION BIOMARKERS FOR DIAGNOSIS OF MALIGNANT MESOTHELIOMA

The diagnosis of a malignant mesothelioma is difficult, and the additional measurement of soluble biomarker levels could be advantageous. Several studies have tried to combine soluble biomarkers to increase the diagnostic accuracy, however, few have succeeded\textsuperscript{136}.

In paper I we evaluated 7 soluble biomarkers, 4 well-known in addition to three novel biomarkers. The 4 more established markers were hyaluronan, two proteolytically generated parts of mesothelin (the N-terminal N-ERC/mesothelin and the C-terminal C-ERC/mesothelin) as well as osteopontin. The three additional biomarkers were syndecan-1, syndecan-2 and thioredoxin. Levels were assessed on an internal sample of 190 patient effusions. Log\textsubscript{10}-transformation was used since it generated distribution more closely resembling normal distribution. By using bootstrapping it was clear that hyaluronan and N-ERC/mesothelin were the strongest predictors of a malignant mesothelioma (ROC curves for each of the 7 biomarkers can be seen figure X in paper I). In paper I ROC curves are calculated to estimate diagnostic utility. Analysis of a combinations incremental value was assessed using calibration slopes, net reclassification improvement, integrated discriminatory improvement and discrimination slopes as suggested by a recent study\textsuperscript{313}.

Syndecan-1 is associated with epithelial cells and the ectodomain is solubilised by shedding, and were detectable in the pleural effusions. Results in paper I indicates that syndecan-1 could function as a general carcinoma marker. Osteopontin did not discriminate a mesothelioma in this study. The protein was shown to be only marginally degraded, and was disregarded as a possible effusion biomarker for malignant mesothelioma.

Several groups have attempted to combine established soluble biomarkers such as hyaluronan and mesothelin without success\textsuperscript{153,158,160,220}. Most comparable to paper I is the study by Grigoriu et al. from 2009 that attempted a logistic regression based model of hyaluronan and mesothelin. Their conclusion was that there was no gain from a combinatorial approach\textsuperscript{153}.

One of the main reasons why a direct joining of these two markers by logistic regression will fail is the phenomenon of linear separation. This means that one biomarker on its own separate the disease group of interest, from control groups to such an extent that another marker will not identify additional patients. Combination in such a scenario could even result in decreased predictability.

So, as expected, in paper I combination of hyaluronan and N-ERC/mesothelin by logistic regression did not yield better predictions with the entire sample material included. However, inclusion of a cut-off based step to select patients with moderate
expression led to avoidance of linear separation. Ascribing an individual cut-off value that defined 100 per cent specificity for each biomarker before applying logistic regression to all patients bellow, led to a significantly increased accuracy over single biomarker analysis. P-values ascribing a probability of having a mesothelioma could then be calculated by incorporating log_{10} values for each biomarker into a logistic regression model. Probability ranged from 0 to 1, where 0 is no likelihood of having a mesothelioma and 1 is 100 per cent likelihood of having a mesothelioma. Besides, all patients with an initial expression level for either of the two biomarkers above their cut-off value automatically resulted in a p-value of 1.

Introduction of a cut-off value to avoid linear separation of high expressing mesotheliomas might have been the most significant contributing factor in enabling combination of hyaluronan and N-ERC/mesothelin. However, when comparing to the study by Grigoriu et al., additional differences are evident. Most notably, they utilized the C-terminal part of mesothelin (as measured by the MesoMark™-kit) and only had pleural effusions from a limited number of patients (20 mesotheliomas and 29 controls)\textsuperscript{153}.

A crucial part of our study involved a large external validation. When applying the two-step model on the external validation material there was shrinkage of predictability. There was still, however, an advantage in combining both biomarkers over single analyses.

The reduced performance upon validation is expected and will partly be due to differences in the study populations: for example, in the external material, less laboratory support for the diagnosis might lead to the inclusion of some more uncertain diagnoses. Furthermore, with a different source of exposure, the Turkish material had a larger proportion of women with mesothelioma (65 per cent compared to 11 per cent in the internal material) and to some extent it also had a different setup of diagnoses in the control group. Additional biases, such as batch effects, seem less likely but could imply a difference as well. Accordingly, a model will almost always perform best on the material it was created in, and therefore it is of utmost importance to include a proper external validation.

This study furthermore indicates that the N-ERC/mesothelin is to be preferred over C-ERC/mesothelin when measuring mesothelin to aid mesothelioma diagnosis. The two mesothelin entities had similar discriminatory capacity, however, N-ERC/mesothelin was marginally better, and more strikingly, was selected over C-ERC/mesothelin in all bootstrap iterations.

This study shows that a combination of hyaluronan and N-ERC/mesothelin was achievable after avoiding linear separation. In this way the sensitivity for a malignant mesothelioma was significantly increased. Still, a fraction of the mesothelioma patients go undiagnosed.
The most established biomarker combinations will not identify all malignant mesotheliomas (paper I), it is therefore an urgent need for new soluble biomarkers to aid diagnosis. In paper II we initiated a discovery phase where we screened the proteome of pleural effusions from 6 epithelioid mesotheliomas, 6 metastatic lung adenocarcinomas and two pools of effusions caused by benign conditions. Sample pre-processing included affinity removal of abundant serum proteins, iTRAQ mass labelling and ultra-narrow IEF fractionation. Two iTRAQ 8-plexes were used, and when analysed with nLC-ESI-MS/MS, 1184 proteins were detected in the first iTRAQ pool and 569 proteins were detected in the second iTRAQ pool. The overlap between the different pools was 382 proteins. The evaluation with biostatistics and bioinformatics focused mostly on the overlapping proteins.

Principal component analysis showed a relatively high homogeneity between samples. OPLS-DA supervised clustering and model building identified 37 proteins in a model with moderate discriminatory capacity of a mesothelioma from a lung adenocarcinoma. SAM analysis identified differentially regulated proteins comparing mesothelioma and lung cancer effusions, while assessing their q-values. It is likely that the influence of biological variance between individuals, and the small number of patients is the cause of rather high q-values (> 20 per cent) found in this study. Nevertheless, several proteins known to be involved in mesothelioma pathobiology and or diagnosis were identified, strengthening our study design.

Candidate biomarkers were chosen based on these analyses and on biological interactions and information about the identified proteins. These candidates were further validated on a larger population of patient effusions. Validation included controls with a composition encountered in the clinical setting. It was unfortunately not uncommon that initial MS findings and ELISA validation did not correlate. This could be due to the protein inference problem with shotgun proteomics or due to varying antibody specificities of the ELISA assays compared to antibody-free MS analysis.

Findings in paper II include kallistatin, an inhibitor of both TNF-α and NFkB signalling. In our MS based screening, kallistatin was lower in mesotheliomas than controls, which is a cross-validation of another recent study on the same topic. Proteins involved in cells ROS response were upregulated in the mesothelioma patients (superoxide dismutase-2 and catalase).

Individual patient pathway analyses indicate well-known mesothelioma and cancer hubs (ERK1/2, EGFR and NFkB). Interestingly, ezrin and moesin were downregulated in pleural effusions from some mesothelioma patients (data not shown). These proteins are closely related and share several functions with merlin. They all interact with CD44.
and suppress cell proliferation\textsuperscript{324}. However, due to few patients and high variance, it is hard to draw any conclusions from this analysis.

One of the major findings includes the excellent discrimination of mesotheliomas and adenocarcinomas by galectin-1. Galectin-1 is a matricellular protein that has been shown to deactivate T-cells by regulating T-cell apoptosis\textsuperscript{325}. Galectin-1 enables carcinoma cells to evade the immune system and invade surrounding tissue and metastasise\textsuperscript{326}. In agreement with this, galectin-1 was highly expressed in the metastatic adenocarcinomas in \textit{paper II}. The difference between mesothelioma cells and benign reactive mesothelium is less pronounced and will probably not be enough for discrimination between these two conditions (see \textbf{figure 4} in \textit{paper II}).

Several proteins showed prognostic trends in the mesotheliomas, including galectin-1 (p-value = 0.17) where high values were associated with shorter survival. However, aldo-keto reductase 1B10 was the only validated prognostic candidate to be considered significant (p-value = 0.01; see \textbf{figure 5} in \textit{paper II}). High levels of aldo-keto reductase 1B10 predicted shorter survival. Aldo-keto reductase 1B10 is an aldose reductase that reduces certain aldehydes\textsuperscript{327}. Interestingly, aldo-keto reductase 1B10 expressions seem to be EGF and AP1 dependent\textsuperscript{328} and has been linked to carboplatin and gemcitabine resistance in bladder cancer\textsuperscript{329}. Information about tumour stage was not available in \textit{paper II}, so a more biological association of aldo-keto reductase 1B10 with survival time could not be properly assessed.

There is a need for novel mesothelioma biomarkers today. Screening the effusion proteome from patients identified thousands of proteins. To filter out and identify possible biomarkers is a difficult task, however, in \textit{paper II} we present several promising candidates including galectin-1 and aldo-keto reductase 1B10.

\begin{subsection}{4.3 \textbf{PAPER III: SPECIFIC SYNDECAN-1 DOMAINS REGULATE MESENCHYMAL TUMOR CELL ADHESION, MOTILITY AND MIGRATION}}

A better understanding of the motility of malignant mesenchymal cells could help to explain the invasive nature of these tumours.

In \textit{paper III} we focus on the possible biomarker syndecan-1, in regard to how different domains affect cell adhesion, motility and migration. We transfected various constructs of syndecan-1 into mesothelioma and fibrosarcoma cells while monitoring adhesion and motility read-outs from wound-healing, chemotaxis and live cell imaging. Additionally, we performed a transcriptomics analysis after syndecan-1 overexpression with focus on gene-changes especially affecting adhesion, migration and/or chemotaxis.

Overexpression of full-length syndecan-1 led to enhanced adhesion in the fibrosarcoma cell line in a dose-dependent manner. Overexpression decreased \textit{serum induced} migration in both cell types, possibly also syndecan-1 dose-dependent.
As measured by time-lapse microscopy, syndecan-1 decreased the final displacement of fibrosarcoma cells, while not affecting the total distance of their migrated path. So, in fibrosarcoma cells, overexpression of syndecan-1 decreased directional migration, but not total movement. These data indicates that overexpression of syndecan-1 in this fibrosarcoma cell line does not decrease motility, but induces adhesion and by doing so affects migration. Furthermore, in vitro wound-closure was impeded after transfection of full-length syndecan-1 in both cell lines.

Constructs of different syndecan-1 domains showed that adhesion was largely achieved through the GAG containing ectodomain. The RMKK construct decreased cell adhesion in the fibrosarcoma cell line. In the same cell line the 78 construct increased cell adhesion. Full-length syndecan-1 increased adhesion dose-dependently. In the B6FS cell line, the 78 construct also decreased final displacement, whilst the 77 construct did not. This indicates the importance of the juxtamembrane DRKE motif. However, all truncated constructs increased final displacement in the mesothelioma cell line.

Furthermore, all constructs, except RMKK, decreased serum initiated cell migration in both cell lines. The RMKK construct, only impaired chemotaxis of fibrosarcoma cells. All constructs seemed to slow down in vitro wound healing.

Deletion of RMKKK furthermore showed a loss of nuclear syndecan-1 (figure 6 in paper III), which is in line with earlier findings, where deletion of RMKKK decreased proliferation261. It can be suggested that the effects of the RMKKK construct on adhesion is linked to nuclear translocation of full-length syndecan-1. It could be that the RMKKK peptide out-competes syndecan-1 for nuclear shuttling, and thereby affects proliferation and also adhesion.

Measuring the global gene changes in the mesothelioma cell line after full-length syndecan-1 overexpression highlighted several interesting genes. MTSS1, one of the most upregulated genes, has been reported to suppress cancer migration330 while the most downregulated gene (SLAMF7) belongs to a family also affecting adhesion331. A large fraction of the deregulated genes were linked to adhesion, migration and chemotaxis (GO terms).

These results indicate varying roles of different syndecan-1 domains as well as cell line specificity. Syndecan-1 seems to increase adhesion on the expense of cell motility and migration in the fibrosarcoma cell line. While in the mesothelioma cell line, adhesion is lost together with increased motility. However, the results are varying in effect and direction. It cannot be excluded that some of the observed effects are influenced by changes in the remaining syndecans (most likely syndecan-2 and syndecan-4), since the levels of the syndecan family members seem to include some extent of redundancy261.

Furthermore, migration is described as having a biphasic migration-velocity response to ECM adhesion284, where too much adhesion will reduce migration, as will too little adhesion. This needs to be taken into account when interpreting the results.
The findings presented in paper III indicate that mesenchymal cell motility is affected by various syndecan-1 domains in cell line specific patterns.

4.4 PAPER IV: NOVEL GENES AND PATHWAYS MODULATED BY SYNDECAN-1: IMPLICATIONS FOR THE PROLIFERATION AND CELL-CYCLE REGULATION OF MALIGNANT MESOTHELIOMA CELLS

The study in paper IV was designed to elucidate gene and pathway changes related to syndecan-1 in the malignant mesothelioma STAV-AB cell line. We modulated the levels of syndecan-1 by overexpression or silencing and then followed changes in the global gene expression.

We know from previous studies that overexpression of syndecan-1 reduces proliferation of STAV-AB cells\textsuperscript{261}. So we determined the effect of syndecan-1 silencing on proliferation and cell cycle distribution. It was evident that silencing of syndecan-1 decreased proliferation, as seen by reduced cell number and extended doubling time. Furthermore, cell cycle distribution was significant altered.

Transcriptome analysis indicated a remarkable effect on the global gene expression after syndecan-1 modulation. The change was most pronounced in the syndecan-1 overexpressing cells: at a cut-off of more or less than two folds differential expression, there were 1,124 DE genes in the syndecan-1 overexpressing cells and only 21 in the syndecan-1 silenced cells (q-value < 0.05). Lowering the threshold to one and a half times up- or downregulated increased these numbers to 2,389 and 103, respectively. Fourteen genes were affected by both silencing and overexpression of syndecan-1 (see table 1 in paper IV).

We employed traditional GSEA and pathway analyses as well as a novel NEA approach to determine structure within this vast list of DE genes. Several transcripts encoding enzymes involved in heparan sulphate sulphation patterning (SULF1, SULT1B1 and SULT1E1) were significantly downregulated after syndecan-1 overexpression, which indicates a changed GAG profile of these cells. This could have effects on many GAG dependent cellular processes.

Genes regulated by syndecan-1 overexpression were involved in cell proliferation and cell cycle progression, cellular adhesion, migration and ECM organisation. Furthermore, pathways that belong to TGFß signalling and growth factor signalling (PDGF, FGF, EGF and VEGF) were affected along with cytokine signalling in syndecan-1 overexpressing cells. Several of these changes could be specific to the mesothelioma cell line used.

DE genes in the syndecan-1 silenced cells were fewer. However, the pathways that they affected were still numerous. Pathway analysis show effects on cellular movement, cell death, growth and proliferation, cell-to-cell signalling and several other processes (figure 6 in paper IV). The fact that syndecan-1 silencing affected a much lower number
of genes could be explained by the initial low amount of endogenous syndecan-1 in these cells.

Functions affected by syndecan-1 modulation showed strongest association with adhesion, motility, proliferation and cell cycle. Moreover, pathway analysis indicated a common effect on interleukin and growth factor signalling. NEA analysis of both syndecan-1 overexpressed and syndecan-1 silenced cells revealed an astounding effect on cell proliferation. Almost all analysed pathways linked to cell cycle were depleted after syndecan-1 overexpression and enriched after syndecan-1 silencing – asserting the role of this proteoglycan on proliferation. NEA moreover, identified mutual effects on several KEGG pathways (such as signalling by EGFR, pathways in cancer and VEGF signalling) and growth factor signalling. Hence, NEA confirmed many findings and also indicated effects on the vast majority of other pathways and cellular functions (figure 9 in paper IV).

In paper IV it is shown that syndecan-1 silencing hampers proliferation. Since a similar effect has been reported earlier for syndecan-1 overexpression\textsuperscript{261}, it indicates that there might be an optimal amount of syndecan-1 to allow maximum proliferation in these mesothelioma cells. It might be a similar bell-shaped curve as is reported to exist between adhesion and migration. TGFβ2 is down regulated upon syndecan-1 overexpression, and interestingly, it has been shown that TGFβ2 has an anti-proliferative effect in mesothelioma cells by delaying syndecan-1 translocation to the nucleus\textsuperscript{332}.

Taken together paper IV shows a great impact of the level of syndecan-1 in this malignant mesothelioma cell line on the global gene expression, especially proliferation.
5 GENERAL CONCLUSIONS

5.1 SUMMARY OF MAJOR FINDINGS

Paper I: Combination of established mesothelioma biomarkers

Σ Combination of biomarkers into a model was possible when a cut-off level was included to avoid linear separation.

Σ Evaluating N-ERC/mesothelin instead of C-ERC/mesothelin in pleural effusions seems to be preferred.

Σ The constructed two-step model, including hyaluronan and N-ERC/mesothelin, increased sensitivity for diagnosing a malignant mesothelioma in pleural effusions. Since pleural effusions are often the first symptom of a mesothelioma, this finding could aid diagnosis.

Paper II: Proteome discovery of de novo biomarkers for malignant mesothelioma

Σ More than a thousand proteins were identified in pleural effusions from mesothelioma patients and controls, with links to several interesting cancer associated pathways.

Σ Galectin-1 is described for the first time as a strong negative predictor of malignant mesothelioma compared to metastatic adenocarcinomas.

Σ Aldo-keto reductase 1810 is described for the first time as a prognostic biomarker for malignant mesothelioma.

Paper III: Syndecan-1 domains with varying influence on adhesion, migration and motility

Σ Adhesion of mesenchymal tumour cells is negatively correlated to migration and altered following syndecan-1 overexpression.

Σ Various parts of the core protein assert different effects; broadly it seems as though the ectodomain is more important for cell adhesion, while the transmembrane and cytoplasmic domain is enough to inhibit cell migration. However, these effects seem to be context dependent.

Σ The novel nuclear localization signal RMKKK might be involved in mesenchymal tumour cells motility by decreasing the amount of full-length syndecan-1 translocated to the nucleus.

Paper IV: The influences of syndecan-1 on the transcriptome

Σ Syndecan-1 levels of a malignant mesothelioma cell line greatly influenced the transcriptome.

Σ Overexpression of syndecan-1 regulated genes involved in cell proliferation, adhesion and migration, which is in line with earlier findings.

Σ Together, silencing of syndecan-1 and overexpression of syndecan-1 affected a multitude of cellular signalling pathways – most strikingly cell cycle networks.

Σ Links to interleukin, growth factor and TGFβ signalling pathways and GAG modifications are identified.
5.2 FUTURE PERSPECTIVES

Combination of biomarkers is an appealing way of reaching sufficient diagnostic accuracy to aid the morphological assessment of a malignant mesothelioma. However, combinatorial efforts have struggled. In paper I we describe the incremental value of hyaluronan and N-ERC/mesothelin, but in the future more biomarkers will have to be added to a growing panel. Such additions could include galectin-1 (paper II), fibulin-3 and others.

If the panel reaches several tens, or hundreds, of proteins, then conventional ELISA techniques will most likely be impractical in the clinical routine. In paper II a model with 37 variables showed moderate discrimination, and it would probably be beneficial to assess such a model using similar mass spectrometry based methods (e.g. multi reaction monitoring) or other multiplex assessment (e.g. Luminex® or SOMAmer technology).

Validation studies of galectin-1 will be performed together with additional biomarkers that are able to separate malignant and benign cases. The finding of a possible prognostic role for aldo-keto reductase 1B10 might be of interest for treatment purposes. High levels of this enzyme can lead to chemo resistance and possibly explain shorter patient survival. There are specific aldo-keto reductase 1B10 inhibitors developed for the treatment of diabetes mellitus (e.g. Tolrestat) that could be given neoadjuvant to patients with high levels of the enzyme. This could sensitisate these patients to chemostatic treatment, resulting in longer survival times. It is not uncommon that prognostic markers go forward to predict treatment.

In both paper I and paper II, there is an interesting sub-group of patients suffering benign asbestos pleuritis. These patients have been exposed to asbestos and developed a chronic inflammation in the pleura, but not mesothelioma. This group has a high risk of developing this tumour and could be the key to identify early, sub-clinical markers for the disease.

It will, however, be in serum that such early markers will be evaluated. The pleural effusions might be the first symptom, but is still a sign of advanced disease. Also, in the case of assessing surrogate endpoints to show efficiency of treatment, serum markers will have to be developed. Some of these studies have already been undertaken in our laboratory.

Syndecan-1’s effect on mesenchymal cell behaviour is not fully understood. The clarification of this proteoglycans role in cell migration and cancer invasion could be rewarding for combatting the disease. Paper III touches upon these questions, but more studies will have to be conducted. The multitude of gene changes described in paper IV furthermore elucidates and highlights syndecan-1’s role in mesothelioma adhesion, migration and cell proliferation. Based on paper III, paper IV and published research, syndecan-1 could be interesting to examine with focus on the EMT character of malignant mesothelioma. For example the role of TGFβ in mesothelioma cells...
transdifferentiation and invasion. Findings in paper IV indicate several growth factors, cytokines and GAG modification that will be exciting to investigate. Moreover, the novel NEA method described seems to be a powerful tool for a holistic view on transcriptomic changes.

Syndecan-1 is investigated in paper I as a biomarker for carcinomas and seems to carry diagnostic information. Syndecan-1 is furthermore identified in paper II as downregulated in mesothelioma effusions compared to effusions from lung cancers (however, with high q-values). These findings together with syndecan-1’s role in tumour cell adhesion, migration and proliferation (paper II and paper IV) makes this proteoglycan interesting to study further in mesenchymal tumours and especially in malignant mesothelioma.

Final remarks
As a whole, this is a translational thesis with diagnostic implications as well as experimental studies of syndecan-1-dependent cell behaviour. The outcomes include increased diagnostic sensitivity for a malignant mesothelioma, possible new biomarkers and an increased understanding of syndecan-1’s role in mesenchymal tumours. In the imminent future are studies to validate mesothelioma biomarker candidates and to follow-up molecular pathways regulated by syndecan-1.
6 ACKNOWLEDGEMENTS

“Join science, see the world!”

– A. Hjerpe

And it has been quite a remarkable journey. I am not only referring to all conferences around the globe, or laboratory visits and courses in Sweden and abroad. But also meeting and interacting with all the people that have helped me along the way!

Life is a bit like science, you try to base your decisions on the information at hand, evaluate the outcome and create a hypothesis (or principles). Then you do it again. And again (that is apparently why it is called re-search). Just like in science, in life you will not get very far without the people around you – your collaborators. I would like to acknowledge them all, but I apologise if I forget anyone.

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😊
7 REFERENCES

28 de Klerk, N. H. et al. Comparison of measures of exposure to asbestos in former crocidolite workers from


Synergy between asbestos and smoking, asbestos exposure, and smoking, asbestos fiber type and lung asbestos fiber type and lung cancer risks. Environ Health Perspect 105 Suppl 5, 1257-1260 (1997).


Bott, M. et al. The nuclear deubiquitinase BAP1 is commonly


106 Fusco, V. et al. Malignant pleural mesothelioma. Multivariate analysis of

53


126 Hjerpe, A. in 11th International Conference of the International Mesothelioma Interest Group p52 (Boston, USA, 2012).


Kobayashi, E., Takai, S. & Hino, O.

Yamashita, Y., Yokoyama, M.

Biomarkers Prev
diagnostic marker.

Hellstrom, I.

12

www.uniprot.org

(1996).

Proc Natl Acad Sci U S A

tumors, and ovarian cancers.


Scholler, N. et al. Soluble member(s) of the mesothelin/megakaryocyte potentiating factor family are detectable in sera from patients with ovarian carcinoma. Proc Natl Acad Sci U S A 96, 11531-11536 (1999).


Prognostic Value of Soluble Mesothelin

Hollevoet, K.

Serum.

Measurement of soluble mesothelin in

The rate is a confounder for the

Hollevoet, K.

Data meta-

Analysis.

Malignant mesothelioma: a meta-

Analysis.

Luo, L.

Prospective longitudinal cohort study.

Asbestos

Of mesothelioma serum biomarkers in

Hollevoet, K.

Med.

Mesothelioma.

Mesothelin

Potentiating factor, and mesothelin

Proteins as markers in the serum of

Potentiating factor, and mesothelin

Onda, M. et al. Megakaryocyte

Potentiation factor cleaved from

Mesothelin precursor is a useful tumor

Marker in the serum of patients with


Shiomi, K. et al. Novel ELISA system for

Detection of N-ERC/mesothelin in the


Creaney, J. et al. Comparison of

Osteopontin, megakaryocyte

Potentiating factor, and mesothelin

Proteins as markers in the serum of


Shiomi, K. et al. Sensitive and specific

New enzyme-linked immunosorbent

Assay for N-ERC/mesothelin increases

Its potential as a useful serum tumor


Hollevoet, K. et al. Diagnostic

Performance of soluble mesothelin and

Megakaryocyte potentiating factor in


Hollevoet, K. et al. Serial measurements

Of mesothelioma serum biomarkers in

Asbestos-exposed individuals: a


Luo, L. et al. Diagnostic value of soluble

Mesothelin-related peptides for

Malignant mesothelioma: a meta-


Hollevoet, K. et al. Serum mesothelin

For diagnosing malignant pleural

Mesothelioma: an individual patient

Hollevoet, K. et al. Glomerular filtration

Rate is a confounder for the

Measurement of soluble mesothelin in


Hollevoet, K. et al. The Effect of Clinical

Covariates on the Diagnostic and

Prognostic Value of Soluble Mesothelin

and Megakaryocyte Potentiating


Shiomi, K. et al. Impact of renal failure

On the tumor markers of

Mesothelioma, N-ERC/mesothelin and


Argani, P. et al. Mesothelin is

Overexpressed in the vast majority of

Ductal adenocarcinomas of the

Pancreas: identification of a new

Pancreatic cancer marker by serial


Launay-Vacher, V., Rey, J. B., Isnard-Bagnis, C., Deray, G. & Daouphars, M.

Prevention of cisplatin nephrotoxicity: state of the art and recommendations

From the European Society of Clinical Pharmacy Special Interest Group on


Weissman, B. & Meyer, K. The Structure of Hyalobiouronic Acid and of Hyaluronic


Itano, N. & Kimata, K. Mammalian


McDonald, J. & Hascall, V. C.


Laurent, T. C. & Fraser, J. R. Hyaluronan.


Meyer, K. & Palmer, J. W. The

Polysaccharide of the vitreous humor. J.

Biol. Chem. 107, 629-634 (1943).

Murphy, J. F. et al. Engagement of CD44

Modulates cyclooxygenase induction, VEGF generation, and proliferation in


Maharjan, A. S., Pilling, D. & Gomer, R.

H. High and low molecular weight

Hyaluronic acid differentially regulate human fibrocyte differentiation. PLoS

One 6, e26078, (2011).

Du, L. et al. CD44 is of functional

Importance for colorectal cancer stem


Ponta, H., Sherman, L. & Herrlich, P. A.

CD44: from adhesion molecules to


Vuskovic, M. I., Xu, H., Bovin, N. V., Pass, H. I. & Huflejt, M. E. Processing and analysis of serum antibody binding signals from Printed Glycan Arrays for...


