Protein biomarkers in chronic disease : proteomics-driven discovery

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PROEFSCHRIFT

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Contents

1 General introduction

Biomarkers

Nowadays, biomarkers are of fundamental importance in medical care as they provide a wide range of clinical information (Table 1.1), like the routine use of cardiac troponin to diagnose myocardial infarction, or brain natriuretic peptide (BNP) to identify patients with heart failure, two established diagnostic biomarkers in heart disease. Furthermore, biomarkers can have a role in risk stratifying patients for preventive interventions, screening populations for early disease detection, sub-typing disease to facilitate therapy, and monitoring response to treatment. Additionally, biomarkers can stimulate the development of new generations of therapeutics.

Table 1.1. Examples of biomarkers and their clinical application.

Clinical application	Example
Diagnosis	Troponin for myocardial infarction [1] and BNP for heart failure [2]
Risk stratification	C-reactive protein: cardiovascular outcomes after preventive statin use [3]
Screening	Phenylalanine for neonatal phenylketonuria [4]
Prognosis	Amyloid β42: from mild cognitive impairment to Alzheimer's disease [5]
Sub-typing to facilitate therapy	Her-2/neu status in women with metastatic breast cancer [6]
Monitoring response to treatment	Hemoglobin A1c in diabetes to assess glycemic control [7]

Although the current clinical biomarker arsenal has convincingly proven track record, there is still room for improvement and refinement. Clinically effective new biomarkers must have high sensitivity (i.e., low false positives), high specificity (i.e., low false negatives) and be sufficiently robust. Besides these requirements, new markers should have an additional improvement compared the current gold standard, for instance a reduced cost or less invasive sampling procedure. However, very few new biomarkers were introduced in routine clinical chemistry laboratories over the last 15 years, on average 1.5 new proteins per year; well-known examples are the immunoassays for lipoprotein(a), cystatin C, BNP and cardiac troponin [8]. Even more, the time frame from discovery to implementation in routine clinical practice is on average 17 years [9], as illustrated by prostate-specific antigen (PSA). Forty years ago, PSA was identified for the first time [10]. However, its clinical utility in prostate

cancer was not proven until 1987 [11]. The 1st International Standard for PSA was established and published in 2000 [12]. Even now, the appropriate clinical application and interpretation of PSA measurements remains controversial, indicating the long and difficult journey from biomarker discovery to acceptance for clinical diagnosis and follow-up of prostate cancer patients.

Although single biomarkers may be useful in selective cases, there is a growing consensus that an integrated panel of multiple disease-specific biomarkers will be required for most clinical applications, such as for example the Framingham formula [13]. This formula, used for risk stratification in coronary disease, takes into account a panel of biomarkers, consisting among other things of age, total cholesterol, systolic blood pressure, smoking status and diabetes mellitus.

For the discovery of new biomarkers and panels of biomarkers, proteomics has proven to make valuable contributions. As these proteomic technologies are excellent tools for unbiased simultaneous detection of a large amount of peptides and proteins [14-19].

Proteomics

In recent years, there has been an enormous growth in the use of genomic information in science. This has greatly expanded the insight into the genetic basis of certain diseases [20-22]. These advanced understandings, however, also stipulated that previously suggested one-gene, one-protein theory is known not to hold [23]. Proteins not only result from the direct translation of genetic material, but also arise from alternative splicing of both messenger RNA and proteins, and from post-translational modifications (PTM). These protein modifications have the potential to alter protein structure and/or function. Proteins are molecular tools responsible for functional output in living organisms by performing most catalytic, structural, and signalling functions. So, the proteome is a dynamic collection of proteins that reflect both the intrinsic genetic programme of the cell and the impact of its immediate environment. Measurements of proteins offer abundant opportunities to detect and characterize molecular malfunctions related to disease and its progression [24-27].

Clinical proteomics

Although currently the main focus is on biomarker discovery for diagnostics, clinical proteomics also attributes to the identification of new therapeutic targets, drugs and vaccines improving therapeutic outcome and successfully preventing diseases. The ultimate goal of biomarker discovery is usually the development of a simple blood test; therefore serum is the fluid of choice in biomarker discovery. It is not only the primary clinical specimen, with

hundreds of millions of tubes withdrawn every year for medical diagnosis, but also represent the most complete informative proteome from a medical point-of-view providing information on both physiological and pathological processes.

Although simple in principle, proteomic analysis of human serum is as such an analytical challenge due to the predominance of several highly abundant proteins and extraordinary dynamic range of proteins in serum. This tremendous dynamic range can be illustrated by two measurements used in current clinical practice: serum albumin (32 – 47 x 10^9 pg/mL) and interleukin-6 (0 – 5 pg/ml). To improve biomarker finding in the lower abundance range different approaches to deplete the high abundant ones have been suggested [28-31], but these strategies have several limitations. First of all, even after a perfect depletion, removing the twenty-two most abundant proteins, the sensitivity of current proteomic technologies is insufficient to adequately detect the proteins in the lowest concentration range. In addition, due to carrier functions of albumin and aspecific removal, interesting less abundant proteins may also be eliminated during the depletion process, resulting in loss of information and potential discrepancies between samples. Currently, there is no general consensus whether depletion of high-abundant proteins is a good strategy for biomarker discovery.

Quantitative proteome profiling

Quantitative proteome profiling is a key step to reveal differentially expressed proteins that are associated with different disease states. This can be done using various proteomic technologies (for more detailed information see [32, 33]). A widely used approach for discovery and identification of differentially expressed proteins from complex proteomic samples is the combination two-dimensional (2D) gel electrophoresis and mass spectrometry (MS) analysis. Samples are first separated by 2D gel electrophoresis, followed by gel image analysis. The differentially expressed spots are excised and in-gel digested with a protease prior to MS analysis in order to determine protein identity (Figure1.1).

Since its introduction in the 70s, 2D gel electrophoresis has been a core method in proteomic research. This technology separates thousands of proteins in a biological sample according to their isoelectric point (pI) and molecular weight (MW). Proteins are amphoteric molecules that carry either a positive, negative or zero net charge, depending on the pH of their environment. The pI of a protein is the pH at which the protein has a zero net charge. At pH values above its pI, a protein carries a net negative charge, and consequently at pH values below the pI, a positive charge. The first stage in 2D gel electrophoresis involves isoelectric focusing (IEF). By placing proteins in a pH gradient with an electric field, proteins will migrate to the pH where they have no net charge (i.e. their pI). Once there, if a protein drifts from its pI, it immediately gains charge and will then migrate back to its pI. In the second dimension, proteins are separated by MW in polyacrylamide gels containing sodium dodecyl sulphate (SDS). This

anionic detergent denaturizes proteins and forms negatively charged protein/SDS complexes. The amount of SDS bound to a protein is directly proportional to MW and proteins are therefore exclusively separated by MW [34-36].

A major limitation of 2D gel electrophoresis is the lack of reproducibility between gels which makes it difficult to distinguish any true biological variation from system variation. Parts of these issues are solved by an improved version of conventional 2D gel electrophoresis, namely two-dimensional differential in-gel electrophoresis (2D-DIGE), which we used in our biomarker discovery studies. The problematic between-gel reproducibility associated with conventional "one sample per gel" 2D gel electrophoresis was addressed by this new technique. It uses fluorescent dyes, which enable simultaneous separation of multiple experimental samples on a single gel (i.e., multiplexing) and as a consequence identical proteins labelled with these dyes will migrate to the same position on a gel [37].

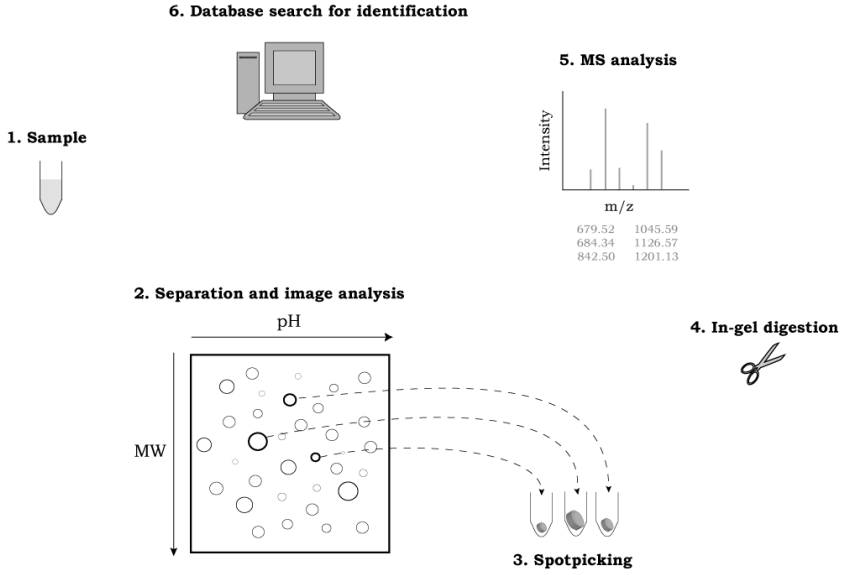

Figure 1.1. General principle of biomarker discovery using the combination 2D gel electrophoresis and mass spectrometry. Complex protein mixtures are separated in two dimensions according to their isoelectric point and molecular weight. Relative levels of expression are compared between samples using computer algorithms to determine differentially expressed proteins. Proteins of interest are excised from the gel, digested with a protease, and subjected to mass spectrometry for identification.

Two-dimensional differential in-gel electrophoresis (2D-DIGE)

In 2D-DIGE, samples are covalently labelled prior to isoelectric focussing with spectrally distinct fluorescent dyes (Cy™2, Cy™3 and Cy™5) and are separated simultaneously on the same 2D gel, as shown in Figure 1.2 [37]. These dyes are structurally similar and their N-hydroxy-succinimide ester reactive group forms an amide linkage with ε-amino group on

lysine residues through nucleophilic substitution. These fluorophores have highly similar molecular masses adding approximately 500 Da to the labelled proteins. The positive charge of the dyes enables matching the charge that is replaced on the lysine residue on proteins present at neutral or acidic pH. This charge and mass matching ensures that all samples essentially co-migrate to the same point during electrophoresis. Attempts to label all lysine residues in a protein led to a large increase in the apparent MW of the proteins and caused protein precipitation [38]. Therefore, the minimal labelling reaction is used and is optimized to deliberately keep the dye-to-protein ratio low (2-5%). Sample multiplexing in DIGE greatly refines the detection of changes at the protein level between samples [39]. The standard design in DIGE comprises of two experimental samples, labelled either with Cy3 or Cy5, and a Cy2-labelled internal standard; and these three samples will be separated on a single gel.

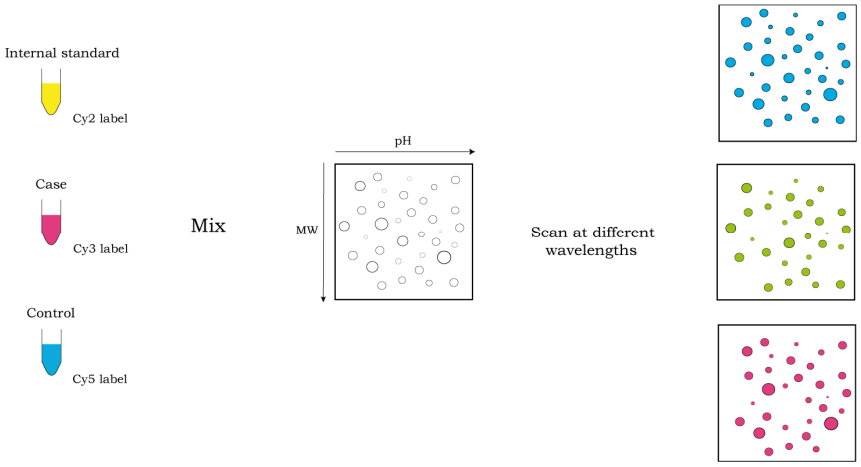

Figure 1.2. Schematic overview of the 2D-DIGE principle. This technique is based on sample multiplexing, which is enabled by covalently labelling experimental samples with fluorescent dyes with different excitation/emission wavelengths. Isoelectric focussing in the first dimension and SDS-PAGE in the second separates thousands of proteins in the samples.

The internal standard is a pool comprising equal amounts of proteins from each experimental sample, ensuring that all protein species are represented in the standard. The pooled standard creates an intrinsic link between internal standard and samples in each gel and enables matching the internal standards between gels. Analyzing the differences in protein abundances is based on comparison of sample-to-standard ratios, rather than direct comparison of raw spot abundances. This normalization of protein abundances simplifies and improves the confidence of inter-gel spot matching and quantification [37, 40, 41]. Variation in spot intensities due to experimental factors (i.e., heterogeneities during acrylamide polymerisation; electrical, pH and thermal fluctuation across the gels during the run; or

variable precipitation of samples during isoelectric focussing) will be the same for each sample within a single DIGE gel, including the internal standard. Therefore, the relative ratio of sample-to-standard will not be affected by such experimental variation. With the conventional "one sample per gel" 2D technique, experimental samples are separated independently on different gels, and consequently spot migration and intensity will differ for each gel and sample in an experiment, increasing the overall experimental variation.
From each image three scans are generated, namely Cy2 for the internal standard and Cy3 or Cy5 for the experimental samples. The visual image of a protein pattern on a 2D gel must be captured in a digital format before computer-based image analysis. The co-detection algorithm in the analysis software co-detects overlaid image pairs, thereby easily visualizing differences in sample composition. Statistical approaches, univariate as well as multivariate methods, are used to reliably detect significant changes in expression for individual spots or to look for patterns in expression changes [38, 42]. Although both gel-based proteomic techniques are able to resolve hundreds to thousands proteins simultaneously on a single gel, they are associated with certain limitations; both methods are laborious, time-consuming and have limited capability in resolving hydrophobic proteins, proteins present at extremely basic or acidic pI values and proteins with MW above or below the molecular size limits of the gels.

Protein identification by mass spectrometry
After separation and detection, proteins of interest must be identified. Therefore, protein spots of interest are automatically excised from the gels and in-gel digested with a sequence-specific protease, such as trypsin. This protease cleaves the proteins at specific amino acids, namely arginine and lysine, generating peptides with an average length of approximately nine residues. These proteolytic peptides are easily eluted from the gel and identified by tandem MS, in particular with matrix-assisted laser desorption/ionization time-of-flight tandem MS (MALDI-TOF/TOF) (Figure 1.3).
The peptides are mixed with an acidic matrix, compromised of small, organic and UV-absorbing molecules and allowed to co-crystallize. The laser energy strikes and excites the matrix causing both matrix and analyte ions to subsequently pass into gaseous phase. The ions, mainly singly charged, are accelerated by high voltage, acquiring a velocity that depends on their mass: light ions acquire a higher speed than heavy ions. Entering a field-free "drift region", each ion keeps the constant velocity it acquired during the acceleration. The timed-ion selector allows high-resolution selection of ions with a particular mass-to-charge (m/z) ratio into the collision cell, while all other ions are rejected. The selected precursor ion collides with a neutral inert gas and during these collisions, the energy of the precursor ion will partly be converted into internal energy and ions become excited to an unstable state. Consequently, precursor ions dissociate or fragment to product ions, which will be separated

in the second TOF section. The reflectron acts as an ion mirror and improves resolution by extending the ion flight path. Consequently, ions of equal mass having slightly different energy will be focussed in time, such that their arrival times at the detector are not different. This leads to a sharper signal, resulting in higher resolution. The detector amplifies and counts the arriving ions; ultimately producing a MS/MS spectrum composed of the precursor mass and charge state, as well as a list of peaks. Each peak is characterized by two values: a measured fragment m/z and an intensity value that represents the number of detected fragments.

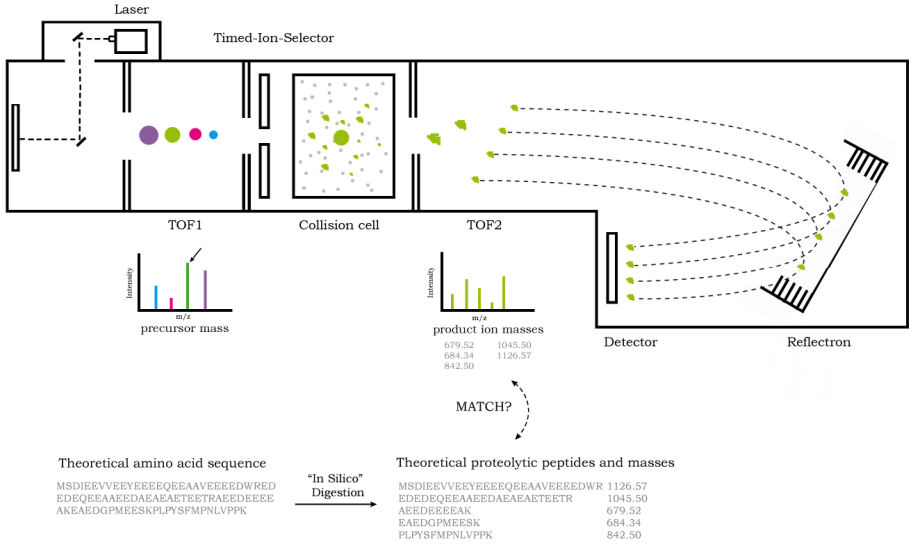

Figure 1.3. Protein identification using tandem mass spectrometry. The tryptic peptides are ionised by matrix-assisted laser desorption ionisation from solid state, and the mass-to-charge ratio of the ions is measured accurately by the time-of-flight analyser, which measures the time for ions to reach the detector. The timed-ion selector selects the precursor ions entering the collision cell, where they will be fragmented. The experimental m/z values can be matched against theoretically obtained mass data from already identified protein sequences contained within databases.

Complex algorithms are used to compare the experimental spectrum with the peptide masses predicted from theoretical (in silico) digestion, using the same protease, of protein sequences contained within databases. Candidate proteins are ranked from a list most closely matched candidates using various scoring algorithms and as such the protein can be identified. Many factors must be taken into account to produce a robust score, like dissimilarities in peak positions due to internal or calibration errors or modified amino acids, noise and contaminant or missing peaks [43, 44]. Following the discovery phase, where potential biomarkers are found and identified, the differential proteins need to be adequately validated, prior to clinical implementation.

Biomarker development pipeline

Successful implementation of a new biomarker into routine clinical practice ideally requires the multidisciplinary collaboration of research laboratories, diagnostic industry, clinical laboratories and clinicians during the whole process from bench to bedside [45].

Input from clinicians is warranted from the start to precisely define the clinical problem, to select and accurately characterize patients and controls, and to provide the clinical specimens. Furthermore, their professional opinion concerning the potential clinical utility is required before further investments are made. Since clinical laboratories are familiar working with standard operating procedures and quality control (QC) protocols, their knowledge regarding these concepts is indispensable. Furthermore, they can cooperate in the analytical (i.e., accuracy, precision, linearity, limit of detection and limit of quantification) and clinical (i.e., sensitivity, specificity, likelihood ratio, ROC curves, positive/negative predictive value) evaluation of potential biomarkers. Multidisciplinary input is essential from the earliest stages, even before research laboratories start with the discovery and subsequent selection of promising biomarkers. This to minimize the risk of introducing methodological bias from the start, such as pre-analytical (i.e., sample collection, processing and storage) and analytical (i.e., QC procedures and reproducibility assessment) errors. The diagnostic industry can share their experiences concerning assay optimalization and ultimately will determine whether the marker is worth pursuing commercially. Many technical, medical, financial and legal factors are considered in making the final decision [9, 46-48].

Although a universal standard workflow for biomarker discovery does not exist, the National Cancer Institute – Clinical Proteomics Technologies for Cancer (NCI-CPTC) Initiative proposed a possible framework; an adaptation of the theoretical model by Rifai *et al.* [46]. The proposed biomarker pipeline compromises four major stages (Table 1.2): (i) biomarker discovery, (ii) biomarker prioritization, (iii) biomarker verification and (iv) clinical validation of the candidate biomarker [33, 49]. Since the discovery phase generally results in the identification of several thousands putative biomarkers, it is necessary to prioritize a subset of these for further investigation. Selection and prioritization of putative biomarkers for verification relies upon clinical relevance. Prioritization assures that several years of time and thousands of Euros are invested preferentially in the most promising biomarkers. During the verification phases, their ability to differentiate cases from controls is quantitatively assessed through alternative analysis techniques in a moderate number of samples. Only a few potentially useful proteins make through the final phases of clinical validation, in which they are tested in a large cohort using assays of high analytical sensitivity and specificity.

The implementation of basic and clinical research findings into healthcare is a challenging and time-consuming task that is often left incomplete. The framework, as described in Table 1.2, and the input from multiple disciplines working together will enable efficient transition of

biomarker candidates from phase to phase; ultimately leading more quickly and efficiently to high-quality biomarkers which can be used in clinical management.

Table 1.2. The biomarker pipeline as proposed by the NCI-CPTC Initiative to improve successful implementation of new biomarkers into routine clinical practice.

Stages	Requirements
Stage I: Discovery	
Genomic/proteomic strategies Adequately screening scientific literature	Define clearly the clinical purpose of the new biomarker. Select an appropriate, well-characterized study cohort. Pre-analytical issue: sample collection, processing, storage.
Stage II: Prioritization	
Select biomarkers with most significant differences between cases and controls Select biomarkers based on biological hypotheses	
Stage III: Verification	
Determine quantitatively whether there is sufficient evidence for potential clinical utility to warrant further investment for clinical validation	Alternative technique (immunoassays, protein microarrays, immunohistochemistry and many more) Estimate sensitivity and specificity QC procedures, reproducibility and precision
Stage IV: Clinical validation	
Confirm and quantify the clinical utility of the candidate in a large study population	Assay optimalization, standardization and automation Quality control procedures, reproducibility and precision Possibly a multimarker approach Improved outcome versus current gold standard

Detection of Biomarkers

In this thesis, we focussed on the discovery of potential biomarkers for three clinical problems; aneurysm of the abdominal aorta, kidney transplantation and multiple sclerosis using 2D-DIGE and a hypothesis-driven approach.

Aneurysms of the abdominal aorta

Aneurysm of the abdominal aorta (AAA) is a potentially life-threatening vascular disease of increasing prevalence; especially among Caucasian men over 65 years (1% to 5%). Today, there are studies showing a substantial decrease of AAA prevalence and evidencing that the incidence of ruptured AAA and mortality from AAA is also declining. This reduction is attributable largely to changes in smoking prevalence, improvement in control of hypertension, a rising use of statins for cardiovascular risk prevention and an increase in elective AAA repair [50-53].

The most accepted definition of an AAA is based on the diameter of the abdominal aorta and is defined as an abdominal aortic diameter of 30 mm or more measured in either anterior-posterior or transverse plane (Figure 1.4). Important risk factors for AAA are advanced age,

male gender, smoking and a positive family history for AAA. This degenerative disease of the abdominal aorta leads to intra-luminal thrombus formation, progressive dilatation and rupture. An AAA is characterized by disruption and degradation of elastin, collagen deposition, smooth muscle cell apoptosis, medial neovascularisation and extensive inflammatory cell infiltration [54]. These inflammatory and degenerative changes of the aortic wall may lead to decreased tensile strength and progressive dilatation, ultimately making the aneurysm more prone to rupture.

Survival associated with AAA rupture is extremely low with rates ranging from 10% to 15%, while elective surgery has an overall survival rate of 95%. It is widely recognized that the risk of rupture increases with increasing diameter. Progression of AAA towards rupture is not linear, but usually presents points of acceleration which can appear at any time [55]. Conversely, aortic dilatations can remain stable and asymptomatic for many years, during which aged patients may die of other causes. In women, AAA are rarer, but represent a higher relative mortality than men [56]. Early detection and elective AAA repair is, for those at risk of rupture, a critical step to limit mortality associated with aneurysm rupture [57]. Besides identifying patients at risk for AAA progression [58-60] and rupture [60], new makers may also help to elucidate the molecular mechanism behind this disease and eventually generate treatments to reduce or prevent AAA progression early in the disease process.

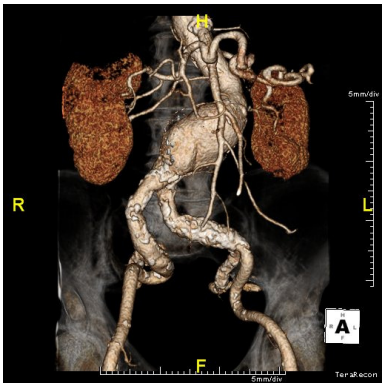

Figure 1.4. Computed tomography angiography depicting an infrarenal abdominal aortic aneurysm

To discover such biomarkers a non-hypothesis driven proteomic technology was used, as described in Chapter 5. In this thesis, we also investigated whether biomarkers, proposed in scientific literature, are associated with aneurysm size (Chapter 2) and are able to predict aneurysm progression (Chapter 3). Furthermore, biomarkers can be used to monitor

successfulness of therapy and we evaluated the diagnostic value of certain biomarkers for endoleakage detection after endovascular aneurysm repair (Chapter 4).

Kidney transplantation

Organ transplantation has evolved rapidly from the first early successes [61] to the widespread use of donated organs for the treatment of end-stage kidney, heart, and liver failure. Kidney transplantation results in longer life expectancy and superior quality of life compared to dialysis treatment [62, 63]. Unfortunately, the number available donor kidneys is not sufficient to treat all patients awaiting transplantation in spite of recent increases in living donor kidney donation [64-67] and expansion of criteria for kidney donation after brain death [68, 69]. Procurement of kidneys from donors after cardiac death (DCD) holds the potential to expand the donor pool 2.5 to 4 times [70], which theoretically should suffice to stabilize or even reduce the waiting lists for kidney transplantation [71].

After organ procurement, kidneys may be preserved by machine pulsatile perfusion or by static cold storage until the time of transplantation [72]. Kidneys preserved by machine perfusion showed reduced incidence of delayed graft function and greater survival at one year after transplantation compared to cold-stored kidneys [73]. In addition to improving graft function after transplantation, machine perfusion may be used to test the viability of kidneys in order to prevent transplantation of grafts that will never regain function. Perfusion characteristics such as flow rate and pump pressure [74] as well as the presence of lactate dehydrogenase, α-glutathione S-transferase, fatty-acid binding protein or redox-active iron in the preservation solution may be useful in selecting viable DCD kidneys for transplantation [75-77]. However, the predictive value of these parameters for adverse transplant outcomes is insufficient to be useful in clinical settings [78-80]. Therefore, new and improved biomarkers are needed to predict kidney viability and in this thesis we focus on finding clinical useful biomarkers using 2D-DIGE (Chapter 7).

Multiple Sclerosis

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS) of unknown aetiology. Typically, MS begins in early adulthood and is potentially the most common cause of neurological disability in young adults with predominance for females. The prevalence of MS in Europe is 0.1%, but varies considerably around the world [81, 82]. This disease is characterized by the formation of a sclerotic plaque particularly localized in the white and gray matter of the brain. It represents the end stage of a process involving inflammation, demyelination and remyelination, oligodendrocyte depletion and astrogliosis, as well as neuronal and axonal degradation [81, 83, 84]. Clinically, multiple

sclerosis manifests as sensory and visual impairment, paralysis and other neurological deficits, sometimes accompanied with considerable cognitive dysfunction.
At disease onset, about 85% to 90% of MS patients suffer from the relapsing-remitting (RR) form, characterized by recurrent relapses followed by complete or partial recovery. RR-MS can transit to a more progressive form, called secondary progressive MS characterized by continuous deterioration. This occurs when the CNS can no longer compensate for additional neuronal loss [85]. Some patients (10% to 15%) immediately display a progressive disease course, which is called primary progressive MS with few or no relapses.
Diagnosing MS is based on the McDonald criteria; a combination of clinical, radiological and laboratory analyses [86]. However, the diagnostic value is limited in terms of predicting long-term clinical outcome and excluding other potentially treatable diseases mimicking the course of MS [82, 87]. Current MS therapy mainly focuses on treating symptoms and slowing the process of neurological disability. Starting the therapy as early as possible increases its efficacy and has important consequences for disease progression [88]. Therefore, the identification of MS-specific biomarkers is needed, since they could have improved diagnostic and prognostic value, could enhance patient-tailored therapy and could possibly elucidate pathophysiological mechanisms. In Chapter 6, 2D-DIGE was used to discover such MS-specific biomarkers.

Outline and aim of this thesis

Current biomarkers provide a spectrum of clinical information; diagnosing a certain disease, predicting disease progression and detecting disease recurrence. However, the medical field shows a growing interest in discovering new and refined biomarkers. These new biomarkers could ultimately improve treatment and reduce health-care costs by providing earlier and more confident diagnoses. As described in *Chapter 1*, proteomics provides opportunities to identify clinically useful biomarkers.
In *Chapter 2* we investigated whether the concentration of potential biomarkers was related to abdominal aortic aneurysm size. In scientific literature, associations between several biomarkers and AAA size, progression and rupture are suggested. Aneurysm size is by lack of a better alternative, a surrogate marker for aneurysm progression. Therefore, biomarkers correlated to aneurysm size represent valuable putative markers for aneurysm progression, but they need to be thorougly validated in a prospective follow-up study.
Chapter 3 describes such a large prospective, standardized follow-up study, in which the potential of predicting aneurysm progression by a panel of biomarkers was evaluated.

Chapter 4 evaluates the plausibility of MMP-9 as marker for success or failure of a endovascular repair of abdominal aortic aneurysm.
Previous studies have identified AAA biomarkers by studying the levels of different molecules potentially related to AAA pathological mechanisms. A different, non hypothesis-driven, biomarker strategy using proteomics will offer new opportunities to gain further insight into the disease processes, including their molecular mechanism, the risk factors involved, and the analysis of disease progression (*Chapter 5*).
Furthermore, this proteomic strategy was used to detect potential biomarkers for diagnosing Multiple Sclerosis (*Chapter 6*) and for assessing the viability of donor kidneys (*Chapter 7*).
Finally, *Chapter 8* discusses the results and conclusions of the above mentioned studies and provides directions for future research.

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2

Biomarkers & Aneurysm size

CIRCULATING BIOMARKERS AND ABDOMINAL AORTIC ANEURYSM SIZE

Background. Abdominal aortic aneurysm (AAA) is a degenerative disease of the abdominal aorta leading to progressive dilatation, intra-luminal thrombus (ILT) formation, and rupture. Understanding the natural history of AAA is essential, because different processes and, therefore, different biomarkers, could be involved at each stage of disease progression. The purpose of the present study was to investigate the relationship between systemic expression of biomarkers of inflammation and extracellular matrix remodelling and aneurysm size in AAA patients.

Methods and Results. All consecutive patients admitted to the (out-) patient clinic of the surgical department of two large community centers were prospectively included. Patients were divided into three groups based on their aneurysm diameter: small (30 – 44 mm; n = 59), medium-sized (45 – 54 mm; n = 64) or large (≥ 55 mm; n = 95) AAA. Linear regression modelling showed that age and serum hsCRP concentration were positively associated, whereas serum HDL and IgG concentrations were negatively associated with aneurysm size. This regression model was corrected for possible bias due to statin use and center of inclusion; and also indicated that in general men have larger aneurysms compared with women.

Conclusions. Different aneurysm sizes showed different expression pattern of HDL, IgG, and hsCRP. These biomarkers may be useful in predicting AAA progression.

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Introduction

Abdominal aortic aneurysm (AAA) is defined as a permanent dilatation of the abdominal aorta. By definition the diameter should exceed 30 mm or 1.5 times the expected normal aortic diameter [1]. The incidence and prevalence of AAA are increasing, due to coincidence finding through general use of imaging diagnostics, longer life expectancy of the population and implementation of ultrasound screening programs. The ultimate complication of AAA is rupture of the aneurysm, which is a life-threatening condition with an approximate overall mortality rate of 80% [2]. Although the risk of rupture increases exponentially with maximal anterior-posterior aortic diameter, aneurysm size does not completely reflect the natural history of AAA, since small aneurysms occasionally rupture and a substantial proportion of large AAA remain stable over time [3-5]. There is a need for patient-specific prediction of AAA progression. Hence, understanding the pathophysiology of AAA is essential. Several risk factors are strongly associated with AAA, including male gender, smoking, age, history of myocardial infarction, hypertension, low high-density lipoprotein (HDL) cholesterol concentration and high concentration of total serum cholesterol [6, 7].

Two key features of AAA pathophysiology are inflammation and extracellular matrix (ECM) destruction [8, 9]. Inflammation is characterized by inflammatory cell infiltrates which include T-cells, B-cells, and macrophages. These cells produce numerous cytokines which can induce the expression and activation of various proteases [10]. Several studies have demonstrated elevated levels of circulating IL-1β, IL-6, TNF-α and IFN-γ in patients with AAA, and also specifically implicated involvement of these cytokines in AAA pathogenesis [11-14]. IL-6 induces the production of C-reactive protein (CRP) in the liver [15]. CRP is a strong predictor of various cardiovascular events and has been associated with aneurysm size, but not with growth rate [16-21]. Hallmarks of ECM degradation in AAA are loss of elastin and change in general aortic wall architecture. Remodelling of the aortic wall is mainly regulated by matrix metalloproteinases (MMP), serine and cathepsin proteases. Proteolytic degradation and remodelling of elastin and collagen are associated with weakening and dilatation of the aneurysm, making the aneurysm more prone to rupture [8, 9, 22]. Circulating levels of MMP have been moderately associated with AAA size [23]. Conflicting results have been reported on the association between plasma MMP concentration and growth rate [21, 24, 25].

The purpose of the here presented study was to quantify several candidate markers and investigate their relationship with aneurysm size. This investigation has the potential to (1) define the most promising factors which could be used in predicting aneurysm growth, since aneurysm size is one of the main predictors of aneurysm progression; and (2) decipher which pathophysiological mechanisms play at various disease stages or various aneurysm sizes. Recently, we adequately reviewed all candidate circulating markers for AAA progression [8, 9],

but how the stages of AAA progression correlate with AAA size is unknown. For the purpose of this study we selected biomarkers based on their pathophysiologic relationship, economical reasons and availability at our research facility. We hypothesize that AAA biomarker profiles vary according to AAA size, with both inflammation and ECM remodelling dominating the first stages of AAA. This should be reflected by an increased expression of inflammatory markers, such as for instance IgG and CRP, and markers of ECM remodelling in patients with small AAA.

Methods

Patient population

From January 2006 to January 2009, all consecutive patients admitted to the (out-) patient clinic of the surgical department of the Maastricht University Medical Centre (MUMC, Maastricht, the Netherlands) and Atrium Medical Centre (Atrium MC, Heerlen, the Netherlands) with an AAA were invited to participate in this study. All consecutive patients with AAA ≥ 55 mm undergoing elective open or endovascular repair were included for pre-operative blood sampling (MUMC and Atrium MC). Patients with AAA < 55 mm were included for a follow-up programme (only MUMC). Nine of the patients invited to participate in the follow-up programme did not present at the first follow-up visit; four patients refused to enter, due to severe Parkinson (n = 1), psychiatric illness (n = 1) and terminal malignant disease (n = 2), and five patients died of non-aneurysmal-related cause before attending the first visit. For the purpose of this study, analyses were only performed on data collected at inclusion from asymptomatic non-ruptured AAA. Patient characteristics, blood samples and aneurysm diameter measurements were collected. Patients were divided into three groups based on their aneurysm diameter; Small (30 - 44 mm; n = 59), medium-sized (45 - 54 mm; n = 64) or large (≥ 55 mm; n = 95) AAA.

Demographic patient characteristics were obtained by interview and included gender, age, smoking habit (current smoker, ex-smoker since one year, never smoked), hypertension (use of antihypertensive drugs), cardiovascular prescription drugs (including statins), familial history, presence of diabetes mellitus (use of insulin or oral antidiabetic drugs) and coronary disease (history of unstable angina pectoris, myocardial infarction, coronary artery bypass grafting or percutaneous transluminal coronary angioplasty).

An age- and gender-matched control group with ultrasound-confirmed, non-dilated (< 30 mm) infrarenal aorta was included (n = 69; mean age: 71.6 ± 5.4 years; range: 65 – 85 year; n = 59 (85.5%) males).

The study was approved by both institutional ethics committees and all patients provided written informed consent. Participants did not receive any form of financial compensation.

Table 2.1. Demographic patient characteristics of 218 patients with asymptomatic non-ruptured abdominal aortic aneurysm

		Controls (n=69)	Small AAA (n=59)	Medium-sized AAA (n=64)	Large AAA (n=95)	ANOVA p-value[1]
Male		85,5%	76.3%	85.9%	93.7%	0.008
Age (years)		71.6 ± 5.4	70.1 ± 7.4	71.7 ± 7.9	72.7 ± 7.5	0.130
Height (cm)		172 ± 5	174 ± 8	173 ± 8	173 ± 7	0.620
Weight (kg)		81 ± 15	81 ± 12	78 ± 13	79 ± 11	0.262
Aorta size (mm)		18.9 ± 3.2	37.4 ± 4.1	49.6 ± 2.9	67.1 ± 10.5	<0.001
Family History		30.4%	19.0%	17.7%	17.0%	0.573
Hypertension		34.8%	68.4%	62.3%	54.8%	0.423
Smoking	Never	43.5%	6.9%	8.6%	4.3%	0.809
	Ex	40.6%	56.9%	55.2%	63.8%	
	Current	15.9%	36.2%	36.2%	31.9%	
Coronary disease		26.1%	37.3%	41.0%	46.2%	0.548
Diabetes		13.0%	6.8%	11.7%	7.7%	0.587
Statin		30.4%	62.7%	59.0%	52.9%	0.478

[1] Differences in categorical variables, expressed as percentages, between the three aneurysm groups were compared using the Pearson Chi-square test. Continuous variables, expressed as mean ± standard deviation, were compared using one-way ANOVA with Bonferroni correction for multiple comparisons.

Imaging modalities

Aneurysm diameter of follow-up patients was assessed by ultrasound using an Ultramark 9, Advanced Technology Laboratories, Bothell, Washington scanner with a curved 3.5 MHz transducer. All ultrasound measurements were performed by one trained operator with an intra-operator variability of two mm. Prior to operation, computed tomography angiography (CTA) was used to determine aneurysm diameter and to establish suitability for endovascular aortic repair. The variability between ultrasound and CTA measurement was 2.8 mm (data not shown).

Blood collection

Venous blood was drawn in patients, fasting overnight, via an anticubital vein puncture without venous stasis and collected in SST (serum) and EDTA (plasma) buffered vacutainers (Becton Dickensen, Plymouth, UK). Exactly thirty minutes after collection, blood was centrifuged (15 minutes, 3000g, 4°C) and multiple aliquots were stored at -80°C pending analysis.

Laboratory measurements

Serum concentrations of α1-antitrypsin (α1-AT), cystatin C, haptoglobin, hsCRP and immunoglobulin G (IgG) were analyzed on the BN ProSpec (Siemens Healthcare Diagnostics,

Den Hague, the Netherlands). Cholesterol, creatinine (Jaffé reaction), HDL and triglyceride concentrations were measured on the Synchron LX 20 (Beckman Coulter, Fullerton, CA). Low-density lipoprotein (LDL) concentration was determined on the Synchron LX20 or calculated using Friedewald's formula when triglyceride concentration was < 4.5 mmol/L. Analyses were performed at the MUMC Department of Clinical Chemistry.

Assays

Commercially available enzyme-linked immunosorbent assay (ELISA) kits were used according to the manufacturer's instructions to measure plasma concentrations of MMP-2, MMP-9 and tissue inhibitor of the matrix metalloproteinases (TIMP)-1 (GE Healthcare, Uppsala, Sweden). The sample was retested in duplicate when the duplicate varied more than 10% in concentration; the average value was taken when the duplicate variation was less then 10%.

After preliminary studies, to establish optimal dilution conditions, all plasma samples were analyzed at 1:70 for MMP-2, 1:5 for MMP-9 and 1:20 for TIMP-1. According to the manufacturer, the intra- and interassay variation for MMP-2 and -9 were < 6% and 10%, respectively. The detection limit of the MMP-2 assay was 0.37 ng/mL and the MMP-9 assay was 0.6 ng/mL, respectively. According to the manufacturer, the intra- and interassay variation for TIMP-1 were < 10% and < 14%, respectively. The detection limit of the TIMP-1 assay was 1.25 ng/mL.

It is known that posture and nutritional state can alter the concentrations of circulating proteins by redistribution of blood volume [26]. A striking decrease in serum concentration of albumin was seen for pre-operative blood samples as compared with the follow-up samples; small AAA: 40.5 ± 2.9 g/L, medium-sized AAA: 37.9 ± 6.3 g/L and large AAA: 29.1 ± 4.6 g/L. The average albumin concentration in controls was 44.8 ± 2.3 g/L, in symptomatic AAA 26.1 ± 5.6 g/L and in ruptured AAA 16.9 ± 6.2 g/L. Pre-operative patients were hospitalized (non-ambulant) and follow-up patients were ambulant, and differences in nutritional state were considered unlikely. To correct for hemodilution differences, biomarker concentrations were adjusted for albumin. This was achieved by multiplying the individual biomarker concentrations by the ratio of average albumin concentration of controls and the individual albumin concentration.

Statistical analysis

Categorical variables, expressed as percentages, were compared using the Pearson Chi-squared test. With respect to the continuous variables, analysis of variance (ANOVA) was used to compare the different diameter groups, applying the Bonferroni correction for

multiple comparisons. Not normally distributed continuous variables were logarithmically transformed and presented as median with interquartile range

Associations between demographic patient characteristics, biomarker concentrations and aneurysm diameter were calculated with linear regression. The variables included in the final model were determined by the backwards selection procedure. The goodness of fit of the regression model was expressed by R^2. Assumptions of the regression model were checked using histograms, residual plots, and scatterplots. The presence of multicollinearity and influential outliers was investigated using Variance Inflation Factors and Cook's distance.

Results with $p<0.05$ were considered statistically significant. All statistical analyses were carried out using SPSS version 16.0 (SPSS Inc, Chicago, IL).

Results

The study population consisted of 218 patients (189 men and 29 women) aged 71.7 ± 7.6 years. The demographic patient characteristics are presented in Table 2.1; only gender (p=0.008) was significantly different between the aneurysm diameter groups, with more men presenting with a large AAA.

The concentration of various biochemical markers, such as α1-antitrypsin, creatinine, cystatin C, haptoglobin and IgG tend to be higher in AAA patients compared with controls (Table 2.2). As shown in Table 2.2, several biochemical parameters showed a different expression pattern associated with aneurysm diameter. Both serum HDL and IgG concentration showed an inverse relationship with aneurysm size (Figure 2.1).

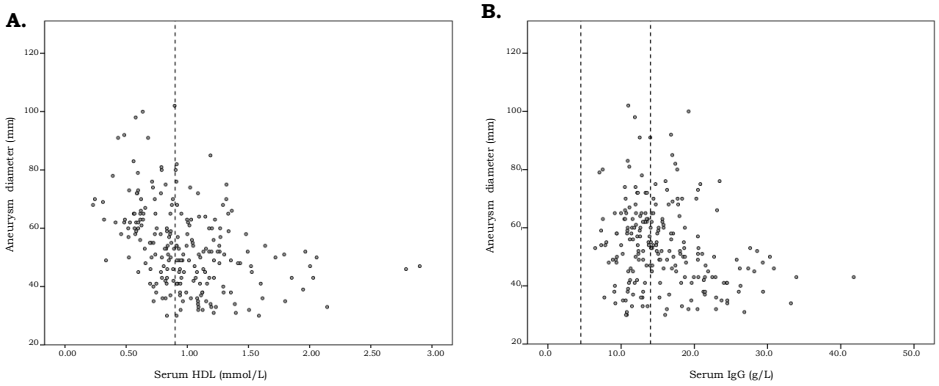

Figure 2.1. Relation between aneurysm diameter and serum HDL (r = -0.39; p < 0.001) and IgG (r = -0.25; p < 0.001) concentration. The dashed lines represent the reference values established at the Clinical Chemistry Department of MUMC.

Patients with small AAA tend to have elevated serum IgG concentrations compared with controls (Table 2.2).

Furthermore, higher serum creatinine and hsCRP values were observed in patients with a large aneurysm compared with patients with either a small or medium-sized aneurysm. Plasma concentration of MMP-9 was higher in patients with a large AAA compared with patients with small and medium-sized aneurysms (60.3 ng/mL *versus* 35.0 ng/mL *versus* 32.3 ng/mL; p<0.001). A strong significant correlation between serum creatinine and serum cystatin C concentrations (r=0.903; p<0.001) was observed in asymptomatic non-ruptured abdominal aortic aneurysms.

Table 2.2. Circulating biomarker concentrations in patients with asymptomatic non-ruptured abdominal aortic aneurysm[1]

	Reference values	**Controls (n = 69)**	**Small AAA (n=59)**	**Medium-sized AAA (n=64)**	**Large AAA (n=95)**	**ANOVA p-value[2]**
α1-AT (g/L)	0.75 – 1.85	1.42 ± 0.23	1.84 ± 0.38	1.85 ± 0.47	1.87 ± 0.47	0.894
Cholesterol (mmol/L)	5.0 – 6.4	5.13 ± 1.33	5.12 ± 1.13	5.01 ± 1.02	5.08 ± 1.28	0.584
Creatinine (μmol/L) [3]	60 - 115	83 (75; 98)	110* (97; 129)	115 (98; 158)	131 (111; 160)	0.012
Cystatin C (mg/L)	0.53 – 0.95	0.57 ± 0.27	1.22 ± 0.62	1.33 ± 0.56	1.47 ± 0.83	0.105
Haptoglobin (g/L)	0.25 – 1.90	1.35 ± 0.48	2.08 ± 0.88	2.05 ± 0.66	2.13 ± 0.90	0.830
HDL (mmol/L)	> 0.9	1.21 ± 0.38	1.14 ± 0.34**	1.14 ± 0.47**	0.80 ± 0.28	< 0.001
hsCRP (mg/L) [3]	< 3.0	1.30 (0.65; 2.61)	3.57* (1.78; 5.70)	4.35 (1.57; 8.56)	5.16 (2.57; 13.4)	0.010
IgG (g/L)	4.5 – 14.0	11.3 ± 6.1	18.2 ± 7.1**	16.5 ± 6.1*	13.8 ± 3.4	< 0.001
LDL (mmol/L)	3.5 – 4.4	3.17 ± 1.22	3.06 ± 0.97	3.02 ± 0.93	3.35 ± 1.17	0.096
MMP-2 (ng/mL)		1240 ± 204	1398 ± 407	1335 ± 418	1459 ± 445	0.343
MMP-9 (ng/mL) [3]		30.7 (23.7; 50.1)	35.0* (24.5; 71.8)	32.3* (19.4; 67.7)	60.3 (34.8; 94.9)	< 0.001
TIMP-1 (ng/mL) [3]		114 (104; 129)	141 (117; 168)	157 (124; 190)	153 (122; 188)	0.084
Triglycerides (mmol/L)	0.80 – 1.94	1.78 ± 1.18	2.04 ± 1.05	2.05 ± 1.20	2.18 ± 1.14	0.672

[1] Biomarker concentrations were corrected for hemodilution using serum albumin (see Methods section)
[2] Bonferroni correction was used for pairwise comparison between different aneurysm groups: *p < 0.05 and **p < 0.001 compared to large AAA.
[3] Non-normally distributed variables (creatinine, hsCRP, MMP-9, and TIMP-1) were logarithmically transformed to calculate ANOVA p-values.

Linear regression was used to asses significant associations between demographic and biochemical parameters and aneurysm diameter. The regression model was corrected for possible bias due to statin use and centre of inclusion (Table 2.3). Age and hsCRP concentration showed a positive association with aneurysm diameter, whereas HDL and IgG concentrations were negatively associated with aneurysm diameter. This model also indicates that in general men have larger aneurysms compared to women.

Table 2.3. Final linear regression model corrected for statin use and centre of inclusion

	β (SE)	p-value
Male gender	5.30 (2.45)	0.032
Age (years)	0.40 (0.11)	<0.001
HDL (mmol/L)	-9.49 (2.18)	<0.001
IgG (g/L)	-0.86 (0.15)	<0.001
Log hsCRP (mg/L)	5.16 (1.53)	0.001

Goodness of fit: R^2=0.365

Discussion

The present study showed that the systemic expression of biomarkers of inflammation and ECM remodelling in patients with AAA were related to aneurysm size. This is evidenced by the linear regression model showing that age and serum hsCRP concentration were positively associated, whereas serum HDL and IgG concentrations were negatively associated with aneurysm size. This regression model was corrected for possible bias due to statin use and centre of inclusion; it also indicated that in general men have larger aneurysms compared with women.

Previous studies have reported on the relationship of systemic inflammatory markers and aneurysm size [12, 20, 27-29]. To our knowledge, we are the first to report an association between serum concentrations of IgG and aneurysm size. IgG is capable of enhancing phagocytosis, fixation of complement and is suitable as a marker of inflammation. Elevated levels of IgG in AAA tissue have been reported. IgG extracted from the aneurysm wall and serum of patients with AAA were immunoreactive with AAA tissue itself. Furthermore, purified IgG of either source were immunoreactive with microfibrillar elements of normal aortic adventitia [30, 31]. A 40 kDA autoimmune protein extracted from the abdominal aorta reacting with IgG isolated from AAA tissue has been partially sequenced [32]. These findings have led to the hypothesis that an autoimmune mechanism is involved in AAA formation [30].

The suitability of CRP as a marker of AAA disease is controversial, as results have been conflicting on the relationship between CRP, AAA status, aneurysm size, growth rate and symptomatology. The positive trend of hsCRP and aneurysm size is in accordance with Vainas *et al.* [20] and Flondell-Sité *et al.* [29], whereas Lindholt *et al.* [28] were not able to determine any relationship. Furthermore, reports on the relationship between MMP-9 and aneurysm size have been inconsistent. One report indicated a positive trend of increased plasma concentration of MMP-9 with increasing aneurysm size, which is in accordance with our data, while others failed to detect such correlation [23-25]. Patients with large AAA showed higher MMP-9 concentration compared with patients with small AAA, possibly reflecting increased ECM degeneration in large AAA.

Several studies have presented evidence for an association between HDL and LDL cholesterol and AAA presence [6, 33-36]. We are the first to report a negative correlation between aneurysm size and HDL serum concentrations. In our study, serum HDL concentrations decreased from 1.14 ± 0.34 mmol/L in small AAA to 0.80 ± 0.28 mmol/L in large AAA. Patients with larger AAA were more likely to be older patients with lower HDL concentrations. Besides its common function for cholesterol transportation, HDL also has anti-inflammatory properties [37]. As serum concentrations of HDL decrease with increasing AAA size, one could postulate that patients with large AAA have a diminished anti-inflammatory response, leading to enhanced effect of pro-inflammatory factors. These pro-inflammatory factors are known to induce MMP expression, which leads to ECM degradation and ultimately AAA progression. Serum HDL concentration has previously been negatively associated with ILT volume [38]. Since ILT volume is associated with aneurysm size [38], this may in part explain our negative relationship between HDL and aneurysm size. In this study we were not able to include ILT volume in the analysis, because all patients with aneurysm < 55 mm did not undergo CTA imaging.

In contrast to previous findings, we were not able to demonstrate a significant inverse correlation between aneurysm size and serum cystatin C concentrations [28, 39]. Interestingly, overall serum cystatin C concentrations were elevated above the upper reference limit. Furthermore, we found a strong, significant correlation between serum creatinine and cystatin C concentration, suggesting that cystatin C reflects renal function instead of inhibition of ECM remodelling.

The stratification of patients in three groups according to aneurysm size was done as described by Flondell-Sité *et al.* [29]. The method of blood collecting, processing and storage of serum and plasma in this study has been carefully chosen. The methodology has been reported by others and plasma is the appropriate matrix for measurement of MMP [40-44]. In serum, MMP-9 is released from neutrophils and leukocytes during clotting, thereby yielding falsely elevated levels. Therefore, the addition of an anticoagulant (e.g. EDTA) to the collected blood was necessary to prevent this in vitro artefact [45, 46]. Collection of serum and plasma was prospectively performed; ELISA and laboratory measurements were performed in bulk after all samples had been collected. The pre-operative patients have been non-ambulant for several days, which can effect their albumin concentration [26]. We had not anticipated hemodilution and do not have hematocrit values for these samples, which is a well established method to correct for hemoditution. Besides hematocrit, hemoglobin and albumin are used to correct for hemodilution. Finally, due to economic reasons and lack of availability at our research facility, we did not include markers representing the ILT, despite the fact that thrombus is an important characteristic of AAA.

The fundamental knowledge about the etiology of AAA remains limited and the cause remains illusive. However, several contributing features, including ECM remodelling, inflammation and environmental factors have been identified. Circulating biomarkers, reflecting these processes, can provide insight into molecular mechanisms underlying AAA pathophysiology. We demonstrated a clear relation between AAA size and serum hsCRP, IgG and HDL concentration. Whether these biomarkers are useful in predicting AAA growth will be investigated in a cohort of AAA patients who are enrolled in a standardized follow-up regime.

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3

Biomarkers & Aneurysm expansion

PREDICTION OF ABDOMINAL AORTIC ANEURYSM PROGRESSION

Background. Abdominal aortic aneurysm (AAA) is a degenerative disease of the abdominal aorta leading to progressive dilatation, intraluminal thrombus formation and rupture. Although rupture risk increases with increasing maximal aneurysm diameter (Dmax), the natural history of AAA differs between individual patients. Several circulating biomarkers are candidates for prediction of AAA natural history. In the present study, we investigated systemic expression of biomarkers of inflammation and ECM remodelling for prediction of natural history of AAA.

Methods and Results. Between January 2006 en January 2009, 287 AAA patients agreed to participate in the presented study. Thirty-eight percent of the patients had an aneurysm < 55 mm, they were invited to participate in an imaging surveillance program. Patients with a large aneurysm (≥ 55 mm) or (symptoms of immiment) AAA rupture (62%) immediately underwent elective open or endovascular repair. We showed that AAA progression is highly dependent on Dmax; a higher initial AAA diameter is associated with higher AAA expansion rate. Furthermore, fast AAA expansion is related to slower than on average increasing cotinine values and faster than on average increasing values of GFR, Cystatin C and MMP-9.

Conclusion. Aneurysm diameter remains the gold standard for prediction of AAA expansion.

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Introduction

Abdominal aortic aneurysm (AAA) is a degenerative disease of the abdominal aorta with a natural history of progressive dilatation with substantial inter-individual differences, intraluminal thrombus formation, and ultimately rupture of the aneurysm. Up to 9% of the western population older than 65 years is affected [1, 2]. Aneurysm rupture is lethal in 90% of the cases [3] and accounts annually for approximately 15.000 deaths in the United States [4]. The principal goal of AAA treatment is preventing AAA rupture [5] and can be accomplished by elective open surgery or endovascular aneurysm repair (EVAR), which have an overall combined mortality of 5% [6]. To date, AAA size is considered the gold standard for prediction of rupture risk, since both AAA expansion [7-15] and rupture risk [16] are associated with aneurysm diameter. Population studies provided solid evidence that surgical repair is the appropriate treatment for aneurysms exceeding 55 mm in diameter [16], since the risk of rupture exceeds surgical morbidity and mortality for these large aneurysms [17]. The risk of rupture in small aneurysm is low and early elective surgery of AAA smaller than 55 mm does not improve survival [17-19]. The ADAM and UKSAT study indicated that surveillance with imaging is a safe treatment strategy for small AAA. However, small AAA can rupture unexpectedly and even large AAA tend to be stable over many years. Consequently, patient-specific prediction of AAA progression is warranted. Circulating biomarkers are a potential tool for predicting aneurysm progression. Furthermore, they could be used to monitor the progress and response to AAA treatment and offer opportunities for targeted therapy [20, 21].

Identification of potential AAA progression biomarkers requires knowledge of processes that contribute to or cause initiation, progression and rupture, since different processes (and, therefore, biomarkers) could be involved at each stage of disease progression [21]. The pathophysiology can roughly be divided into two inextricably connected processes; extracellular matrix degeneration and inflammation [22]. Furthermore, biomarker expression profiles can vary according to the Dmax of the aneurysm [23, 24]. Recently, we have reviewed candidate circulating biomarkers for AAA progression proposed in literature [21, 22].

Besides biomarkers, patient characteristics can be of help in predicting AAA progression. Several patient characteristics are associated with AAA presence; smoking and diabetes mellitus can predict AAA progression [20]. Current cigarette smoking is associated with AAA expansion resulting in 15% to 20% estimated increase in expansion rate [15, 25]. Conversely, diabetes has been negatively associated with expansion [12, 15].

Better insight into predictors of AAA progression may lead to future improvements in the follow-up efficiency, future therapies to slow AAA expansion, and better selection criteria for

surgery preventing AAA rupture [26]. The aim of this study is to investigate the association of a predefined set of demographic characteristics and biomarkers with AAA progression.

Methods

Patients

Patients with AAA admitted to the department of Vascular Surgery of the Maastricht University Medical Centre (MUMC) between January 2006 and January 2009 were invited to participate in the study. Two hundred and eighty-seven AAA patients provided written informed consent, 38% (n=110) of the patients presented with an aneurysm smaller than 55 mm and 62% (n=177) had a large aneurysm (≥55 mm) or (symptoms of imminent) AAA rupture. Patients with either an inflammatory or a mycotic aneurysm (n=3) were excluded. The study was approved by the medical ethical committee of the MUMC.

Follow-up

Patients with an aneurysm diameter between 30 and 55 mm (n=110) were invited to participate in an imaging surveillance program. A total of 101 (92%) patients formally entered the follow-up program; four patients refused to enter the follow-up due to severe Parkinson's disease (n=1), psychiatric illness (n=1), and terminal malignant disease (n=2) and five patients died of a non-aneurysmal related cause before attending the first follow-up visit.

Follow-up program

Every six months patients visited the hospital and underwent ultrasonography. Patients whose aneurysm exceeded 55 mm in diameter during follow-up were offered surgical repair. Expansion rate was calculated as the change in anteroposterior diameter relative to the observation time in years. Infrarenal aortic diameter was measured by the same operator using an Ultramark 9 scanner (Advanced Technology Laboratories, Bothell, Washington) equipped with a curved 3.5 MHz transducer. The limits of intra-operator variability of the measurement of the infrarenal aortic diameter were less than 3 mm, which correlates well with other published data [27-29]. The operator was blinded for the previous measurement.

At every visit all patients had a standard medical examination by the same observer, including measurements of height, weight, and blood pressure. Blood pressure was measured in a supine position in right and left arm using a Dinamap 1846SX/P device (Critikon, Kettering UK), after 15 minutes of rest. Body mass index (BMI) was calculated as kg/m^2.

Details of current smoking status, smoking history, family history of AAA, medical history and drug history were obtained through a questionnaire. The following definitions were used

in this study: smoking habits were classified as current smoking, never smoked, or seized smoking for more than 1 month. A positive family history indicated that a patient had one or more first degree family members affected by AAA. A positive history of ischemic heart disease was recorded if the patient had experienced a myocardial infarction, had hospital treatment for angina, had undergone a coronary artery bypass operation, or coronary angioplasty. Angina presence was diagnosed according to the criteria of the Rose questionnaire [30]. Hypertension was defined as a systolic blood pressure >160 mmHg, diastolic blood pressure >95 mmHg, or ever used of antihypertensive medication. Diabetes was defined as current use of anti-diabetic medication. The glomerular filtration rate (GFR) was calculated using the MDRD formula. Renal impairment was defined by a MDRD < 50 mL/min.

Blood collection

At each visit, blood samples were taken for biomarker measurement. Venous blood was drawn at baseline and 6-monthly thereafter in all patients, fasting overnight, via an antecubital vein puncture. A brief venous stasis applied to the upper arm was released before blood sampling. Blood was collected in SST™ (serum) and EDTA (plasma) buffered vacutainers® (BD, Plymouth, UK). Exactly thirty minutes after collection, blood was centrifuged (15 minutes, 3000g, 4°C) and exactly one hour after sampling multiple aliquots were stored at -80 °C pending analysis.

Laboratory measurements

Serum concentrations of (high sensitive measured) C- reactive protein (CRP), alpha-1 antitrypsin (α_1-AT), cystatin C, haptoglobin and immunoglobulin G (IgG) were analyzed on the BN Prospec (Siemens Healthcare Diagnostics, Den Hague, the Netherlands). Cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglycerides, creatinine (Jaffé reaction assay) were measured on the Synchron LX 20 (Beckman Coulter, Fullerton, CA). LDL concentration was estimated by Friedewald's formula, when triglyceride concentration was < 4.5 mmol/L. Analyses were performed at the MUMC department of Clinical Chemistry. Reference values established at this department are shown in Table 3.1.

Assays

To measure EDTA buffered plasma concentrations of MMP-2, MMP-9 and tissue inhibitor of the matrix metalloproteinases (TIMP)-1 commercially available enzyme-linked immunosorbent assay (ELISA) kits were used according to the manufacturer's instructions (GE Healthcare, Uppsala, Sweden). When the duplicate varied >10% in concentration, the plasma sample was retested in duplo and averaged when the duplicate variation was <10%. After preliminary studies, to establish optimal dilution conditions, all plasma samples were analyzed at 1:70 for

MMP-2, 1:5 for MMP-9 and 1:20 for TIMP-1. According to the manufacturer, for both MMP-2 as well as -9 the intra- and interassay variation was <6% and <10%, respectively. The detection limit of the MMP-2 assay was 0.37 ng/mL and for the MMP-9 assay 0.6 ng/mL, respectively. According to the manufacturer, the intra- and interassay variation for TIMP-1 were <10% and <14%, respectively. The detection limit of the TIMP-1 assay was 1.25 ng/mL.

Table 3.1. Biomarker concentrations at baseline

	Reference value	**Follow-up group (n = 101)**
α1-antitrypsin (g/L)	0.75 – 1.85	1.7 (0.3)
Creatinine (μmol/L)	60 - 115	109.5 (35.4)
Cystatin C (mg/L)	0.53 – 0.95	1.1 (0.5)
Haptoglobin (g/L)	0.25 – 1.90	1.8 (0.6)
hsCRP (mg/L)	< 3.0	3.1 (1.4 – 5.3)
Immunoglobulin G (g/L)	4.5 – 14.0	16.7 (5.3)
MMP-2 (ng/mL)		1244 (344)
MMP-9 (ng/mL)		28.6 (20.9 – 54.5)
TIMP-1 (ng/mL)		127 (39.8)
Total Cholesterol (mmol/L)	5.0 – 6.4	4.4 (1.1)
HDL-Cholesterol (mmol/L)	> 0.9	1.0 (0.4)
LDL (mmol/L)	3.5 – 4.4	2.6 (0.9)
Triglycerides (mmol/L)	0.80 – 1.94	1.8 (0.9)

Concentrations are expressed as mean (SD). Except for MMP-9 and CRP (not normally distributed): median (25-75 quartiles)

Statistical analysis

- Prediction of Dmax

We hypothesized that given Dmax at visit t, we are able to accurately predict Dmax at the next visit (t + 6 months). Next, we investigated whether prediction of Dmax at t + 6 months could be improved by individually incorporating other measured variables. The final multivariate regression model was developed using the backward selection method.

- Clustering of patients

Using a conventional growth model and a growth mixture model (GMM), we investigated whether the 100 patients in the study could be clustered together such that Dmax of patients in a group evolved similarly in time.

Conventional growth models can describe the individual and average trajectory of the Dmax in time and are able to capture individual differences by introducing patient-specific parameters such as random intercept and random slope. Furthermore, the measured variables were examined individually to determine their effect on the random parameters (intercept and slope). The log-likelihood, Akaike's information criterion (AIC), Bayesian information criterion (BIC) indices were used to identify the model with superior fit. The final multivariate regression model was developed using the backward selection method. The

random intercept captures the heterogeneity between patients, for the measured variable, at baseline. The random slope is the deviation in a patient his measured evolution of the variable as compared to the average variable evolution. The random diameter slope was related with the random measured variable slope using linear regression and we hypothesized that a slower or faster growing diameter can be related to a slower or faster rate of a measured variable.

General growth mixture modelling (GGMM) is a method used to identify profiles and trajectories of subgroups within a heterogeneous sample. Latent class growth analysis (LCGA) was used to model the heterogeneity in our data by identifying discrete growth trajectories (classes) and to test predictors of membership in these classes. LCGA does not assume a single population and can test for the presence of multiple classes of individuals that represent distinct multivariate normal distributions. To obtain the final model, we first employed simple growth models to obtain some initial insights in the growth factor variation. Second, we compared two- and three-class unconditional growth mixture models (no measured variables). Third, we extended the model by including the measured variables as predictors of class membership. Based on the BIC, AIC and the likelihood ratio test the final model with the best fit was selected.

Results

Primary aortic repair

Based on Dmax and absence of symptoms, 135 (78%) patients were offered elective AAA repair. Open repair was performed in 54 (40%) and EVAR in 81 (60%) of patients. At surgery the mean Dmax was 67 mm and mean age of the patients was 72 years. Thirty-nine patients presented at the emergency department with ruptured (n = 23), or symptomatic (n = 16) AAA and underwent emergency repair (29 (74%) open repair and 10 (26%) EVAR). The in-hospital survival was 94% for the elective group and 87% for the emergency group. Survival at the end of the study was 85% for the elective group and 82% for the emergency group.

Follow-up patients

During follow-up 18 patients underwent elective surgery either, open (n=9) or endovascular repair (n=9) of their AAA. The number of patients available for ultrasound follow-up were 90 at 6 months, 74 at 12 months, 53 at 18 months, 46 at 24 months, 19 at 30 months, 7 at 36 months, and one at 42 months. The median progression rate and the number of patients requiring surgery during FU are summarized in Table 3.2. Patients with larger aneurysms at baseline require more early an elective surgery compared to patients with small aneurysms at

baseline. Biomarker concentrations at baseline are summarized in Table 3.1. Patient characteristics at every follow-up moment are summarized in Table 3.3.

Table 3.2. Three year follow-up: Expansion rate and need for surgery

		30 – 34 mm (n = 15)	35 – 39 mm (n = 17)	40 – 44 mm (n = 23)	45 – 49 mm (n = 23)	50 – 54 mm (n = 23)
Expansion rate*		1.0 (0.6)	2.0 (0.7)	3.0 (0.4)	2.0 (0.8)	1.0 (0.8)
One year FU	- EVAR	-	-	-	-	4
	- Open	-	-	-	1	3
Two years FU	- EVAR	-	-	-	2	2
	- Open	-	-	1	2	2
Three years FU	- EVAR	-	-	-	1	-
	- Open	-	-	-	-	-

* Expansion rate is expressed as median (SE)

Table 3.3. Patient and aneurysm characteristics

		FU 1 (n=101)	FU 2 (n=90)	FU 3 (n=74)	FU 4 (n=53)	FU 5 (n = 46)	FU 6 (n = 19)	FU 7 (n = 7)	FU 8 (n = 1)
Gender	♂	81 (80)	71 (79)	54 (74)	41 (80)	36 (78)	15 (79)	7 (100)	1
Age (years)		71 (7.7)	71 (7.7)	72 (7.3)	73 (7.2)	72 (7.0)	73 (6.5)	73 (6.7)	76
Height (cm)		172 (7.1)	172 (7.3)	172 (7.5)	172 (7.9)	173 (8.1)	172 (7.1)	173 (6.1)	175
Weight (kg)		80 (12.4)	79 (12.5)	79 (12.3)	80 (12.8)	81 (13.2)	79 (13.2)	77 (5.6)	79
BMI (kg/m²)		26.9 (3.7)	26.7 (3.7)	26.8 (3.6)	27.0 (3.8)	27.2 (4.1)	26.6 (3.1)	25.8 (2.1)	25.8
Dmax (mm)		43 (6.7)	44 (7.0)	45 (7.3)	47 (7.4)	47 (8.1)	45 (7.5)	47 (8.2)	43
Expansion (mm/year)		1.9 (2.7)	2.4 (3.1)	2.5 (2.9)	2.0 (1.9)	1.6 (2.7)	-	-	-
GFR		64.8 (24.2)	66.6 (25.1)	65.1 (24.1)	62.7 (24.1)	64.7 (23.4)	60.8 (20.7)	57.5 (25.4)	71.4
Family history	Yes	20 (20)	17 (19)	11 (15)	8 (15)	6 (13)	5 (26)	1 (14)	0
Hyper-tension	Yes	64 (63)	62 (70)	53 (72)	40 (76)	36 (78)	12 (63)	3 (43)	0
Smoking	Never	8 (8)	8 (9)	7 (10)	5 (9)	4 (9)	2 (11)	0	0
	Quitted	56 (55)	51 (57)	41 (55)	33 (62)	30 (65)	12 (63)	5 (71)	1
	Current	37 (37)	31 (34)	26 (35)	15 (28)	12 (26)	5 (26)	2 (29)	0
Coronary disease	Yes	41 (41)	41 (46)	35 (47)	27 (51)	23 (50)	6 (32)	3 (43)	1
Statin use	Yes	58 (57)	55 (61)	48 (65)	33 (62)	30 (65)	8 (42)	1 (14)	0
Diabetes mellitus	Type I	1 (1)	1 (1)	0	0	0	0	0	0
	Type II	7 (7)	6 (7)	6 (8)	5 (9.4)	4 (9)	2 (11)	1 (14)	

Categorical variables, expressed as number (percentage). Continuous variables, expressed as mean (standard deviation).

Prediction of AAA natural history

- Prediction of Dmax

Figure 3.1 clearly shows that Dmax at visit t + 6 months exhibits a strong linear relationship with Dmax at visit t. The effect of Dmax measured at the previous visit was highly significant (R^2= 92%; p <0.001) and indicated that Dmax at t + 6 months is equal to 1.014 times the Dmax at visit t. Corrected for the diameter at time y, the model showed that only plasma concentration of LDL, measured at time t, showed a suboptimal statistically significant

(quadratic) relationship with the diameter at time t + 6 (p = 0.071). The diameter increases with increasing LDL, up to a LDL concentration of 2.45 mmol/L, where after Dmax decreases with increasing LDL.
The R^2 of the model with LDL incorporated is almost equal to the R^2 of the model only taken into account Dmax at the previous visit. Taken together, these data suggest that modelling of AAA progression can be performed with Dmax only.

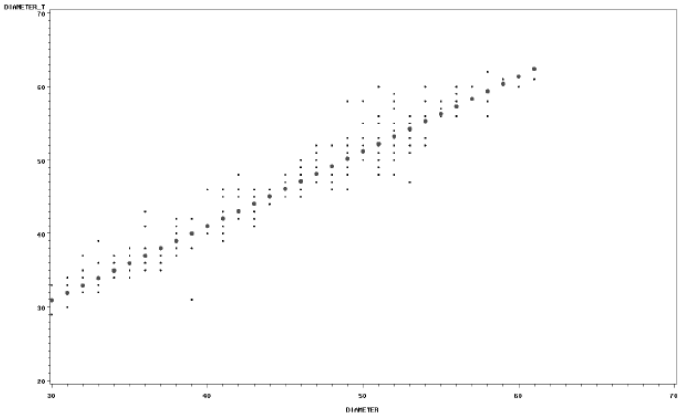

Figure 3.1. Relation between the diameter at visit t and t + 6. The dots represent individual data, the red line represents a smoothed function through the observed data.

- Clustering of patients by conventional growth modelling

Individual patient profiles for Dmax in time are shown in Figure 3.1. The observed heterogeneity between Dmax at baseline (t = 0) and deviation of a specific patient from the overall average Dmax evolution were captured by random intercepts and random slopes, respectively. The average value of AAA Dmax at baseline was 42.84 mm and the average yearly increase was 2.25 mm. The covariance between random intercepts and random slopes was 3.57, suggesting that patients with large AAA at baseline in general grow faster.

- Model for the heterogeneity in Dmax and expansion rate

The variability observed among the random intercepts was much larger than the variability in the slopes. This means that the heterogeneity between patients for their Dmax at the first visit is larger than in their expansion rate. This heterogeneity at baseline was related to age (p = 0.066), BMI (p = 0.022), HDL (p = 0.048), GFR (p = 0.0998), and MMP-9 (p = 0.011) in univariate regression model. For BMI and HDL the random intercept first decreased with increasing covariate, and thereafter started to increase again. These results suggest that older patients with decreased GFR and decreased MMP-9 plasma concentration are not

heterogeneous. For BMI the minimum value for the random intercept is predicted at BMI of 28.3. For HDL the random intercept changes quadratically as a function of HDL, with a minimum at HDL concentration of 1.31 mmol/L. Model reduction resulted in a final multiple regression model for the random intercept with a significant effect of MMP-9 and BMI. After correcting for MMP-9, the random intercept reaches a minimum value for BMI of 27.7. The final model explained 13% of the variability in the random intercept.
The heterogeneity in expansion rate was related to cotinine ($p = 0.030$), total cholesterol ($p = 0.752$) and LDL ($p = 0.056$) serum concentrations at baseline. These univariate regression results indicate that AAA patients with increased total cholesterol and LDL serum concentrations have an increased expansion rate. For cotinine the random slope first increases with increasing covariate and thereafter starts to decrease again. The maximum random slope is predicted for a cotinine of 207.5 ng/mL. At a 10% level of significance also the relation with total cholesterol and LDL is quadratic. The maximum value of the random slope was reached at a cholesterol concentration of 4.06 and a LDL concentration of 2.51 mmol/L.
Model reduction resulted in a final multiple regression model for the random slope, and included only the value of the random intercept. When relaxing the significance level to 10% then the LDL and cotinine stayed in the final model (quadratic relationships). The R^2 value for this model equalled 23.7%, the adjusted value equalled 19.2%.

- Growth models for the predictors

Linear regression was used to relate the random slope of Dmax with the random slope of the diameter. These results showed that a fast growing AAA can be related to slower than on average increasing values of cotinine ($p = 0.024$), and faster than on average increasing values of GFR ($p = 0.030$), cystatin C ($p = 0.076$) and MMP-9 ($p = 0.097$).

- GMM One-class model

In the statistical analyses discussed in this section only seven time points are considered, one time point had to be removed since only one diameter measurement was available (Table 3.3). Model fitting procedures for the single-class mixture model, with constant residual variance, resulted in an estimated intercept of 42.84. This means that on average, a patient had a diameter of 42.84 mm at the start of the study. The estimated slope was 2.248 ($p < 0.0001$) which indicated that the diameter yearly grows 2.25 mm. The estimated intercept variance of 41.57 ($p < 0.0001$) for the diameter, and the variance of the latent slope score of 3.38 ($p < 0.0001$), indicated that substantial variation existed among the patients, mainly in initial status, at time zero (see also Figure 3.1), but also in change over time, referring to expansion.

The covariance between the random intercepts and random slopes was 3.55, indicating that patients with a large diameter at the start of the study, in general increased faster.

- GMM Two-class model

Based on the intercept and slope factor, these classes were labelled: (1) 'Small diameter (= Small AAA) and slowly growing' and (2) 'Large diameter (= Large AAA) and faster growing'. The variances of the intercepts were relatively large; indicating that even within a class there still was heterogeneity. This heterogeneity seemed to be more pronounced in class 2. The same was seen in terms of the random slope. The variance of the slope for class 2 was larger than this for class 1. So patients of class 2 differed more in their expansion than patients of class 1. For both classes the covariance between the random intercept and slope was not statistically significant. Indicating, that conditionally on class-membership, the value of the diameter at the start of the study and the expansion were not independent.

The variance of the intercept and slope is significant for both classes. Forty percent (n = 40) of the patients was allocated to class 1 and 60% (n = 60) to class 2. This allocation was based on the patient's most likely latent class-membership. Table 3.4 show the average posterior probabilities to belong to the two classes from the model. High diagonal and low off-diagonal values indicate good classification.

Table 3.4. Agreement between the classification probabilities based on the average class probabilities and latent class membership for the two-class model

	Latent class 1	**Latent class 2**
Average class probability 1	0.879	0.121
Average class probability 2	0.098	0.902

* Expansion rate is expressed as median (SE)

The highest probability for 'correct' classification, in the sense of agreement between the latent class membership and status in terms of average class probability, occurred for class 2, the patients with large diameters at the start of the study (= Initial Dmax) and fast growing. Patients of this class had 90% chance to be assigned to class 2. Twelve percent of the patients with smaller diameters at the start of the study, was 'misclassified' into class 2.

Discussion

Several studies have previously investigated AAA expansion rates, outcomes, and natural history, and various surveillance protocols have been recommended [12, 31-35].

Our major finding is that AAA progression is highly dependent on Dmax; a higher initial AAA diameter was associated with higher AAA expansion rate. The importance of diameter in predicting subsequent aneurysm expansion has been clearly identified in studies of small AAA [15, 17, 18, 25, 36].

We also studied whether implementation of clinical parameters and/or biomarkers would improve the prediction of AAA expansion. Plasma concentrations of LDL, incorporated in a prediction model, barely improved the prediction compared to a model only taken into account Dmax at the previous visit. Based on these results we therefore confirm that AAA progression can best be predicted by Dmax only. Flondell-Sité *et al.* previously reported a moderate correlation between initial Dmax and AAA progression (r = .386; P = .001), and also found no additional predictive value of biomarkers (MMP-2, MMP-9, TIMP-1, serpine-1, tPA-serpine-1 and APC-PCI complex)[24].

The heterogeneity between patients' Dmax at the first follow-up visit was larger than the expansion rate that they exhibited the following 6 months. This heterogeneity was related to age, BMI, GFR and circulating levels of HDL and MMP-9. Older patients with decreased GFR and lower plasma levels of MMP-9 were not heterogeneous. Heterogeneity of expansion rate was related to circulating levels of cotinine, total cholesterol and LDL at baseline. In this study, we confirm that AAA expansion rate is exponential [7, 23, 26] as initial diameter was related to yearly AAA expansion. Fast AAA expansion was related to slower than on average increasing values of cotinine and faster than on average increasing values of GFR, cystatin C and MMP-9.

We reported a mean Dmax at baseline of 43 mm and an average yearly progression of 2.25 mm. Others have reported expansion rates of 3.5 mm/year, with no mention of initial Dmax [24], a linear expansion of 2.81mm/year with a baseline median Dmax of 35 mm [37], a median expansion rate of 2.61 mm/year with a median initial Dmax of 33 mm [38]. One other study reported expansion rates of 2.0 mm, 3.4 mm, and 6.4 mm per year for AAA that started with an initial Dmax between 30-39 mm, 40-49 mm, and 50-59 mm, respectively [33]. Schlösser *et al.* found that expansion rates increased with 0.94 mm per year with every mm increase in initial AAA diameter [26].

Of all the potential biomarkers we tested in this study, the members of the lipid spectrum showed the most promising results. LDL levels provided a small improvement of the basic AAA expansion prediction model. Furthermore, AAA patients with increased circulating levels of total cholesterol and LDL displayed increased expansion rate. The association between LDL

cholesterol and AAA formation has been shown by Hobbs *et al.*[39]. A case-control study has demonstrated a significant association between elevated LDL cholesterol and the presence of small AAA, even after adjusting for other relevant confounders [39]. For every 0.1 mmol/L increase in LDL the relative risk of having an AAA increases by 3%. They hypothesized that the association of LDL cholesterol and small AAA found in this study suggests that LDL cholesterol may be important as one of the initiating factors associated with aneurysm formation [39].

Data published on the association between lipid abnormalities and AAA risk have been conflicting. Several epidemiological studies have demonstrated an association between lipid levels and AAA [1, 40-42] whilst others have failed to do so [43-45]. It is not clear whether these observations represent a true association with AAA or simply reflect the higher incidence of atherosclerotic disease found in this group of patients. Schlösser *et al.* showed that LDL, HDL cholesterol and triglycerides did not significantly influence AAA expansion rate (univariate analysis); they suggested that the decreasing AAA expansion was due to the pleiotropic effects of statins [26].

Several experimental studies in mice and rats have reported the effect of statins on AAA expansion rates [46-48]. A recent randomized placebo-controlled trail in only 21 patients undergoing elective open repair demonstrated that simvastatin reduced MMP-9 levels in the AAA wall by 40% [49]. MMP play a pivotal role in the development of AAA, and the reduction in concentration and activity of these enzymes is believed to explain the mechanism behind the observed association of statins and reduced AAA expansion [26]. In our study, approximately 60% of the follow-up patients were taking statins. The use of statins, however, did not influenced expansion rate in our population. Gottsäter *et al.* have shown, in a cross-sectional study, that statin-treated patients showed lower values of homocyteine, MMP-9, and ET-1 [50]. Later, in a longitudinal study the same group showed that statin-treated patients displayed lower expansion rate after two and four years of follow-up, whereas mean yearly AAA expansion rate during the whole follow-up did not differ between AAA patients with and without statin therapy [23]. Statins are a complex group of drugs that in addition to their lipid lowering effects may have a role in reducing the destructive remodelling of the aortic wall that occurs in aneurysms by altering endothelial function and reducing the expression of MMP via their anti-inflammatory and immune modulatory properties [51-53]. Schouten *et al.* showed a 1.16 mm per year lower AAA expansion rate in users of statins compared to nonusers [7]. Sukhija *et al.* reported that the sizes of AAA of patients not treated with statins significantly increased after two years of follow up, but the AAA sizes of patients treated with statins for the same period did not significantly change [54].

We found no association between smoking habit and AAA progression. However, observed heterogeneity in AAA expansion rate was related to circulating levels of cotinine. Others have

also failed to associate AAA expansion to smoking [26]. While more substantial evidence clearly associates smoking to increased expansion rate [11, 15].

Reports on gender dependent expansion rate have been conflicting [7, 14, 15, 55]. Our group of 101 follow-up patients consisted of 17 females, and we found no gender related differences in AAA expansion rate, which is in accordance with the studies of Brown *et al.* [14] and Brady *et al.* [15].

A biomarker, ideally should have (1) a causal relationship with the disease, (2) be involved in the pathophysiological pathways, and (3) reflect disease severity and progression in its concentration [22]. Identification of biomarkers for AAA is complicated by the fact that AAA is a multifactorial disease with a complex pathophysiology, which has not yet been elucidated. The AAA expansion can only be accurately determined when one imaging modality is consistently used for each patient, and the intra-operator and inter-operator measurement variation is less than the average expansion rate. In addition, investigations are needed to determine the minimum follow-up time after blood sampling for the purposes of biomarker measurement in patients with AAA and whether multiple blood samples are necessary. Previous investigations have shown that the histopathological characteristics of AAA were not related to Dmax, ILT thickness, expansion rate, and clinical presentation (rupture vs. non-rupture). These findings suggested that progression of AAA is mainly determined by parameters other than histopathological features of the diseased vessel wall [56]. Irrespective of the size, expansion rate or clinical presentation of the AAA composition on a histological level was similar between different AAAs. In addition, smaller (50-70 mm) AAA shows comparable destruction to larger AAA (>70 mm). We found the same lack of histopathological indifference between ruptured and non-ruptured AAA. Whether or not these findings can be extrapolated to small AAA (30-50 mm) needs to be ascertained [56].

The therapeutic goal is to prevent small aneurysms from reaching a size at which the risk of rupture is high. The optimal treatment strategy for patients with small AAA is not known. However, since it is known that there is a low risk of rupture for AAA less than 50 mm in diameter [57], it has been proposed that watchful waiting with the use of imaging might be a better long-term treatment strategy than early surgical repair of small AAA [5]. UK Small Aneurysm Trial (UKSAT) and the Aneurysm Detection and Management Veterans Affairs Cooperative Study Group have shown that early elective surgery for small AAA (40-55mm) does not improve survival compared with ultrasound surveillance, despite low operative mortality [17, 18]. Lederle *et al.* found a risk of 0.6% per year for patients with AAAs between 40 and 55 mm [18]. Early surgery could have beneficial biologic or lifestyle-related effects [19]. Twelve months after early surgery, patients' perceptions of their health were improved [58]. There is a higher rate of smoking cessation among patients who underwent early surgery. Major surgery is recognized as an important stimulus to smoking cessation, the

survival benefits of which do not become apparent for five or more years [59-61]. Smoking cessation results in a particular reduction in mortality from cardiovascular causes [62]. The UKSAT showed a smaller number of deaths from myocardial infarction and stroke in the early-surgery group and patients who reported that they were former smokers had a lower risk of death than those who reported that they were current smokers [19]. Favourable biologic results of early surgery could include reduction of IL-6, a marker of cardiovascular risk [63]. In the UKSAT two thirds of the 496 deaths were attributed to a cardiovascular cause [19]. The diameter of the aneurysm is an independent marker of the risk of cardiovascular disease [64] Thus, a combination of lifestyle-related and biologic effects of early repair of an AAA could underlie the long-term survival benefit of early surgery [19].

Biomarkers found in patients with more extensive AAA expansion rate may be of importance for the selection of (pharmacological) treatment for decelerate AAA expansion rate and postponing the need for repair. Different pharmacological agents, such as specific MMP inhibitors [65], doxycycline [66], and a CD40L pathway inhibitor [67] have been evaluated concerning their ability to influence inflammatory processes and matrix degrading enzymes to halt AAA expansion rate with the aim of preventing AAA rupture and surgery [23]. Effective medical therapy must reduce the rate of expansion by more than 50% in order to keep a 40 mm AAA from reaching the 55 mm threshold within five years [68].

This and other studies concerning the expansion of AAA presents a number of methodological challenges [20]. Firstly, the change in diameter over time is small and characterized by periods of rapid expansion and quiescence. Simple estimates of expansion usually overestimate the rate of progression, and, given that losses to follow-up are inevitable, estimation techniques such as a Bayesian multilevel random effect model have been recommended [15]. Secondly, the measurement of aortic diameter using ultrasound (and to a lesser extent CT) has an error margin of 2 to 3 mm [15, 25, 69], which is larger than the annual expansion of many small AAA. Finally, larger AAA are regularly lost to surveillance when clinicians recommend surgical intervention on the basis of risk of rupture. This is a particular problem in studies of 40 to 50 mm AAA (the group most likely to require surgical treatment) where the intervention rate is around 10, 25 and 40% at 1, 2, and 3 years, respectively, even in centres with conservative surgical protocols [1, 17].

In conclusion, prediction of AAA expansion rates is highly dependent on initial aneurysm diameter. Taken together, AAA size remains the gold standard for prediction of AAA expansion.

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4

MMP-9, marker of successful EVAR

PLASMA LEVELS OF MATRIX METALLOPROTEINASE-9: A POSSIBLE DIAGNOSTIC MARKER OF SUCCESSFUL ENDOVASCULAR ANEURYSM REPAIR

Objective. Evaluating the diagnostic value of plasma matrix metalloproteinase (MMP)-2, -9, and tissue inhibitor of MMP (TIMP)-1 for endoleak detection after endovascular aneurysm repair (EVAR).

Report. Consecutive EVAR patients (n=17) with endoleak and matched controls without endoleak (n=20) were prospectively enrolled. Increased levels of MMP-9 were observed in patients with endoleak ($p<0.001$). Regression analysis showed no significant influence of age, sex and AAA size. The receiver operating characteristic (ROC) curve of plasma MMP-9 showed that a cut-off value of 55.18 ng/mL resulted in 100% sensitivity and 96% specificity with an AUC value of 0.988 ($p<0.001$) to detect endoleak.

Conclusion. Plasma MMP-9 levels appear to discriminate between patients with and without an endoleak with high sensivity and specificity.

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Introduction

Endovascular aneurysm repair (EVAR) is a safe and effective method to treat suitable patients with an abdominal aortic aneurysm (AAA), and compared to conventional open aortic aneurysm repair EVAR has lower early mortality rates and length of hospital stay [1-3]. Endoleak is the most common complication of EVAR with a reported incidence varying from 2% to 45% [1, 4, 5]. Re-interventions are frequently required since endoleak may cause enlargement with eventually rupture of the aneurysm sac. Computed tomography angiography (CTA) is considered the gold standard for detection of these complications, despite known disadvantages such as cumulative radiation dose, nephrotoxic contrast agent and high costs [2]. Therefore, a less harmful and less costly alternative for follow-up after EVAR is desirable.

Increased circulating levels of plasma matrix metalloproteinase (MMP)-2, -9, and tissue inhibitor of MMP (TIMP)-1 are associated with presence and size of abdominal aortic aneurysm [6]. It has been shown that decreased MMP levels during post-EVAR surveillance might indicate successful EVAR whereas an increase may help to identify patients with aneurysm sac growth and/or endoleak [7, 8]. However, reports on the diagnostic value of these biochemical assays are lacking and hence none of these assays are clinically applied. The objective of this study was to evaluate the potential clinical applicability of MMP-2, -9, and TIMP-1 as a diagnostic tool for endoleak presence.

Methods

Following endovascular AAA repair at either Maastricht University Medical Centre (Maastricht, the Netherlands) or Atrium Medical Centre (Heerlen, the Netherlands), patients underwent routine CTA follow-up. Between January and July 2008, thirty-seven patients were included in the study. Seventeen patients had an endoleak as detected on CTA, which included four type I, twelve type II, and one type III endoleak. The other twenty patients were matched controls. Blood was sampled via an antecubital vein puncture and exactly thirty minutes after collection, blood was centrifuged at 3000 g for fifteen minutes at 4°C. Both institutional ethics committees approved the research protocol and all participants gave written informed consent to their involvement in the study.

CTA was performed by a multidetector 16 slice helical CT scanner with an in-plane resolution of 512x512 pixels and a slice-thickness of either 1 mm or 3 mm due to the use of different protocols. Maximal anterior-posterior diameter of the aneurysm sac, presence of endoleak,

migration, or component separation was determined. Plasma levels of MMP-2, -9, and TIMP-1 were determined in duplicate using a commercial ELISA (GE Healthcare).

Results

Patients and aneurysm characteristics as well as plasma levels of MMP-2, -9, and TIMP-1 are listed in Table 4.1. Higher MMP-9 levels were observed in patients with an endoleak as compared to patients without endoleak ($p<0.001$). The ROC curve represents the relationship between the specificity and the sensitivity of plasma MMP-9 levels in detecting endoleak presence. The area under the curve (AUC) was 0.99 with a sensitivity of 100% (95% CI; 80.5 - 100) and a specificity of 96% (95% CI; 75.1 – 99.9) using a cut-off value of 55.18 ng/mL.

Table 4.1. Summary of patient and aneurysm characteristics

		Endoleak		p-value
		No (n=20)	**Yes (n=17)**	
Male gender		19 (95%)	16 (94%)	n.s.
Age in years (mean ± SD)		74 ± 9.5	73± 7.0	n.s.
Smoking history	- Current smokers	5 (25%)	4 (24%)	n.s.
	- Ex smokers	15 (75%)	13 (76%)	
Statin use		7 (35%)	11 (65%)	n.s.
Time between EVAR and CTA (months)		18 ± 15	21 ± 15	n.s.
Initial AP AAA diameter (mm)		57 ± 17	72 ± 19	0.038
Right groin introduction of main device		18 (90%)	17 (100%)	n.s.
Neck diameter (mm)		24 ± 4.2	24 ± 3.7	n.s.
Unfavorable angulation of neck		3 (15%)	2 (12%)	n.s.
Diameter ipsilateral CIA (mm)		17 ± 6.1	15 ± 3.2	n.s.
Diameter contralateral CIA (mm)		18 ± 7.3	17 ± 4.5	n.s.
Tortuosity of iliac axis		3 (15%)	4 (24%)	n.s.
Patent IMA		5 (25%)	4 (24%)	n.s.
MMP-2 (ng/mL)		1007 ± 286	1110 ± 370	n.s.
MMP-9 (ng/mL)		25.0 ± 13.4	89.5 ± 26.5	<0.001*
TIMP-1 (ng/mL)		138 ± 69	143 ± 63	n.s.

AP = Anterior-posterior, CIA = Common iliac artery, IMA = Inferiro mesenteric artery, n.s., not significant
* Additional regression analysis showed that sex, age and AAA size (Dmax) do not influence the significant effect of MMP-9 on predicting endoleak.

Plasma MMP-9 levels can not differentiate between different endoleak types. Anterior-posterior aneurysmal diameter (Dmax) was significantly larger in the endoleak group (72 mm vs 57 mm; $p = 0.038$); however, plasma MMP-9 levels were not associated with Dmax ($r^2 = 0.30$) or intraluminal thrombus (ILT) volume ($r^2 = 0.20$).

Two patients who underwent intervention to eliminate type II endoleak showed at one month post-intervention a decrease in plasma MMP-9 levels (102.95 to 16.23 ng/mL and 121.97 to 20.28 ng/mL). Furthermore, we determined MMP-9 levels in fluid aspirated from the aneurysm sac of these patients, showing greatly increased levels of MMP-9 (respectively 386.34 and 343.78 ng/mL).

Discussion

The present study showed that plasma MMP-9 levels can accurately discriminate between patients with and without and endoleak with both high sensitivity and specificity. The ROC and AUC demonstrated that plasma MMP-9 is an excellent test to determine endoleak presence. Implementing a blood test to differentiate between patients with and without an endoleak is clinically important. Patients without an endoleak could be spared to undergo CTA with the aforementioned additional hazards and costs.

Sangiorgi *et al.* and Lorelli *et al.* previously suggested that plasma MMP-9 levels can be used to monitor the successfulness of EVAR procedures and showed proof of concept [7, 8]. The current study is the first to report the diagnostic value of the MMP-9 assay in post-EVAR surveillance. We also showed that MMP-9 levels were associated with endoleak presence and not with Dmax or ILT. Although this case control study has a limited number of patients, sensitivity and specificity rates are impressive and the confidence intervals are reassuring. Nevertheless, an adequately powered prospective clinical trial is necessary to validate the applicability of plasma MMP-9 levels to differentiate between patients with and without endoleak in clinical practice and the potential role of plasma MMP-9 levels in selecting EVAR patients requiring CTA. Furthermore, standardization of the assay (e.g. monoclonal antibody and calibrators) will be necessary for the application of one standard cut-off value.

In conclusion, the present study suggests that plasma levels of MMP-9 can accurately discriminate between patients with and without an endoleak with both high sensitivity and specificity. A prospective clinical trial in order to validate the clinical applicability of this assay is in progress.

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5 Biomarker discovery in AAA

DIFFERENTIAL PROTEIN EXPRESSION IN SERUM OF ABDOMINAL AORTIC ANEURYSM PATIENTS – A PROTEOMIC APPROACH

Objective. To investigate the differential expression of proteins in serum of abdominal aortic aneurysm (AAA) patients in relation to aneurysm size (Dmax) and progression.

Methods. Two-dimensional differential in-gel electrophoresis (2D-DIGE) together with tandem mass spectrometry (MS/MS) was used to analyze the serum proteome from patients with small (Dmax 30 – 54 mm) AAA, either stable (increase Dmax < 5 mm/year; n = 8) or progressive (increase Dmax ≥ 5 mm/year; n = 8) or large (Dmax ≥ 55 mm; n = 8) AAA. The identified proteins were quantitatively validated in a larger population (n = 80).

Results. Several proteins were differentially expressed in serum of small stable, small progressive and large AAA. Three validated proteins (IgG, α1-antitrypsin and Factor XII activity) showed strong correlation with Dmax. Size combined with either Factor XII activity or α1-antitrypsin had minimal effect on the prognostic value in predicting aneurysm progression compared to size alone (AUC, 0.85; 95% CI, 0.73-0.97; p<0.001 and AUC, 0.85; 95% CI, 0.72-0.98; p<0.001 vs AUC, 0.83; 95% CI, 0.71-0.96; p<0.001 respectively)

Conclusion. The present study indicates that both Factor XII and α1-antitrypsin are found in differentially expressed in the serum of patients with AAA. However, combination of either Factor XII or α1-antitrypsin with aneurysm diameter had little effect on prediction of aneurysm progression versus diameter alone.

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Introduction

Abdominal aortic aneurysm (AAA) is a degenerative disease of the abdominal aorta leading to progressive dilatation, intra-luminal thrombus (ILT) formation and rupture. Survival associated with AAA rupture is extremely low with rates ranging from 10 to 15%, while elective surgery has an overall survival rate of 95%. It is widely recognized that the risk of rupture increases with increasing diameter, with the threshold for elective repair at ≥55 mm in diameter (Dmax). However, a significant number of AAA ≥55 mm never experience rupture [1], while small AAA can rupture unexpectedly [2]. Early detection and elective AAA repair is, for those at risk of rupture, a critical step to limit mortality associated with aneurysm rupture [3]. Better approaches are needed for follow-up of patients with small asymptomatic AAA. Most patients with small AAA are prospectively followed by serial imaging until elective repair is indicated [3-7], although growth rate differs substantially between individuals [8]. Besides identifying patients at risk for AAA progression [9, 10] and rupture [11], new biomarkers may also help to elucidate the molecular mechanisms behind this disease and eventually generate treatments to reduce progression rate.

Proteomics provides powerful opportunities to examine protein expression patterns of tissues or body fluids [12-15]. Compared to conventional 2D gel electrophoresis, 2D differential in-gel electrophoresis (2D-DIGE) enables quantitative comparison of protein expression and reduces the analytical variability through the use of an internal standard [16].

Aim of the present study was to detect differential serum protein expression related to aneurysm size and progression. To do so, we utilized 2D-DIGE together with matrix-assisted laser desorption ionization time-of-flight tandem mass spectrometry (MALDI-TOF/TOF) to detect and identify differentially expressed proteins between small stable, small progressive and large AAA. Differential expression of the identified proteins was quantitatively validated using routine immunochemical techniques.

Materials and Methods

Sample collection

Between January 2006 and January 2009, blood samples (e.g., serum, EDTA and citrate plasma) were obtained from patients with small asymptomatic AAA who were invited to participate in a standardized six-monthly follow-up study. Furthermore, pre-operative blood samples were obtained from patients undergoing elective or emergency reconstruction of the infra-renal abdominal aorta at the Department of Vascular Surgery, Maastricht University Medical Centre+ (MUMC+, The Netherlands). The Medical Ethical Committee approved the

research protocol and all participants gave written informed consent. The present study enrolled forty-eight consecutive patients with small (Dmax 30–54 mm) asymptomatic AAA, either stable (increase Dmax <5 mm/year; n=27) or progressive (increase Dmax ≥5 mm/year; n=21), and 32 consecutive AAA patients undergoing elective open or endovascular repair (Dmax ≥55 mm). Patients with symptomatic, mycotic or ruptured AAA were excluded. Demographics of the study subjects at baseline were obtained by interview and examination (Table 5.1). These included sex, age, smoking habit (current smoker, ex-smoker since one year, never smoked), hypertension (use of antihypertensive drugs), family history, coronary disease (history of unstable angina pectoris, myocardial infarction, coronary artery bypass grafting or percutaneous transluminal coronary angioplasty), chronic obstructive pulmonary disease (COPD; based on the Global initiative for chronic Obstructive Lung disease (GOLD) criteria [17]), peripheral arterial disease (PAD; ankle/brachial pressure index <0.9), chronic renal failure (Glomerular filtration rate <15mL/min/1.73m^2) and statin use.

Table 5.1. Baseline demographics of patients with stable, progressive and large AAA

		Stable AAA (n=27)	**Progressive AAA (n=21)**	**Large AAA (n=32)**	**ANOVA p-value**
Male sex		21 (77.8 %) [1]	15 (71.4 %) [2]	31 (96.9 %)	0.029
Age (years)		69.9 ± 6.3 [3]	71.7 ± 8.2	74.4 ± 6.0	0.040
Range		(61 – 83)	(54 – 82)	(63 – 87)	
AAA diameter (mm)		36.6 ± 4.2 [4]	44.9 ± 6.5 [4]	72.1 ± 10.1	<0.001
Range		(31 – 43)	(30 – 53)	(55 – 92)	
Family history		6 (22.2 %)	1 (4.8 %)	7 (21.9 %)	N.S.
Hypertension		21 (77.8 %)	15 (71.4 %)	19 (59.4 %)	N.S.
Smoking history #	Never	1 (3.7 %)	3 (14.3 %)	3 (11.5 %)	N.S.
	Quitted	18 (66.7 %)	8 (38.1 %)	14 (53.9 %)	
	Current	8 (29.6 %)	10 (47.6 %)	9 (34.6 %)	
Coronary disease		12 (44.4 %)	11 (52.4 %)	16 (50.0 %)	N.S.
COPD		2 (7.4 %)	2 (9.5 %)	5 (15.6 %)	N.S.
PAD		13 (48.1 %)	6 (28.6 %)	5 (15.6%)	0.030
Chronic renal failure		3 (11.1 %)	2 (9.5 %)	3 (9.4%)	N.S.
Statin use		18 (66.7 %)	14 (66.7 %)	17 (53.1 %)	N.S.

Significantly different compared to large AAA: [1] p=0.03; [2] p=0.01; [3] p=0.04; [4] p<0.001; N.S. not significant different.
Smoking history is available for 26 patients with large AAA

Blood was allowed to coagulate at room temperature for thirty minutes and centrifuged at 3000g for fifteen minutes at 4°C. Exactly one hour after blood collection samples were stored in aliquots at -80°C pending analysis. Maximal anterior-posterior Dmax was measured by one observer, with intra-observer variability of 2 mm (data not shown); using ultrasound for follow-up patients and computed tomography angiography before surgery.

Sample preparation

For 2D-DIGE, serum samples with no freeze-thaw cycle were selected from patients with either small stable (n=8), small progressive (n=8) or large (n=8) AAA. Samples from time point *t* were used to discover factors prospectively predicting aneurysmal progression in one year. Aneurysmal progression was calculated as the change in Dmax from time point *t* to *t*+12 months.

To improve the DIGE analysis, individual serum samples were fractionated based on their biophysical properties using Expression Difference Mapping Kit (Bio-Rad Laboratories Inc., Hercules, CA). Six fractions were obtained per patient; fractions pH3, pH4 and pH5 were pooled together (denoted fraction A), and fractions pH7 and pH9 were mixed (denoted fraction B). The organic fraction was discarded, since isoelectric focusing was carried out on strips with pH range of 3-10. The pH of the fractions was adjusted to 8.5 by 50mM NaOH to obtain optimal labelling conditions.

2-D DIGE

Fraction A and B were separately analyzed, comparing protein expression differences between the groups within each fraction. Fractions from individual participants were randomly labelled with Cy3 or Cy5 according to the manufacturer's instructions (GE Healthcare, Uppsala, Sweden). Dye-swapping was performed to control for preferential labelling by one of the dyes. Labelling reaction was quenched on ice in the dark for ten minutes by scavenging non-bound dye with 10mM lysine (Sigma-Aldrich, St. Louis, MO). Three labelled samples, two analytical and a Cy2-labeled internal standard, were pooled and an equal volume of 2x lysis buffer (7M urea, 2M thiourea, 4% CHAPS, 0.04% bromophenol blue, 2% DTT, 2% IPG-buffer pH 3-10) was added.

Samples were focussed on an IPGphor using immobilized DryStrip gels (3-10 pH range, non-linear, 18 cm), which were passively pre-rehydrated for six hours with DeStreak Rehydration Solution supplemented with 0.5% IPG-buffer. Strips were equilibrated for fifteen minutes with gentle shaking in equilibration solution (6M urea, 2% SDS, 50mM Tris pH 8.8, 0.02% bromophenol blue, 30% glycerol) supplemented with 1% DTT, followed by 2.5% iodoacetamide in fresh equilibration solution for additional fifteen minutes incubation with gentle shaking. Second dimension SDS-PAGE was carried out on polyacrylamide gels (12.5% T, 3% C) using the Ettan DALT twelve. Gels were run at 20°C, 0.5 W/gel for one hour and 15 W/gel until the bromophenol blue frontier reached the bottom of the gel and subsequently scanned on the Ettan DIGE imager using CyDye-specific excitation/emission wavelengths. Gels were analysed using DeCyder 7.0 software (GE Healthcare). Differentially expressed protein spots present in >70% of the gels were selected for identification using MS/MS.

Protein identification and in-gel digestion

Preparative gels, loaded with 300 µg unlabelled protein, were run using previous conditions. Gels were fixed for thirty minutes in 10% CH_3OH and 7% CH_3COOH followed by an overnight incubation in Sypro Ruby (Bio-Rad). Thereafter, gels were again fixed in 10% CH_3OH and 7% CH_3COOH for forty-five minutes followed by a thorough rinse with milliQ. Protein spots of interest were picked from the Sypro-stained gel using the automated Ettan Spot Picker (GE Healthcare) into 96-well plates and in-gel digestion was carried out on the MassPREP digestion robot (Waters, Manchester, UK).

Gel plugs were destained twice in 100mM NH_4HCO_3, 50% (v/v) CH_3CN for ten minutes, then dehydrated in 100% CH_3CN for five minutes, after which supernatant was removed, and gel plugs were allowed to air dry for ten minutes. Cysteines were reduced with 10mM DTT in 100mM NH_4HCO_3 for thirty minutes followed by alkylation with 55mM iodoacetamide in 100mM NH_4HCO_3 for twenty minutes. Gel plugs were washed with 100mM NH_4HCO_3 and subsequently dehydrated with 100% CH_3CN. Trypsin suspended in 50mM NH_4HCO_3 was added (12 ng/µL) and allowed to digest at 40ºC for five hours. Peptides were extracted twice with 1% (v/v) formic acid, 2% (v/v) CH_3CN.

MS/MS and database analysis

Peptide solutions were mixed at a 1:1 ratio with 5 mg/ml α-cyano-4-hydroxycinnamic acid (CHCA) matrix in 50% CH_3CN, 0.1% TFA and spotted in duplo. Spots were allowed to air dry for homogeneous crystallization. Spectra were acquired in positive ion reflectron mode on 4800 MALDI-TOF/TOF analyzer (Applied Biosystems, Foster City, CA). Tandem MS fragmentation spectra were acquired for each sample, averaging 500 laser shots per fragmentation spectrum on each of the eight most abundant ions present in each sample. The generated peak list was searched with MASCOT search engine by GPS Explorer v.3.9. Software (Applied Biosystems) against the Swiss-Prot protein database with taxonomy at *Homo sapiens*; trypsin and keratin peaks were excluded. One miss-cleavage was tolerated; carbamidomethylation was set as a fixed modification and oxidation of methionine as an optional modification. Protein charge was set at 1+, mass tolerance for precursor ion at 150 ppm and MS/MS tolerance at 0.2 Da. No restrictions were made on protein mass. Identification was considered confident when total ion and protein score were above the 95% confidence interval (CI).

Laboratory validation measurements

Quantitative validation measurements were carried out using native serum or citrate plasma from consecutive patients with stable (n=27), progressive (n=21) and large (n=32) AAA. Serum concentrations of alpha-1 antitrypsin (α1-AT) and immunoglobulin G (IgG) were analysed on

the BN ProSpec (Siemens Healthcare Diagnostics, The Hague, The Netherlands). Analyses were performed at the Department of Clinical Chemistry, MUMC+ with following reference values: α1-AT (0.75–1.85 g/L) and IgG (4.5–14.0 g/L). Citrate plasma Factor XII activity and D-dimer concentrations were analysed on the Sysmex CA-7000 (Siemens Healthcare Diagnostics) at the Department of Haematology, MUMC+ with following reference values 60-140% and <500 ng/mL, respectively. D-dimer was not found as one of the discriminating proteins using DIGE, but was included as a "positive control" measurement testing the validity of our population, since recent literature has evidenced a clear relationship with AAA presence, diameter and progression [18-21]

Statistical analysis

Data were analyzed with PASW Statistics 18.0 Software (IBM Corporation, Somers, NY). Categorical variables, expressed as percentages, were compared using the Pearson Chi-squared test. ANOVA was used to compare continuous demographic variables, expressed as mean ± standard deviation, applying the Bonferroni correction for pairwise comparison. Associations between biomarker concentrations and aneurysm diameter were calculated with linear regression. Multivariate linear regression was used to correct the associations for possible bias due age, sex and PAD. One-way ANOVA with Bonferroni post-hoc testing (DeCyder 7.0 software, GE Healthcare) was used to detect significant differences in protein abundances between the three experimental groups.

Results

Identification of differentially expressed proteins in serum from AAA patients

On average, approximately 700 spots were detected on each gel. In total, twenty-six spots were differentially expressed between patients with stable and large AAA; with two up-regulated and nine down-regulated spots in fraction A and three up-regulated and twelve down-regulated spots in fraction B of patients with stable AAA. Fraction A as well as B showed one up-regulated spot in patients with large AAA compared to patients with progressive AAA. Only fraction B revealed significant differential expression between progressive and stable AAA, with eight spots up-regulated in stable AAA.

All spot features of interest were trypsin-digested and submitted to MS/MS for identification. We were able to identify seven spots representing five unique proteins that were significantly different expressed between the experimental groups (Table 5.2).

Table 5.2. Differentially expressed proteins in serum of stable, progressive and large AAA identified by MALDI-TOF/TOF after 2D-DIGE analysis.

Name	Total Ion Score (95% CI)	Stable AAA	Progressive AAA	Large AAA	ANOVA
		Mean standardized abundance ± standard deviation			
Albumin	401 (100%)	1.27 ± 0.36 [1]	1.98 ± 0.80	2.54 ± 0.62	0.009
Complement C3	215 (100%)	1.29 ± 0.20	1.01 ± 0.35 [1]	1.93 ± 1.12	0.022
α-1 antitrypsin	341 (100%)	1.89 ± 0.75 [1]	1.14 ± 0.28	1.03 ± 0.51	0.026
Factor XII	126 (100%)	0.09 ± 0.04 [1]	0.36 ± 0.36	0.99 ± 0.59	<0.001
Ig κ chain C region	65 (100%)	1.06 ± 0.10 [1]	0.98 ± 0.15	0.88 ± 0.08	0.043
Ig κ chain C region	39 (99.95%)	1.06 ± 0.17 [1, 2]	0.87 ± 0.10	0.87 ± 0.12	0.025
Ig κ chain C region	309 (100%)	1.17 ± 0.18 [2]	0.95 ± 0.14	0.97 ± 0.11	0.021

[1] Significantly different compared to large AAA (p<0.05); [2] Significantly different compared to progressive AAA (p<0.05)

Validation of differentially expressed proteins in serum from AAA patients

To confirm the 2D-DIGE differential protein expression, quantitative validation of the results was carried out on serum or citrate plasma using a larger sample set including the samples analyzed with DIGE (n=80). Although the difference in Ig spot abundance between large and stable AAA was minimal, significantly higher serum IgG concentrations were found in stable and progressive AAA compared to large AAA (18.4 ± 4.8 and 17.5 ± 4.9 vs 8.9 ± 2.6 g/L, p<0.001, Figure 5.1A).

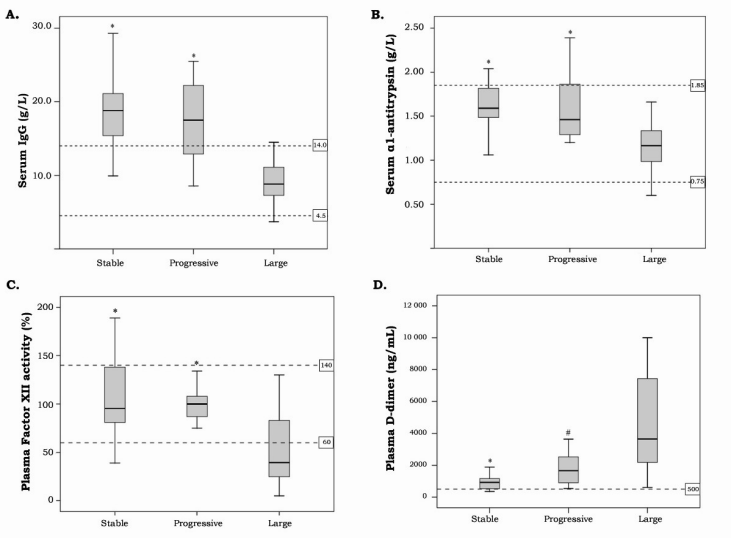

Figure 5.1. Validation of differential (A) serum IgG, (B) serum α-1 antitrypsin, (C) plasma Factor XII activity, and (D) plasma D-dimer expression in eighty patients with abdominal aortic aneurysm. Dashed lines show the reference values, as established at our hospital. Significant difference compared to large AAA is indicated by * p<0.001 or # p = 0.002.

Serum α1-AT concentrations were also increased in patients with stable and progressive AAA relative to patients with large AAA (1.62 ± 0.25 and 1.62 ± 0.37 vs 1.16 ± 0.25 g/L, p<0.001, Figure 5.1B). Notably, serum IgG concentrations were elevated above the upper reference limit in almost all small AAA and were positively correlated (r = 0.67; p<0.001) with serum α1-AT concentrations. Factor XII was down-regulated in stable AAA, as indicated by the DIGE analysis. Interestingly, Factor XII activity was significantly lower in plasma of large AAA compared to stable and progressive AAA (53.6 ± 34.9 vs 104.5 ± 36.3 and 97.5 ± 24.9 %, p<0.001, Figure 5.1C). In almost all AAA patients, plasma D-dimer concentrations were elevated above the reference limit and were significantly different between patients with stable, progressive and large AAA (Figure 5.1D).

Aneurysm size was significantly correlated with serum IgG (r = -0.65; p<0.001), serum α1-AT (r = -0.55; p<0.001), plasma D-dimer (r = 0.62; p<0.001) concentrations and plasma Factor XII activity (r = -0.48; p<0.001). Even after correcting for possible bias due to differences in age, sex and PAD, the associations remained significant (Table 5.3).

As expected aneurysm size shows relatively good prognostic value in predicting aneurysm progression (AUC, 0.83; 95% CI, 0.71-0.96; p<0.001).

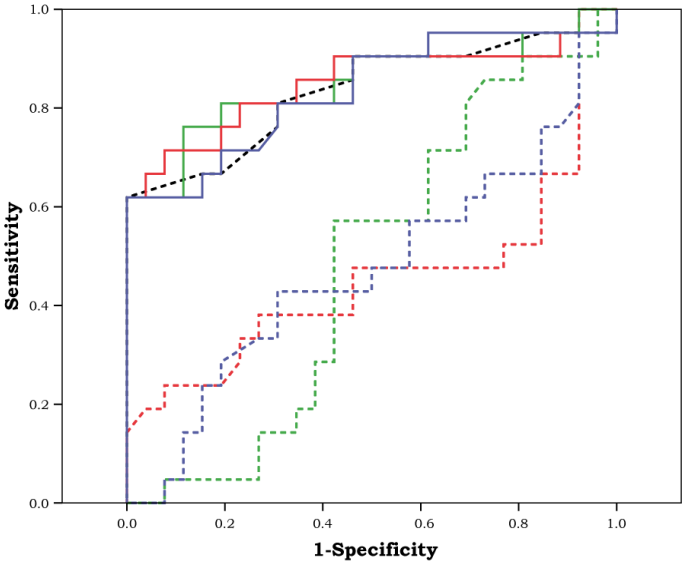

Figure 5.2. Prediction of aneurysm progression by the individual parameters (dotted line) and parameters combined with aneurysm size (full line). IgG (**blue**), α-1 antitrypsin (**red**), Factor XII (**green**) and aneurysm size (**black**).

The prognostic value of the individual markers was not significant, but aneurysm diameter combined with either Factor XII activity or α1-AT, by regression, had minimal effect on the

prognostic value in predicting aneurysm progression (AUC, 0.85; 95% CI, 0.73-0.97; p<0.001 and AUC, 0.85; 95% CI, 0.72-0.98; p<0.001, respectively; Figure 5.2). Although Ig spot abundance differs significantly between stable and progressive AAA, making this a putative marker for aneurysm progression, serum IgG concentration combined with Dmax did not improve the ability to predict aneurysm progression (AUC, 0.83; 95% CI, 0.71-0.96; p<0.001).

Table 5.3. Linear regression assessing the individual relation between biomarker and Dmax.

	B (SE)	p-value	Goodness of fit
α1-antitrypsin	-21.29 (4.80)	<0.001	0.381
IgG	-1.66 (0.25)	<0.001	0.505
D-dimer	0.004 (0.001)	<0.001	0.482
Factor XII activity	-0.17 (0.04)	<0.001	0.336

Corrected for possible bias due to age, sex and PAD.

Discussion

We detected differentially expressed proteins in the serum from patients with stable, progressive and large AAA. These differential proteins are involved in pathophysiological key processes of AAA, such as inflammation (e.g. immunoglobulins), extracellular matrix (ECM) remodelling (e.g. α1-antitrypsin) or coagulation and fibrinolysis (e.g. Factor XII).

In a recent review, members of the "Fighting Aneurysmal Disease" Consortium highlighted the role of ILT in AAA pathogenesis [22]. The ILT is biologically active with at the luminal interface active fibrinolysis and coagulation; markers of these processes correlate with AAA size [18, 19, 23, 24]. D-dimer is likely to be released as a breakdown product of continuous thrombus remodelling [25]. Furthermore, D-dimer is known to stimulate synthesis and release of pro-inflammatory cytokines from neutrophils and monocytes *in vitro* [26]. Plasma D-dimer concentrations show strong association with AAA presence, diameter and progression [18-21]. This association between D-dimer concentrations and AAA diameter was confirmed in our patient population. We are the first to correlate Factor XII with AAA size. Factor XII is involved in the intrinsic coagulation cascade and is not required for physiological coagulation *in vivo* since Factor XII deficiency does not result in abnormal bleeding. It is hypothesized that Factor XII plays a role in stabilizing the fibrin clot. We found an increased expression of Factor XII, combined with a lower Factor XII activity in large compared to small AAA. The fact that both Factor XII and its activity are highly significantly related to aneurysm size suggests an excess consumption of Factor XII. Further research to the role of Factor XII in AAA is required to validate our findings.

Neutrophils predominate within the ILT and are a major source of proteases associated with ECM degradation. In this study, we detected and validated higher serum α1-AT

concentrations in patients with small compared to large AAA. Alpha-1 antitrypsin is an acute-phase protein and serine protease inhibitor, which inactivates enzymes such as elastase. Decreased amounts of α1-AT in serum facilitate proteolytic ECM degradation, weakening the vascular wall, resulting in dilatation and making it more prone to rupture. In agreement with our study, Schachner *et al.* demonstrated that α1-AT concentrations are reduced in the vascular wall of ascending aortic dissections compared to healthy aortas [27]. Furthermore, Lindholt *et al.* and Vega de Céniga *et al.* both showed a positive relation between AAA progression and serum α1-AT concentrations, but were not able to demonstrate any relation with AAA diameter [28, 29]. In contrast to α1-AT, IgG measurements in addition to aneurysm size did not improve the ability to predict aneurysm progression. Correlation between serum α1-AT and IgG concentrations suggests that α1-AT up-regulation in small AAA is associated with inflammation and to a lesser extent with ECM degradation. Inflammation is a key process in AAA formation, as demonstrated by extensive inflammatory cell infiltration [30], but it remains unclear whether this inflammation is causal or simply a reaction on ECM degradation. Here, we showed the increased expression of serum IgG in small AAA. Based on positive associations between AAA size and cytokines as well as other inflammatory markers [29, 31-33], higher serum IgG concentrations would be expected in large AAA. This discrepancy could be explained by the hypothesis that inflammation has diminished in large AAA and progression is more likely due to hemodynamic factors. This hypothesis is partly supported by a recent publication of Parry and colleagues, in which they observed elevated CRP and fibrinogen levels in men with small AAA reflecting a pro-inflammatory state [21].

However, some limitations should also be addressed. This proteomic technique has a low resolution for alkaline, low-abundant and high molecular weight proteins, and a number of differential proteins could not be identified with the presently available techniques. The tremendous dynamic range of protein concentrations in serum limits biomarker discovery using 2D-DIGE and depleting the most abundant proteins has received considerable attention. However, the removal process must be close to 100% efficiency to be interesting and less abundant proteins may also be eliminated during the depletion process, due to carrier function of albumin or aspecific removal, resulting in loss of reliable information and potential discrepancies between samples. Furthermore, investigating the correlation of biomarker concentrations, especially Factor XII, and ILT volume would be valuable. Unfortunately, data on ILT volume are only available for a limited amount of patients with small AAA, since aneurysm size was assessed using ultrasound in the majority of patients.

In summary, we report a proteomic study on serum samples of AAA patients. The differentially expressed proteins (including Factor XII and α1-antitrypsin) are involved in several key features of AAA pathophysiology. Incorporating either Factor XII activity or α1-antitrypsin measurements together with aneurysm diameter did not have a marked effect on

the prediction of aneurysm progression. Further research, with larger sample sizes, is necessary to investigate whether these or other serum markers of AAA reflect causative factors for aneurysm formation and progression, or are merely secondary effects of aneurysm presence.

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6

Biomarker discovery in MScl

DETECTION OF DIFFERENTIAL PROTEIN EXPRESSION IN CEREBRO-SPINAL FLUID OF PATIENTS WITH MULTIPLE SCLEROSIS THROUGH 2D-DIGE

Background. Multiple sclerosis (MScl) is an inflammatory demyelinating disease of the central nervous system, which preferentially affects young adults. Cerebrospinal fluid (CSF) proteins may provide important clues about the pathophysiological mechanisms behind this disease and may be used as biomarkers for MScl.

Methods. Differential proteins in CSF pooled from five MScl patients, five patients with clinically isolated syndrome (CIS) and five controls were detected and identified using two-dimensional differential gel electrophoresis (2D-DIGE) and tandem mass spectrometry (MS/MS).

Results. Several proteins were differentially expressed in the CSF of MScl patients, patients with CIS and controls. Higher abundances of immunoglobulin peptides were found in CSF of MScl patients and this was confirmed using routine diagnostic analyses. Furthermore, increased asialotransferrin was found in CSF of both patients with MScl or CIS compared to controls (22.5 ± 4.3 % and 24.3 ± 4.0 % vs 16.6 ± 3.3 %, p=0.022).

Conclusions. Transferrin glycosylation analysis holds diagnostic potential and suggests that aberrations in glycosylation play a role in the pathophysiology of MScl.

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Introduction

Multiple sclerosis (MScl) is a chronic inflammatory demyelinating disease of the central nervous system (CNS). It is the most frequent cause of neurological disability in young adults and affects approximately 0.1% of people in Western communities. Women are affected about twice as often as males. Multiple sclerosis is a multifactorial disease influenced by genetic predispositions, environmental factors and immunological mechanisms which damage the CNS [1-3]. Pathological hallmarks of MScl are focal lesions, which represent a complex process involving the infiltration of lymphocytes and macrophages in the CNS, myelin loss, astrogliosis, axonal degeneration, resulting in inflammatory sclerotic plaques in the white and gray matter [1, 4, 5]. These pathogenic processes give rise to the typical, but highly variable clinical manifestations such as visual and sensory impairment, limb weakness, bladder and bowel disturbances and fatigue [6]. The most widely accepted hypothesis states that MScl is an autoimmune disease which leads to destruction of CNS myelin. However, the underlying mechanism of the widespread axonal degeneration remains to be elucidated [4].

Diagnosing MScl is based on the McDonald criteria; a combination of established clinical, radiological and laboratory analyses [7]. However, their diagnostic value is limited in terms of predicting long-term clinical outcome and excluding other potentially treatable diseases [2, 8]. Therefore, biomarkers for clinical subtyping and monitoring disease progression are needed. These biomarkers could have improved diagnostic and prognostic value, could enhance patient-tailored therapy and could possibly elucidate molecular mechanisms. The cerebrospinal fluid (CSF) is in direct contact with the brain interstitial fluid, often reflecting the biochemical changes related to the underlying neurological disease. Therefore, CSF is considered a promising source of potential biomarkers for neurological diseases. Since MScl has a complex pathogenesis, no single biomarker with 100% sensitivity and specificity for MScl is likely to be discovered. However, panels of biomarkers will enhance the ability to predict outcome and prognosis of MScl.

Proteomics provide powerful opportunities to examine the entire protein expression pattern of tissues or body fluids. Several proteomic techniques have been consistently used to identify potential MScl-specific biomarkers in blood and CSF [9-23].

In this pilot study, we utilized 2D-DIGE together with matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometry (MALDI-TOF-MS/MS) to detect and identify differentially expressed proteins in MScl. The differential expression patterns were validated using traditional immunochemical techniques.

Materials and Methods

Sample collection

We included in this study fifteen subjects by approaching consecutive patients who visited the outpatient clinic of our MScl centre in the period 2005-2009. Along with the specimen for diagnostic purposes, additional serum and CSF samples were collected. For the proteomic analysis, CSF samples were selected from five relapsing-remitting MScl (RR-MScl) patients all in clinical remission state, five individuals with clinically isolated syndrome (CIS) and five individuals who where diagnosed not to have a neurological disease. RR-MS patients were all in clinical remission state and all on immunomodulatory drugs. Lumbar puncture was performed through standard procedure as a part of the diagnostic evaluation. MScl diagnosis was confirmed based on the diagnostic criteria described by McDonald [24]. Immediately after lumbar puncture, CSF samples were centrifuged at 500 g (4°C) for ten minutes, aliquoted and stored at -80°C pending analysis. This study was approved by the local Medical Ethics Committee according to the Declaration of Helsinki. All participants provided written informed consent. Clinical and laboratory features of the participants are summarized in Table 6.1.

Table 6.1. Clinical and laboratory data of RR-MScl patients, CIS patients and controls

	Reference value	**RR-MScl (n = 5)**	**CIS (n = 5)**	**Controls (n = 5)**
Female		4 (80%)	4 (80%)	5 (100%)
Age (years)		28.4 ± 6.7	30.8 ± 11.7	37.2 ± 8.9
CSF IgG (mg/L)	20-50	55.5 ± 30.3	29.4 ± 14.8	25.5 ± 9.9
Serum IgG (g/L)	4.5 – 14.0	10.3 ± 2.0	9.1 ± 2.0	10.7 ± 1.4
CSF OGB				
- Yes		5 (100%)	2 (40%)	1 (20%)
- No		-	3 (60%)	4 (80%)

Sample preparation

All fifteen CSF samples were mixed with ice-cold acetone (1:4) (v/v) and incubated at -20°C for 60 minutes to remove salt from the samples. Subsequently, the mixture was centrifuged at 12000g for 30 minutes at 4°C. The supernatant was discarded and the air-dried pellet was solubilised in labelling buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris; pH 8.5). The protein concentrations were measured on the Synchron LX 20 (Beckman Coulter, Fullerton, CA). Three pools (i.e. RR-MScl, CIS and controls) were generated by combining equal amounts CSF from five individuals per group. The pH was adjusted to 8.5 by 50 mM NaOH to obtain optimal labelling conditions.

2-D DIGE

CSF from individual participants and sample pools were randomly labeled with Cy3 or Cy5 according to the manufacturer's instructions (GE Healthcare, Uppsala, Sweden). Dye-swapping was performed to control for preferential labeling by one of the dyes. Fifty µg of CSF protein belonging to either RR-MScl patients, CIS patients or controls were minimally labeled. A Cy2-labelled internal standard was generated by combining equal amounts of CSF from all samples in the proteomic analysis. Labeling reaction was quenched by incubation with 10 mM lysine (Sigma-Aldrich, St. Louis, MO, USA) on ice in the dark for ten minutes. Three labeled samples, two analytical and an internal standard, were then pooled and an equal volume of 2x lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.04% bromophenol blue, 2% DTT, 2% IPG buffer pH 3-10) was added.

Samples were focused on an IPGphor using immobilized DryStrip gels (3-10 pH range, non-linear, 18 cm), which were passively pre-rehydrated for six hours with DeStreak Rehydration Solution supplemented with 0.5% IPG buffer. These strips were equilibrated for fifteen minutes with gentle shaking in equilibration solution (6 M urea, 2% SDS, 50 mM Tris pH 8.8, 0.02% bromophenol blue, 30% glycerol) supplemented with 1% DTT, followed by 2.5% iodoacetamide in fresh equilibration solution for additional fifteen minutes incubation with gentle shaking. Second dimension SDS-PAGE was carried out on polyacrylamide gels (12.5% T, 3% C) using the Ettan DALTtwelve. Gels were run at 20°C, 0.5 W/gel for one hour and 15 W/gel until the bromophenol blue frontier reached the gel bottom and subsequently scanned on the Ettan DIGE Imager using CyDye-specific excitation/emission wavelengths. Gels were analyzed using DeCyder 7.0 software (GE Healthcare). Differentially expressed protein spots, present in more than 70% of the gels, were selected for identification using tandem mass spectrometry.

Protein identification and in-gel digestion

Preparative gels, loaded with 150 µg Cy2-labelled proteins, were run using previous conditions. Gels were fixed three times for one hour in 30% CH_3OH and 10% CH_3COOH followed by an overnight incubation in MQ-water.

Protein spots of interest were picked from the preparative gel using the automated Ettan Spot Picker (GE Healthcare) into 96-well plates and in-gel digestion was carried out on the MassPREP digestion robot (Waters, Manchester, UK). Gel plugs were destained twice in 100 mM NH_4HCO_3, 50% (v/v) CH_3CN for ten minutes, then dehydrated in 100% CH_3CN for five minutes, after which supernatant was removed, and gel plugs were allowed to air dry for ten minutes. Cysteines were reduced with 10 mM DTT in 100 mM NH_4HCO_3 for thirty minutes followed by alkylation with 55 mM iodoacetamide in 100 mM NH_4HCO_3 for twenty minutes. Gel plugs were washed with 100 mM NH_4HCO_3 and subsequently dehydrated with 100%

CH_3CN. Trypsin suspended in 50 mM NH_4HCO_3 was added (12 ng/µL) and allowed to digest at 40°C for five hours. Peptides were extracted twice with 1% (v/v) formic acid, 2% (v/v) CH_3CN.

MS/MS and database analysis

C18 ZipTip pipette tips (Millipore, Bedford, MA) were first washed three times with 10 µL 0.1% trifluoroacetic acid (TFA) in 100% ACN and thereafter three times with 10 µL 0.1% TFA in 50% ACN. Following these wash steps, peptide digests were loaded through ten up-down pipette draws with the C18 ZipTips which had been equilibrated with 0.1% TFA. Peptides were eluted through three up-down pipette draws in 0.1% TFA in 50% ACN. Peptide solutions were mixed at a 1:1 ratio with 5 mg/ml α-cyano-4-hydroxycinnamic acid (CHCA) matrix in 50% CH_3CN, 0.1% TFA and spotted in duplo. Spots were allowed to air dry for homogeneous crystallization. Mass spectra were acquired in positive ion reflectron mode on 4800 MALDI-TOF/TOF analyzer (Applied Biosystems, Foster City, CA). Tandem MS fragmentation spectra were acquired for each sample, averaging 500 laser shots per fragmentation spectrum on each of the eight most abundant ions present in each sample. The generated peak list was searched with MASCOT search engine by GPS Explorer v.3.9. Software (Applied Biosystems) against the Swiss-Prot protein database for protein identification with taxonomy at *Homo sapiens* and exclusion of trypsin and keratin peaks. One miss-cleavage was tolerated; carbamidomethylation was set as a fixed modification and oxidation of methionine as an optional modification. Protein charge was set at 1+, mass tolerance for precursor ion at 150 ppm and MS/MS tolerance at 0.2 Da. No restrictions were made on the protein mass. Identification was considered confident when the total ion and protein score were above the 95% confidence interval.

Total transferrin and isoelectric focussing transferrin isoforms

Transferrin concentration in CSF was analyzed on the BN ProSpec (Siemens, Munich, Germany). Isoelectric focussing (IEF) of human CSF transferrin was performed by PhastSystem (GE Healthcare). CSF samples were incubated for 30 minutes with 20 mM ferric citrate and 0.5 mM sodium bicarbonate to saturate transferrin with iron. The iron-saturated CSF transferrin was applied to a hydrated dry Phastgel containing carrier ampholytes (pH 5-7). After IEF, the transferrin isoforms were detected by adding rabbit anti-human transferrin antibody (Dako, Glostrup, Denmark) and thereafter gels were stained with silver-nitrate. The relative amounts of the transferrin isoforms were determined by scanning the stained gel using a Scanjet Scanner 5530 (Hewlett-Packard, Palo Alto, CA) and quantified using Image Quant TL software (GE Healthcare).

Statistics

Patient data were analyzed with PASW Statistics 18.0 software (IBM Corporation, Somers, NY). Categorical variables, expressed as percentages, were compared using the Pearson Chi-squared test. ANOVA was used to compare continuous variables, expressed as mean ± standard deviation, applying the Bonferroni correction for pairwise comparison. Protein spots abundances were compared using ANOVA with Bonferroni post-hoc testing (DeCyder 7.0 software, GE Healhtcare).

Results

Detection and identification of differentially expressed protein spots in CSF

The differential protein expression between RR-MScl, CIS and control pools, where equal amounts CSF from five participants per group were pooled, was analyzed using 2D-DIGE with two technical replicates (Figure 6.1).

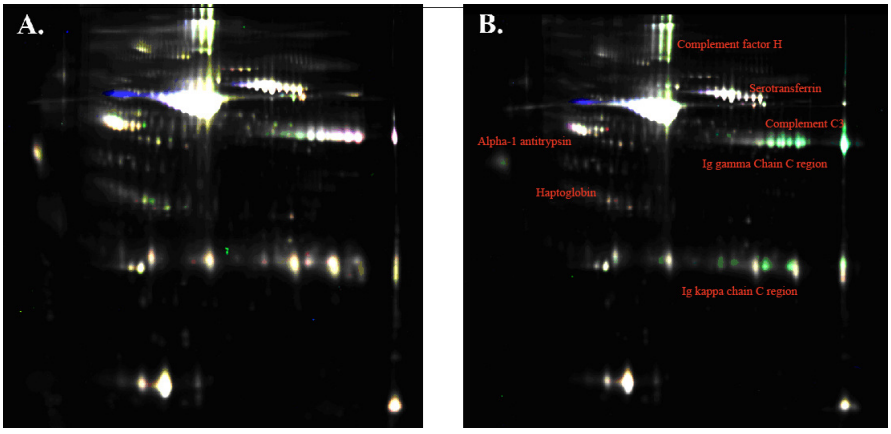


Figure 6.1. Representative 2D-DIGE gels of CSF proteins from (A) CIS patients *versus* controls and (B) MS patients *versus* controls.

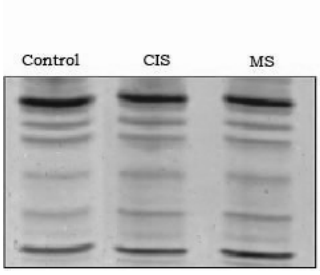
In total, a hundred spots were significantly differential between the three study groups and twenty-two of them were identified; complement C3, complement factor H, transferrin, α-1 antitrypsin, Ig γ chain C region, Ig κ chain C region and haptoglobin. Three identified spots, representing transferrin, showed lower abundances in CSF of MScl patients compared to CIS and control patients. Abundance of spots representing complement factor H as well as complement C3 were significantly lower in MScl patients. Furthermore, MScl patients showed decreased expression of α-1 antitrypsin. Both haptoglobin and almost all immunoglobulin

peptides were upregulated in the CSF of MScl patients. Analysis of the individual samples showed only significant higher abundances of spots representing Ig γ and κ chain in MScl patients compared to either control or CIS patients.

Confirmation of differential protein expression

Proteomic analysis revealed significant higher abundances of immunoglobulin peptides in CSF of MScl patients which was confirmed using clinical routine diagnostics (MS: 55.3 ± 30.3 mg/L *versus* 29.4 ± 14.8 and controls: 25.5 ± 9.9, p=0.075, Table 6.1).

Although transferrin spot abundances were significantly lower in MScl compared to both CIS and control patients, no significant differences in CSF transferrin concentrations were found (MS: 19.9 ± 3.7 mg/L *versus* CIS: 20.1 ± 10.1 mg/L *versus* Controls: 18.0 ± 7.0 mg/L). Furthermore, we investigated the glycosylation pattern of CSF transferring in the three study groups. Both MScl and CIS patients showed relatively more asialotransferrin in CSF compared with controls (22.5 ± 4.3 % and 24.3 ± 4.0 % *versus* 16.6 ± 3.3 %, p=0.022, fraction 0 in Figure 6.2).



	Relative distribution (%)		
	Co	CIS	MS
← 4	44.8 ± 4.6	45.5 ± 5.6	45.3 ± 2.1
← 3	17.3 ± 3.2	13.4 ± 2.7	15.2 ± 2.9
← 2	10.1 ± 3.2	9.2 ± 3.2	9.6 ± 1.5
← 1	11.2 ± 4.9	7.5 ± 2.6	7.5 ± 2.0
← 0*	16.6 ± 3.3	24.3 ± 4.0	22.5 ± 4.3

Figure 6.2. The relative distribution (%) of sialotransferrin fractions. The number of sialic acids per molecule transferrin (0-4) is indicated on the right, representing a- (cathodal), mono-, di-, tri- and tetrasialotransferrin (anodal end), respectively. ANOVA was used to compare differences in relative sialotransferrin distributions between the three experimental groups (*p<0.05)

Discussion

In diseases with complex pathogenesis, such as MScl, an individual biomarker is likely to reflect only one of many ongoing pathogenic processes, thus its specificity and predictive value are likely to be very low. Proteomics provide powerful opportunities to examine the entire protein expression pattern of tissues or body fluids and as such can aid in finding panels of potential biomarkers. In this study, we analyzed the CSF proteome of MScl, CIS and control patients and searched for differentially expressed proteins.

Currently, the presence of oligoclonal IgG bands or an increased IgG index in CSF of MScl patients is the only laboratory-based test for MScl. Immunoglobulins are involved in the inflammatory process and have been repeatedly reported in MScl pathogenesis. Caroscio and colleagues suggested the use of CSF IgG levels as marker of disease activity [25]. Free κ light chains of immunoglobulins in CSF may be used as marker for disability prognosis [26]. Furthermore, extra oligoclonal IgG bands can be used as a potential marker for progression from CIS to MScl [27]. Intrathecally generated immunoglobulins, specifically IgG, are found in 95% of MScl patients [28]. Considering the fact that immunoglobulin levels in the MScl samples used in this study were higher than in controls, it was expected that some immunoglobulin peptides would be identified as differentially expressed using 2D-DIGE.

Complement system plays a central role in the innate immune system, providing an important defense against infection and immune complex disease. The role of complement in immunopathogenesis of multiple sclerosis is intensively investigated, as recently highlighted by Ingram and colleagues [29]. However, conflicting results exist on the validity of these complement components, such as C3 and C4, as biomarkers for MS [12-14, 16-18, 29]. Complement factor H, a single chain serum glycoprotein, regulates the formation and function of complement C3 and C5 convertase enzymes. A recent study showed significantly higher CSF factor H concentrations in MScl [30], in contrast we found using DIGE significant lower concentrations of factor H in CSF of MScl. Although complement activation is not specific to MScl, patterns of activation, assessed in combination with other inflammatory and immune markers, may be of value as biomarkers in MScl subgroups.

Transferrin, an iron-binding protein present in serum as well as CSF, was previously found significantly decreased in CSF of MScl patients [12]. This is in accordance with our DIGE results; unfortunately no difference was found in total CSF transferrin concentration by clinical laboratory diagnostics. This discrepancy may be related to the lack of specificity for transferrin fragments or isoforms of the pan-antitransferrin antibodies used in the immunochemical clinical assay. A recent paper by Kim *et al*, suggested an important role of post-translational modifications (PTM) in multiple sclerosis. They studied the post-translational modifications of myelin basic protein and found increased methylation as well as deimination and greatly reduced phosphorylation of this protein in MScl samples [31]. A possible pathway of PTM pathogenesis is that self-antigens may represent modified peptides. Furthermore, the extent and nature of PTM may be related to the extent of demyelination and disease heterogeneity in MScl as suggested by the studies on citrullinated myelin basic protein [32, 33]. Therefore, we investigated the glycosylation pattern of CSF transferrin. The distribution of transferrin isoforms in serum has been well characterized since it is the gold standard for the diagnosis of congenital glycosylation disorders due to an N-glycosylation defect [34]. In contrast, relatively little is known about transferrin isoforms in the central

nervous system and CSF. Normal CSF contains both sialo and asialo forms of transferrin, but their origin is unknown [35, 36]. Increased asialotransferrin relative to total transferrin was found in CSF of patients with cerebellar ataxia with free sialic acid, suggesting that the sialyation of key central nervous system proteins, such as transferrin, is altered [37]. Furthermore, changes in the sialic acid content of surface glycoconjugates are known to influence the activation of monocytes and subsequent cytokine production upon stimulation; removal of sialic acid from glycoconjugates on the surface of monocytes enhances their respons to bacterial lipopolysaccharide [38, 39]. Hence, we hypothesize that the increase in CSF asialotransferrin in patients with multiple sclerosis and CIS is attributable either to an hyperactivity of a sialidase, which represents an defense mechanism also targeting the transferrin glycosylation.

The use of 2D-DIGE permitted an accurate quantification of changes in protein expression associated with MScl. However, our approach was not limitation-free. First, the strength of our observations is limited by the small sample size, and requires further rigorously validation with quantitative immunoassays in a larger independent cohort of patients and controls in combination with well-characterized clinical and imaging data. Second, depletion of high-abundant proteins, such as albumin and IgG, could enhance the representation of low-abundant proteins. But depletion of high-abundant proteins is associated with concomitant removal of non-targeted proteins and can subsequently influence reproducibility [40]. Therefore, we used untreated CSF samples, which can reflect the actual CSF protein information. Furthermore, this approach enables measurement of IgG as a positive control, since the presence of oligoclonal IgG bands or an increased IgG index in CSF is used in diagnosing MScl. Third, the samples were pooled to reduce potential inter-individual differences of CSF content between individual patients with may be due to a high degree of disease heterogeneity in MScl. Such a pooling strategy is employed in several other publications reporting on proteomics and MScl [11-13, 15]. Furthermore, we also analyzed all our samples individually using 2D-DIGE, but due to the high degree of heterogeneity a limited amount of differential proteins were found.

In conclusion, 2D-DIGE analysis of pooled CSF proteome showed significant differences between MScl patients, CIS patients and controls. Among these proteins were important factors involved in the immune response, such as IgG, complement C3 and factor H. Furthermore, increased asialotransferrin was found in CSF of patients with MScl, suggesting that the sialyation of proteins in the CNS is altered. This observation could provide new clues in understanding the underlying MScl pathophysiology, but requires thorough validation with a larger number of patient samples.

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7

Perfusate proteome of donor kidneys

CHARACTERIZATION OF THE PERFUSATE PROTEOME OF HUMAN DONOR KIDNEYS

Background. Preservation of deceased donor kidneys by hypothermic machine perfusion results in superior transplant outcomes as compared to static cold storage and provides the opportunity to measure biomarkers of cellular injury in perfusate samples. Unfortunately, only limited success has been achieved in identifying biomarkers for predicting early graft dysfunction.

Methods. Two-dimensional difference gel electrophoresis and tandem mass spectrometry were used to explore the proteome of perfusate samples from machine-perfused human donor kidneys (n = 18) and to discover potential biomarkers of ischemic acute kidney injury.

Results. Thirty-two protein spots were successfully identified, representing nineteen unique proteins that were derived from renal tissue and from residual plasma in de renal microvasculature. Two unidentified protein spots were significantly up-regulated, whereas one protein spot (identified as haptoglobin) was significantly down-regulated in the perfusate of ischemically injured kidneys from donors after cardiac death as compared to kidneys from brain-dead donors that had not suffered warm ischemic injury.

Conclusion. We provide the first description of the renal perfusate proteome and present preliminary evidence of three differentially expressed biomarkers in human donor kidneys with different levels of acute ischemic injury. Their diagnostic value for the selection of marginal kidneys in clinical transplantation should be determined in future studies.

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Introduction

Preservation of deceased donor kidneys by hypothermic machine perfusion results in superior transplant outcomes as compared to static cold storage [1]. Furthermore, hypothermic machine preservation provides the opportunity to study the renovascular resistance to perfusate flow and to measure biomarkers of cellular injury in samples of the perfusion solution. High vascular resistance and increased levels of 'viability markers' such as lactate dehydrogenase (LDH), glutathione S-transferase (GST), fatty acid-binding proteins (FABP) and redox-active iron are associated with early graft dysfunction and are used by transplant centers to decide whether or not to accept marginal donor kidneys for transplantation [2-7]. However, it has recently been demonstrated that these parameters have insufficient predictive value to be clinically useful in identifying adverse transplant outcomes [8-10].
Given the ongoing interest in the diagnostic potential of perfusate biomarkers, we explored the proteome of perfusate samples from human donor kidneys using two-dimensional difference gel electrophoresis (2D-DIGE) and tandem mass spectrometry (MS/MS) to discover potential biomarkers for ischemic acute kidney injury. This unbiased approach may provide novel insight into the application of hypothermic machine perfusion as a diagnostic tool to identify marginal kidneys with a low risk of early graft failure that can safely be used for transplantation.

Methods

Study design
This observational cohort study included eighteen donor kidneys preserved by hypothermic machine perfusion at our transplant center from May 2007 until April 2009. All kidneys were recovered from different donors between 18 and 65 years and were transplanted within the Eurotransplant area. Among the twenty-one donor kidneys fulfilling these criteria, six kidneys from donors after brain death (DBD), six kidneys from donors after cardiac death (DCD) with controlled withdrawal of supportive treatment in the intensive care unit and six kidneys from uncontrolled DCD donors after failed cardiopulmonary resuscitation (CPR) were selected. The perfusate proteome of these kidney types was compared to discover novel biomarkers of ischemic acute kidney injury. This sample size is considered sufficient for statistical analysis of proteomic findings for biomarkers [11].
Graft function in the early postoperative period was defined as immediate function (no dialysis treatment after transplantation), delayed graft function (temporary dialysis treatment

initiated in the first week after transplantation), or primary non-fuction (continuous dialysis treatment after transplantation).

Hypothermic machine perfusion

DBD kidneys were recovered according to standard multi-organ procurement techniques [12]. DCD kidneys were recovered after rapid laparotomy and direct aortic cannulation for controlled donors and after *in situ* perfusion with double-balloon triple-lumen catheters (Coloplast A/S, Humlebaeck, Denmark) for uncontrolled donors [13]. After recovery, donor kidneys were transported to our institution for hypothermic perfusion on a LifePort machine with 1 L UW-MPS preservation solution (Organ Recovery Systems, Des Plaines, IL). Maximum perfusion pressure was 30 mmHg for DBD and controlled DCD kidneys and 40 mmHg for uncontrolled DCD kidneys. The LifePort machine contains a 20 µm filter to clear large particles such as white blood cells from its circulation, leaving all proteins in the perfusate.
At one hour of perfusion, a sample of the preservation solution was taken from the machine and centrifuged at 900 g at 4°C for ten minutes. Within thirty minutes from sampling, 100 µL and 1 mL aliquots of the perfusate were stored at -80°C until further analysis. Renovascular resistance was recorded and the perfusate concentrations of LDH and GST were measured. LDH was determined by standard colorimetric assay (Roche Diagnostics, Mannheim, Germany) and total GST activity was measured as described previously [14]. All perfusion characteristics were adjusted for kidney weight.

Two-dimensional Difference Gel Electrophoresis (2D-DIGE)

CyDye minimal labelling was performed according to the manufacturer's instructions (GE Healthcare, Uppsala, Sweden). Five microgram perfusate protein from three experimental groups was randomly labelled with either 400 pmol Cy3 or Cy5. A Cy2-labelled internal standard was generated by combining equal amounts of each perfusate sample in the proteomic analysis. Labelling was performed on ice in the dark for thirty minutes at pH 8.5. Labelling reaction was quenched by incubation with 10 mM lysine (Sigma-Aldrich, St. Louis, MO) on ice in the dark for ten minutes. Subsequently, three labelled samples, two analytical and an internal standard, were pooled and an equal volume of 2x lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.04% bromophenol blue, 2% DTT, 2% (v/v) IPG buffer pH 3-10) was added. Immobiline DryStrip gels (18 cm; pH 3-10 non-linear; GE Healthcare) were passively pre-rehydrated for six hours with DeStreak Rehydration Solution (GE Healthcare) supplemented with 0.5% (v/v) IPG buffer pH 3-10. Consequently, perfusate samples were applied on these strips via cup-loading and iso-electric focussing was carried out at a constant temperature of 20°C as follows: 150 V for three hours, 300 V for three hours, gradient to 1000 V in six hours, gradient to 8000 V in one hour, 8000 V for two hours.

Proteins on the focussed strips were equilibrated for fifteen minutes with gentle shaking at room temperature in equilibration solution (6 M urea, 2% SDS, 50 mM Tris pH 8.8, 0.02% bromophenol blue, 30% glycerol) supplemented with 1% DTT, followed by 2.5% iodoacetamide (IAA) in fresh equilibration solution for fifteen minutes incubation with gentle shaking at room temperature. Second dimension SDS-PAGE was carried out on polyacrylamide gels (12.5% T, 3% C) using the Ettan DALTsix (GE Healthcare). The IPG strips were loaded on these gels and fixed with 0.5% agarose containing a trace of bromophenol blue. Gels were run at 20°C, 0.5 W/gel for one hour and 15W/gel until the bromophenol blue frontier reached the bottom of the gel. After completing the second dimension, gels were scanned on the Ettan Dalt Imager (GE Healthcare) using CyDye-specific excitation/emission wavelengths. Gels were analysed using DeCyder 7.0 software (GE Healthcare) according to the manufacturer's recommendations. Briefly, Differential In gel Analysis (DIA) and Biological Variation Analysis (BVA) modules were used to calculate normalized protein abundances.

In-gel digestion

Preparative gels, loaded with sixteen µg unlabelled protein, were run using previous conditions. These gels were fixed for thirty minutes in 10% methanol and 7% acetic acid followed by an overnight incubation in Sypro Ruby (Bio-Rad, Hercules, CA). Subsequently, gels were incubated for 45 minutes in 10% methanol and 7% acetic acid and then thoroughly rinsed with MilliQ water. Protein spots of interest were picked from the Sypro-stained gel using the automated Ettan Spot Picker (GE-Healthcare) into 96-well plates and in-gel digestion was carried out on the MassPREP digestion robot (Waters, Manchester, UK). The two mm diameter gel plugs were destained twice in 100 mM ammonium bicarbonate (NH_4HCO_3), 50% (v/v) acetonitrile (ACN) for ten minutes. Gel plugs were then dehydrated in 100% ACN for five minutes, supernatant was removed, and gel plugs were allowed to air dry for ten minutes. Cysteines were reduced with 10 mM DTT in 100 mM NH_4HCO_3 for thirty minutes followed by alkylation with 55 mM IAA in 100 mM NH_4HCO_3 for twenty minutes. Spots were washed with 100 mM NH_4HCO_3 and subsequently dehydrated with 100% ACN. Trypsin suspended in 50 mM NH_4HCO_3 was added to the gel plugs (12 ng/µL) and allowed to digest at 40°C for five hours. Peptides were extracted twice with 1% (v/v) formic acid, 2% (v/v) ACN.

Protein identification by MS/MS

C18 ZipTip pipette tips (Millipore, Bedford, MA) were first washed three times with 10 µL 0.1% trifluoroacetic acid (TFA) in 100% ACN and thereafter three times with 10 µL 0.1% TFA in 50% ACN. Following these wash steps, peptide digests were loaded through ten up-down pipette draws with the C18 ZipTips which had been equilibrated with 0.1% TFA. Peptides

were eluted through three up-down pipette draws in 0.1% TFA in 50% ACN. Peptide solutions were mixed at a 1:1 ratio with 5 mg/mL α-cyano-4-hydroxycinnamic acid (CHCA) matrix in 50% ACN, 0.1% TFA and spotted on stainless steel MALDI sample plate (Applied Biosystems). The spots were allowed to air dry for homogeneous crystallization. MALDI-TOF mass spectra were acquired in positive ion reflectron mode on 4800 MALDI-TOF/TOF analyzer (Applied Biosystems). Tandem MS fragmentation spectra were acquired for each sample averaging 200 laser shots per fragmentation spectrum on each of the eight most abundant ions present in each sample. The generated peak list was searched with the MASCOT search engine (www.matrixscience.com) by the GPS Explorer v.3.9. Software (Applied Biosystems, Foster City, CA) against the Swiss-Prot protein database for protein identification with taxonomy set at *Homo Sapiens*; trypsin and keratin peaks were excluded. One miss-cleavage was tolerated; carbamidomethylation was set as a fixed modification and oxidation of methionine as an optional modification. The protein charge was set at 1+. Mass tolerance for precursor ion was set to 150 ppm and MS/MS tolerance was set at 0.2 Da. No restrictions were made on the protein mass.

Table 7.1. Transplant characteristics of machine perfused human donor kidneys

	Uncontrolled DCD kidneys (n = 6)	**Controlled DCD kidneys (n = 6)**	**DBD kidneys (n = 6)**	**p-value**
Donor characteristics				
Age (years)	51 ± 3	56 ± 3	49 ± 1	0.29
Male	83%	33%	17%	0.05
Cause of death (neurological/cardiovascular)	0/100%	100/0%	83/17%	0.001
Serum creatinine (μmol/L)	93 ± 16	84 ± 14	65 ± 8	0.34
Hypertension	20%	0%	20%	0.50
Preservation characteristics				
CPR time (min)	45 ± 10	-	-	-
Ventilator switch-off time (min) [1]	-	28 ± 8	-	-
Warm ischemia time (min) [2]	37 ± 10	18 ± 2	-	0.001
Cold ischemia time (hours)	22 ± 1	17 ± 2	31 ± 3	0.007
Anastomosis time (min)	41 ± 5	43 ± 6	68 ± 15	0.17
Renovascular resistance (mmHg/mL/min/100g)	0.20 ± 0.01	0.24 ± 0.03	0.37 ± 0.09	0.43
LDH (U/L/100g)	339 ± 20	193 ± 45	188 ± 31	0.03
GST activity (U/L/100g)	48 ± 9	19 ± 4	17± 7	0.02
Recipient characteristics				
Age (years)	65 ± 2	49 ± 6	43 ± 6	0.02
Male	83%	83%	33%	0.11
Early graft function (immediate/delayed/never)	0/67/33%	50/50/0%	50/50/0%	0.13
Duration of delayed graft function (days)	13 ± 2	16 ± 6	8 ± 5	0.52
GFR at 3 months (mL/min) [3]	48 ± 6	31 ± 6	52 ± 7	0.10

[1.] Ventilator switch-off time was defined as the time from withdrawal of supportive treatment until circulatory arrest.
[2.] Warm ischemia was defined as the time from circulatory arrest until initation of hypothermic organ perfusion.
[3.] Glomerular filtration rate was estimated using the MDRD formula [15].

Ethics

Patient data were collected, stored and used in agreement with the code of conduct 'Use of data in health research' put forward by the federation of Dutch medical scientific societies (www.federa.org).

Statistics

Continuous variables were expressed as means with standard errors and categorical variables as percentages. Differences between groups were compared with Kruskal-Wallis tests for continuous variables and with Fisher Exact tests for categorical variables. Spot abundances were compared with one-way ANOVA after logarithmic transformation and standardization. Results with $p < 0.05$ were considered statistically significant.

Results

Transplant characteristics

Donor, preservation and recipient characteristics of the machine perfused kidneys are presented in Table 7.1. As expected, uncontrolled DCD donors died significantly more often from cardiovascular causes than controlled DCD and DBD donors and warm ischemia times of DCD kidneys were significantly longer than those of DBD kidneys. Furthermore, DBD kidneys were associated with significantly longer cold ischemia times, since our policy was to preserve DBD kidneys by hypothermic machine perfusion only for recipients residing in the Dutch Antilles with expected prolonged cold ischemia. Patients who received uncontrolled DCD kidneys were significantly older than recipients of controlled DCD and DBD kidneys, which may be explained by the tendency to transplant grafts of perceived lower quality into older recipients with a worse prognosis on dialysis therapy. Other baseline characteristics were similar between the study groups.

During machine perfusion, renovascular resistance of the three kidney types was comparable, whereas LDH and GST concentrations in the perfusate were significantly higher for uncontrolled DCD kidneys compared to controlled DCD and DBD kidneys. Immediate graft function was observed in 50% of controlled DCD and DBD kidneys but not in uncontrolled DCD kidneys, whereas 50% of controlled DCD and DBD kidneys and 67% of uncontrolled DCD kidneys suffered from delayed graft function. Primary non-function was observed in two grafts from uncontrolled DCD donors. The relatively high incidence of delayed graft function in DBD kidneys can be explained by the long cold ischemic times needed to transfer their recipients across the globe. At three months after transplantation, estimated GFR of functioning grafts was not significantly different between the study groups.

Profiling of the renal perfusate

To characterize the renal perfusate proteome, MS/MS was used to identify proteins present on 2D-DIGE gels. No protein spots were detected in the preservation solution sampled before the start of renal perfusion (data not shown). In contrast, sixty-eight protein spots were detected in the renal perfusate. Thirty-two of these spots (47%), representing nineteen unique proteins, were successfully identified (Figure 7.1 and Table 7.2).

Table 7.2. Proteins identified in renal perfusate from human donor kidneys

SwissProt ID	Name	Function	Sequence coverage (%)*
Blood coagulation			
P02679	Fibrinogen γ-chain [1]	Fibrin polymerization and platelet activation	16.1
P08758	Annexin A5 [1]	Anticoagulant	6.3
Cellular iron homeostasis			
02787	Transferrin [1]	Iron transport	21.8
P02790	Hemopexin [1]	Heme transport	18.4
P00738	Haptoglobin [1]	Hemoglobin binding	20.2
Cytoskeleton			
P63261	Cytoplasmic 2 actin [2]	Cell motility	16.0
Energy and metabolism			
P00325	Alcohol dehydrogenase, subunit β [2]	Alcohol metabolism	17.9
P04406	Glyceraldehyde-3-phosphate dehydrogenase [2]	Glycolysis	12.8
P06733	α-enolase [2]	Glycolysis	7.8
P15121	Aldose reductase [2]	Carbohydrate metabolism	7.9
Q03154	Aminoacylase [2]	Amino acid metabolism	4.2
Immune response			
P01834	Immunoglobulin κ-chain [1]	Adaptive immunity	48.1
P01857	Immunoglobulin γ1-chain [1]	Adaptive immunity	16.7
P01859	Immunoglobulin γ2-chain [1]	Adaptive immunity	13.8
P01860	Immunoglobulin γ3-chain [1]	Adaptive immunity	8.8
P01009	α1-antitrypsin [1]	Acute phase response	38.8
Transport			
P02766	Transthyretin [1]	Carrier for thyroxine and retinol	8.8
P02768	Albumin [1]	Carrier for diverse molecules	52.9
P02774	Vitamin D binding protein [1]	Carrier for vitamin D and metabolites	7.6

[1.] Blood borne proteins. [2.] Cytoplasmic proteins

* Amino acid coverage of matched peptides

Of these proteins, only six (32%) cytoplasmic proteins are known to be present in renal tissue whereas the remaining thirteen (68%) proteins are probably derived from plasma that remained in the renal microvasculature after the initial flush-out.

Perfusate biomarker discovery with 2D-DIGE

To find potential biomarkers of ischemic injury, the differences in renal perfusate proteome between three types of donor kidneys was investigated using 2D-DIGE. To reduce the chances of random associations due to multiple comparisons, protein spots were only considered relevant when the differential abundance had at least a 1.5 fold change and the p-value was less than 0.05.

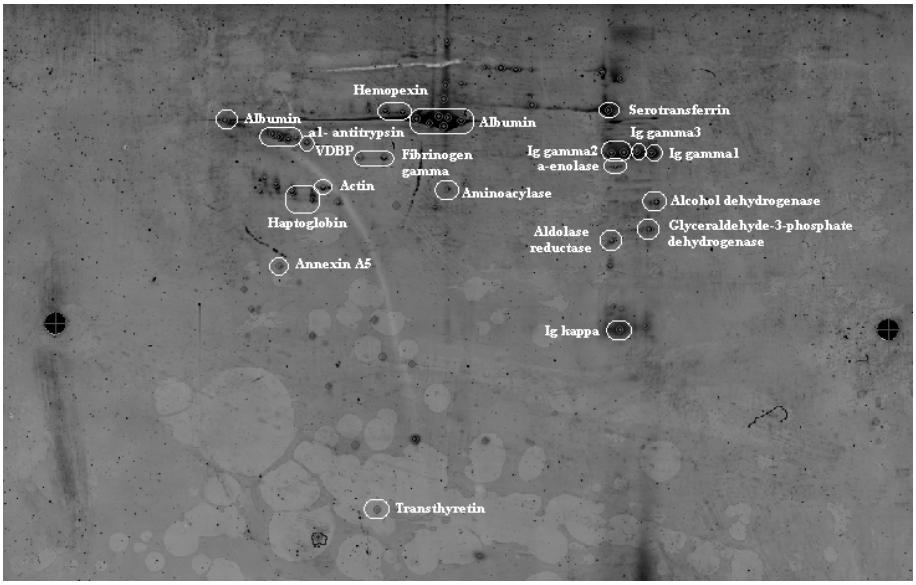

Figure 7.1. 2D-gel of perfusate samples from machine perfused human donor kidneys. Thirty-two protein spots were identified by MS/MS representing nineteen unique proteins. Proteins were visualised by Sypro Ruby post-staining.

Two protein spots were up-regulated in the perfusate of controlled and uncontrolled DCD kidneys as compared to DBD kidneys, whereas one protein spot was down-regulated in the perfusate of uncontrolled DCD kidneys as compared to DBD kidneys (Table 7.3).

Table 7.3. Differentially expressed proteins in perfusion solution of donor kidneys*

	Uncontrolled DCD kidneys	Controlled DCD kidneys	DBD kidneys	ANOVA p-value
Spot 1: Haptoglobin	0.68 ± 0.08	0.98 ± 0.20	1.95 ± 0.51	0.025
Spot 2: Not identified	3.86 ± 0.71	3.83 ± 1.18	1.67 ± 0.29	0.012
Spot 3: Not identified	2.82 ± 0.43	2.54 ± 0.48	1.33 ± 0.14	0.007

* Data are presented as mean standardized abundance ± standard error. ANOVA p-values are calculated using log standardized abundances.

Confounding by recipient age or gender was excluded because these variables were not associated with donor type or spot abundance (data not shown). These three spots were

automatically picked from a preparative gel and subjected to trypsin in-gel digestion for subsequent identification by MS/MS. The down-regulated spot in the perfusate of DCD kidneys was identified haptoglobin, whereas the two up-regulated spots could not be identified. In a preliminary analysis of spot abundance according to graft function after transplantation, the perfusates of the two donor kidneys with primary non-function contained greater quantities of spots 2 and 3 compared with functioning kidneys (data not shown).

Discussion

Hypothermic machine preservation of donor kidneys provides the opportunity to study the renovascular resistance to perfusate flow and to measure biomarkers of cellular injury in samples of the perfusion solution. In the current study, we provide the first description of the renal perfusate proteome and present preliminary evidence of three potential biomarkers of ischemic acute renal injury in human donor kidneys. Using 2D-DIGE and MS/MS, nineteen unique proteins were identified that were derived both from renal tissue and from residual plasma in the renal microvasculature. Two unidentified protein spots were up-regulated, whereas a third protein spot representing haptoglobin was down-regulated in the perfusate of ischemically injured DCD kidneys as compared to DBD kidneys that had not suffered warm ischemic injury. These potential biomarkers may provide valuable information for the assessment of ischemic injury in human donor kidneys before transplantation.

Until now, hypothesis-driven selection of parameters for evaluation of ischemic injury to donor kidneys has failed to produce clinically useful predictors of graft viability. Characteristics of hypothermic machine perfusion such as renovascular resistance and perfusate concentrations of enzymes released from damaged tubular epithelial cells (LDH, GST and FABP) did not predict early graft failure with sufficient accuracy to justify the discard of scarce donor kidneys [8-10]. Since the hypothesis-driven identification of biomarkers for acute ischemic injury so far had only limited success, we decided to take an alternative approach by performing unbiased data-driven analysis of the perfusate proteome. Rather than building on previous experimental findings, this approach may generate novel hypotheses about the pathophysiology and diagnosis of ischemic acute kidney injury [16]. In renal transplant recipients, analyses of the urinary proteome have previously been successful in identifying biomarkers for acute rejection, chronic allograft nephropathy and BK virus nephropathy [17-23]. Furthermore, acute kidney injury after cardiopulmonary bypass or administration of iodinated contrast agents was also reflected by changes in the urinary proteome [24-26].

A major strength of the current proteome analysis is the use of preservation solution from isolated perfused human donor kidneys. Since the preservation solution did not contain proteins before perfusion, all protein spots in the gels must have been derived from the donor kidneys, increasing the chances of discovering tissue-specific biomarkers. Interestingly, the majority of identified proteins in the renal perfusate were not synthesized by the kidney but were blood-borne proteins which remained in the renal microvasculature in spite of manual flush-out of the vasculature before machine perfusion. Proteome analysis has been criticized because of its low reproducibility between laboratories. Indeed, some techniques are sensitive to seemingly minor differences in the analytical protocol [27]. We therefore collected, processed and stored the perfusate samples in a highly standardized fashion. Moreover, the reproducibility of gel electrophoresis was improved by normalizing the spot intensities to an internal standard that was run on each gel. Furthermore, separating proteins by 2D-DIGE generally does not permit resolution of proteins with high (> 150 kDa) or low (< 10 kDa) molecular mass, nor of very basic or hydrophobic proteins, limiting the proteomic coverage of biological samples. However, in contrast to other proteomic techniques, 2D-DIGE can be used to detect post-translational protein modifications.

After an unbiased assessment of the perfusate proteome of machine perfused donor kidneys, haptoglobin was identified as a potential biomarker of ischemic acute kidney injury. Lower concentrations of haptoglobin in the perfusion solution were associated with more extensive ischemic injury to the donor kidneys. Haptoglobin is an acute-phase plasma protein that scavenges free hemoglobin with high affinity, thereby preventing oxidative stress mediated by the redox-active iron in its heme group [28]. We have previously reported that higher concentrations of redox-active iron in the renal preservation solution are associated with increased risk of primary non-function in clinical kidney transplantation [5]. Since lower levels of haptoglobin will lead to higher levels of free hemoglobin – containing redox-active iron in its heme group – our current findings have biological plausibility and fit into an established theoretical framework. Although we were able to identify almost 50% of the spots picked from the Sypro Ruby-stained gel, the identity of the other two different protein spots unfortunately could not be identified by MALDI-TOF/TOF, nor by additional attempts using ESI-MS/MS.

Validation of proteomic-based findings is an important next step in biomarker discovery research. Preliminary attempts to validate haptoglobin with ELISA were inconclusive. However, proteomic results can sometimes not be confirmed with antibody-based techniques due to epitope reactivity of antibodies and post-translational modifications of proteins. Mass-spectrometry based assays provide an excellent alternative approach for quantitative biomarker validation and circumvent some limitations of antibody-based methods. Selected

reaction monitoring can be used toe specifically select and quantify protein biomarkers in serum and tissue [29].

In conclusion, after unbiased exploration of the protein content of perfusate samples from machine perfused kidneys, we provide the first description of the renal perfusate proteome and present preliminary evidence of three potential biomarkers of ischemic acute renal injury in human donor kidneys. In this biomarker discovery study, perfusate proteomes of kidneys from different donor types were compared, whereas the clinical use of these biomarkers will eventually be to predict graft outcome rather than organ donor category. Therefore, their diagnostic value for the selection of ischemically injured kidneys in clinical transplantation should be determined in future studies.

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8 General discussion

Proteomics

Currently, optimal patient management is impeded by the enormous variability in disease manifestation and treatment outcome between patients. To overcome these hurdles, the medical field is moving towards individualized medical approaches, in which diagnostic and therapeutic tools are customized to the patient's profile.
Proteomics provides opportunities to identify new clinically useful tools or biomarkers. For example the discovery of OVA1, a proteomics-discovered diagnostic test, recently approved by the US Food and Drug Administration (FDA) and based on a panel of proteins that aid in the preoperative identification of malignant ovarian tumours [1]. Various proteomic technologies are able to detect differentially expressed proteins that are associated with different disease states (Table 8.1) [2, 3].

Differential proteomic technology for biomarker discovery
In this thesis, the combination 2D-DIGE with MALDI-TOF/TOF mass spectrometry was used in *Chapter 5, 6 and 7* for discovery and identification of differentially expressed proteins, which could represent new clinically useful biomarkers. For several reasons, DIGE can be seen as an improved version of conventional 2D gel electrophoresis. The dyes used in 2D-DIGE afford great sensitivity, capable of detecting as little as 25 pg of protein. This detection system shows a linear response to protein concentration up to five orders of magnitude (10^5). In contrast, post-electrophoretical staining methods, used in conventional 2D gel electrophoresis, only show a linear response to protein concentration over a very limited range ($10^2 – 10^3$) and have substantially lower sensitivity (1-100 ng of protein) [4].
A major concern associated with conventional "one sample per gel" 2D gel electrophoresis is its reproducibility. The standard design in DIGE comprises two experimental samples, labelled either with Cy3 or Cy5, and a Cy2-labeled internal standard. These three labelled samples will be separated on a single gel. Every protein in the sample is intrinsically linked to

the corresponding spot in the internal standard. The same internal standard is incorporated on each gel, which enables accurate inter-gel matching and comparison of protein abundances. Differences in protein abundances are analyzed by comparing the ratios of sample-to-standard, rather than direct comparison of raw spot volumes. Consequently, differences in spot intensity that may arise due to experimental factors during the separation process will be the same for each sample within a single gel, including the internal standard; therefore the relative ratio of sample-to-standard will not be affected by such experimental variation [5, 6]. Furthermore, recent research demonstrated that DIGE is a robust technology, displaying good within-laboratory and across-laboratory reproducibility [7]. Taken together, 2D-DIGE delivers the opportunity of internal standardization, reduces the number of gels that must be run, has a wide dynamic range for quantitation and shows improved sensitivity.

Table 8.1. Summary of differential and quantitative proteomic technologies applicable to bio-fluids as well as to tissue and cell lysates

Method	Year introduced	Types of sample
Gel electrophoresis		
2D gel electrophoresis	1974	Tissue/cell lysates; bio-fluids
2D DIGE	1997	Tissue/cell lysates; bio-fluids
Time-of-flight mass spectrometry (TOF-MS)		
MALDI-TOF	1988	Tissue/cell lysates; bio-fluids
SELDI-TOF	1993	Tissue/cell lysates; bio-fluids
Liquid chromatography mass spectrometry (LC-MS)		
ESI-LC-MS/MS	1989	Tissue/cell lysates; bio-fluids
Label-free MS	2004	Tissue/cell lysates; bio-fluids
Chemical labelling		
ICAT	1999	Tissue/cell lysates; bio-fluids
iTRAQ	2004	Tissue/cell lysates; bio-fluids
TMT	2006	Tissue/cell lysates; bio-fluids
mTRAQ	2008	Tissue/cell lysates; bio-fluids

Challenges in clinical proteomics

Despite significant technological advances, the field of clinical proteomics is still maturing and faces many challenges. The translation of proteomics-discovered biomarkers into clinically meaningful assays is impeded by several factors, from fundamental research design to more secondary issues, such as logistical and financial restrains. These fundamental limitations comprise among others lack of standardization throughout the biomarker pipeline, introducing biases that affect the final results. Serious investments and a universal proteomics workflow are required to accelerate the translation of proteomics discoveries to practical applications in the area of health and medicine [8]. To improve the path from

biomarker discovery to clinical implementation, Mischak *et al.* suggested essential requirements for clinical proteomics investigations, as shown in Table 8.2 [9].

Table 8.2. Requirements or guidelines for clinical proteomics investigations.

Aspect	Information required
Study aims	Concise definition of the overall and specific clinical aims
Study groups	Description of group size and composition (all relevant demographic and clinical data)
Samples	Sample type, collection and processing
Sample size and design	How the numbers in each group were selected
Analysis	Full details on the analytical approach.
Quality control and protein identification	
Data processing, data analysis and data reporting	
Validation	In an independent sample set using the original technique or an alternative assay such as an immuno-based assay

In the subsequent sections, the attempts undertaken in our proteomics discovery studies to fulfil the above requirements will be discussed, as well as the encountered challenges and the lessons learned.

Study aims and groups

New biomarkers are only clinically meaningful if they provide, compared to the current situation, additional benefits to the patients and preferably at reasonable costs. The involvement of clinicians is indispensable from the beginning of each biomarker discovery study. These specialists are suited to define well-framed clinical problems or unmet needs; and to select and accurately characterize patients and controls. Selecting well-defined patients and searching appropriate matched controls is essential to minimize the risk of introducing bias and consequently possible misinterpretations. This is illustrated by a study of Villanueva *et al.*, in which a serum proteomics test was discovered with approximately 100% sensitivity and specificity in diagnosing prostate cancer. Unfortunately, patients and controls were not well matched. Persons with prostate cancer were men with an average age of 67 years, while the control group was on average 35 years old and over 50% was female [10]. Randomization and matching of potential confounding variables, such age and sex as was done in *Chapter 4*, prior to data analysis could prevent biases in the obtained results. Unfortunately, it is not always possible to collect samples from a sufficient number of sex- and age-matched study subjects. Sometimes samples are collected from not optimally matched study populations (*Chapters 2 and 5*). In *Chapter 5*, we investigated whether biomarker expression was related to aneurysm size and progression. To do so, we stratified the study population in three groups (i.c. stable, progressive and large AAA) and found significant associations of aneurysm size with IgG, α1-antitrypsin, Factor XII activity and D-

dimer. However, the study populations also differed based on the frequency of males (p=0.029) and peripheral arterial disease (p=0.030), and also on the age of the patients (p=0.040). We used regression analysis to control for these differences in baseline demographics. The found associations of IgG, α1-antitrypsin, Factor XII activity and D-dimer remained significant (all p<0.001). So, multiple regression analysis can be used to control for potential differences due to inappropriate matching; the found associations of biomarkers with aneurysm size in *Chapters 2 and 5* remained significant, regardless of the differences in baseline demographics, such as age, centre of inclusion, gender and peripheral arterial disease.

Samples and pre-analytical conditions

For biomarker discovery purposes it's critical to employ the most suitable biological materials to identify new biomarkers. These biomarkers ought to be indicative of the investigated clinical problem and hence should be identified in representative samples of the disease condition. With the focus on non-invasive diagnostics, blood-based biomarkers or biomarker panels are likely to be the preferred form of a clinical test. A big debate in the proteomics community is which type of sample – blood or diseased tissue - should be used to discover biomarkers. Blood is an exceptional proteome; it is a rich source of biochemical analytes which can be measured as indicators of the physiological or pathological status of a patient. For instance, serum levels of hormones, cholesterol, enzymes and other proteins provide information regarding conditions as diverse as pregnancy, cardiovascular or nutritional status, and cancer. In addition, it's the most difficult proteome due to the wide dynamic range of protein abundances, the large amount of albumin (± 55%) present and the enormous heterogeneity of its predominant glycoproteins [11]. The sample complexity of blood can by reduced by depletion of high-abundant proteins, thereby yielding an enrichment of the low-abundant ones.

Different types of affinity chromatography methods for protein and peptide depletion are available [12-17], but are also associated with several limitations. Firstly, the efficiency of several depletion strategies ranged from 96.0 to 99.5%, as reported by Björhall and colleagues [13]. After depletion of plasma containing average 41 g/L albumin using the depletion method with the highest efficiency, 2.1 10^5 µg/L albumin would still be present; the remaining albumin concentration still exceeds by far the concentration of known low-abundant biomarkers, such as for example carcinoembryonic antigen (CEA; 2.5 µg/L). Secondly, removal of high-abundant proteins can result in concomitant removal of non-targeted proteins. Albumin, for instance, acts as a carrier protein for several low-abundant proteins, protecting them from clearance by the kidneys. Unfortunately, at the same time these potentially low-abundant proteins bound to albumin can be co-depleted [18]. Furthermore,

we experienced that insulin growth factor binding protein-3 (IGFBP-3; 1-9 mg/L) was almost completely removed by aspecific binding when using a kit depleting the twenty most abundant proteins in serum (data not shown). In *Chapter 6*, untreated cerebrospinal fluid (CSF) samples were used to discover new biomarkers, which reflect the actual CSF protein information. Specifically, this non-depleting approach enables IgG measurement as a positive control, since the presence of oligoclonal IgG bands or an increased IgG index in CSF is used in diagnosing multiple sclerosis (MScl). To conclude, it is imperative to balance benefits against additional sources of variability that might be introduced.

On the other hand, primary sites of disease, such as tumours or affected tissue, represent an interesting sample resource for biomarker discovery. The challenge of human tissue is its cellular and individual heterogeneity, but a possible solution to control for this individual biological variability is by differential analysis of paired samples (i.e., diseased and adjacent non-diseased tissue from the same patient). Furthermore, laser capture microdissection (LCM) is an excellent tool for isolating pure cell populations, providing homogeneous protein samples [19]. Although LCM is very efficient, it is also very laborious and proteins extracted in this way are mostly inactive. Currently, cell secretome analysis represents a potential strategy for successful biomarker discovery [20-22]. The cell secretome is the collection of proteins released into conditioned media of cultured cells. The dynamic range is lower and protein population is less diverse as compared to serum and tissue. However, this approach is not limitation-free. First, cell line cross-contamination or misidentification is shown to present 15 to 20% of the time. Second, tumours represent often a heterogeneous mixture of cells with various interactions between cell types. Therefore, multiple cell lines should be analysed to capture the molecular diversity and subsequent range of proteins secreted into the conditioned media. Lastly, cell cultures are an *in vitro* system and can be different from the *in vivo* situation [23].

Besides widely used diagnostic specimens such as serum (*Chapter 5*) and CSF (*Chapter 6*), we used alternative fluids for the discovery of new biomarkers. In *Chapter 7* the preservation solution of donor kidneys was utilized to discover new biomarkers. No protein spots were found in 2D-DIGE gels from preservation solution that was sampled before the start of renal machine-perfusion. Therefore, all protein spots in the solution after one hour of perfusion are likely to be derived from the donor kidneys, enhancing the chances of discovering biomarkers specific for renal injury. All samples used in this thesis were collected, processed and stored in a highly standardized way, as suggested by Bons *et al.* [24]; this to minimize the bias due to pre-analytical factors. Exactly 30 minutes after blood collection, samples were centrifuged at 3000 g for 15 minutes at 4°C and stored in multiple aliquots at -80°C within one hour after collection.

Standardization of sample collection and processing procedures is crucial, since they can have a dramatic effect on quality and reproducibility of the obtained results. This was highlighted in 2006 by a study of Irani *et al.*, in which they proposed that a cleavage product of Cystatin C was a biomarker for multiple sclerosis [25]. However, soon after the appearance of this article, Del Boccio and colleagues showed that this marker was formed by degradation of the first eight N-terminal residues of Cystatin C. This truncated form was always absent in fresh samples or in samples stored at -80°C, but present in samples left for more than 10 days at -20°C [26]. Truncation occurred in all investigated CSF samples, regardless of the underlying neurological status, indicating a storage-related phenomenon causing false interpretations as a biomarker for multiple sclerosis. Besides storage conditions, factors such as length of time in which the tourniquet is applied or the posture of the patient (i.e., supine or seated position) can affect the concentration of certain analytes up to 15% [27]. In *Chapter 2*, we observed a striking decrease in serum albumin concentration in pre-operative blood samples as compared to samples of follow-up patients. These pre-operative patients were hospitalized (i.e. non-ambulant) for several days, which can affect their albumin concentration. Besides hematocrit, hemoglobin and albumin can both be used to correct for such hemodilution. We corrected our measurements using the albumin concentration of control patients; their concentration (i.c. 44.8 ± 2.3 g/L) was within the normal reference range i.c. 32.0 – 47.0 g/L). Furthermore, the effects of pre-analytical variables, such as type of collection tube, clotting time, storage time and temperature, transport/incubation time, freeze/thaw cycles on sample quality are intensively investigated [28-32]. A recent study showed that the plasma, platelet and peripheral blood mononuclear cell proteomes are hugely influenced by methods of blood sampling and sample preparation, leading to potentially large within and between subject background variation [33]. These and other studies emphasize the need for standardized protocols concerning sample collection, processing and storage; this will be especially important to improve the collection and storage of high-quality, well annotated bio-specimens in bio-banks [34]. Taken together, focus is not on which procedure is better, but rather on using standardized protocols to obtain comparable and reproducible results.

Sample size and design

As proteomic technologies are rather expensive, many biomarker discovery studies are based on a modest number of replicates. Most important, however, is that the power of the study is sufficient to ensure a high probability of detecting true differential expression. As described by Karp *et al.* [35], three to five biological replicates are sufficient to determine a 1.5 to 2.0 fold change in protein expression relying on the DIGE-inherent systemic variance of <20%. Several other publications on 2D-DIGE showed that on average six samples per group for 2D-

DIGE screening are sufficient to find statistically sound results. For the proteomic analyses, described in *Chapters 5, 6 and 7*, five to eight representative patients were selected from each study population. Proteomic discovery results all require extensive validation with preferentially other analytical methodologies and larger cohorts of study subjects, as was done in *Chapter 5*.

Proteomic methodology and quality control

Quality control (QC) is becoming increasingly important in proteomic investigations to enable and maintain consistent results. The 2D-DIGE analyses summarized in this thesis (*Chapters 5, 6 and 7*) were performed following written step-by-step standard operating procedures (SOP) to ensure uniform analysis. Digestion and identification of protein spots using tandem mass spectrometry was rigorously subjected to several quality control procedures. Bovine serum albumin (± 100 ng per gel plug) proceeded together with the experimental samples from digestion to identification; and as such can be seen as QC sample monitoring the whole procedure. Furthermore, to enable accurate calculation of the *m/z* values and subsequent confident identification, the mass spectrometer was externally calibrated using a calibration mixture before every identification analysis. External calibration is performed by fitting the square of the observed flight times of the components of a polymeric mixture (i.e. six lyophilized proteins or peptides), which covers the entire mass range of interest (i.e. 900 – 3600 Da), to their expected *m/z* values. These and other stringent procedures ensure high quality analytical performance of the used proteomic technologies.

Validation methods

The most important and probably also most difficult phase in biomarker discovery research is validation of proteomics-discovered results. Frequently, antibody-based approaches such as for instance ELISA, Western Blotting or immunohistochemistry, are used to validate the differential protein expression. This validation is preferentially performed with all samples used in the discovery phase as well as with independent samples. Since ELISA enables quantitative validation of potential biomarkers, it was traditionally the gold standard for targeted protein quantification. Unfortunately, a suitable antibody is not always available. Especially not, when the potential biomarker is not the whole protein, but only a fragment of it. Consequently, validation is biased towards proteins with antibodies available, impeding the validation of potential new biomarkers. Development of specific antibodies for all putative protein biomarkers is a very expensive (i.e., 50 000 – 1.5 million Euros per biomarker candidate) and time-consuming (i.e., more than a year) process [36]. Furthermore, ELISA-based assays are known to be associated with false positive and false negative results due to for instance aspecific binding of antibodies. This approach is also unable to detect multiple

analytes in the same bio-specimen due to the cross reactivity of antibodies and each combination of antibodies needs to be evaluated for interferences. Nonetheless validation experiments are essential to assign those potential protein biomarkers that merit the effort and expense of full clinical validation.

Mass spectrometry-based assays provide an excellent alternative approach for quantitative biomarker validation and circumvent some limitations of antibody-based methods. Selected reaction monitoring (SRM) can be used to specifically select and quantify promising protein biomarkers in serum or tissue [37]. This technique combines the enhanced sensitivity of immunoassays with the specificity of mass spectrometry, while maintaining the capability to simultaneously target and measure thirty to hundred candidate biomarkers. In addition, SRM can measure the levels of post-translational modifications including phosphorylation, glycosylation and ubiquitination [2]. SRM is commonly performed on triple quadrupole mass spectrometers. In brief, the instrument has the ability to select precursor ions of targeted proteins based on their *m/z* using the first quadrupole. The selected precursor ion is dispersed into several fragment ions in the second quadrupole using collision energy. Only the fragment ions of interest can pass into the third quadrupole and quantitation is subsequently achieved by measuring the intensity of the fragment ions. Although SRM provides a powerful method for biomarker validation, its sensitivity remains constrained by sample complexity. Detection of low-abundant proteins in serum requires depletion of abundant proteins, with the associated problems described above. Recently, two methodologies were developed to enrich the low-abundant target analytes from complex samples for SRM analysis, namely stable isotope capture by antipeptide antibodies (SISCAPA) [38] and immuno-MALDI (iMALDI) [39]. However, these SRM-based methods are also not limitation-free, as evidenced by the high costs and extensive efforts associated with the development of high quality reagents (i.e., heavy isotope versions of target proteins, internal standard peptides and antipeptide antibodies) and by concerns on sample recovery throughout the procedures.

Validation of potential biomarkers

Validation experiments in *Chapters 5 and 7* were carried out using quantitative immuno-based methods available at our research facility. The differential expression of serum IgG and α1-antitrypsin in serum of abdominal aortic aneurysm patients (*Chapter 5*), detected with 2D-DIGE, was confirmed using the available methods. Although these validated biomarkers showed strong associations with aneurysm size, their value in predicting aneurysm progression was limited. Aneurysm size remains by lack of a better alternative the main predictor of progression. As described *Chapter 7*, 2D-DIGE revealed differential expression of transferrin in CSF samples of multiple sclerosis and clinically isolated syndrome (CIS)

patients, and controls. However, no difference in total CSF transferrin concentration was found by clinical laboratory diagnostics. This discrepancy may be related to the lack of specificity for transferrin fragments or isoforms of the pan-antitransferrin antibodies used in the immunochemical clinical assay. Since Kim *et al.* [40] suggested an important role of post-translational modifications in MScl, we investigated the glycosylation pattern of CSF transferrin. We showed for the first time that asialotransferrin was increased in CSF of patients with multiple sclerosis, suggesting that the N-glycosylation of proteins in the central nervous system is altered. Several other promising biomarkers were not further validated, such as the differential expression of Complement H in CSF of multiple sclerosis patients (*Chapter 7*). At the moment, there is no other approach available with the same reasonable cost, speed, and sensitivity. Development of SRM methods for validation of potential biomarkers is promising, but is very time-consuming and costly.

Besides validation of newly discovered potential biomarkers, several biomarkers suggested to be related with abdominal aortic aneurysm in literature were extensively validated using immuno-based methods. Although single biomarkers may be useful in selective cases, there is a growing consensus that an integrated panel of multiple biomarkers will be required for most clinical applications. First, we investigated whether biomarker concentrations were related to aneurysm size (n=218). *In Chapter 2*, we showed that HDL-cholesterol, CRP and IgG concentrations are strongly correlated to aneurysm size and are consequently potentially valuable markers for prediction of aneurysm progression. Secondly, we collected samples from patients with an AAA enrolled in a standardized follow-up study and investigated using adequate statistical testing the potential of biomarker panels for predicting aneurysm progression. Patients with elevated total cholesterol and LDL-cholesterol concentrations at baseline showed an increased AAA growth rate. However, aneurysm size remained the most accurate predictor of aneurysm progression (*Chapter 3*).

Deja-vu of proteins

In this thesis, some proteins were reported as differentially expressed in serum of abdominal aortic aneurysm patients (*Chapter 2, 3 and 5*), in CSF of multiple sclerosis patients (*Chapter 6*) and even in the preservation fluid of donor kidneys (*Chapter 7*). Proteins like haptoglobin, IgG and α1-antitrypsin are unfortunately not disease-specific and possibly originate from common disease mechanisms, such as inflammation. Furthermore, Petrak *et al.* and Wang *et al.* suggest that the frequent detection of these proteins may be linked to the used proteomics methodology [41, 42]. Some physiochemical characteristics of peptides govern the ion yield in MALDI: peptides containing arginine, phenylalanine or tyrosine enhance ion yield and are hence more easily detected compared to for instance hydrophobic peptides which ionize poorly. Furthermore, the detected and validated differential proteins were all relatively high-

abundant proteins, inherent to the used techniques. These findings emphasize the need for more sensitive proteomic technologies for biomarker discovery in bio-fluids; and the need for a protein equivalent of polymerase chain reaction (PCR) to specifically amplify low-abundant proteins.
Quite surprisingly, large amounts of blood-borne proteins were detected in the solution used to preserve donor kidneys till transplantation (*Chapter 7*). Their presence might indicate that an amount of blood is still present in the renal vasculature, even after thoroughly flushing the kidney following organ procurement. Therefore, sample collection protocol of preservation fluid needs to be optimized; possibly prolonging the flushing time after organ procurement and increasing the centrifugation speed to minimize contamination of blood-borne proteins.

Directions for future research

To move the field forward, fundamental biological questions remain to be addressed, and experimental designs need to be improved. Most study designs so far have been based on intuitive concepts and assumptions. For example, one assumption is that a subset of proteins present in the blood reflects, reproducibly and specifically, a single disease at a particular stage, which is not always so in case of multi-morbidity. We also assume that we can identify disease-specific signals over noise in the blood.
At present the most promising application for proteomics is the detection of specific subsets of protein biomarkers for certain diseases, rather than large scale full protein profiling. For instance, studying specifically post-translational modifications, such as the glycosylation pattern of transferrin (*Chapter 7*), which have been demonstrated relevant to disease pathology and useful targets for therapeutics. Furthermore, proteomics holds promise in routine clinical practice: identification of bacteria strains [43], testing the quality of biological medicines [44] and also for quality control in transfusion medicine [45]

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Summary

Clinical proteomics is a scientific discipline that identifies proteins associated with a disease by means of their altered levels of expression between the healthy control and disease state. These protein signatures or biomarkers can be used as a diagnostic or prognostic marker or as an indicator of the response to a certain treatment. Furthermore, biomarkers can attribute to the development of new therapeutics and can help to elucidate both physiological and pathological processes. Various proteomic technologies are suitable to discover and identify differentially expressed proteins, which could represent new potential biomarkers. These technologies can be roughly divided into gel-based and mass spectrometry-based approaches.

The aim of this thesis was to detect and identify potential biomarkers for abdominal aortic aneurysm (AAA), multiple sclerosis, and viability of transplantable kidneys. To do so, the combination two-dimensional differential in-gel electrophoresis (2D-DIGE) and matrix assisted laser desorption/ioniziation time-of-flight tandem mass spectrometry (MALDI-TOF/TOF) was used. Furthermore, AAA biomarkers identified on pathophysiological grounds, were measured in a large patient cohort to evaluate their usefulness as markers of aneurysm size, progression and success of surgical AAA repair.

In *Chapter 1* we discussed the approach employed to detect and identify potential biomarkers, and the subsequent steps necessary to successfully implement new biomarkers into routine clinical practice. Furthermore, background information on the three clinical problems (i.e., abdominal aortic aneurysm, multiple sclerosis, and viability of transplantable kidneys) is summarized in this chapter.
Abdominal aortic aneurysm, defined as an enlargement of the abdominal aorta with a diameter of 30 mm or more, is the tenth leading cause of death in the Western world. If left untreated, an AAA will increase in size until rupture of the aortic wall occurs, causing a life-threatening haemorrhage. Aneurysm size is fairly good in prospectively predicting aneurysm progression; consequently biomarkers associated with aneurysm size are promising indicators of aneurysm progression. In *Chapter 2* we concluded that HDL-cholesterol, CRP and IgG concentrations are potentially valuable markers for prediction of aneurysm

progression. Thorough validation of these and other serum/plasma markers in a large prospective standardized follow-up study showed that patients with elevated total cholesterol and LDL concentrations at baseline have an increased AAA growth rate. However, aneurysm size remained the most accurate predictor of aneurysm progression (*Chapter 3*).

To find new and improved serological markers for prediction of aneurysm progression, a non-hypothesis driven strategy using 2D-DIGE was employed in *Chapter 5*. The differentially expressed proteins found in serum of patients are involved in several key processes of AAA pathophysiology, such as inflammation, extracellular matrix remodelling, coagulation and fibrinolysis. The differential expression of three proteins (i.e., IgG, α1-antitrypsin and Factor XII activity) was validated and these proteins showed strong correlation with aneurysm size. However, combination of either α1-antitrypsin or Factor XII activity measurements with aneurysm size had little effect on prediction of aneurysm progression versus diameter alone.

Surgical repair of an abdominal aortic aneurysm is indicated when aneurysm exceeds 55 mm in diameter and can be accomplished by elective open surgery or endovascular aneurysm repair (EVAR). EVAR has decreased early mortality and length of hospital stay, but requires long term imaging surveillance for detection of stent graft related complication such as endoleak. Computed tomography angiography is considered the gold standard for detection of complications, but has known disadvantages. Therefore, a less harmful and cheaper alternative for post-operative follow-up is desirable. We showed in *Chapter 4* that plasma MMP-9 concentration is able to detect endoleak with high sensitivity (i.e., 100%) and specificity (i.e., 96%). The clinical applicability of this assay will be validated in a prospective clinical trial.

In *Chapter 6* we detected and identified - using 2D-DIGE and tandem mass spectrometry - differentially expressed proteins in cerebrospinal fluid of patients with multiple sclerosis, patients with clinically isolated syndrome or patients who were diagnosed not to have a neurological disease (i.e., controls). Higher IgG concentration was found in cerebrospinal fluid of multiple sclerosis patients compared to both controls and patients with clinically isolated syndrome. Furthermore, an increased amount of asialotransferrin was found in cerebrospinal fluid of both patients with multiple sclerosis or clinically isolated syndrome compared to controls. This observation suggests that alteration in N-glycosylation of cerebrospinal fluid proteins could be associated with pathogenesis of neurological diseases and could be useful in diagnostics.

The study described in *Chapter 7* aims at finding biomarkers indicative of graft viability in the preservation solution from machine-perfused human donor kidneys. Such viability markers could identify donor kidneys with a low risk of early graft failure and prevent transplantation of kidneys that will never regain function. Using 2D-DIGE and tandem mass spectrometry, nineteen unique proteins were identified that were derived both from renal tissue and from

residual plasma in the renal vasculature. Lower haptoglobin concentrations in the perfusion solution were associated with more extensive ischemic injury, indicating decreased viability of donor kidneys. However, further investigation is required to evaluate the potential of haptoglobin in assessing donor kidney viability.

Finally, the major findings of this thesis and the encountered challenges in clinical proteomics are discussed in *Chapter 8*, which also contains directions for future research.

Samenvatting

Clinical proteomics is een tak van de wetenschap die zich focust op de identificatie van ziekte-geassocieerde eiwitten, met name door een veranderd expressie niveau tussen ziek en de gezonde controle. Deze eiwit signaturen of biomerkers kunnen gebruikt worden als diagnostische of prognostische merkers, of zelfs als een indicator van de reactie op een bepaalde therapie. Daarnaast kunnen biomerkers bijdragen aan de ontwikkeling van nieuwe therapieën, alsook aan de ontrafeling van zowel fysiologische als pathologische processen. Verscheidene proteomics technologieën zijn geschikt voor de ontdekking en identificatie van eiwitten met een differentiële expressie, mogelijk nieuwe potentiële biomerkers. In grote lijnen kan men deze proteomics technologieën opsplitsen in gel-gebaseerde technieken en technieken gebaseerd op massa spectrometrie.

Deze thesis richtte zich op de detectie en identificatie van potentiële biomerkers voor aneurysmata van de abdominale aorta (AAA), multiple sclerose, en de viabiliteit van nieren die getransplanteerd worden. Hiervoor werd gebruik gemaakt van de combinatie tweedimensionale differentiële in-gel elektroforese (2D-DIGE) en matrix-assisted laser desorptie/ionisatie time-of-flight tandem massa spectrometrie (MALDI-TOF/TOF). Daarnaast werd de expressie van AAA biomerkers, geïdentificeerd op pathofysiologische basis, geanalyseerd in een groot patiëntencohort, om zo hun bruikbaarheid als merkers voor aneurysma grootte, groei en succes van een bepaalde chirurgisch ingreep aan het AAA te evalueren.

In *Hoofdstuk 1* werd de gebruikte strategie voor de detectie en identificatie van potentiële biomerkers bediscussieerd, alsook de daaropvolgende stappen die nodig zijn om nieuwe biomerkers met succes te implementeren in routine klinische praktijken. Tevens wordt er dieper ingegaan op de drie klinische problemen (i.e. aneurysmata van de abdominale aorta, multiple sclerose en de viabiliteit van nieren die getransplanteerd worden).
Vergroting van de abdominale aorta met een diameter van 30 mm of meer wordt gedefinieerd als AAA. Deze aneurysmata zijn de 10e belangrijkste oorzaak van overlijden in de westerse wereld. Wanneer een AAA niet behandeld wordt, zal ze toenemen in grootte totdat de aortawand ruptureert en zo een levensbedreigende bloeding veroorzaakt. Op basis van de

grootte van een aneurysma kan men prospectief redelijk goed de progressie van het aneurysma voorspellen; dus biomerkers geassocieerd met de aneurysma grootte zijn veelbelovende indicatoren van aneurysma progressie. In *Hoofdstuk 2* concludeerden we dat HDL-cholesterol, CRP en IgG concentraties potentiële merkers zijn voor het voorspellen van aneurysma progressie. Grondige validatie van deze en andere serum/plasma merkers in een grote prospectieve gestandaardiseerde follow-up studie toonde aan dat patiënten die bij de start een verhoogde totale cholesterol en LDL concentratie hadden, het aneurysma sneller groeide. Desalniettemin blijft de grootte van het aneurysma de meest accurate voorspeller van aneurysma groei (*Hoofdstuk 3*).

Om nieuwe en betere serologische merkers te vinden voor het voorspellen van aneurysma groei, werd 2D-DIGE gebruikt (Hoofdstuk 5). De eiwitten met differentiële expressie in het serum van de AAA patiënten zijn betrokken bij verschillende processen van de AAA pathofysiologie, zoals inflammatie, hermodelleren van de extracellulaire matrix, stolling en fibrinolyse. De differentiële expressie van drie eiwitten (i.e. IgG, α1-antitrypsine en Factor XII activiteit) werd gevalideerd; en deze eiwitten vertoonden een sterke relatie met de grootte van een aneurysma. Toch had de combinatie aneurysma grootte en enerzijds α1-antitrypsine metingen en anderzijds Factor XII activiteit metingen weinig additief effect op het voorspellen van aneurysma progressie vergeleken met de voorspellingen enkel gebaseerd op de aneurysma grootte.

Chirurgisch herstel van een aneurysma van de abdominale aorta is aangewezen wanneer de diameter van het aneursysma groter is dan 55 mm. Chirurgisch herstel kan gebeuren door middel van een electieve open chirurgische ingreep of door een endovasculaire ingreep (EVAR). De endovasculaire ingreep vertoont een verminderde mortaliteit en verminderde hospitalisatieduur, maar vereist wel herhaaldelijke beeldvorming gedurende een lange tijd. Dit om complicaties gerelateerd aan de stent op te sporen zoals endolekkage. Computed tomografie angiografie wordt beschouwd als de gouden standaard voor het opsporen van complicaties, maar er zijn ook enkele nadelen aan verbonden. Daarom is er nood aan een minder schadelijk en goedkoper alternatief voor de post-operatieve follow-up. In *Hoofdstuk 4* toonden we aan dat de MMP-9 concentratie in het plasma een endolekkage met een hoge sensitiviteit (i.e., 100%) en specificiteit (i.e., 96%) kan opsporen. De klinische toepasbaarheid van deze test zal gevalideerd worden in een prospectieve klinische trial.

In Hoofdstuk 6, detecteerden en identificeerden we, door middel van 2D-DIGE en tandem massa spectrometrie, eiwitten met een differentiële expressie in het cerebrospinaal vocht van patiënten met multiple sclerose, patiënten met clinically isolated syndrome of patiënten die geen neurologische aandoeningen hadden (i.e., controles). Hogere IgG concentraties werden gevonden in het cerebrospinaal vocht van patiënten met multiple sclerose vergeleken met zowel de concentraties in het cerebrospinaal vocht van controles en patiënten met clinically

isolated syndrome. Daarenboven, werd er meer asialotransferrine gevonden in het cerebrospinaal vocht van zowel patiënten met multiple sclerose als patiënten met clinically isolated syndrome vergeleken met controles. Deze observatie suggereert dat veranderingen in de N-glycosylatie van eiwitten in het cerebrospinaal vocht gerelateerd kunnen zijn met de pathogenese van neurologische aandoeningen en dus ook bruikbaar kunnen zijn in de diagnostiek.

De studie beschreven in Hoofdstuk 7 richt zich op het zoeken naar biomerkers in de perfusie oplossing van humane donornieren, geperfuseerd door een machine, die indicatief zijn voor de viabiliteit van transplantaten. Zulke merkers kunnen donornieren identificeren met een laag risico op early graft failure en kunnen de transplantatie van nieren voorkomen die nooit hun functie herwinnen. 2D-DIGE en tandem massa spectrometrie identificeerden negentien unieke eiwitten die hun oorsprong vinden in zowel het nierweefsel als het resterende plasma in de nier vasculatuur. Lagere haptoglobine concentratie in de perfusie oplossing is geassocieerd met een meer uitgebreide ischemische schade, wat wijst op een verlaagde viabiliteit van de donornieren. Doch is verder onderzoek nodig om het potentieel van haptoglobine als merker voor viabiliteit verder te analyseren.

Tot slot, worden de belangrijkste bevindingen van deze thesis en de tegengekomen hindernissen van clinical proteomics bediscussieert in Hoofdstuk 8. Daarnaast worden ook enkele richtlijnen geven voor verder onderzoek.

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Bianca

Curriculum Vitae

Bianca Pulinx was born on December 19th, 1985 in Bilzen, Belgium. In 2003 she completed secondary school (Latin-Mathematics) at the Heilig-Grafinstituut in Bilzen, Belgium. The same year she started with her academic education Biomedical Sciences at Hasselt University, Belgium. Her graduation project was conducted at the department of Medical Microbiology, Maastricht University Medical Centre under supervision of Prof. dr. C.A. Bruggeman, Dr. F.R. Stassen and Dr. E. Boelen, where she investigated the influence of *Chlamydia pneumoniae* infection on the uptake of amyloid-β in Alzheimer. In July 2007, she received her Master Degree in Clinical Molecular Sciences. In September 2007 she started her PhD project at the department of Clinical Chemistry, Maastricht University Medical Centre, under supervision of Prof. dr. M.P. van Dieijen-Visser, Prof. dr. G.W.H. Schurink and Dr. K.W.H.W. Wodzig. As of September 1st 2011 she is working as a quality control manager at the department of Clinical Biology, Sint-Trudo ziekenhuis in Sint-Truiden, Belgium.

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