CHAPTER VII

Summary and perspectives
The analysis of patient samples to assist in the diagnosis and management of disease is pivotal to modern medicine, and the outcomes of such analyses have a profound influence on clinical decision-making.\textsuperscript{1,2} Laboratory medicine, the field of medicine devoted to analyzing endogenous compounds in these samples, continuously aims at implementing novel tests and improving the quality of already available tests, and this field has always acted on developments in science and technology, accordingly.\textsuperscript{3} For example, doctors do not need to taste urine to diagnose diabetes anymore following the advent of quantitative methods based on chemical reactions that produce measurable, glucose concentration-dependent color changes or electrical currents.\textsuperscript{4-6} Blood group classification based on mixing blood from a patient with blood from different individuals represents another diagnostic technique of the past, as this technique was gradually replaced by standardized tests using commercial antisera (e.g., reagents containing anti-A, anti-B, or anti-Rhesus factor antibodies).\textsuperscript{7} Progress has also been made on the diagnosis of infectious diseases, and the introduction of the matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometric (MS) platform for microbial species identification is a noteworthy advancement in this respect. This platform offers a rapid alternative to traditional identification strategies that are based on phenotypic methods (e.g., microscopic examination of bacterial cell shape and size) which have limited discriminatory power and involve subjective judgments to some extent.\textsuperscript{8-10} Obviously, these examples only scratch the surface of how science and technology advanced the field of laboratory medicine in recent history. Despite these advances, however, many diseases still lack specific tests to assist in their diagnosis and/or management which thus provides incentives for science and technology to continue responding to corresponding needs in laboratory medicine.

The successful clinical implementation of MALDI-TOF mass spectrometry for rapid microbial species identification is testament to this technique’s qualitative possibilities, and this success may pave the way for the clinical implementation of MALDI-TOF MS for quantitative purposes. In the past decade, this technique proved to be useful for the quantification of a wide range of compounds including proteins, lipids, and metabolites in human samples (e.g., plasma, serum, urine). Such methods do, however, require that a target of interest is adequately enriched from the sample matrix, which is typically rather complex containing many other compounds that interfere with MALDI-TOF analysis. In addition, proper internal standards need to be incorporated in MALDI-TOF MS-based methods due to the (spatial) heterogeneity of MALDI samples and a high detection variability in consequence. When taking both these requirements into account, MALDI-TOF MS-based quantification can become an attractive analytical methodology from a clinical point of view owing to the simplicity and high throughput capabilities of this mass spectrometric platform. In Chapter 2 of this thesis, we presented such a quantitative method for insulin-like growth factor 1 (IGF1)
in human plasma. For this method we developed an automated IGF1 enrichment procedure using antibodies to selectively enrich this protein from plasma. In addition, we employed a fully $^{15}$N-labeled version of IGF1 as internal standard to correct for MALDI-TOF MS detection variability, yet also for any source of variation arising from the sample preparation procedure. With this IGF1 assay we could not only quantify this protein in human plasma samples but we could also indicate the presence of two IGF1 variants originating from single-nucleotide polymorphisms (SNP) in the IGF1 gene (of unknown clinical relevance). In the MALDI-TOF spectra of two different samples, the IGF1 peak at m/z 7,650 was accompanied by a peak at m/z 7,680 suggesting the presence of the rs17884626 or the rs151098426 SNP, which give rise to alanine-to-threonine substitutions at different positions in the C-terminal region of the IGF1 protein.\textsuperscript{11,12} For both samples this region was examined further by liquid chromatography-mass spectrometry (LC-MS) following Glu-C proteolytic digestion to produce a readily measurable C-terminal IGF1 peptide, and both SNPs were subsequently localized. These IGF1 variants may be missed by the IGF1 immunoassays that are currently used in clinical practice, and thereby lead to falsely low IGF1 levels being reported.\textsuperscript{12} IGF1 levels below specific cutoff values are, however, decisive for whether or not the costly treatment for growth hormone-related disorders is indicated\textsuperscript{13}, hence the ability to measure different IGF1 variants represents a distinctive and crucial feature of our IGF1 assay. Another distinctive feature of this assay was derived from additional peaks in the MALDI-TOF spectra besides the peaks of IGF1, $^{15}$N-IGF1, and the occasionally present IGF1 variants arising from SNPs in the IGF1 gene. In the MALDI-TOF spectra, these proteins were accompanied by +16 m/z mass shifted forms which likely arose as a result of oxidation of IGF1’s sole methionine residue. We found that the intensities of these oxidation peaks relative to those of their nonoxidized counterparts were indicative of the performance of the MALDI-TOF measurements and reflected the accumulation of matrix deposits in the MALDI source in particular. Through monitoring of the extent of IGF1 oxidation we could establish criteria for regular cleaning of the MALDI source which contributed positively to the quality of the MALDI-TOF spectra and the method’s weekly throughput, accordingly. Admittedly, methionine oxidation cannot be monitored for all proteins as some smaller proteins, such as human insulin, lack methionine residues in their mature forms but also because MALDI spectra of larger proteins typically feature one broad peak which envelopes the oxidation products. We also found that variation between the results obtained for different MALDI spots belonging to the same sample was indicative of MALDI-TOF performance as well. This indicator of measurement quality is not dependent on an analyte’s chemical composition (e.g. whether a protein contains methionine residues) and thus represents a more generic tool to ensure consistent performance of MALDI-TOF MS-based assays.
IGF1 is a 7.65 kilodalton (kDa) protein which is present in human plasma in the range of 10 to 1000 ng/mL (i.e. 1.3-130 nM) and is thereby considered to be a small and medium abundant protein. MALDI-TOF MS accordingly is a suitable technique to analyze this protein while this technique is considerably less applicable to larger and lower abundant proteins. For such proteins, LC-MS-based methods represent more suitable mass spectrometric alternatives. These methods typically do not need a target of interest to be enriched from its complex matrix as an adequate degree of separation of the analyte from interfering matrix compounds can often be achieved in the LC part of LC-MS instrumentation. For many low abundant proteins, however, LC separation alone is not effective enough, and these proteins require the use of an upfront analyte enrichment procedure to reduce matrix complexity. We found that this requirement also held true for the soluble receptor of advanced glycation end-products (sRAGE) which is addressed in the Chapters 3, 4, and 5 of this thesis. This 34 kDa protein is present in human serum in the range of 0.1 to 10 ng/mL (i.e. 2.9-290 pM) and is thereby more than 4 times larger and 400 times lower in abundance compared to IGF1. sRAGE is a promising biomarker candidate for chronic obstructive pulmonary disease (COPD) which is a prominent example of a disease lacking specific laboratory tests to assist in diagnosis and management. sRAGE has been evaluated in several large-scale clinical studies using research-grade enzyme-linked immunosorbsorbent assays (ELISA) from a single vendor, and all studies underlined the potential clinical applicability of this biomarker.\textsuperscript{14-18} We reasoned that disposing of different bioanalytical assays would aid in gaining a deeper understanding of sRAGE and its possible implications as biomarker in COPD. We developed an LC-MS method for quantification of serum sRAGE accordingly, which is presented in Chapter 3 of this thesis. This method employs an antibody-based sRAGE enrichment procedure using the adsorptive microtiter plates that are commonly used for immunoassays, as solid support to which antibodies were bound through passive adsorption. These adsorptive microtiter plates proved to be a more convenient alternative to magnetics beads, the mostly-used solid support for antibody-based enrichment procedures, in terms of protocol simplicity, throughput, and cost per analysis. Moreover, this procedure did not only form the basis of an LC-MS-based sRAGE method for serum sRAGE quantification at low to sub ng/mL levels, but was also used in our lab to enrich other proteins from serum, plasma, and even cell lysates as part of discovery proteomics experiments. The microtiter plate-based affinity enrichment procedure accordingly is an interesting procedure to be incorporated in LC-MS-based methods for other (low abundant) proteins of clinical interest and may potentially even contribute to expanding the footprint of mass spectrometry as analytical assay platform for protein quantification in clinical laboratories.
The microtiter plate-based affinity enrichment procedure that we developed represented a major milestone in our sRAGE project; however, the foremost goal in this project was to develop an LC-MS-based method for serum sRAGE quantification with the aim of contributing to the development of sRAGE as biomarker for COPD. As part of the work described in Chapter 3, we compared the LC-MS-based sRAGE method with the two most commonly used sRAGE ELISAs, with one of them being a ready-to-use ELISA kit which cannot be modified, and the other one requiring users to coat the plates themselves thereby having a somewhat modifiable assay format. This method comparison experiment indicated that correlation between all three methods was rather good ($R^2 = 0.72-0.79$) which suggests that the methods measure the same, or at least a comparable, fraction of circulating sRAGE molecules. However, 2 and 4 times higher sRAGE levels were reported by the LC-MS method compared to ready-to-use and modifiable ELISAs, respectively. For the modifiable ELISA, we found that using more antibody per sample than is recommended by the supplier led to higher sRAGE levels being reported and thereby to a smaller difference between the results of this assay and those of the LC-MS assay. A similar effect may be observed when increasing the amount of antibody per sample for the other ELISA as well, though this hypothesis could not be tested due to the unmodifiable format of this assay. Nevertheless, these results indicate that assays should be considered as being noninterchangeable unless proven otherwise. These results furthermore teach us to be more careful when using the phrase 'absolute quantification' in reference to the methods we use for protein quantification, since absolute quantification only applies to methods that are calibrated against certified reference materials or against protein standards of known exact quantities as can be determined by amino acid analysis.

With the development of the antibody-based LC-MS method, an alternative method for sRAGE quantification became available which provides a direct readout that relies on protein-specific, confined chemical information rather than an indirect readout that relies on the ability of a detection antibody to bind the target molecule in order to generate a signal, as holds true for the ELISAs. This method has already been applied to 1,000 patient samples from different clinical studies, which, for example, led to the conclusion that a patient's smoking status prior to blood sampling necessitates tight control. More specifically, cigarette smoking just before blood sampling was found to decrease sRAGE levels by a quarter (interquartile range from -33% to -16%) in both healthy subjects and COPD patients. This reduction was also reported when these same samples were analyzed by ELISA, by which we aimed to rule out method-specific artifacts underlying the acute smoking effect we observed. Obviously, this finding puts the previously reported, ELISA-based associations between sRAGE and specific COPD characteristics or outcomes into a different perspective as none of the publications that reported on the analysis of sRAGE in large-scale clinical studies thus far disclosed the
control of smoking status prior to sampling. Further studies are therefore needed to elucidate the mechanism behind this decrease and also to assess the impact of cigarette smoking on previously published data and the conclusions drawn from those data.

Relevant tools to assist in future studies on sRAGE are the two antibody-free LC-MS methods for sRAGE quantification which are presented in the Chapters 4 and 5 of this thesis. With respect to these methods, we felt the need to develop antibody-free sRAGE enrichment procedures as knowledge on sRAGE would otherwise remain solely based on antibody-based methods. Admittedly, antibodies are highly efficient, versatile, and indispensable tools in (bio) medical research, yet these proteins are also associated with complex production procedures resulting in batch-to-batch consistencies that are difficult to control.20 The limitations of antibodies inspired researchers to develop non-antibody affinity ligands and corresponding research endeavors yielded a considerable number of alternatives to antibodies in the past decades. The ‘affimer’ scaffold based on the cysteine protease inhibitor Cystatin A is one example of such an alternative, which we employed for the LC-MS-based method for sRAGE quantification that is described in Chapter 4 of this thesis. Solid-phase extraction (SPE-) based enrichment procedures furthermore represent attractive alternatives to antibody-based procedures or to affinity ligand-based procedures in general. Still, only few publications described the successful application of SPE-based enrichment procedures in quantitative methods for low to sub ng/mL (or sub-nanomolar range) proteins such as sRAGE.21 On the basis of the very unusual, bipolar charge distribution of the sRAGE protein, we were, however, able to establish such an SPE-based procedure for serum sRAGE, which is described in Chapter 5 of this thesis. We found that sRAGE could be enriched by strong cation exchange (SCX) SPE at pH 10, a pH at which most serum proteins do not bind, and subsequently be quantified in serum at clinically relevant levels down to 0.2 ng/mL by LC-MS. When comparing the three sRAGE methods, correlation between the antibody- and the affimer-based methods was very good ($R^2 = 0.88$), though somewhat (approximately 25%) lower sRAGE levels were reported by the affimer-based method. Correlation between the SCX-based method and the other two methods was, in turn, only moderate ($R^2 = 0.46-0.48$) with the SCX-based method reporting on average 18% and 42% higher levels compared to the antibody- and affimer-based methods, respectively. With respect to these differences, it is conceivable that the methods capture a different subset of sRAGE forms, so-called ‘proteoforms’ or ‘protein species’, as binding sites may, for example, be absent in some of these forms or blocked by sRAGE binding proteins. Thereby, our findings indicate that measured protein concentrations must be seen in the context of the measuring principle. Disposing of different (validated) bioanalytical assays accordingly forms the basis for gaining a deeper understanding of protein heterogeneity, its role in disease pathophysiology, and its potential implications on the development of biomarkers.
As for the exact nature of the differences between the assays, we were limited by the sensitivity of currently available instrumentation to elucidate the reasons behind these differences. To illustrate this, the lowest sRAGE level we measured in a clinical sample thus far corresponded to less than 150 attomoles (approximately 90 million molecules) of sRAGE being detected by LC-MS on the basis of the two best performing sRAGE-derived peptides with respect to their detection characteristics (e.g., complete digestion, high ionization efficiency), as we determined empirically. These peptides are furthermore present in most of the predicted sRAGE splice variants and do not contain known posttranslational modifications (PTM) nor encompass highly prevalent SNP-sites. Differentiation between sRAGE variants arising from SNPs, alternative splicing, and/or PTMs, however, comprises the monitoring of peptides which will inevitably be lower in abundance and likely have less favorable detection characteristics compared to the two peptides we selected for the sRAGE methods presented in these thesis. To this regard, the continuously improving sensitivity of novel LC-MS instruments represents a promising development, and the possibility to differentiate between different forms of the sRAGE protein will likely be feasible in the foreseeable future.

Rationalization of how we process samples and target proteins of interest, which in our case resulted in the SCX-based method for sRAGE enrichment, may likely contribute to expanding the possibilities of LC-MS-based workflows for biomarker quantification purposes. Such a concept may, however, be worthwhile extending to LC-MS-based biomarker discovery research as well. In the corresponding field of research, various sample preparation methods are being used that are determining for the potential outcomes of a biomarker discovery experiment. For example, the extraction efficiencies of individual proteins and the incidence of sample preparation artifacts (e.g., methionine oxidation, asparagine/glutamine deamidation) vary between different methods and impact the subset of proteins that are reliably identified in these experiments, as we show in Chapter 6 of this thesis. The data presented in this chapter also indicate that no single sample preparation method will perform best for all types of samples or experiments and thereby argue against the existence of a “universal sample preparation for proteome analysis”\(^2\). Consequently, these data led us to the conclusions that the outcomes of a biomarker discovery experiment, too, must be seen in the context of the measuring principle and that selecting sample preparation methods on the basis of a fit-for-purpose evaluation, rather than using the same method for all samples and experiments, represents a rational strategy to expand the potential of discovery proteomics experiments.

At last, with this thesis I aimed to contribute to the advancement of promising protein biomarker candidates as well as mass spectrometry-based methodologies towards clinical implementation. Work described in this thesis, for example, demonstrates the high throughput capabilities of MS-based biomarker assays and their ability to provide more information than
solely a protein concentration thereby exceeding the possibilities of (most) assay platforms that are used in clinical laboratories these days. In addition, an easy way to set-up and use sample preparation methods was presented which can simplify and accelerate the development of MS-based biomarker assays, which currently represents a major bottleneck in biomarker development research. Some of the limitations of mass spectrometry-based workflows for analyzing proteins in complex human samples were highlighted as well and leads were provided for expanding the capabilities of such workflows accordingly. All in all, this thesis may represent a relevant contribution to biomarker development research, though I cannot deny that the road ahead will be long and that climbs will be steep. Along this road, the added value of mass spectrometric assays over conventional assay platforms needs to become more apparent, for example by following up on developments regarding the analysis of intact proteins or by further exploring the multiplexing and profiling capabilities of MS-based workflows. In addition, tangible success stories are needed to build momentum for the widespread clinical implementation of this technology. Such implementation, in turn, will lead to even more success stories and will remove certain barriers to the translation of promising biomarker candidates to clinical tests.
References


