Immunocapture LC-MS as a multiplexing tool for combined determination of protein variants using SCLC-markers ProGRP and NSE as models

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by

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Oslo, February 2014

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LIST OF PAPERS

The thesis is based on the following papers which will be referred to by their roman numeral in the text:

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II. Torsetnes, S. B.; Nordlund, M. S.; Paus, E.; Halvorsen, T. G.; Reubsaet, L., Determining ProGRP and isoforms in lung- and thyroid cancer patient samples: Comparing an MS method with a routine clinical immunoassay. *Anal Bioanal Chem* **2014**, 406, (11), 2733-8.

III. Torsetnes, S. B.; Løvbak, S. G.; Claus, C.; Lund, H.; Nordlund, M. S.; Paus, E.; Halvorsen, T. G.; Reubsaet, L., Immunocapture and LC-MS/MS for selective quantification and differentiation of the isozymes of the biomarker neuron-specific enolase in serum. *J Chromatogr B* **2013**, 929, 125-32.

IV. Torsetnes, S. B.; Levernæs, M.S.; Nordlund, M. S.; Paus, E.; Halvorsen, T. G.; Reubsaet, L., Determining ProGRP and isoforms in lung- and thyroid cancer patient samples: Comparing an MS method with a routine clinical immunoassay. Manuscript submitted to *Anal Chem*.

V. Torsetnes, S. B.; Levernæs, M.S.; Nordlund, M. S.; Paus, E.; Halvorsen, T. G.; Reubsaet, L., Pre-treatment factors affecting protein determination using bottom-up approach and immunoaffinity extraction prior to LC-MS/MS. Manuscript submitted to *Anal Bioanal Chem*.

Papers not included in the dissertation:

- Lund, H.; Torsetnes, S. B.; Paus, E.; Nustad, K.; Reubsaet, L.; Halvorsen, T. G., Exploring the complementary selectivity of immunocapture and MS detection for the differentiation between hCG isoforms in clinically relevant samples. *J Proteome Res* **2009**, 8, (11), 5241-52.
- Hustoft, H. K.; Brandtzaeg, O. K.; Røgeberg, M.; Misaghian, D.; Torsetnes, S. B., Greibrokk, T.; Reubsaet, L.; Wilson, S. R.; Lundanes, E.; Integrated enzyme reactor and high resolving chromatography in "sub-chip" dimensions for sensitive protein mass spectrometry. *Sci Rep* **2013**, 3, 3511.
- Quader, A. A.; Urraca, J.; Torsetnes, S. B.; Tønnesen F.; Reubsaet, L.; Sellergren, B.; Molecular Imprinted Polymer with nonapeptide target analogs for selective extraction of NLLGLIEAK from digestion of ProGRP in biological samples. Manuscript in preparation.

LIST OF ABBREVIATIONS

ABC	Ammonium bicarbonate
ACN	Acetonitrile
AQUA	Absolute Quantification Peptides
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
CA	Cancer antigene
CEA	Carcinoembryonic antigen
CI	Confidence interval
CID	Collision induced dissociation
CNS	Central nervous system
CYFRA 21-1	Cytokeratin fragment 21-1, a cytokeratin 19 fragment
ED-SCLC	Elevated disease small cell lung cancer
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
ESI	Electrospray Ionization
GI	Gastro intestinal
GRP	Gastrin releasing peptide
hCG	Human chorionic gonadotropin
HCl	Hydrochloric acid
HCOOH	Formic acid
HLOQ	Higher limit of quantification
HPLC	High-performance liquid chromatography
IAE	Immunoaffinity extraction
IEF	Isoelectric focusing
IRMA	Immunoradiometric assay
IS	Internal standard
IVD	In vitro diagnostics
LC	Liquid chromatography
LCNSC	Large-cell neuroendocrine carcinoma
LC-SCLC	Limited disease small cell lung cancer
LLOQ	Lower limit of quantification
LOD	Limit of detection

LTQ	Linear trap quadrupole
MALDI	Matrix-assisted laser desorption ionization
MRM	Multiple reaction monitoring
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MSE	Muscle specific enolase
MS/MS	Tandem mass spectrometry
MTC	Medullary thyroid cancer
m/z	Mass-to-charge ratio
NCBI	National Center for Biotechnology Information
NNE	Non-neuronal enolase
NSCLC	Non-small cell lung cancer
NSE	Neuron-specific enolase
PPT	Protein precipitation
ProGRP	Progastrin releasing peptide
PSAQ	Protein standards for absolute quantification
QqQ	Triple quadrupole detector
r	Correlation coefficient
r^2	Coefficient of determination
RIA	Radioimmunoassay
RP-HPLC	Reversed-phase high-performance liquid chromatography
RSD	Relative standard deviation
SCC	Squamous cell carcinoma
SCLC	Small cell lung cancer
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SID	Stable isotope dilution
SIM	Selected ion monitoring
SQ	Single quadrupole
SPE	Solid phase extraction
SRM	Selected reaction monitoring
S/N	Signal-to-noise
TR-IFMA	Time-resolved immunofluorometric assay
UniProtKB	UniProt Knowledgebase

ABSTRACT

The main objective of this thesis was to demonstrate properties, potential and use of immunocapture LC-SRM-MS methods in a targeted quantitative approach for protein biomarker determination. SCLC-markers ProGRP and NSE were models for the approach, and the aim was to prove the methods superior qualities to the existing and current standard methods for protein marker verification and clinical analyses. The end goal was a tailored multiplexing approach for the two markers with simultaneous differentiated determination of their reported isoforms and isoenzymes.

In *Paper I* a validated method for differentiation of ProGRP isoforms is presented. The work is based on research by Winther et al. which used short calibrator ProGRP(31-98) as standard, employment of the antibody mAb E146 for immunocapture and use of the bottom up LC-MS to determine total ProGRP by signature peptide NLLGLIEAK[1]. This approach was modified to introduce features of quantitative differentiation of the three ProGRP variants termed isoform 1, 2 and 3, by employment of full length recombinant standards for ProGRP and assignment and addition of the following additional two signature peptides to the method: LSAPGSQR and DLVDSLLQVLNVK for isoform 1 and 3, respectively. In addition, the immunocapture format was altered from using 96-well plates to magnetic beads, resulting in reduction of LOD from 200 pg/mL[1] to 8 pg/mL. The method was validated for ProGRP quantification through the signature peptide NLLGLIEAK (for total ProGRP), LSAPGSQR (for isoform 1) and DLVDSLLQVLNVK (for isoform 3) and evaluated for indirect determination of isoform 2. All signature peptides showed acceptable linearity (R^2>0.974), intra-day precision (<18% RSD) and accuracy deviation (≤25%). The obtained LOD for total ProGRP was lower than healthy endogenous serum levels (8 pg/mL=1 pM at S/N = 3) enabling detection of endogenous ProGRP levels in serum from healthy donors.

In *Paper II* performance of the validated ProGRP method from *Paper I* was demonstrated using clinical samples. A number of sixty patient samples were analyzed with two main objectives; 1.) To compare conformity of the MS method with a routine assay, the automated TR-IFMA, for quantification of total ProGRP, and 2.) To perform novel exploration of the pathological isoform expression in the various cancer diseases. When comparing the absolute concentration values obtained by analysis of the two different methods for total ProGRP, the determinations were found to correlate and no unsystematic differing values were identified. The results were though shown to not be directly interchangeable as the MS method determined the total ProGRP concentration systematically approximately 30 % lower than the

TR-IFMA. The MS method supplemented with quantitative determination of two ProGRP isoforms which were found to have more heterogeneous protein expression than was previously reported for mRNA synthesis. Additionally, the expression of isoform 3 was found to dominate over isoform 1 which also differed from the previously reported mRNA ratios. The method from *Paper I* proved valid for a future larger patient study.

In *Paper III* a similar targeted approach as for ProGRP (in *Paper I*) was developed for NSE. Two signature peptides, ELPLYR and TIAPALVSK, specific for the γ-subunit and α-subunit, respectively, were assigned to allow differentiation between these two relevant subunits for the NSE isoenzymes. Sample preparation using mAb E21-coated magnetic beads for selective immunocapture of the γ-subunit was performed, and all reported NSE isoenzymes were extracted: the homo-(γγ) and heterodimer (αγ), and the γ-monomer. The method was validated for NSE quantification using the γ-signature peptide ELPLYR, with excellent linearity (R^2>0.999 at range 5–500 ng/mL) and good intra-day precision (<13% RSD) and accuracy (>95%). The obtained sensitivity (LLOQ of 38 pg/mL at S/N = 10) was lower than healthy endogenous serum levels. In addition, the method allowed detection of the αγ-heterodimer by the α-signature peptide TIAPALVSK.

In *Paper IV* a method for multiplexing of tumor markers was exemplified by combining the methods presented in *Paper I* and *Paper III* for co-determination of the two model markers ProGRP and NSE. These particular markers were chosen for their reported complementary clinical value for SCLC, as combined measurement of ProGRP and NSE then would increase clinical reliability. In addition, co-determination reduced both time, reagent and sample consumption compared to individually performed measurement. For the combination, some alterations were necessary for determination of NSE (compared to the method in *Paper III*), as the calibration matrix used here was ProGRP-depleted serum (5% BSA in *Paper III*), and the endogenous level of NSE in the calibration matrix altered the γ-NSE determination. In addition, the calibration curve for γ-enolase and α-enolase was here harmonized to allow use of the γ-calibration curve to indirectly estimate the α-subunit. The immunocapture SRM method presented determination of the following signature peptides for the protein variants: LSAPGSQR (for ProGRP isoform 1), DLVDSLLQVLNVK (for ProGRP isoform 3), NLLGLIEAK (for total ProGRP), TIAPALVSK (for the α-subunit deriving from the αγ-NSE in case of anti-γ immunocapture) and ELPLYR (for the γ-subunit with possible origin in the homodimer (γγ) or monomer (γ) of NSE).

In *Paper V* NSE and NNE were used as model compounds to investigate some aspects which can affect sensitivity and protein determination when using the bottom-up MS approach after immunocapture. The αα-, γγ-, and αγ-enolase calibrators, and mAbs specific for each of the the two subunits were used. The following was concluded: Trypsin activity was not affected by the presence of mAb coated magnetic extraction beads. However, the binding of NSE markers to mAb coated magnetic beads contributed to a decrease in signature peptide yield, and the cause for this was assigned steric hindrance and availability of trypsin cleavage sites. Denaturation, reduction and alkylation as predigest treatments showed positive effect on both α- and γ-signature peptide production. This was despite the absence of disulfide bridges in NSE, which indicate partial release of marker from the magnetic beads as the assigned cause. And, finally, the non-covalently linked α- and γ-subunits of the NSE heterodimer standard was proven to be partly dissociated, showing that control of standard stability is of utter importance.

1 INTRODUCTION

The subject of this thesis is included in the field termed *clinical proteomics*, which may be defined as study of proteomics activities in the field of medicine. This research also fall under the terms molecular diagnostics and *in vitro diagnostics* (IVDs), where the terms are used for proteomics research of marker that hold promise of being translated into clinical bioanalytical tests. More specifically, this thesis compromise absolute quantification of diagnostic tumor markers in serum samples. The introduction is therefore divided into a clinical part and an analytical part.

Thus, the first chapter (1.1) focus on the clinical aspect of IVDs. It begins with explaining the term and the features of tumor markers, and then it focuses on a few acknowledged tumor markers, depicts characteristics of small cell carcinomas of the lung and reviews in-depth two small cell lung cancer markers; progastrin releasing peptide (ProGRP) and neuron-specific enolase (NSE).

The subsequent chapter (1.2) clarifies the term proteomics and the analytical aspects. This chapter stresses the importance of reliable methods for measurement of tumor markers, explains features of the traditional assays used, and compares these assays with methods which include a targeted MS approach. Further, a typical workflow of targeted MS is explained, and finally the foundation of UiO-performed MS related work on ProGRP and NSE is explained.

1.1 CLINICAL ASPECTS

1.1.1 Tumor markers in biological samples

The first known identified molecular tumor marker in modern medicine was a monoclonal globulin protein found acidified urine from a patient with multiple myeloma, described by Henry Bence Jones in 1848[2]. Biomarkers may be indicators of a variety of health and disease characteristics, while the term tumor marker can be defined as a substance whose concentration or structure is altered in pre-cancerous or cancerous conditions. These substances may offer insight and understanding of pathological mechanisms and be clinically useful in diagnostic confirmation, prognosis and prediction of therapeutic response, and monitoring disease and recurrence, as well as screening and early cancer detection[3].

A biomarker eligible for clinical use must be expected to enhance the ability of a clinician to optimally manage the patient. In this respect, important qualities for an ideal marker is to provide adequate *diagnostic accuracy*, represented by *diagnostic sensitivity and specificity*[4]. *Diagnostic sensitivity* is defined as the ability to correctly classify a malignancy, and the *diagnostic specificity* as the ability to correctly classify non-malignancy. For both 100 % sensitivity and 100 % specificity, a so-called positive result, with the marker passing a defined threshold, must occur in all cases of malignant state, and never in healthy states, respectively[4]. Such an ideal marker is yet to be identified, as the markers known generally are both or either affected by states other than that single malignancy and/or not affected at the early stages of a disease. Characteristics such as long lead-time, level correlation with tumor burden, practical half-life, simple and cheap tests, and easy obtainable specimens are also important features pertaining to an ideal marker[4].

Diagnostic sensitivity and selectivity is also a challenge for diagnostic tools not concerning molecular marker determination. This is why, generally, several different tests are performed and the results considered combined to best manage the patient. Thus, despite their limitations, tumor markers are valuable and extensively used together with other diagnostic tools. Similarly combined determination of several markers adds reliance to the information basis for clinical interventions. The rise of the term multiplexing, a modern term for combined investigation of two or more markers, derives from this.

Molecular markers are rarely used for early diagnosis and screening due to relatively low disease prevalence in combination with limited diagnostic sensitivity and specificity. The primary uses are monitoring of therapy, prediction of therapeutic response, prognosis and help for diagnosis, and surveillances for recurrence of cancerous diseases[4, 5]. Examples of such markers are: α-fetoprotein (AFP), human chorionic gonadotropin (hCG) and lactate dehydrogenase for testicular cancer, recommended used as aids in diagnosis, staging, prognosis determination, recurrence detection, and therapy monitoring[6-9]. Prostate-specific antigen (PSA), a sensitive, but not very specific marker for prostate cancer, is FDA-approved for population screening, however, not universally accepted for this use, but acknowledged for detection of disease recurrence and monitoring therapy[5, 9, 10]. Carcinoembryonic antigen (CEA), with low specificity in gastrointestinal and colorectal cancer, is recommended for prognosis indication, postoperative surveillance, and therapy monitoring in advanced disease[9, 11, 12]. Cancer antigene 125 (CA125) for ovarian cancer is recommended for both detection of cancer recurrence and early detection of women at high risk, therapy monitoring, and for

determination of prognosis, as well as for differential diagnosis of suspicious pelvic masses in postmenopausal women[9]. For breast cancer, CA15-3, CA27.29 or CEA may be used for therapy monitoring in advanced disease, while estrogen and progesterone receptors are mandatory for predicting response to hormone therapy, and human epidermal growth factor receptor-2 measurement is mandatory for predicting response to immunotherapy with trastuzumab, while urokinase plasminogen activator/plasminogen activator inhibitor type 1 may be used for determining prognosis in lymph node–negative patients[9].

1.1.2 Lung cancer, classification and tumor markers for clinical use

World wide, carcinomas that derive from epithelial cells in the lung are found with high incidence and poor prognosis, and it was in the year 2005 the reported leading cause of cancer-related death[13, 14]. The treatment and prognosis for a lung cancer patient depends on the extent of tumor development at the time of diagnosis and the histological subtype of the carcinoma. Primary lung cancer can be divided into two histological subtypes; *non-small cell lung carcinoma* (NSCLC) and *small-cell lung carcinoma* (SCLC).

The NSCLCs dominate in prevalence and incidence, and consists of several subtypes, predominantly adenocarcinoma, squamous-cell carcinoma (SCC), and large-cell lung carcinoma (LCLC), which generelly are treated in the same manner[13]. The survival rates to the NSCLC patients varies from 1% to 67% at 5 years depending on development and metastases of the tumor[15, 16], with an median survival of about 2 years. The focus of this thesis, however, is SCLC and will therefore be discussed in more detail.

SCLC comprises approximately 15-20 % of the bronchogenic carcinomas and is associated with the poorest prognosis of all histological types. Despite greatly improved therapeutic regimes over the last decades, the 5-years survival rate of less than 10%, the median survival is of less than 1 year for late stage carcinoma (termed elevated disease, ED), and less than 2 years for early stage carcinoma (termed limited disease, LD)[13]. This is due to early and rapid doubling time and aggressive metastasizing of the carcinoma. Thus, most patients are diagnosed with ED and not responding well to therapy[13].

SCLC typically display phenotypic features of neuroendocrine character which are not exclusively occurring in SCLC nor in the lung[17]. An estimate of about 30% of lung tumors are neuroendocrine, implying that NSCLC patients may also exhibit neuroendocrine differentiation, and it has been suggested that these subgroups may benefit from treatment regimes similar to those of SCLC[18]. The 2004 WHO classification recognizes four major

subtypes of neuroendocrine pulmonary tumors arising in the bronchial mucosa termed typical carcinoids (TC), atypical carcinoids, (AC), small cell lung cancers (SCLC) and large cell neuroendocrine carcinomas (LCNEC)[17, 19, 20]. This overlap of features may be considered as part of the histological gray zones related to disease heterogeneity in disease progression[21] and the described similar patterns of differentiation of certain pulmonary carcinomas[20]. Biological factors succeeding from the tumor may relate to this heterogeneity[22], as suggested by a study of phenotypically different cells in a mouse model[23]. To elucidate and add these parameters to guide the selection of appropriate therapy may significantly improve disease management. Thus, differentiation of pulmonary tumors is important, and might influence survival[24-26].

Accurate differential pathological diagnosis, staging and disease monitoring is essential for assigning and adjusting to the most effective treatment for a lung cancer patient. Several diagnostic tools are used because no single tool is fully sensitive nor specific. For lung carcinomas, progastrin-releasing peptide (ProGRP) and neuron-specific enolase (NSE) have complementary clinical value for diagnosis and treatment purposes when used together[26-32].

1.1.2.1 Progastrin-releasing peptide (ProGRP)

ProGRP is the precursor of the biologically active end products of gastrin releasing peptide (GRP) which is a member of the bombesin family. The highest concentrations of GRP in humans are found in fetal lung, neurons in the CNS and GI tract. These neuropeptides regulate several functions of the GI system and CNS, including release of GI hormones, contraction of smooth muscles, and proliferation of epithelial cells, and they are likely to play a role in human cancers such as those of the lung[33-36], colon[37-39], stomach[40, 41], and prostate[42, 43].

Variants of ProGRP proteins, termed isoforms, are expressed on mRNA level through alternatively spliced mRNAs from the human GRP gene[44-46]. Three mRNA types 1, 2 and 3 separately encode the known ProGRP proteins coined ProGRP isoform 1, 2 and 3, respectively[44, 47], which each have molecular weights of about 16 kDa (UniProtKB/Swiss-Prot: P07492 for all three isoforms). The mRNA types 1, 2 and 3 are reported to appear in approximate relative ratios of about 60:5:35, respectively, in both healthy[47, 48] and neoplastic tissue[47]. All mRNA types encode precursor ProGRP, preProGRPs, which are attributed by an additional N-terminal signal sequence (residue -23-1), and a common GRP sequence, but differ in the sequence encoding the C-terminal extension peptide[46]. Compared to ProGRP isoform 1, a 21-base pair deletion in mRNA type 2 results in corresponding deletion of amino acids 105-111 for isoform 2, and for isoform 3 a 19-base pair deletion in mRNA type 3

introduces a frame shift and creation of a premature stop codon which produce an unique C-terminal heptadecapeptide starting from amino acid 99[46]. The common amino acid sequences (the N-terminal signal peptide, GRP, and the rest of the carboxyl-flanking peptide through residue 98), as well as the differing parts for the isoforms, are shown in Table 3.1 in chapter 3.1.

During the initial processing of the ProGRP isoform 1, the precursor preProGRP is converted to ProGRP by cleavage of the N-terminal signal peptide. Further processing by endoproteolytic cleavage combined with carboxypeptidase B-like activity produces glycine extended GRP_{1-27} (GRP_{1-27} Gly), as well as the C-terminal extension peptide (residue 31-125). Amidation of the neuropeptide to the mature GRP_{1-27} is aided by peptidylglycine α-amidating monooxygenase (PAM), and an additional endoproteolytic cleavage forms the mature GRP_{18-27}, however, it is not known if the last cleavage occurs before or after amidation. Originally, only the mature amidated form of GRP was considered biological active, but recent studies have proven the nonamidated GRP_{18-27} Gly, as well as recombinant and synthetic C-terminal extension peptide (residue 31-125) and its fragments to be biologically active in a range of tissues and in cancer cell lines[49, 50]. The receptor for the latter, the C-terminal extention peptides and its fragments, is not yet established, however, the different GRP neuropeptides can activate three known mammalian reseptors; the GRP preferring receptor (GRPR), the neuromedin B preferring receptor (NMBR), and the bombesin receptor subtype (BRS-3)[34, 51, 52].

The discovery of production of GRP in SCLC encouraged attempts to establish methods for determination of GRP in blood derived samples[53, 54], but rapid elimination of GRP made this challenging[55]. However, the precursor, ProGRP, proved more stable and resulted in determining ProGRP. This was first demonstrated by Holst et al. in 1989 and used to prove increased $ProGRP_{42-53}$ in plasma and spinal fluid from SCLC patients[56, 57]. The first radioimmunoassay (RIA) for ProGRP was developed two decades ago[58], shortly followed by an enzyme linked immunoassay (ELISA)[59].

These, and other similar assays, have demonstrated good diagnostic sensitivity and specificity for ProGRP in SCLC[29, 58-61], especially for the limited disease stage, higher than both NSE and the other more commonly used lung cancer markers[29, 30, 60, 62, 63]. ProGRP is considered a good prognostic marker, and has been shown useful as an indicator for disease extent, with ability to discriminate between limited and extensive disease[64-66], and treatment response[32, 55, 60, 67, 68], and tumor regression or progression[65, 67-70], though reported not to correlate well with

tumor extent. Overall, the general prognostic impact of ProGRP is better than the established NSE marker[55, 65, 71].

As already mentioned, ProGRP is a neuroendocrine marker and a valued marker for both detection and monitoring of SCLC, and considered a good tool for discriminating SCLC from NSCLC[32]. However, high serum ProGRP concentrations are observed in some NSCLCs. These NSCLCs often also express other neuroendocrine markers and show different clinical characteristics than typical NSCLCs, such as improved response to treatment adjusted for lung cancers with neuroendocrine features[72] similar to LCNEC[73]. Thus, ProGRP may be used to reveal neuroendocrine characteristics of histological diagnosed NSCLC[74].

1.1.2.2 Neuron-specific enolase (NSE)

NSE is a term for γ-isoenzymes which belong to the enzyme class of enolases (or 2-phospho-D-glycerate hydro-lyases, EC 4.2.1.11) which are glycolytic multifunctional proteins. The mammalian enolases; non-neuronal enolase, muscle-specific enolase and neuron specific enolase (NNE, MSE, and NSE, respectively), are encoded by separate genes, and are composed of one or two of the three possible subunits; the α-, β-, and γ-enolase also termed Enolase 1, Enolase 3 and Enolase 2, respectively. These combine to form the five most referred isoenzymes in homo- and heterodimers. The α-homodimer, termed non-neuronal enolase (NNE), is expressed in embryo and most tissues, while the hetero- and homodimers of β-enolase (αβ- and ββ-enolase), termed MSE, are found in striated muscle tissue[75]. As for the γ-enolase, the hetero-, and homodimer as well as the monomer of this subunit (αγ-, γγ- and γ-enolase, respectively) they all comprise NSE and are produced by and located in nervous tissue and neuroendocrine cells, as well as found in erythrocytes and platelets[76]. The active enzymes are dimers of non-covalently linked subunits, and these two relevant subunits for human NSE, the α- and γ-enolase, each have a molecular weight of 47 kDa[77] and consist of 434 amino acids (UniProtKB/Swiss-Prot: P06733.2 and P09104.3, respectively). These subunits are distinguished by 72 replacements (no deletions or insertions) in the sequences of AA 271-285, 298-316 and 416-433 as shown by the marked green amino acids in Table 3.1 in chapter 3.1.

The reported function of NSE is neurotrophic properties for a range of CNS neurons and cell survival for neocortical neurons[78]. The NSE concentration in serum from healthy humans is below 10-20 ng/mL, however, the NSE level can be influenced by a broad range of diseases and disorders[79, 80]. In addition to being a useful lung cancer marker[81, 82], NSE has proven to be a marker for acute cell damage in human CNS[83, 84], Creutzfeldt-Jakob disease[85], ischemic and

hemorrhagic stroke[86], and other brain injuries[87, 88]. Elevated expression of NSE is also found in cases of neuroendocrine tumors[89], such as neuroblastoma, carcinoid tumors, malignant melanoma[90], seminoma[91, 92] and SCLC[82].

NSE is considered a valuable marker for both staging and monitoring treatment response of SCLC patients[93] and is the primary marker for SCLC with weak predicative values for NSCLC[94]. The prognostic value is demonstrated in several multivariate trials for both SCLC[30, 95-98] and NSCLC[99-101] where NSE relate well to treatment response[96] and tumor mass extension[95], and to have high specificity for ED-SCLC[95]. Though NSE does not have the sufficient specificity or sensitivity to be used in screening or as a sole marker for lung cancer differentiation, some utility for differential diagnosis of SCLC from NSCLC has been demonstrated, especially in combination with other markers[32]. In addition, similar to ProGRP, regarding both disease progression and the complex and mixed histological features of lung cancer[102], NSE may be used for general identification of carcinomas of the lung which exhibit neuroendocrine features[103, 104], and then to discriminate these from other lung diseases.

In short, NSE is the established tumor marker for monitoring SCLC. The newer and promising lung cancer marker, ProGRP, has shown higher sensitivity and specificity for SCLC than NSE. In general, ProGRP and other SCLC and lung cancer markers such as NSE, CYFRA 21-1 and CEA have improved diagnostic sensitivity when combined[26, 62]. Excellent specificity for SCLC was reported when combining the markers with respective cut-off values; NSE (>35ng/mL), ProGRP (>100pg/mL) and SCC antigen (<2ng/mL)[25]. NSE and ProGRP have complementary clinical information and in particular high sensitivity for SCLC[32, 69] in both limited and extensive disease status[32], and the combination of the two further increase diagnostic sensitivity for SCLC[29, 62]. This increased clinical value when combining selected markers will be a subject of explanation under chapter 1.2.1.

1.2 ANALYTICAL ASPECTS

1.2.1 Proteomics

The term “proteomics”, coined in early 1990s, derives from the merging of “protein” and “genomics”. Proteomics is a post-genomic discipline, compromising a large field of studies with common incentives to unravel information regarding expression, modification and interaction of proteins related to roles or functions in a biological system. The proteomics field protruded from the realization that the final product of a gene is more complex and closer linked to function than the gene itself, and by this, cancer markers can be used not only to identify the presence of a tumor, but they may also be applicable to determine stage, subtype and ability to respond to therapy. The aim of studies related to this area of proteomics, also termed clinical proteomics, usually belong to the fields termed discovery and quantitative targeted proteomics, with purpose to discover new targets for therapeutics, and to screen for and verify biomarkers for immediate assessment of "real-time" health and disease status.

Verification of the clinical utility value of such markers rely on sufficient *test accuracy* to elucidate and eventually also benefit from the *diagnostic accuracy* (mentioned in section 1.1.1) of a marker. The term *test accuracy* include both precision and trueness of the measurement, which is essential for reliable determination[4]. To illustrate the importance of both diagnostic accuracy and test accuracy, Figure 1.1 depict a biomarker measurement performed with the objective to separate patients into two groups based on the presence or absence of a specific disease. Here the diagnostic accuracy, decision threshold and predictive values are linked to test accuracy showing its significance in IVD.

The field targeted quantitative proteomics is in essence the study and analysis of one or several preselected proteins to deliver more precise, quantitative and sensitive data, and is increasingly used for establishing biomarkers and for development and validation of clinical methods. Emerging from the introduction of the radioimmunoassay in the 1960s[105], targeted proteomics has for a long time relied on antibodies as analytical tools for determination. However, the improvements of technologies for peptide/protein separation, MS analysis, isotope labeling for quantification, and bioinformatics data analysis has further expanded the possibilities within this field. Developments within and related to MS technology and pertaining tools within the last decades have increasingly made it become an established strategy, the method of choice for analysis of complex protein samples and considered a mainstream technology. Features such as multiplexing capability, the shorter and cheaper

process of method development compared to other alternatives, and the ability to discriminate between protein variants such as isoforms and post-translational modifications (PTMs), are valued properties of the SRM-based proteomics[106, 107] (see also Figure 1.4 under section 1.2.3.3). Effectively, MS is widely used in academia and in pharmaceutical and biotechnology industries for both discovery and targeted proteomic analyses[108].

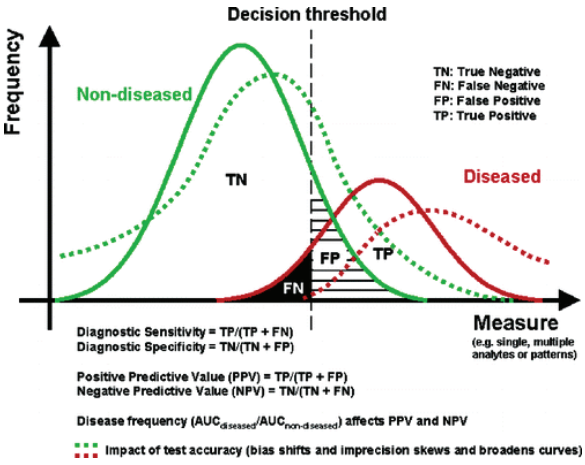


Figure 1.1: Important parameters in IVD. A hypothetical produced example of the dependence of the frequency of a non-diseased and a diseased population versus biomarker measurement to illustrate the relationship between diagnostic accuracy (sensitivity and specificity), decision thresholds (cutoff points), predictive values (PVs), and test accuracy. Sensitivity is defined as true positive/true positive + false negative; TP/(TP + FN). Specificity is defined as true negative/(true negative + false positive); TN/(TN + FP). As shown, PVs, sensitivity and specificity is determined by the position of the decision thresholds (cutoff points). These will be affected by the test accuracy in cases of uncertainty or if compared to a pathognomonic test (gold standard). This figure is adapted with permission from reference [4]. Copyright © 2005 American Chemical Society.

The potentials of MS based experimental research for use in routine clinical practice has been demonstrated by many. In example, a study by Petricoin et al. in 2002 showed impressing findings when investigating proteomic patterns in serum in relation to early-stage ovarian cancer by surface-enhanced laser desorption/ionization-time-of-flight (SELDI-TOF) mass spectrometry. By establishing a multiplexed list of biomarker in combination together with a algorithm acquired by marker pattern-recognition, the study showed a positive predicative value (PPV) of 94 % against the comparable PPV of 34% for the widely used ovarian cancer marker CA125. As the PPVs of low incidence diseases, such as ovarian cancer, should be close to 100% for population screening to avoid high numbers of false positives, the study suggest to combine the MS approach with, in example, ultrasonography to reach a prospective population-based assessment of this technology as a screening tool for all stages of ovarian cancer in both high-risk and general populations[109].

As this example demonstrates, several different tests are generally performed and their results considered combined for the clinical evaluation of pathologies. This is due to challenges of

sensitivity and selectivity which are typical for diagnostic tools also when they not concern molecular marker determination. Diagnostic investigation can involve multiple tests which can be performed and considered combined in a series manner or in a parallel manner to support decisions on interventions. For a series manner, test A is applied first, and, if positive, re-tested with test B. While for a parallel manner, both test A and test B are applied simultaneously. The approaches have their strengths and weaknesses. The advantage of serial testing is cost-effectiveness through the typically positive impact on pre-test probability and PVs, with a potential disadvantage of false negative values as well as a potential of delay in treatment initiation. While for the parallel testing, rapid and comprehensive results are produced at a potential cost of resources.

However, the mutual purpose for considering multiple results for both series and parallel testing is to add assurance to the diagnostic evidence to improve the PVs and thus the clinical sensitivity and/or specificity. For this either OR rules or AND rules may be applied for the combined interpretation of results, which affect the PVs (the clinical sensitivity and specificity values) differently[110]. Relative to considering the test results alone, the OR rule for considering the two or several results gives higher sensitivity and lower specificity than either test individually, whilst for the use of the AND rule the specificity is higher and sensitivity lower. Consequently, when the OR rules are used the approach is very predicative in the confirmation or rule-in for a particular disease, whilst for the AND rules, increased sensitivity can be useful for ruling out a disease[4]. So there is a trade-off between sensitivity and specificity when combined evaluating different test results. However, to prevent the impact of this trade off, a set of results can also be considered and interpreted as one single multivariate observation as in the mentioned study by Petricoin et al.[109], which will not be discussed here.

Equations OR-rule: $SeA + SeB - SeA \times SeB > SeA \cup SeB$

$SpA \times SpB < SpA \cup SpB$

Equations AND-rule: $SpA + SpB - SpA \times SpB > SpA \cup SpB$

$SeA \times SeB < SeA \cup SeB$

1.2.2 Immunoassays for cancer marker measurements

Antibodies have been utilized in clinical diagnostic immunoassays for several decades. Both for research and clinical purposes, the need to further improve analytical specificity and sensitivity has driven continuous refinements of this methodology, bringing many methods from benchtop to bedside, and making these tests the gold standards for protein quantification in clinical use[111].

1.2.2.1 Immunoassay principle and design

Immunoassays are biochemical tests that exploit analytical specificity of antigene-antibody reactions to measure presence or concentration of analyte such as a protein tumor marker. These methodologies can employ a variety of different labels for detection, such as enzymes in enzyme-linked immunosorbent assays (ELISAs) or enzyme immunoassays (EIAs), radioactive isotopes in radioimmunoassays (RIAs) or immunoradiometric assays (IRMAs) and fluorogenic reporters in various immunoassays as in immunofluorometric assays (IFMAs). Their different designs may roughly be categorized as either competitive binding or non-competitive binding immunoassays, where the common feature is that a catcher antibody binds to an area on the analyte termed an epitope. In a competitive design the analyte is measured indirectly and usually by detection of a marked analyte which competes for binding-sites on the limited amount of catcher antibodies. For the non-competitive design, excess catcher antibody binds the antigen and excess of labeled antibody binds to analyte to determine the marker. This latter design, often called two-site or sandwich immunometric assays, has improved assay kinetics and enhances sensitivity through the favoring of antigen-antibody complex formation[112].

1.2.2.2 The advantages and shortcomings of immunoassays

The combined economical and analytical qualities of immunometric assays has been highly competitive to other methods, as shown by its great extend of use. This is because the modern immunometric assays generally hold high good sensitivity and specificity at high throughput in par due to ease of automation. However, these assays also have limitations and drawbacks both related to development and use.

Concurrent with evolvement in “omics” technologies the introduction of thousands of biomarker candidates the last decades has strained a bottleneck in the biomarker pipeline. The need for an interface between biomarker discovery and clinical validation has increased with increased data. Traditionally, immunobased assays have been developed for this purpose,

however, due to high cost and time-consuming developments, production of an assay may not always be justified, considering the rather low success rate of biomarker candidates. The main factors for development are availability or cost of production of both high quality antibodies and assay optimization.

A typical ELISA development generally costs between hundred thousand and two million dollars per biomarker candidate (number from year 2009) and takes more than a year to develop[113]. In this context, targeted proteomics by SRM-MS is suggested as a well-suited preceding or complementary method in the development[114, 115]. In terms of pre-clinical biomarker testing, SRM-MS is highly specific, has short lead time, and multiplexing capacity. The use of such faster, less expensive and more straightforward multiplexing application can relieve the bottleneck of verifying putative markers and is of increasing use[116, 117]. The workflow and features of such a method will be addressed in later (see section 1.2.3 and 1.2.4).

Despite the great analytical sensitivity of many immunoassays, cases of lack of adequate specificity and accuracy have led to false results by analyte-independent and analyte-dependent interferences. These are effects on the measurement of an analyte caused by presence of a substance in the sample that alters the assay response. Possible interferences can in example derive from endogenous substances, such as heterophile antibodies or autoantibodies, or be caused by lipaemia, cross-reactivity and exogenous substances[112, 118]. This may lead to falsely elevated[119, 120] or false low [119, 121-123] response depending on the nature of the interfering substance and the assay design[118] and may lead to misdiagnosis[124].

To mention one example, the hook effect is a possible analyte-dependent interference in immunoassays. It involves assay saturation due to high analyte concentration where constituents in the sample interact with reagent antibodies. Careful assay design and performance may, however, minimize the probability of these effects[125-127], which is the case for most of the modern immunometric assays. It should also be mentioned, that when other methods utilize antibodies as part of their method, such as in IA extraction (described under section 1.2.3.3), these must also be carefully designed to not give false results.

Another limitation concerning the specificity of the immunoassays is their inability to distinguish between different variants of a marker. However, there are exceptions where different epitopes allow for differentiation. For NSE isoenzymes different mAbs have shown to have different selectivity for the two possible subunits α- and γ-enolase[128]. The SRM-MS approach can however selectively and simultaneously determine marker isovariants not

possible with traditional immunoassays, and this will be addressed how the under section 1.2.3 and 1.2.4.

However, it should be mentioned that both immunoassays and SRM-MS are unable to detect markers when they occur in certain unforeseen altered forms. These altered states may in example be polymorphisms derived from exons or post translational modifications (PTMs) which can affect either the accessibility of antibody epitope or the yield of recognized signature peptide.

1.2.2.3 Current clinical measurement of NSE and ProGRP

Most of the existing clinical methods used for cancer marker measurements are immunometric competitive assays, as are the ones for NSE and ProGRP. There are several commercially available kits for serum NSE determination, while quite few companies offer assays for ProGRP.

The first commercialized assay for ProGRP, the manual sandwich ELISA, was developed[59, 60] and patented by Yamaguchi et al. and subsequently licensed to Abbott (Abbott Diagnostics, Germany). Years later, the same research group developed the ARCHITECT® ProGRP[129] (Abbott Diagnostics), an automated two-step multiple site quantitative chemiluminescent microparticle immunoassay (CMIA)[130, 131]. Two other immunoassays to measure total ProGRP are the CanAg® ProGRP EIA (Fujirebio Diagnostics, Inc., Japan), a solid-phase, one-step, non-competitive immunoassay using the mAb E146 and the newly released Elecsys® ProGRP (Roche, Switzerland), an automatic heterogeneous immunoassay with electro chemiluminescence (ECL) measurement[132]. These specific tests either use plasma or serum samples with volume between 30-150 µL, and report to measure ProGRP(31-98) within an upper measurable concentration span in the range 1000-5000 pg/mL, with sensitivity of ≤4 pg/mL and with assay cut-offs for suspiciously elevated levels between 70-86 pg/mL[132-134].

For NSE, the existing immunoassays are non-competitive, heterogeneous sandwich based, such as DELFIA (PerkinElmer, USA), Elecsys 2010 (Roche, Switzerland), Kryptor (BRAHMS GmbH for Thermo Fisher Scientific Inc, USA), the ELISA (DRG International Inc., USA) or immunoradiometric assays (IRMAs) (DiaSorin, Italy, and Immunotech Laboratories Inc, USA). These tests use sample sizes between 25-300 µL, the sensitivities are ≤1 ng/mL (LLOQ), the upper measurable concentration span the range 100-1000 ng/mL, and as of the reference cut-off limit for suspiciously elevated levels these are 12.0-16.3 ng/mL[135, 136].

There has been reported significant discrepancies in correlation between and in the performance characteristics of a selection of the commercially offered NSE immunoassays (n=7)[135]. NSE is, as mentioned earlier, present as homodimeric, heterodimeric and monomeric γ-enolase, and the main probable reason for discrepancies are assigned the use of different mAbs with different affinities for the different isoenzymes[135]. This is of known relevance as studies have shown significant variation of the relative proportion of the different NSE isoenzymes between individual samples[137], and NSE mAbs (n=12) from various companies and research groups have therefore thoroughly characterized in ISOBM-initiated workshops[138].

1.2.3 Targeted MS workflow

MS is an analytical tool which utilizes ionization in gas phase to measure molecules by their masses and relative concentrations of atoms and molecules. However, targeted proteomics experiments typically consist of several stages before MS determination of a low abundant analyte in a biological sample. They can be divided into following stages[139]: 1.) Protein isolation/fractionation, 2.) Degradation of proteins to peptides using the bottom-up approach, 3.) Peptide separation by on-line LC, and, finally, 4.) MS determination by selected reaction monitoring (SRM). The following sections will comprehend these stages in a reversed order, and, in addition, end with a section (1.2.4) on quantitation strategies.

1.2.3.1 LC-ESI-MS in SRM mode

The core of modern targeted quantitative MS is to use MS in an MS^n mode. A powerful approach is to use the selected reaction monitoring (SRM) technique for sensitive and precise quantification of targeted proteins with complex backgrounds. The specific predetermined analytes with known fragmentation properties may with this technique be measured across multiple samples in a consistent, reproducible and quantitative manner. The most common is to use a QqQ instrument with an ESI ion source[140] for applicability of analysis of complex samples. Compared to another ion source, the matrix-assisted laser desorption/ionization (MALDI), the MALDI is normally used to analyze relatively simple peptide mixtures, while the ESI-MS systems allow for integrated liquid separation tools, typically LC-ESI-MS, to perform simplification of the sample before MS introduction. Further on, ESI is a soft ionization technique, leaving the peptide intact prior to entering the mass analyzer, and, as for the mass analyzer, the QqQ system perform very well for quantitative purposes with high throughput, selectivity and sensitivity.

The following main events occur in the QqQ in SRM mode: On the MS^1-level scans, the first quadrupole of the QqQ transmits only targeted species by their preselected specific *m/z* value. In the next step, collision induced dissociation (CID), trough ion-activation and breakage of the weakest peptide bonds, produce reproducible fragments of the marker, which are most often y- and b- fragments. Subsequently, on the MS^2-level scans, the last quadrupole of the QqQ transmits only the preselected fragments by their preselected specific *m/z* value to the detector.

The described two-stage filtering of SRM enhances selectivity, which makes it a highly specific MS strategy, however, it is sensitive to interference from other components that have very similar precursor- and fragment transitions. The LC therefore performs a MS complementary pre-separation of peptides and interferences, which is important for the potential of multiplexing of complex samples by LC-MS analysis. Due to band separation of peptides regarding to their physiochemical properties in the LC-system, distinct peptide ions and their transitions can be monitored at their respective time periods of elution, termed segments, reducing issues of limitation of simultaneous MS capacity. The measurement of specific peptide/fragment *m/z* pairs is continuously repeated over a defined time period (within the segment) and usually two or more peptide fragment are detected for each peptide. Out of these, one fragment transition is typically used for the basis of quantification, termed the quantifier, while the one or two other are for verification of peptide identity based on their relative signal intensity, and are termed qualifiers.

1.2.3.2 The bottom-up approach

When performing quantitative LC-MS analysis, it is common to produce proteolytic peptides of the proteins using the so-called bottom-up strategy, with top-down and middle down being the alternative strategies. The bottom-up approach produces peptides of marker proteins, and it is commonly used for accurate measurement of protein concentration to circumvent challenges associated with intact protein separation, ionization and MS characterization.

The bottom-up process involves selective proteolysis of proteins, and detection of specific proteolytic peptides as surrogates for their parent protein. These are termed *signature peptides* (or alternatively proteotypic peptides) when their sequence is unique to the marker protein and, when in addition, a method enables them to serve as a quantitative stoichiometric measure of marker protein concentration.

Both chemical and enzymatic proteolysis may be used to create such peptides, however, the latter, for cleavage of peptide bonds between individual amino acids, is most common. The biochemical specificity and characteristics as well as availability of trypsin makes it the gold standard for proteomics. Before a tryptic digest, proteins containing cystein (C) residues are often unfolded by both thermal and chemical aid, because cystein residues may form inter-covalent bonds. Trypsin specifically cleaves peptide bonds C-terminally to arginine (R) and lysine (K) residues, unless blocked by an adjacent proline (P) residue. The products are generally of moderate length, and carry two or three charges when ionized by ESI, and these tryptic peptides generally have better front-end separation, and are more suited for very sensitive and selective detection by ESI-SRM-MS than the intact proteins they derive from.

1.2.3.3 Sample preparation: fractionation aided by immunocapture

The complexity and abundance of proteins in most clinical relevant matrixes exceed the capacity of the typical LC-MS systems. A method for clinical application of targeted serum protein analysis therefore requires an efficient sample preparation to ensure a sample of significantly lower complexity than that of most biological fluids to reach sufficient marker sensitivity. Despite high sensitivity and specificity of LC-SRM-MS, the dynamic range of proteins in serum can exceed 10 orders of magnitude, and highly selective sample purification, as well as enrichment, may in many cases be necessary to quantitatively determine low-abundance markers in a reproducible manner, and it may additionally decrease LC cycle time and allow higher throughput.

The approaches for sample preparation are diverse, and lack of quality to allow for direct subsequent quantitative LC-MS analysis of the very-low abundant protein markers are common, however, they have uses in other aspects of the proteomics field. A traditional sample preparation technique for proteomics is the gel electrophoresis, in either one or two dimensional mode where proteins are typically separated either or both by isoelectric point by isoelectric focusing (IEF) and by length/molecular weight by SDS-PAGE. Other basic sample preparation techniques are, filtration and protein precipitation, both for non-specific protein purification by removal of high abundant protein, and solid phase extraction (SPE) which fractionate proteins or peptides by adjustment of the SPE system.

However, the need for improved sensitivity and specificity, as well as sample throughput has driven the sample preparation towards other more specific approaches. The advantage of using immunoaffinity (IA) extraction[141, 142] in combination with LC-MS for analytes in biological samples[143-145] was emphasized many years ago. However, the application techniques and accomplishments of use, especially that of clinical use, is still in its early stages. Van den Broek et al. has compiled sensitivities for methods with different sample preparation strategies preceding LC-MS/MS to illustrate which LLOQs these approaches typically can obtain and is shown in Figure 1.2.

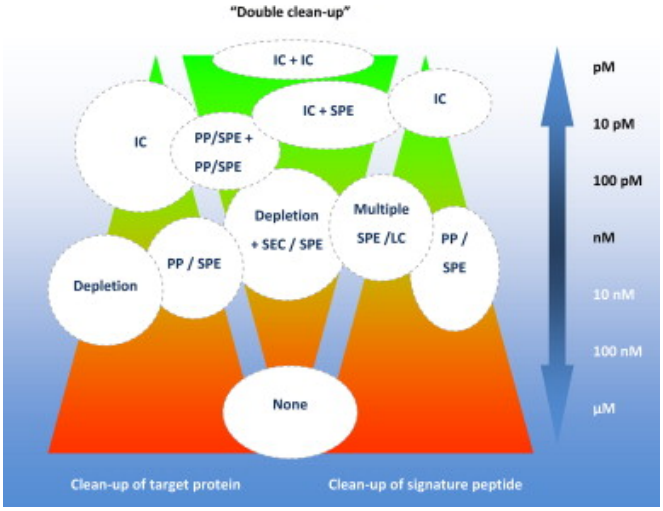


Figure 1.2: Résumé of achieved sensitivities by use of different sample preparation strategies in targeted MS approaches. A number of fifty-two targeted quantification methods for proteins in serum or plasma have various sample preparations combined with LC-MS determination. The different clean-up approaches are divided into being for either proteins, signature peptides or a combination of the two, and their compiled obtained LLOQs are shown. Symbol explanation; IC: immunocapture, LC: liquid chromatography, PP: protein precipitation, SEC: size exclusion chromatography, SPE: solid phase extraction. This figure was adapted with permission from reference [146]. Copyright © 2013 Elsevier.

IA extraction may be used in sample preparation in different formats. Immunocapture is a common term for extraction based upon molecular recognition of antibodies directed against epitopes of one selected protein or a group of proteins[1, 147-149] . When the antibodies instead are directed against tryptic peptides, the technique is often termed stable isotope standards with capture by anti-peptide antibodies (SISCAPA®)[150-152]. As an alternatively to use for isolating target molecules, the immunoaffinity strategy can also be used for subtraction of the most abundant proteins[153]. The immunobased approaches as means of sample preparation may be used in different overlapping formats such as off-line[1, 154-156], on-beads[152, 157], on-column[150], on-line[143, 158-161] and in-line bead trap[162]. A typical workflow for a bottom-up on-beads immunocapture and SISCAPA approach is shown in Figure 1.3, in part A and B respectively.

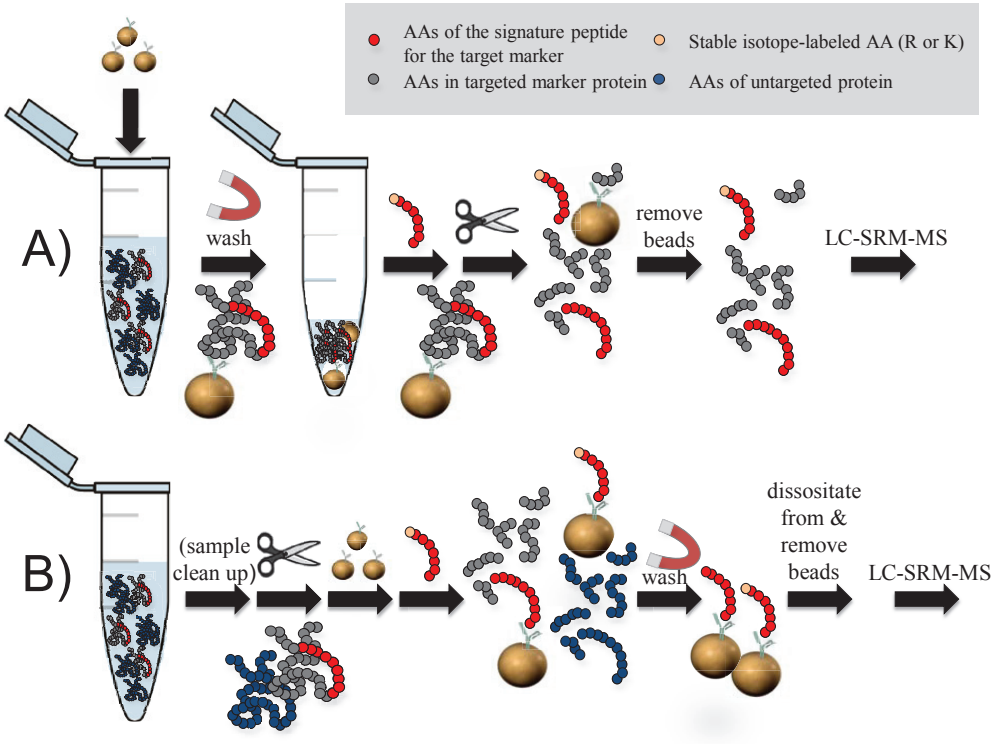


Figure 1.3: A typical workflow of A) an immunocapture part and B) a SISCAPA approach by use of AQUA peptides (isotope-labeled signature peptides) for SID. As the figure shows, the point in the process where the antibody-coated magnetic beads are added differ between the approaches. The scissors illustrate enzymatic digestion by trypsin, and the magnets illustrate magnetism used in the detainment of magnetic beads with mAb-bound target markers.

Affinity approaches and SRM assays have different performance profiles as illustrated in Figure 1.4. Combining the IA extraction with LC-MS detection may increase sensitivity and capacity. Many of the IA formats for marker fractionation allow for enrichment, circumventing the sensitivity limitations of the MS. Another bottleneck for the LC-MS system is its capacity limitations, and antibody selectivity, which its orthogonality to LC, may allow for higher throughput by reducing the LC cycle time, and improving the detection limits by reduction of introduced interferences into the MS. The MS on the other hand contributes with superior specificity compared to the immunoassays. The setup can typically enable study of differences between very similar proteins, such as post-translational modifications (PTMs) or isoforms, in which pure antibody strategies, such as immunoassays, have limitations for. However, if the PTMs are on the epitope or otherwise affect the immunoextraction affinity, this will of course affect the LC-MS method similar to the immunological methods,

highlighting the importance of control of antibody selectivity. It should also be mentioned that non considered alterations pertaining to the signature peptide also may affect the SRM-MS determination.

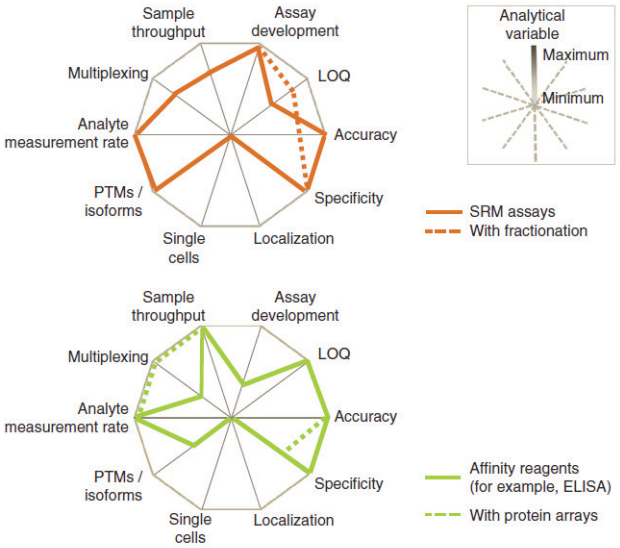


Figure 1.4: Performance profile of SRM- and affinity assays to target protein quantitation. The analytical variables are represented on axes starting from the same point, and the length of a spoke for a variable (from the center) is proportional to the magnitude the variable relative to the maximum magnitude across all the techniques compared. This figure was adapted with permission from reference [163]. Copyright © 2012 Nature Publishing Group

This was proven by Lund et al. in the development of a combined immunocapture SPE LC-SRM-MS approach to distinguish between known hCG isoforms and disease related enzyme-degraded (nicked) variants in which immunoassays are less able to differ between[156, 164]. The approach was additionally used for testing and proving difference in antibody reactivity with six reference regents[165]. The combination immunocapture SPE LC-SRM-MS has by Winther et al. also shown to be an unique tool in the sampling area with the marker ProGRP(31-98)[1] as shown in section 3.2.1.1.

1.2.4 Quantification strategies for SRM

The use of SRM in the area of quantitative MS is at present an established strategy[163, 166]. At the dawning of the field of proteomics, experiments mainly concerned qualitative purposes. The shift towards quantitative experiment may be dated to around the turn of the century and is linked to facilitation of different techniques of stable isotope labeling to allow mass resolution of proteolytic peptides of identical sequences using MS[163]. Further on, the strategies for quantification are generally divided in to two; relative or absolute quantification strategies, with the latter gaining increasing interest and focus. While relative strategies study

the relation of expression of one analyte compared to another within one or between samples, an absolute strategy aim to determine the amount of analyte present in a sample.

The foundation for both strategies is to use quantification based on the signal intensities of specific SRM transitions. To singly use this signal in a so-called label-free quantification is considered challenging to make reproducible, as there can be many causes to fluctuations between analyses. These problems can be tackled by use of synthetically modified imitations of endogenous counterparts, such as the already mentioned stable isotope analogs. Determination of absolute amount of a specific protein using stable isotope dilution (SID) theory and MS was explored decades ago[167, 168], but recent subsequent improvements in MS technology has advanced the implementation of such synthetic markers which is linked to the increasing interest for absolute quantitative approaches.

There are several strategies to involve chemical derivatization of protein or peptide with a synthetic agent for absolute purposes. However, the earliest and perhaps most straight forward approach for absolute quantification is the AQUA (absolute quantification peptide) approach [168-171], using chemically synthesized peptides. These so-called AQUA peptides contain amino acid residues enriched with heavy isotopes to allow mass resolution from the target unlabelled (light) analyte and are added either before or after the proteolytic digest. In addition to similar approaches to the AQUA peptides, other central stable isotope standard alternatives to the approach are the techniques involving introduction of heavy labeled versions of the signature peptides assigned the one or multiple proteins of interest incorporated in proteins. Some of these strategies will now be mentioned. Quantification concatemer termed QconCAT is an artificial protein composed of the different heavy signature peptides[172, 173], while protein epitope signature tag termed PrEST[174] are shorter fragments of the protein produced by the Human Protein Atlas (http://www.proteinatlas.org). Protein standard absolute quantification termed PSAQ[175-177] involves having the entire target protein in stable isotope-labeled form as the internal standard, which also is the design of the full-length expressed stable-isotope labeled proteins for quantification termed FLEXIQuant[178]. However, FLEXIQuant additionally flank the protein analogues with a novel peptide (a heavy labeled FLEX peptide^{H}) for internal calibration against a the non-heavy FLEX peptide^{L},

One of the key-differences between these mentioned SID strategies is where they allow for the IS to be introduced, which is illustrated in Figure 1.5. However, they all have their advantages and disadvantages and all are readily used. The AQUA peptides are commercially available and ready for use, and circumvent some potential issues of uncompleted digestion.

However, the financial cost may limit possibilities of use in extensive multiplexing experiments and the AQUA peptide IS' are generally not compatible with initial fractionation of the biological sample and added close to the digest step (see Figure 1.5). In comparison, the QconCAT obviates the need to handle multiple peptide standards, offer possibility of decreased costs by biosynthesis and is intended to introduce equimolar amounts of undigested signature peptides. As for disadvantages, these proteins can both fail to be expressed and the potential of uncompleted digestion of both the QconCAT IS and target marker must be handled with care. PSAQ is designed to handle differential digestion and may be added prior to even highly selective sample fractionation such as immunocapture (see Figure 1.5). However, the PSAQ IS' are not commercially available and the demand of recourses for production must be weighed against the potential gain of such standards. This because standard proteins usually are expressed heterologously by differing folding and PTMs which may compromise the anticipation of strict stoichiometry and thus limit the benefit of both PSAQ and FLEXIQuant.

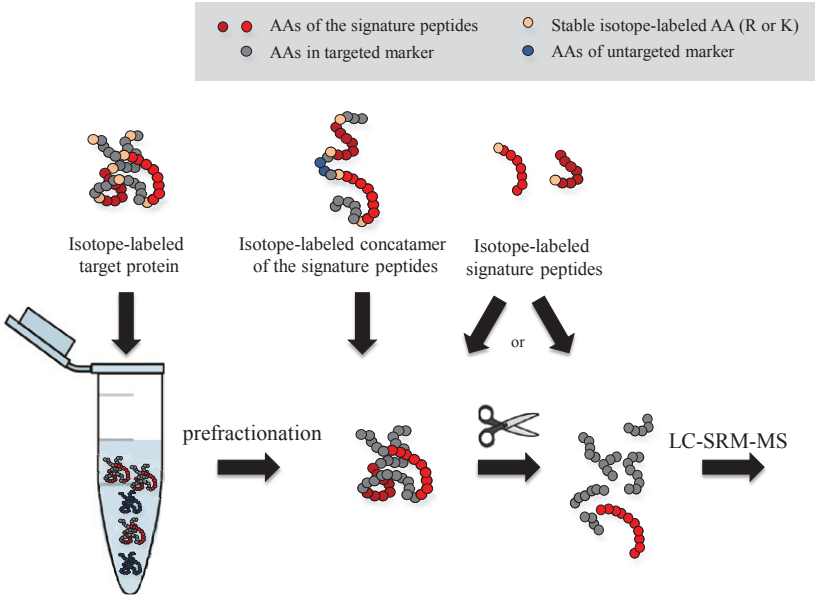


Figure 1.5: The different SID approaches for quantification allow the internal standard to be introduced at different steps of the sample preparation process. The isotope-labeled target proteins (such as PSAQ and FLEXIQuant) are compatible with target marker selective prefractionation and may be added immediately. The isotope-labeled concatemers of the signature peptides (such as QconCAT or PrEST) are generally added subsequent to prefractionation, and before enzymatic digest to pass information of trypsin activity in the generation of signature peptides. The isotope-labeled signature peptides (such as AQUA-peptides) are normally added either before the enzymatic digestion or right before LC-SRM-MS analysis.

2 AIMS OF THE STUDY

In this thesis, the main objective was to demonstrate properties, potential and use of immunocapture LC-SRM-MS methods in a targeted quantitative approach for protein biomarkers. ProGRP and NSE were chosen as model markers with aim of proving usability of this set up as a multiplexing strategy. The goal was to design LC-SRM-MS methods with ability to individually and simultaneously quantify protein isovariants of these SCLC-markers which conventional immunoassays are unable to. In this process, two methods were developed for the two markers and the methods were finally combined.

The following was explored:

- Differentiated quantification of isovariants through assigned signature peptides for two out of three ProGRP isoforms, for total ProGRP and one for each of the two possible subunit for the NSE isoenzymes (*Paper I, III and IV*)
- Establishment of immunocapture procedures to purify and enrich serum samples for the very low abundance markers (*Paper I, III and IV*)
- Validation of two immunocapture LC-SRM-MS methods, one for ProGRP and one for NSE (*Paper I and III*)
- Novel exploration of pathological isoform expression of serum ProGRP in selected neuroendocrine carcinomas (*Paper II*)
- Establishment of a multiplexing method for co-determination of both ProGRP and NSE isovariants (*Paper IV*)
- Comparison of ProGRP and NSE determination by immunocapture LC-SRM-MS against routine clinical assays (*Paper II and IV*)
- Exploration of mechanisms and parameters affecting signature peptide yield from immunocapture (*Paper V*)

3 RESULTS AND DISCUSSION

3.1 IDENTIFICATION AND QUALITATIVE DIFFERENTIATION OF PROGRP ISOFORMS & NSE ISOENZYMES

The potentials of MS based experimental research for use in routine clinical practice has been demonstrated by many. To improve the value of immunocapture LC-SRM-MS for targeting biomarkers in clinical samples, it was preferred that the method should be able to distinguish between the reported variants, the isoforms and isoenzymes, of the SCLC markers ProGRP and NSE at clinical relevant levels. The investigation and designation of signature peptides was for this purpose performed through the following steps (*Paper I and III*):

1) *in silico* investigation of signature peptide candidates
2) investigation of generation and detection of signature peptide candidates
3) optimization of an LC-SRM-MS method for selecting the final signature peptides

3.1.1 Step 1: in silico investigation of signature peptide candidates

The isoforms of ProGRP have large similarities in their structure, as have the isoenzymes of NSE. However, the ProGRP isoforms differ in their C-terminal end, while there are 72 amino acid replacements between the α- and γ-enolase. These differences in primary structure are shown in Table 3.1.

To explore if a tryptic bottom up approach could generate isoform- and isoenzyme specific peptides of appropriate length, an *in silico* experiment was carried out. For this, the computer software ProteinProspector (5.4.2) simulated a tryptic proteolysis of the three isoforms of ProGRP, and the two subunits, α- and γ-enolase, for NSE. From this, a list of peptides with characteristics of no missed tryptic cleavages and with length of at least 6 amino acids was produced. To investigate and identify the marker-specificity, protein BLAST searches were performed on the organisms; homo sapiens, mouse and bovine. The two latter organisms were included in the search because some of the reagents used in the final method derive from these organisms. From the BLAST search, many of the peptides were found to solely origin from the homo sapiens protein markers, and this verified that tryptic proteolysis could produce signature peptide candidates eligible for isoform and isoenzyme differentiation. The step 1-investigated tryptic peptides are listed in Table 3.2.

3.1.2 Step 2: generation and detection of signature peptide candidates

When signature peptide candidates eligible for differentiation were elucidated *in silico*, a real tryptic digest and LC-MS/MS analysis was performed to investigate if these were both generated and detected. *In-solution* digests of the three recombinant ProGRP standards (isoform 1, 2 and 3) and the NSE standards (αα- and γγ-enolase) were separately produced by diluting each protein standard with 50 mM ABC-buffer and adding bovine derived trypsin before over-night incubation at 37˚C. In addition, for the NSE standards, these were also added treated with heat, DTT and IAA prior to trypsin digestion to ensure that their cysteine residues did not interlink and to aid denaturation (*Paper III*). Due to absence of cysteine residues in ProGRP, and in accordance with earlier investigations[179], reduction and alkylation was avoided for ProGRP at this stage (*Paper I*).

The proteins were digested in concentration levels of µg/mL to ensure detection of as many peptides as possible. After an over night incubation at 37˚C, the solution was analyzed on an LC-MS system with an LTQ or an LTQ Orbitrap MS. Separation was carried out on an Aquasil C18 column (50 x 1mm x 3µm). For the final methods an Aquasil C18 precolumn (10 x 1mm x 5um) was also included. A standard linear gradient increasing the ACN-to-20 mM formic acid-ratio from 1:99 (v/v) to 99:1 (v/v) was applied over 60 minutes to get well-separated analytes. The MS was set to perform in a data dependant mode, consisting of two constantly alternating MS events. In event 1 a full scan of all values between 200-1500 *m/z* was performed in MS^1. In event 2, the highest *m/z* value in event 2 was isolated in MS^1, CID produced fragments of selected *m/z* analyte, and a full scan MS^2 of the analyte fragments was acquired. The latter spectra was used for the peptide fingerprinting performed by Proteome Discoverer using the IPI human as search database, as well as for manual confirmation against fragments generated by ProteinProspector (5.4.2). The generated and identified tryptic peptides that coincided as passed marker specific signature peptide candidates in step 1 narrowed the selection of signature peptide candidates, as annotated in Table 3.2. In some cases, missed cleavages were also observed and the tryptic peptides included in these were also discarded as signature peptide candidates.

3.1.3 Step 3: optimization of an LC-SRM-MS method for selecting the final signature peptides

In this step, the aim was to point out the signature peptides which after an *in-solution* digest allowed sensitive and reproducible detection by LC-SRM-MS analysis. Then, the LC-program was adjusted, anticipating the increased complexity of the samples when immunocapture digests was analyzed and also considering the time of analysis.

Initially, the MS, in a SIM mode, was used to investigate which peptides that was readily produced and detected by the QqQ. This involved to alternately allowing analytes of the *m/z* values pertaining to the candidates to transit the MS^1. The peptides producing the highest signals in SIM mode were then further analyzed to elucidate the fragments suited for a final SRM mode. To ensure sensitivity, selectivity and reproducibility, the following preferences were set:

- To ensure sufficient sensitivity of marker determination the fragments showing the highest yield were preferred.
- To reduce background, fragments of higher *m/z* value than the intact parent signature peptide *m/z* were preferably chosen.
- For reliable determination the regarded most stable fragments, the y-fragments, were preferably chosen, and in all cases definite reproducible fragmentation was investigated and assured.
- To ensure peak identity, as well as to set a limit for inferring signals, two to three transitions were chosen for each peptide for dedicating one transition to aid as a quantifier and at least one other transition as a qualifier.

The further investigation involved both automatic and manual tests and adjustments to best meet these criteria, and to optimize the SRM-MS method. For the initial automatic test, SPE was performed on *in-solution* digests of the markers to remove salts and contaminants, to ensure correct pH and to individually infuse the candidates in aliquots by gradual increase of SPE eluate strength. Automatic compound optimization by Xcalibur adjusted multiple MS parameters for a stable and high signal in SRM mode. For both automatic and manual tests, the energy applied in CID was varied between 10-35 V to find the optimum for the transitions. For the manual test, the *in-solution* samples were injected as a normal sample to the LC-MS system and, in both product ion mode and SRM mode, collision energy was finely tuned to give high stable fragment signals.

Based on the described investigation and analyses of *in-solution* samples, signature peptides were selected and confirmed using the final immunocapture LC-SRM-MS method to test yield and stoichiometric relation to parent markers. The signature peptides were chosen to function as specific surrogates for each of the different isovariants of the markers. All the signature peptides are listed in Table 3.1-3.3, and in Table 3.4, with their optimal CID and transitions in SRM mode.

For ProGRP isoforms, the two signature peptides LSAPGSQR and DLVDSLLQVLNVK were assigned for isoform 1 and isoform 3, respectively (*Paper I*). For ProGRP isoform 2, which is very sparsely expressed on mRNA level[47, 48], no signature peptide candidate was considered suited, and an alternative approach for its determination is described later (in section 3.2.2.1). In addition to the assigned signature peptides for the isoforms, the signature peptide NLLGLIEAK was confirmed for total ProGRP, which used to increase sensitivity for ProGRP (see Table 3.5 for LLOQs) (*Paper I*).

For NSE isoenzymes, one signature peptide was assigned each of the two possible subunits for NSE (*Paper III*). Several candidates were eligible; however, the two which best fitted to the listed preferences (see previous page), were chosen. The signature peptide ELPLYR was assigned for γ-enolase, which can derive from either of the two dimeric forms of NSE (αγ or γγ) or the free γ-monomer (see NCBI-BLAST search results in Table 3.4). The signature peptide for α-enolase was TIAPALVSK, which occur both in the NNE-form (αα) and heterodimeric NSE-form (αγ) of human enolases. The immunocapture process with NSE specific anti-γ mAbs would however selectively extract the NSE, involving that the signature peptide could only derive from the α-subunit in heterodimeric NSE.

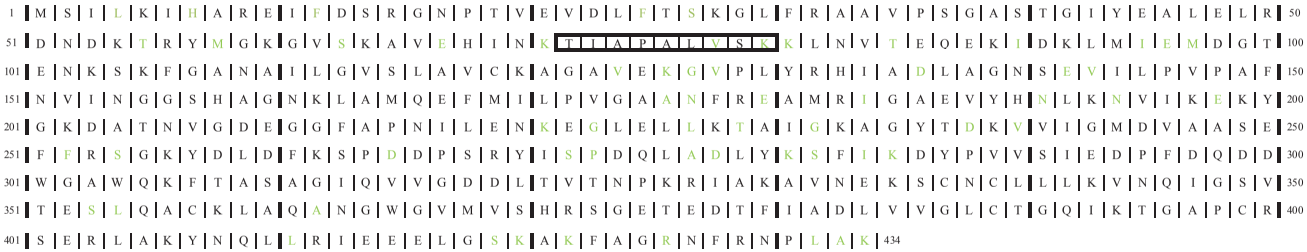
Table 3.1: The primary structures of reported isovariants for ProGRP and NSE. Each letter represents one amino acid residue. The differing AA-residues between the two different subunits (α- and γ-enolase) for NSE isoenzymes and the three isoforms of ProGRP are marked green, and the chosen signature peptides are emphasized by frames. The sequences are according to UniProtKB/Swiss-Prot: P07492 (for all three isoforms), P06733.2 (α-enolase) and P09104.3 (for γ-enolase).

Isoform 1 (-23-125 AA)

Isoform 2 (-23-118 AA)

Isoform 3 (-23-115 AA)

α-enolase (1-434 AA)



γ-enolase (1-434 AA)

Table 3.2: Steps in the process to identify and choose signature peptides for ProGRP. Step 1 lists all *in silico* generated tryptic peptides composed of 6≥amino acid residues. Step 2 lists the variant specific sequences from Step 1 that was detected by LC-MS analyses of tryptic digests. Step 3 lists the chosen signature peptides in **bold**. The sequences marked grey were not regarded eligible in the different steps.

Tryptic peptide	Step1	Step2	Step3
iso1T3[b]	ELPLVLLALVLCLAPR[c]		
iso1T5[b]	AVPLPAGGGTVLTK[c]		
iso1T7[a,b]	GNHWAVGHLMGK		
iso1T9[b]	STGESSSVSER	STGESSSVSER	
iso1T13[a,b]	WEEAAR		
iso1T14[b]	**NLLGLIEAK**	**NLLGLIEAK**	**NLLGLIEAK**
iso1T16[b]	NHQPPQPK[c]		
iso1T17[b]	ALGNQQPSWDSEDSSNFK	ALGNQQPSWDSEDSSNFK[d]	
iso1T21	**LSAPGSQR**	**LSAPGSQR**	**LSAPGSQR**
iso1T23[b]	NPQLNQQ	NPQLNQQ[d]	
Iso2T3[b]	ELPLVLLALVLCLAPR[c]		
Iso2T5[b]	AVPLPAGGGTVLTK[c]		
Iso2T7[ab]	GNHWAVGHLMGK		
iso2T9[b]	STGESSSVSER	STGESSSVSER	
iso2T13[a,b]	WEEAAR		
iso2T14[b]	**NLLGLIEAK**	**NLLGLIEAK**	**NLLGLIEAK**
iso2T16[b]	NHQPPQPK		
iso2T17[b]	ALGNQQPSWDSEDSSNFK	ALGNQQPSWDSEDSSNFK[d]	
iso1T23[b]	NPQLNQQ	NPQLNQQ[d]	
Iso3T3[b]	ELPLVLLALVLCLAPR[c]		
Iso3T5[b]	AVPLPAGGGTVLTK[c]		
Iso3T7[a,b]	GNHWAVGHLMGK		
iso3T9[b]	STGESSSVSER	STGESSSVSER	
iso3T13[b]	WEEAAR		
iso3T14[b]	**NLLGLIEAK**	**NLLGLIEAK**	**NLLGLIEAK**
iso3T16[b]	NHQPPQPK[c]		
iso3T17[b]	ALGNQQPSWDSEDSSNFK	ALGNQQPSWDSEDSSNFK[d]	
iso3T18	**DLVDSLLQVLNVK**	**DLVDSLLQVLNVK**	**DLVDSLLQVLNVK**

Symbol description: [a] not found exclusively in ProGRP deriving from homo sapiens (with swissprot database), [b] not isoform specific, [c] not detected in step 2, [d] detected with missed cleavage**.**

Table 3.3: Steps in the process to identify and choose signature peptides for NSE. Step 1 lists all *in silico* generated tryptic peptides composed of 6≥amino acid residues. Step 2 lists the variant specific sequences from Step 1 that was detected by LC-MS analyses of tryptic digests. Step 3 lists the chosen signature peptides in **bold**. The sequences marked grey were not regarded eligible in the different steps.

Trypticpeptide	Step1	Step2	Step3
αT3[a]	EIFDSR		
αT4	GNPTVEVDLFTSK	GNPTVEVDLFTSK	
αT6[b]	AAVPSGASTGIYEALELR		
αT11	AVEHINK[c]		
αT12	**TIAPALVSK**	**TIAPALVSK**	**TIAPALVSK**
αT14	LNVTEQEK[c]		
αT16	LMIEMDGTENK[c]		
αT18[b]	FGANAILGVSLAVCK		

αT19[a]	AGAVEK		
αT20[a]	GVPLYR		
αT21	HIADLAGNSEVILPVPAFNVINGGSHAGNK	HIADLAGNSEVILPVPAFNVINGGSHAGNK	
αT22	LAMQEFMILPVGAANFR	LAMQEFMILPVGAANFR	
αT24	IGAEVYHNLK	IGAEVYHNLK	
αT28	DATNVGDEGGFAPNILENK	DATNVGDEGGFAPNILENK	
αT29	EGLELLK	EGLELLK	
αT31[a]	AGYTDK		
αT32	VVIGMDVAASEFFR	VVIGMDVAASEFFR	
αT34[b]	YDLDFK		
αT35	SPDDPSR[b]		
αT36	YISPDQLADLYK	YISPDQLADLYK	
αT38	DYPVVSIEDPFDQDDWGAWQK	DYPVVSIEDPFDQDDWGAWQK	
αT39	FTASAGIQVVGDDLTVTNPK	FTASAGIQVVGDDLTVTNPK	
αT43	SCNCLLLK[c]		
αT44	VNQIGSVTESLQACK[c]		
αT45	LAQANGWGVMVSHR	LAQANGWGVMVSHR	
αT46[a]	SGETEDTFIADLVVGLCTGQIK		
αT50	YNQLLR	YNQLLR	
αT51	IEEELGSK	IEEELGSK	
γT3[a]	EILDSR		
γT4	GNPTVEVDLYTAK	GNPTVEVDLYTAK	
γT6[b]	AAVPSGASTGIYEALELR[c]		
γT11	AVDHINSTIAPALISSGLSVVEQEK[c]		
γT12	LDNLMLELDGTENK[c]		
γT14[b]	FGANAILGVSLAVCK		
γT15[a]	AGAAER		
γT16	**ELPLYR**	**ELPLYR**	**ELPLYR**
γT17	HIAQLAGNSDLILPVPAFNVINGGSHAGNK[c]		
γT18	LAMQEFMILPVGAESFR	LAMQEFMILPVGAESFR	
γT20	LGAEVYHTLK	LGAEVYHTLK	
γT24	DATNVGDEGGFAPNILENSEALELVK[c]		
γT26[a]	AGYTEK		
γT27	IVIGMDVAASEFYR	IVIGMDVAASEFYR	
γT29[b]	YDLDFK		
γT30	SPTDPSR[c]		
γT31	YITGDQLGALYQDFVR	YITGDQLGALYQDFVR	
γT32	DYPVVSIEDPFDQDDWAAWSK[c]		
γT33	FTANVGIQIVGDDLTVTNPK	FTANVGIQIVGDDLTVTNPK	
γT37[a]	ACNCLLLK		
γT38	VNQIGSVTEAIQACK[c]		
γT39	LAQENGWGVMVSHR[c]		
γT40[a]	SGETEDTFIADLVVGLCTGQIK		
γT41[a]	TGAPCR		
γT44[a]	YNQLMR		
γT45	IEEELGDEAR	IEEELGDEAR	
γT46	FAGHNFR	FAGHNFR	

Symbol description: [a] not found exclusively in NSE-subunits α- or γ-enolase (homo sapiens as only searched organism, with swissprot database), [b] not isoenzyme specific, [c] not detected in step 2.

Table 3.4: Summary of the selected signature peptides and internal standards. The table includes the possible origins, E-values from the NCBI-BLAST search, occurrence in other proteins and the final MS parameters for the chosen peptides.

Peptide	Origin	E-value[a]	Occurs in other human, bovine or mouse proteins	CE[b] (V)	Fragment transitions
TIAPALVSK	α-enolase in Homo sapiens	5E-04	No	16 16	450.6 → 614.4 (y_6)[c] 450.6 → 685.4 (y_7)[d]
ELPLYR	γ-enolase in Homo sapiens	2E-02	No	14 14	395.7 → 274.7 ($y4^{+2}$)[c] 395.7 → 548.3 (y4)[d]
ELPLY[R_$^{13}C_6$_$^{15}N_2$]	N.A.	N.A.	No	14 14	401.0→279.5 ($y4^{+2}$)[c] 401.0→544.4 (y4) [d]
LSAPGSQR	preProGRP isoform 1 in Homo sapiens	6E-03	No	17 14	408.2 → 272.6 (y_5^{2+})[c] 408.2 → 544.4 (y_5)[d]
NLLGLIEAK	preProGRP isoforms 1, 2 and 3 in Homo sapiens	4E-04	No	15 16	485.8 → 630.3 (y_6)[c] 485.8 → 743.4 (y_7)[d]
NLLGLIEA[K_$^{13}C_6{}^{15}N_2$]	N.A.	N.A.	No	15 16	489.9 → 638.3 (y_6)[c] 489.9 → 751.4 (y_7)[d]
DLVDSLLQVLNVK	preProGRP isoform 3 in Homo sapiens	2E-08	No	34 29 18	728.6 → 200.8 (a_2)[c] 728.6 → 228.8 (b_2)[c] 728.6 → 359.9 (y_3)[d]

Symbol description: [a] From NCBI-BLAST search. [b] Collision energy, [c] Quantifier transition. [d] Qualifier transition. N.A.: Not applicable.

3.2 FURTHER OPTIMIZING THE LC-SRM-MS METHODS FOR BIOLOGICAL SAMPLES

3.2.1 Beads-aided immunocapture prior to MS analysis

Targeted sample preparation by immunoaffinity extraction (IAE), often termed immunocapture in some designs, was aimed to be the sample preparation of choice to use for clinical serum samples. This highly selective samples preparation approach can both aid enrichment which may be necessary for the markers occurring at very low concentration levels, and enable simultaneous capture of several markers (multiplexing). Former extensive and thorough investigation of several mAbs with different properties for ProGRP[180, 181] and NSE[138] led to the choice of two antibodies; mAb E146 for ProGRP (also termed anti-ProGRP, *Paper I*) which binds to aa 48-52 as shown by epitope study[180], and mAb E21 (also termed anti-γ and anti-γ enolase, *Paper III*) with selective affinity for γ-enolase, with binding epitope

close to aa 416-433 (see Table 3.1 for primary structure). In a few experiments mAb anti-ENO1 supplied by Abcam (also termed anti-α or anti-α enolase), with selective affinity for α-enolase (for which the epitope is not reported), was used (*Paper V*).

3.2.1.1 ProGRP

The approach of using immunocapture for extraction of ProGRP from serum as sample preparation for the ProGRP(31-98)-standard was evaluated by Winther et al., who also investigated alternative sample preparations in form of using monoclonal imprinted polymers (MIP, unpublished data) and protein precipitation (PPT)[182]. The immunocapture format was mAb E146-coated microtiter plates with 96-wells which was used to extract ProGRP from serum followed by SPE and LC-MS analysis. Because the well-format limited the sample volume to 200 µL[1], *Paper I* introduced a modified approach using mAb E146-coated magnetic beads where a higher sample volume could be used, as illustrated in Figure 3.1. This new approach was used to extract recombinant ProGRP standards for ProGRP isoform 1, 2 and 3 from 1000 µL serum instead of the ProGRP(31-98)-standard from 200 µL. Selected chromatograms obtained by use of these three methods with different sample preparations (PPT, in-well immunocapture and on-beads immunocapture) are illustrated in Figure 3.2 showing superiority of the use of immunocapture in a beads design for serum samples.

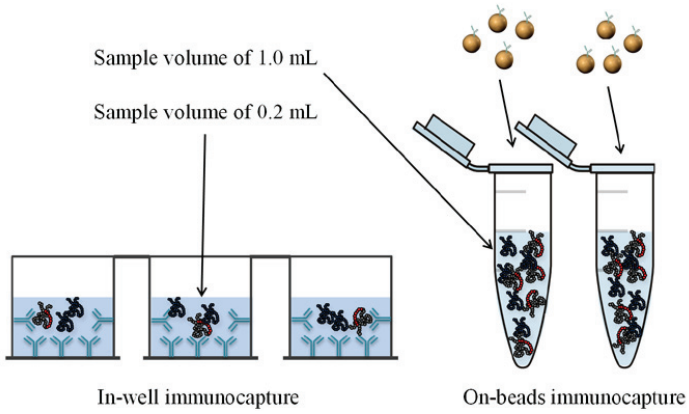


Figure 3.1: An illustration of the immunocapture approaches in the in-well and on-beads formats. The in-well format is restricted to the size of the wells, and in addition the wells are generally only coated with one antibody. For the magnetic beads, different aliquots can be coated separately with their different antibodies, and thus, without challenging the coating process, offer a more flexible format for multiplexing purposes. In addition, this format serves flexibility in consumption of sample volume and possibilities for enrichment.

The beads compared to the wells design improved sensitivity due to higher sample volume and increased enrichment factor, as well as sufficient purification to circumvent the time- and

labor consuming SPE-step. The well immunocapture LC-MS approach reported LODs and LLOQs of 200 and 330 pg/mL[1], respectively, for determination of ProGRP (31-98) in serum. This does not include the reference limit for healthy endogenous levels, which is reported to be 58.9 pg/mL at a 97.5 percentile, estimated using the standard ProGRP (31-98) corresponding to about 7.2 pM ProGRP[181]. While for the beads-design immunocapture LC-MS approach presented in this thesis, the achieved LODs and LLOQs where 1 and 10 pM (*Paper I and II*), respectively, (corresponding to about 8 and 82 pg/mL of ProGRP (31-98)). These limits are below the reported cut-off value (for positive classification of SCLC versus non-small cell lung cancers and benign lung diseases at >95% specificity) for the TR-IFMA being 10.3 pM (85 pg/mL)[183].

The method allowed not only determination total ProGRP, but also simultaneous quantification of isoform 1 and isoform 3. *Paper I* described this possibility to, for the first time, measure these isoforms. The novel ability to differentiate between these different forms of ProGRP now offers a tool to investigate if they hold individual clinical information (see section 3.4.1.1). As mentioned earlier (under chapter 3.1), no specific tryptic peptides for isoform 2 was found, which rendered direct determination of this isoform impossible, whilst an indirect determination will be described in section 3.2.2.1.

3.2.1.2 NSE

For NSE, no prior investigation of immunocapture has been performed. However, a method strategy for plasma samples was earlier tested in a master thesis by Lund[184]. In this design protein precipitation, tryptic digest and on-line RAM-trap with back-flushing on to a LC-MS system showed neither reproducible nor sufficient sensitive detection. The beads-design immunocapture LC-MS approach for NSE (*Paper III*), however, passed the validation (see chapter 3.3) for quantification of γ-NSE with LOD and LLOQ of 11 and 38 pg/mL, respectively (corresponding to about 0.3 and 0.8 pM of γ-enolase with Mw of 48 kDa). These limits are well below the reported reference levels for NSE which varies between 7-20 ng/mL[185, 186]. Selected chromatograms obtained by use of these two methods with different sample preparations (PPT and on-beads immunocapture) are illustrated in Figure 3.3 where the superior serum samples clean-up by use of immunocapture is evident.

Another quality of the immunocapture MS method can be seen from Figure 3.3; in addition to determine the γ-enolase, the method may also determine the α-enolase. Antibodies for immunometric assays for NSE may, in comparison, have different affinities for

NSE-isoenzymes (see 1.2.2.3); however, to the writers knowledge, no assay can simultaneous differentiate between them. The immunocapture MS method will also be unable to distinguish between γγ- and γ-enolase, but by measuring α-enolase, it may give knowledge about the amount of αγ-enolase in a sample (see section 3.4.1.2 and 3.4.2.2). The determination of α-enolase was an indirect estimation which is described under section 3.2.2.2.

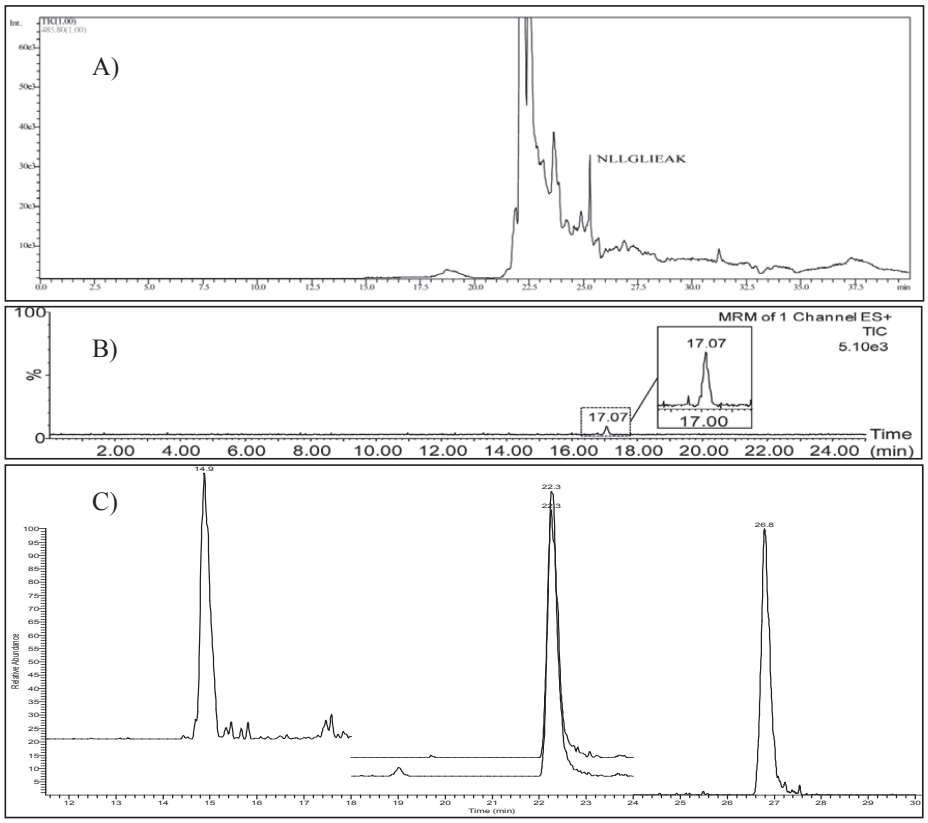


Figure 3.2: Chromatograms from LC-MS analysis of different sample preparations of serum samples being either patient serum samples or healthy donor serum which was added ProGRP-standards.

- **A)** The analyzed sample was a 1 mL healthy serum sample with added ProGRP (31–98)-standard to give a concentration of 30 ng/mL. The method set up was PPT-RAM LC-MS with determination in SIM mode (*m/z* 485.8) with reported LOD of 1500 pg/mL. This chromatogram is adapted with permission from reference [179]. Copyright © 2007 WILEY-VCH Verlag GmbH & Co.
- **B)** The analyzed sample was patient serum with concentration of 720 pg/mL ProGRP. The method set up was in-well immunocapture of 0.2 mL serum sample, and LC-MS determination in SRM mode (*m/z* 486.01 → 743.74) with reported LOD of 200 pg/mL. This chromatogram is adapted with permission from reference [1]. Copyright © 2009 WILEY-VCH Verlag GmbH & Co.
- **C)** The analyzed serum sample was from a SCLC patient with total ProGRP concentration of 2318 pg/mL (a sample from *Paper I*). The method set up was on-beads immunocapture of 1 mL sample, and LC-MS determination in SRM mode of LSAPGSQR (*m/z* 408.2→544.4) at 14.9 min, DLVDSLLQVLNVK (*m/z* 728.6→359.9) at 26.8 min, NLLGLIEA[K_ $^{13}C_6{}^{15}N_2$] (*m/z* 489.9→751.4) at 22.3 min (top peak), and NLLGLIEAK (*m/z* 485.8→743.4) at 22.3 min (bottom peak), with reported LOD of 8 pg/mL for the total ProGRP (*Paper I*).

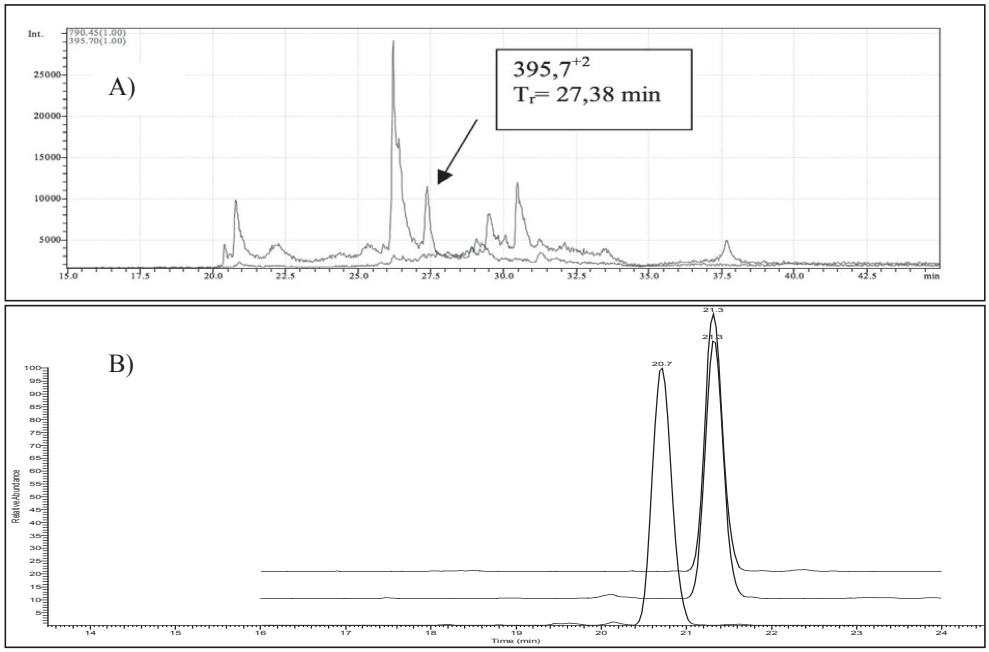


Figure 3.3: Chromatograms from LC-MS analysis of two different sample preparations of serum samples with endogenous NSE.

A) The analyzed patient serum sample had RIA-determined NSE concentration of 2350 ng/mL. The method set up is PPT-RAM LC-MS with detection in SIM mode of γ-enolase (*m/z* 395.7 for ELPLYR) of a 1 mL serum sample with no reported LOD due to irreproducibility of the trypsin step. This chromatogram is adapted with permission from reference [184]. Copyright © 2006 Hanne Lund.

B) The analyzed serum sample was from a healthy donor with normal concentration levels of about 14 ng/mL. The method set up (from *Paper IV*) was on-beads immunocapture of 1 mL sample, and LC-MS determination in SRM mode of TIAPALVSK (*m/z* 450.2→685.4) at 20.7 min, ELPLY[R_$^{13}C_6$_$^{15}N_2$] (*m/z* 401.0→558.3) at 21.3 min (top peak), and ELPLYR (*m/z* 395.7→548.3) at 21.3 min (bottom peak), with reported LOD of 11 pg/mL for the γ-subunit of NSE.

To conclude; the advantage of immunocapture over protein precipitation was demonstrated for both markers. The benefits of using antibody based extraction by magnetic beads of these markers are that: 1) the selective antibodies can ensure a high degree of purification, 2) the flexibility in final added solution ensures downstream compatibility with trypsin digestion conditions, 3) use of larger sample volume and simultaneous enrichment can be arranged, 4) the strategy is applicable to a selection of specified markers which is favorable when aiming for a multiplexing method.

3.2.2 Determining ProGRP isoform 2 and α-NSE

The methods for ProGRP and NSE as described in section 3.2.1.1 and 3.2.1.2, respectively, allow us to measure:

- ProGRPs isoform 1 and isoform 3 as well as the total ProGRP
- γ-enolase from NSE

A shortcoming was the inability to determine isoform 2 of ProGRP and the α-subunit of NSE, directly. For the determination of these two variants, modifications in modes of conduct, compared to the other marker variants, was necessary for determination. This will now be described.

3.2.2.1 The isoform 2 of ProGRP

The lack of signature peptide for ProGRP isoform 2demanded an alternative approach for its determination. For this, the feasibility of an indirect measurement was investigated through a blinded experiment with added standards of each of the three recombinant ProGRP isoforms (*Paper I*). This determination was based on the assumption that ProGRP only consisted of the three isoforms (isoform 1, 2, and 3, see section 1.1.2.1 and chapter 3.1) and that the contribution to the signal of signature peptide for total ProGRP (NLLGLIEAK) was equal for each of the ProGRP isoforms using the immunocapture SRM method. The assumptions founded Equation I, which show that the amount of ProGRP isoform 2 was calculated from subtracting the measured amount of two other ProGRP isoforms from the measured amount of total ProGRP.

Equation I: $[\text{ProGRP isoform 2}] = [\text{total ProGRP}] - [\text{ProGRP isoform 1}] - [\text{ProGRP isoform 3}]$

The indirect determination was evaluated by its ability to determine relative presence of this isoform in the sample compared to the other isoforms. The correlation between added and measured relative amounts was considered to be acceptable and to be the same for the three isoform determinations, though deviations was observed (*Paper I*). This indirect determination was however not used for patient samples.

3.2.2.2 The α-subunit of NSE

For NSE, it was not possible to determine the amount of α-enolase from its heterodimer (αγ) directly due to standard instability (*Paper V*). This heterodimer standard, the αγ-standard, was obtained from ion-exchange chromatography of human brain homogenate, as described elsewhere[187]. The heterodimer was attempted applied as a standard as it would allow

determination of both the α- and γ-subunit in a straightforward manner by use of one single standard. Analyses of a single immunocapture of the heterodimer standard from an ABC-buffer solution showed presence of both subunits (see Figure 3.4); however, the non-covalently linked subunits of this heterodimer proved to be dissociated, and could therefore not be used as a standard when using the immunocapture method which relays on one of the subunits. This was concluded after performing extraction of the αγ-standard added to ABC buffer solution using anti-γ coated magnetic beads and separately digesting the beads-bound fraction and the not-extracted markers left in the ABC buffer solution shown in Figure 3.4. The figure shows the relative yield for these two conditions in comparison to an *in-solution* digest. Here, the signature peptides for the two enolases show that a large degree of α-subunit and very little γ-subunit was left in the solution implying that storage had impaired the standard to dissociate the subunits.

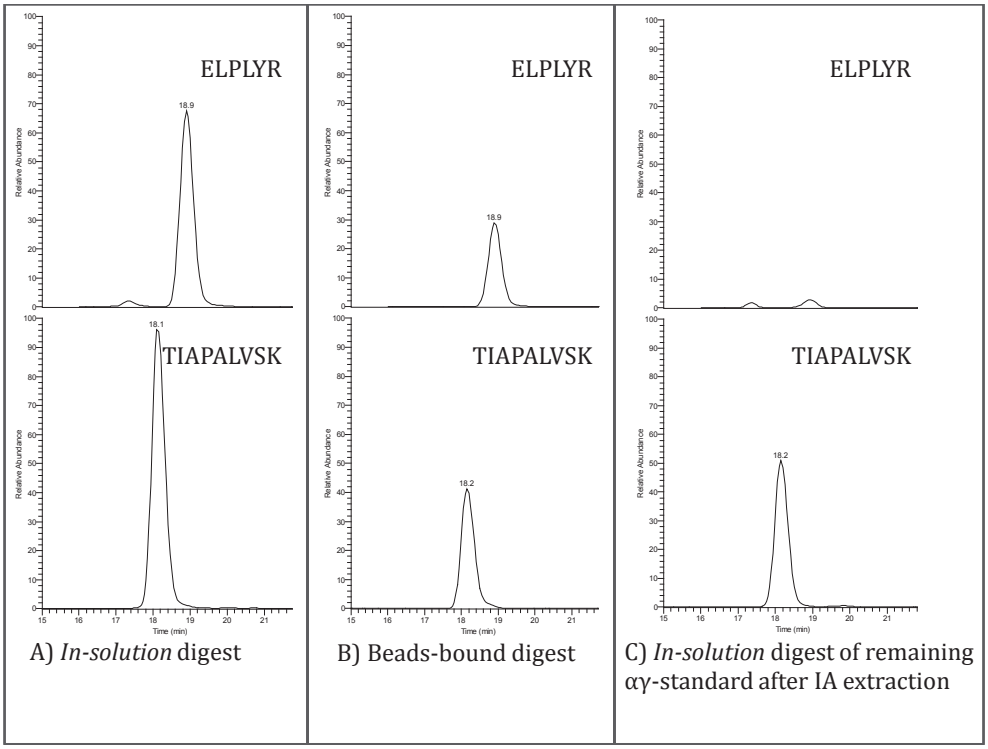


Figure 3.4: Different digests of a αγ-calibrator prove instability of the standard. In **A)** a solution of ABC-buffer added αγ-standard was digested, in **B)** an identical solution was added anti-γ coated magnetic beads and the captured (beads-bound) markers were digested, and in **C)** the solution of remaining non-bound markers was digested. The scale of the y-axis is similar for all the chromatograms.

As the intended standard was considered unsuited, alternative approaches were considered. The approach of choice was an indirect determination of heterodimer NSE, where the calibration curve for γ-enolase was used to estimate α-enolase (*Paper IV*). In this process, the calibration curve for γ-enolase and α-enolase was harmonized by obtaining the average ratio of the calibration curves of an *in-solution* digest of the αα-standard and γγ-standard.

This resulted in an average ratio of TIAPALVASK:ELPLYR close to 1:0.8 implying that the yield of both peptides, and thus a combination of both tryptic digestion completeness and ionization efficiency of these, were comparable (see Figure 3.4). By assuming similar signature peptide yield from the immunocapture process, the γ- signature peptide standard curve may be applied for an indirect estimate of the α-enolase concentration. The calibration curve for γ-signature peptide was thus used as a surrogate by modifying the signal for α-signature peptide by multiplying it with the ratio factor. Ultimately, this approach was used to estimate the level of α-enolase in patient samples as shown in section 3.3.3.1. It should be noted that later experiments indicated that the immunocapture process may affect production signature peptide (*Paper V*); and thus have effects for such a determination. This will briefly be discussed in section 3.3.3.1.

3.3 EVALUATION OF THE QUANTITATIVE METHODS

The main objective of method validation is to demonstrate the reliability of a particular method for the determination of an analyte concentration in a specific biological matrix. Two validations were performed using the ICH-guideline *Validation of Analytical Procedures*[188] and EMA's *Guideline on bioanalytical method validation*[189] as guides (*Paper I and III*). The validations were carried out to evaluate the two individual method's ability to 1.) Determine both the concentration of ProGRP isoforms 1 and 3, and the total ProGRP concentration (*Paper I*), and 2.) Determine the concentration of the γ-subunit and to identify the α-subunit of NSE (*Paper III*). Ultimately, the extraction and determination was performed simultaneously involving a fusion of the two validated methods into one multiplexing method. This method was evaluated based on comparison with the two validated methods (*Paper IV*).

3.3.1 Performance parameters

From EMA's guideline[189] the following main characteristics is stressed to be essential to ensure the acceptability of the performance and the reliability of analytical results of a

bioanalytical method: selectivity, lower limit of quantification (LLOQ), the response function and calibration range, accuracy, precision, matrix effects, and stability of the analytes. The tested validation parameters and obtained values are listed in Table 3.5, which show that the methods pass strict validation criteria. The conditions of validation and evaluation are described in section 3.3.2 and 3.3.3.

As Table 3.5 show, LODs and LLOQs for the two isoforms did not reach the concentration limits for healthy serum samples, but the LOD for total ProGRP at 1 pM did. This is as also shown in Figure 3.5. This limit would theoretically allow a LLOQ of 3 pM, however, due to demand for sufficient accuracy and precision the obtained LLOQ was 13 pM in *Paper I*, though revised to be 10 pM in *Paper II*.

Table 3.5: Validation parameters for the ProGRP- and the NSE-methods. The overview is a reproduction of information from *Paper I and III*.

Parameters for validation

Protein	*Sample matrix*	*Range*	*LOD*	*LLOQ*	R^2		*Precision (RSD%)*		*Accuracy (bias%)*
							Intraday	Interday	
ProGRP isoform 1	ProGRP-depleted serum	35-3468 pM	10 pM	35 pM	0.983	LLOQ	10%	19%	9%
						MQ	11%	20%	5%
						HLOQ	8%	26%	1%
ProGRP isoform 3	ProGRP-depleted serum	20-2048 pM	5 pM	20 pM	0.977	LLOQ	9%	10%	23%
						MQ	32%	12%	13%
						HLOQ	9%	25%	4%
Total ProGRP	ProGRP-depleted serum	10-7631 pM	1 pM	10 pM*	0.974	LLOQ	6%	21%	25%
						MQ	11%	14%	2%
						HLOQ	9%	33%	7%
γ-subunit of NSE	5% BSA	5-500 ng/mL	11 pg/mL	38 pg/mL	0.999	LQ	10%	20%	4%
						MQ	3%	20%	9%
						HQ	13%	4%	1%

Protein	*Sample matrix*		*Freeze-thaw stability*			*Bench-top stability*
			Cycle 1	Cycle 2	Cycle 3	4 hours
ProGRP isoform 1	Healthy donor serum	LQ	98%	84%	81%	98%
		HQ	111%	97%	74%	86%
ProGRP isoform 3	Healthy donor serum	LQ	52%	66%	61%	52%
		HQ	97%	85%	84%	88%
Total ProGRP	Healthy donor serum	LQ	65%	72%	74%	87%
		HQ	91%	95%	103%	97%
γ-subunit of NSE	Healthy donor serum	-	102%	106%	108%	88%
	5% BSA	LQ	83%	85%	81%	102%
		HQ	88%	97%	90%	101%

* *LLOQ from* Paper II. BSA: Bovine serum albumin

3.3.2 Selectivity, choice of matrix & choice of internal standard

The *selectivity* was extensively tested and assured by the assigned signature peptides determined by the presented LC-MS method in SRM mode combined with the highly selective antibody based sample preparation approach. The LC method was adjusted to separate the peaks from the signature peptides to allow for MS segments, as well as to avoid co-elution with inferring compounds deriving from the beads-extraction of the different samples.

The calibration standards were created by adding standard solutions of the markers to the chosen *matrixes*. See Table 3.6 for the relevant matrixes for the different methods. Both validation guides[188, 189] recommend to aspire use of a identical or similar blank matrix as the biological matrix spiked with the reference standards for preparation of calibration standards, quality control samples and stability samples, and to investigate for matrix effects.

For ProGRP, healthy donor serum was attempted as a blank matrix, but as endogenous ProGRP could be detected in these samples, the serum needed to be depleted for ProGRP to serve as a blank matrix, as can be seen in Figure 3.5. The depletion was performed by performing an extraction of endogenous ProGRP with immunocapture using the E146 mAb coated beads. This resulting ProGRP-depleted serum was ultimately was used as calibration matrix for the validated method (*Paper I*).

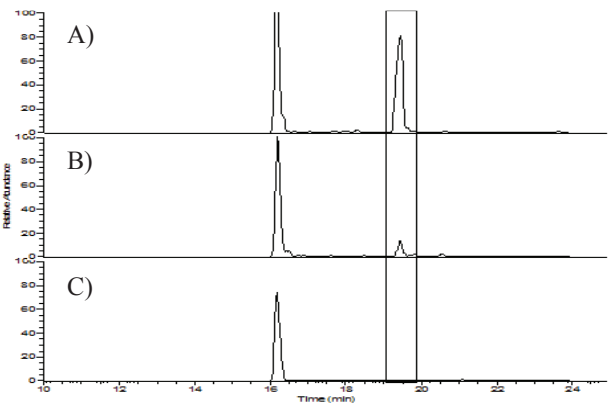


Figure 3.5: ProGRP detected in serum from healthy donor. The immunocapture MS method for ProGRP performed on: **A)** ProGRP added to healthy serum (500 pM), **B)** healthy donor serum, and **C)** ProGRP-depleted serum. The signature peptide for total ProGRP was detected in the healthy sample (B), but not in the ProGRP-depleted sample (C).

Endogenous NSE was also present in serum from healthy donors. However, as several cycles to attempt to deplete entirely for the marker did not create a blank matrix (performed as for ProGRP-depletion of healthy serum), a 5 % bovine serum albumin (BSA) solution was chosen as calibration matrix (*Paper III*).

Firstly, it was assured that no inferring peaks were found in LC-MS methods. For this, immunocapture of "blank samples" of the relevant calibration matrixes (samples not added standards) was performed to investigate ProGRP-depleted serum for ProGRP signature peptides and 5 % BSA for NSE signature peptides. For the test of *matrix effects*, post-column infusion of the heavy internal standards was performed for analyses of serum samples from healthy donors. The AQUA peptides NLLGLIEA[K_$^{13}C_6{}^{15}N_2$] (*Paper I*) and ELPLY[R_$^{13}C_6$_$^{15}N_2$] (*Paper III*) as surrogates for their light variant as it was assumed that the heavy version of a signature peptide would have the same ionization properties as its light signature peptide. Both methods passed this test as the signal did not change around their respective retention time. Matrix effects for the other signature peptides were not directly evaluated; however, they were indirectly evaluated by testing of linearity and accuracy, as well as by monitoring of agreement of parallel.

For the absolute quantification methods, the AQUA peptides were chosen as *internal standard* due to commercially availability and the possibility of quick and easy implementation. It is commented that incomplete digestion of the target protein[190, 191], partial modification of the target peptide or partial loss of the synthetic peptide before addition can affect the accuracy of such an approach. However, for these method designs this is not a relevant weakness, as the internal standards are merely used to correct for variance caused by the auto injector or the MS and not for direct quantification purposes.

3.3.3 The merging of two methods to demonstrate multiplexing potential

One of the aims of this thesis was to merge the ProGRP method and NSE method. The purpose was to exemplify the strength of the combination of immunocapture LC-MS by establishment of one single multiplexing method. The aim was both to improve throughput and utilization of the available sample without compromising on the separate methods performance. Such a method should have higher diagnostic accuracy (see section 1.2.1) than the individual methods due to simultaneous measurement of ProGRP and NSE, and thus be a more valuable diagnostic tool for SCLC-marker determination.

The method for co-determining ProGRP isoforms & NSE isoenzymes was not validated itself, however evaluated based on the full validations of the two individual methods (*Paper I and III*). In the merging process, some alterations were necessary as a few conditions differed between the methods (see Table 3.6). The effects of these modifications were evaluated (*Paper IV and V*) and will be addressed in these subsequent sections.

Table 3.6: Key parameters form the immunocapture MS methods. Three methods were developed for determination of each or both ProGRP and NSE. The main differing parameters between them are in *italic* and underlined.

	***ProGRP-method* (*Paper I*)**	***NSE-method* (*Paper IV*)**	***Combined NSE and ProGRP method* (*Paper IV*)**
Quantifiable markers	ProGRP: isoform 1, isoform 3, & total ProGRP	NSE: the γ-subunit	ProGRP: isoform 1, isoform 3, & total ProGRP NSE: *the α-* & the γ-subunit
Sample matrix	ProGRP-depleted serum	*5% BSA*	ProGRP-depleted serum
Immunocapture	mAbE146-coated magnetic beads	mAbE21-coated magnetic beads	mAbE146-coated magnetic beads *plus* mAbE21-coated magnetic beads
Post-immunocapture treatment	*Trypsin digest*	Reduction heat & alkylation, trypsin digest	Reduction, heat & alkylation, trypsin digest
LC-MS/MS	Aquasil C18 column, standard gradient elution with 40 µL/min flow at 30℃, ESI-SRM-MS in positive mode	Aquasil C18 column, standard gradient elution with 40 µL/min flow at 30℃, ESI-SRM-MS in positive mode	Aquasil C18 column, *2-step gradient elution with 45 µL/min flow at 45℃*, ESI-SRM-MS in positive mode

3.3.3.1 Choice of extraction matrix and its implications for NSE determination

As NSE and ProGRP were to be extracted from the same sample, the intended approach was to add both anti-NSE and anti-ProGRP beads to the patient sample. It was also found convenient to use the same calibration sample and thus *identical calibration matrix* for both markers. ProGRP-depleted serum was chosen as sample matrix to produce the calibration curves. This had no implications for the initial calibration approach for ProGRP; however, for γ-NSE a standard addition variant of the calibration became necessary (*Paper IV*).

For the determination of the patient samples, the determination involved a calibration regression where the *contribution of endogenous γ-NSE* in the calibration matrix had to be considered. From the standard linear regression (see Equation II and Figure 3.6 A), where x is set as the concentration of *added γ-NSE standard*, this endogenous γ-NSE contribution is found by extrapolating the produced regression and setting the y-value to zero (given by the symbol d in Equation III). For the patient samples, the endogenous contribution needed to be added to the estimated concentration from the standard linear regression. In the case of the

calibration matrix in Figure 3.6, this endogenous contribution was: $\frac{0.7727}{0.05755} = 13.4$ ng/mL. The linear equation can in principle thus be shifted to go through origo to be used for patient samples. This involve using the same slope, a, and setting the constant term, b, to zero for Equation II .

In addition, as introduced in section 3.2.2.2, an estimation of α-subunit from heterodimer NSE was included by adjusting and using the calibration curve for γ-enolase based on an *in-solution* digest of NSE standards giving the approximately ratio of 1:0.8 between the signature peptides TIAPALVASK and ELPLYR, respectively. By making assumptions of similar signature peptide production under immunocapture conditions of the different isoenzymes of NSE, the calibration curve for γ-NSE could be used for estimation of concentration of α-NSE. Equation IV shows the calculated adjustment of the signal for the α-signature peptide, given by the symbol w, with the ratio factor. This y-value could then be used for the calibration curve for γ-enolase where the regression must be shifted to go through origo as described above.

Equation II: $y = ax + b$

Equation III: $d = \frac{b}{a}$

Equation IV: $y = w \times 0.8$

This indirect estimation of α-enolase may be a valuable, though the assumption of similar signature peptide production under immunocapture conditions of the different isoenzymes of NSE may be incorrect. In addition, though it is also is a necessary assumption of similar γ-signature peptide production from monomeric, homodimeric and heterodimeric NSE, this may also not be the case. In *Paper V* it was found that immunocapture of γγ-standard gave lower measurements of ELPLYR compared to non-captured. The assumed reason was steric hindrance of the mAb-bound γγ-homodimer caused antibody-hampered trypsin availability. Different measures to reduce this steric hindrance of trypsin, was tested for the αγ-standard of NSE. The effect on the γ-subunit was here larger than for the α-subunit, indicating that that signature peptide production from the bound γ-subunit may be hampered by the trypsin availability to larger degree than the unbound. Different signature peptide contribution from the two subunits of the homodimeric and the monomeric form of NSE will have implications in terms of uncertainty for both the determination of total γ-enolase by the γ-signature peptide, as well as to add to the uncertainty of indirect determination of α-NSE which relies on a set ratio between the signature peptides of the two subunits.

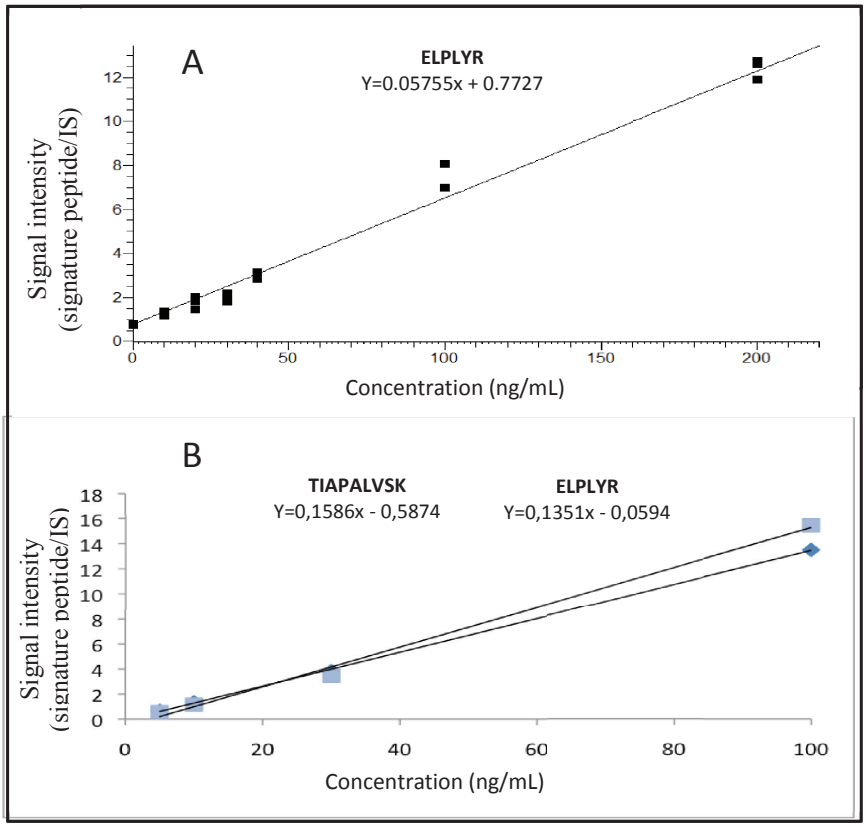


Figure 3.6: Calibration regressions A) immunocapture MS method performed on ProGRP-depleted which was added γγ- standard, and B) in-solution digestions of αα- and γγ- standards. The x-axis annotates the added amount of standard. The y-axis annotates the measured signature peptide to internal standard-ratio.

3.3.3.2 Presence of two different mAb beads and varying levels of individual markers

Addition of differing mAb coated beads, as well as differing levels of the other marker, could theoretically have an effect on both or either the immunocapture extraction and the tryptic yield. Three experiments were performed to investigate these matters (*Paper IV*).

Firstly, the effect of introducing different mAb coated beads than used for the target marker was tested for both immunocapture extraction from human serum and 5% BSA. The relative yields obtained from the simultaneous extraction and LC-MC determination against the yields from use of the separate methods are shown in Figure 3.7. This indicates minimal effect of co-extraction and trypsin activity in the presence of magnetic extraction beads for the other marker, as well as implies non-altered MS signal and no matrix effects when introducing IA extraction beads for the other marker.s

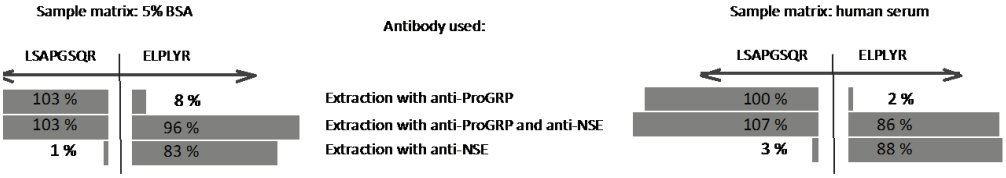


Figure 3.7: Yield after immunocapture with varying presence of ProGRP isoform 1 and γγ-NSE as well as varying presence of anti-ProGRP coated magnetic beads and anti-NSE coated magnetic beads. ProGRP and NSE were individually and simultaneously extracted from both 5% BSA or ProGRP depleted serum and digested with different anti-marker coated magnetic beads. The bars show the signature peptide yield relative to the extraction yield from a sample added only the single marker and extracted with its respective antibody (see supplementary data for *Paper IV*)

Secondly, to further test the effect of co-extraction on signature peptide yield, the level of the other marker was varied and an unpaired *t*-test was performed on the results. The H_0 hypothesis was no effect on yield of signature peptide signal by varying the concentration of the other marker. Two stagnant concentration levels (for the values see *Paper IV*) of each marker were tested against two different concentrations of the other marker. The H_0 hypothesis was not rejected ($P>0.064$, $\alpha=0.05$) which indicated that immunocapture of each marker is unaffected by various levels of the other marker.

As a final test, the linearity for each signature peptide measurement, in cases of both constant and varying levels of the other marker were produced, as shown in Figure 3.8. These had comparable calibration regressions, and good values for linearity ($r^2>0.970$). Together, the experiments proved that neither introduction of the others markers IA extraction beads nor high amounts of the other marker affected their determination. These results also imply no matrix effects.

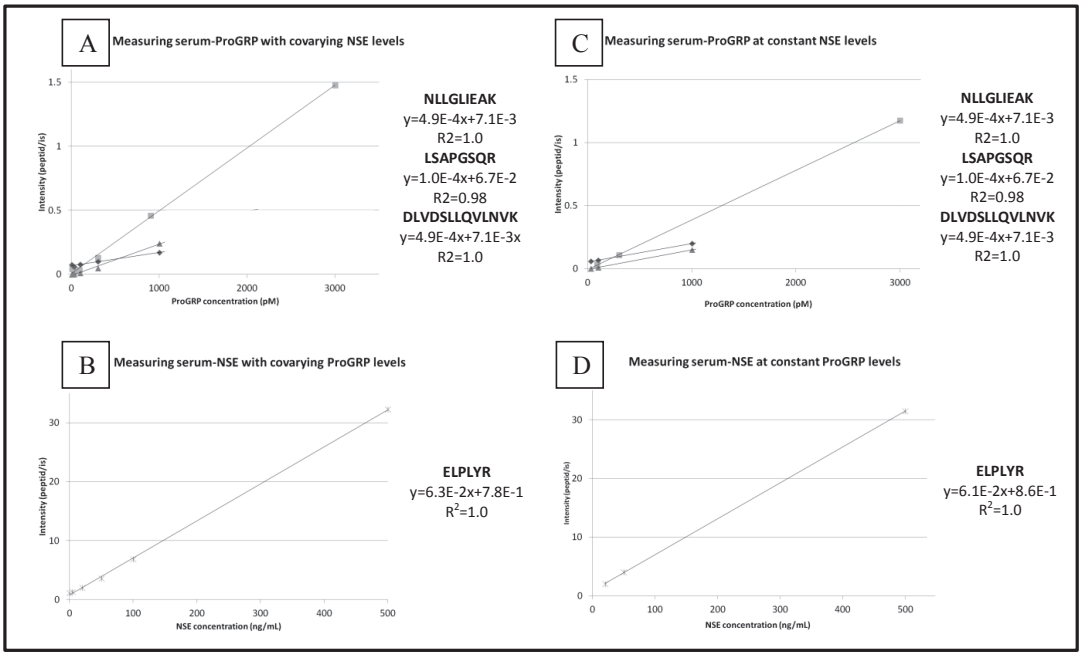


Figure 3.8: The markers are extracted and measured both at stagnant levels of the other marker and co-varying levels in serum. The stagnant levels were 30 pM for ProGRP and 20 ng/mL for NSE. The co-varying values can be found in Supplementary data in *Paper IV*.

3.3.3.3 Reduction and alkylation

As NSE contains several cystein residues the assumed need for reduction, heat and alkylation (*Paper III*) was the reason for this applied pre-digest treatment in the combined method (*Paper IV*). The effects of two pre-digest treatments were evaluated; either only heat treatment or both heat, reduction and alkylation (*Paper V*). In earlier work by Winther et al. it was shown that both reduction, heat and alkylation had limited or no effect on the yield of the signature peptide NLLGLIEAK (for total ProGRP) from the *in-solution* digest of ProGRP(31-98) standard[179], and similar results was also obtained for the recombinant full-length ProGRP standards for *in-solution* digest.

However, to test this effect for immunocapture digestions the following experiment was carried out: SCLC standards were added 5% BSA samples and performed immunocapture on. The IA beads then underwent different pre-treatments before digested. One parallel underwent reduction, heat and alkylation, a second parallel underwent only heat, and the third parallel underwent no pre-digest treatment. Before initiating tryptic digest, the supernatants were was in all cases separated from the beads, thus separating unbound and bound markers.

Figure 3.9 shows the effect of these pre-treatments on the yield of signature peptide TIAPALVSK and ELPLYR for the determination of the two subunits of NSE (Figure 3.9 and *Paper V*). The trends of increased yield when using pre-treatment conditions compared to direct tryptic digest after immunocapture (no pre-digest treatment) were even larger for signature peptides for total ProGRP and it isoform 1 and 3 (data not shown). This was, according to the initial hypothesis, unexpected, as NSE is the marker to contain cystein residues. As ProGRP does not contain cystein residues, the increase in yield had to be caused by another mechanism than the direct reduction and alkylation of the marker. This supports that signal increase for *on-beads* digests was related to reduction of external steric hindrance and not the markers cystein residues.

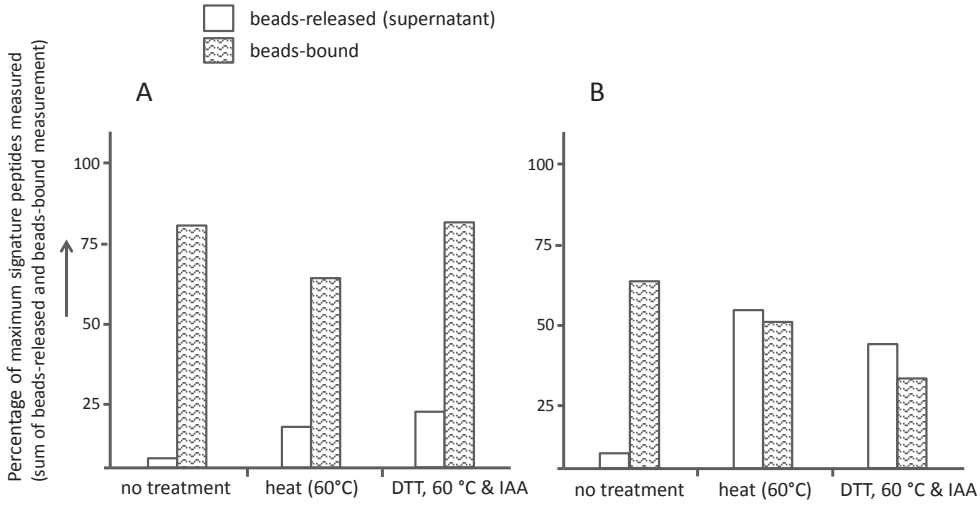


Figure 3.9: Effect of pre-digest treatments on α-signature (A) and γ-signature peptide (B). An αγ-standard was from a 5% BSA solution extracted by use of anti-γ extraction beads and either given no pre-digest treatment, heated, or reduced, heated and alkylated. The magnetic beads and the supernatant were subsequently separated to allow separate digestion of the beads-bound and post-treatment beads-released NSE. The bars show the yield of signature peptides representing the α-subunit in A) and the γ-subunit is shown in B) (n=4).The white bars represents the relative yield of signature peptides from the unbound markers and the other bar represents the same from the beads-bound markers. Adapted from *Paper V* (manuscript submitted to a journal in American Chemical Society).

3.3.3.4 Adjustment of the LC-MS method

Another adaption to be made for co-determination of ProGRP and NSE was adjustment of the LC-MS program. The LC programs for the two separate validated methods were identical (see Table 3.6 and *Paper I and III*); however, for the combined methods, the LC program had to be further optimized due to increased complexity of the sample to be analyzed. Figure 3.10 shows a chromatogram of combined immunocapture using the same LC program as used for the two separate methods and a corresponding chromatogram with the adjusted and final LC program (see Table 3.6). These alterations in the LC-method was done to avoid co-elution of the signature peptide LSAPGSQR (for ProGRP isoform 1) and a possible inference, as well as to better separate the signature peptides in MS segments.

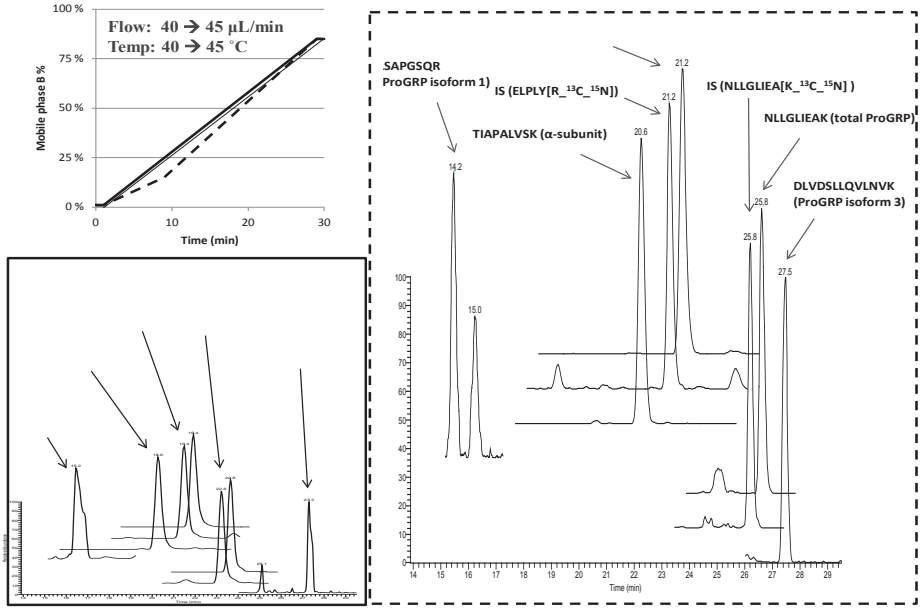


Figure 3.10: Chromatograms obtained using two different LC-MS methods to analyze immunocapture of two different patient serum samples. The chromatogram to the left shows the use of the LC-MS method for the two individual validated methods (*Paper I and III*), while the chromatogram to the right was used for the combined marker determination (*Paper IV*). The differences between the LC programs are displayed in the top graph; a slight alteration in the gradient (straight line belongs to the graph to the left, and dashed line to the graph on the right), and an increase in both the temperature of the column and the flow rate (the highest values belong to the graph on the right).

3.4 APPLICATION OF SRM MS METHODOLOGY ON CLINICAL SAMPLES: THE PROOF OF PRINCIPLE

ProGRP and NSE were analysed in patient samples to investigate and demonstrate the clinical applicability, value and implication of absolute quantitative proteomics with SRM MS methodology. Serum samples from patients with carcinomas with neuroendocrine character were analyzed with both the ProGRP method (*Paper I and II*) and the final method for combined ProGRP and NSE determination (*Paper IV*) with aim to display both variant-differentiating and multiplexing features of SRM MS methodology. These isovariants are not individually quantified with immunoassays; however, total ProGRP and γ-NSE were measured with the clinical established immunometric assays and compared with the immunocapture MS methods (*Paper II and IV*).

3.4.1 Variant differentiation: proof of principle

Three immunocapture MS methods, which allowed for differential determination of defined marker variants termed isoforms and isoenzymes, were developed. Two of these, the individual ProGRP method and the multiplexing MS method for both ProGRP and NSE, were used on patient samples.

3.4.1.1 ProGRP isoforms

The ProGRP proteins are expressed as three isoforms determined by the isoform encoding mRNAs[44, 48]. The mRNA expressions have been investigated in tissue by others[47, 48], only total ProGRP previously been had determined on protein level. To explore if the immunocapture MS method for ProGRP was able to detect the isoforms, six patient serum samples were analyzed (*Paper I)*. All signature peptides for the isoforms were found in the four samples from SCLC patients, and total ProGRP was found in all six samples, including the two samples from NSCLC patients. Thus, the presence of ProGRP isoforms on protein level had been revealed for the very first time. The analyses indicated higher relative levels of isoform 3 compared to isoform 1 in all six samples, and in addition, the concentrations of one isoform relative to the other and to total ProGRP, differed substantially between the patients indicating possible difference in isoform expression between patients.

This differences in isoform expression provoked curiosity to explore this further. Thus, 60 samples from patients with different neuroendocrine carcinomas were collected and analyzed (*Paper II*). These results confirmed the trend from *Paper I*; the concentrations were relatively higher for isoform 3 than isoform 1 for 27 out of 29 samples which had quantifiable

levels of both isoform 1 and isoform 3. This is the opposite trend for protein ratio expression of isoform 1 and 3, compared to that reported earlier on mRNA level[41, 45], as also shown in Table 3.7. In addition to different ratios from mRNA expressions, the protein isoform heterogeneity was higher than previously shown for mRNA[41, 45] (see Table 3.7). One of the plausible explanations for both of these findings is different ProGRP protein isoform stability.

The displayed heterogeneity imply that differing assay affinities for isoforms can be a potential source for between-assay discrepancies, and should be revised for clinically used assays as specificity is essential for interpretation and true absolute quantification, especially if ProGRP values are compared. To examine if this heterogeneity can be linked to differences in pathology, a much larger study population is needed.

Table 3.7: ProGRP isoform expression measured on mRNA level (studied by others[47, 48]), and protein levels (from *Paper II*).

A) The listed mRNA expressions (in % relative to total expression) are in this table reproduced and summarized based on the report from the two referred studies by Spindel et al. and Uchida et al.

B) The listed ratios on protein level are based on the quantifiable isoform levels found by the MS analysis of the sixty patient samples. The top row for protein level shows the results from all the quantifiable samples, and the bottom three rows are values sub-grouped to some of the respective pathologies.

A)

Specimen	mRNA type 1 to total ± RSD (%)	mRNA type 3 to total ± RSD (%)	Reference
Neoplastic tissue from various pathologies (MCT, SCLC, & a pulmonary carcinoid tumor)	63.1±3.2%	28.6±2.1%	Spindel et al.[47]
Neoplastic tissue (only SCLC)	55.4±7.6% (n=5)	42.8±4.3% (n=5)	Uchida et al.[48]

B)

Serum samples	Protein isoform 1 to total-ratio ±SD	Protein isoform 3 to total-ratio ±SD	Protein isoform 1 to isoform 3-ratio ±SD	Reference
All patient samples	0.24±0.24 (n=29)	0.65±0.29 (n=53)	0.44±0.40 (n=29)	*Paper II*
SCLC	0.21±0.14 (n=10)	0.56±0.15 (n=15)	0.38±0.23 (n=10)	*Paper II*
Adenocarcinoma	0.47±0.61 (n=3)	0.53±0.43 (n=4)	0.79±0.35 (n=3)	*Paper II*
MTC	0.16±0.09 (n=8)	0.74±0.31 (n=19)	0.25±0.10 (n=8)	*Paper II*

3.4.1.2 NSE isoenzymes

NSE consists of the hetero-, and the homodimer (αγ-enolase or γγ-enolase) and the monomer (γ-enolase). Two-site immunoradiometric assays (NSE IRMAs) has been reported to different affinity for the hetero- and homodimeric form of NSE, as shown in ISOBM TD-7 workshop epitope characterization of NSE mAbs[138, 185]. However, to the authors' knowledge, no existing assays fully differ between the NSE isoenzymes. In comparison, the two developed immunocapture MS methods for NSE has shown to differ between the two possible subunits of NSE more directly in its quantification of γ-enolase in the individual NSE method (validated in *Paper III*) and indirect estimation of α-enolase from NSE in the multiplexing method (evaluated in *Paper IV*) performed as described in detail in section 3.3.3.1.

With determination limits well below the defined reference levels for NSE, both α- and γ-enolase are detected in serum samples from healthy subjects with both the individual NSE method (*Paper III*) and the multiplexing method (*Paper IV* and Table 3.8). Quantitative measurement of α- and γ-enolase in serum was performed (*Paper IV* and Table 3.8), but the samples are too few to investigate or relate the levels of the enolases to each other. This may be interesting to investigate on protein level in a larger study, similar to the study for ProGRP (see 3.4.1.1 and *Paper II*). Alteration of enolase expression has earlier been a subject of study on gene level, where transitions in gene expression between isoform enolases in rat heart were different between normal and pathological growth[192].

3.4.2 Comparison with established assays: proof of principle

The validity of the immunocapture MS approach was confirmed by comparing the developed methods to two clinically used conventional immunometric assays; the ProGRP TR-IFMA and the NSE IRMA. These assays were thus also used to analyze patient serum samples in *Paper II and IV*.

3.4.2.1 ProGRP

First, the individual ProGRP method as well as the automated TR-IFMA was used for 60 patient samples (*Paper II*). This established immunoassay for ProGRP which measures the total ProGRP in serum samples, and the test was used as a reference to evaluate the performance of the immunocapture MS method. The total ProGRP values from the MS method compared to those obtained from the automated TR-IFMA were systematically determined approximately 30 % lower than the reference method restricting possibilities for interchangeability. The can be many reasons for differences between methods, as listed by the

National Academy of Clinical Biochemistry Laboratory Medicine Practice Guidelines[193]. Different method designs and different calibrators are two probable explanations for the systematic deviations in this comparison. The results individual method and TR-IFMA showed good correlation (R^2=0.887) and in the Bland-Altman plot for method agreement only four out of the sixty samples (< 7%) fell outside the 95% CI. It was suggested from these corresponding results that comparable ProGRP values are obtain which imply applicability of the individual ProGRP method.

Secondly, both the multiplexing method and the ProGRP TR-IFMA were used for six patient samples (*Paper IV*). However, the multiplexing method, one of the samples fell below the LLOQ of the MS method, and the other was above the calibrated range (see the values emphasized by boxes in Table 3.8). However, if these are excluded from a brief comparison the methods, there was a similar tendency of higher estimated absolute concentrations of ProGRP using the assay than the MS method for these very few samples concurrent with the findings in the larger study (n=60) in *Paper III*. (see Table 3.8)

The two out of the six patients showed as mentioned considerable deviating values between the multiplexing MS method and the TR-IFMA (Table 3.8). No similar deviating results were observed in the larger comparison study (n=60) were the individual ProGRP method was compared to the TR-IMFA (see 3.4.1.1 and *Paper III*), which encouraged to considered the most possible reason for a potential erroneous measurement Data investigations to reveal matrix effects for the multiplexing method was thus performed, though the number of samples were too few to draw any definite conclusion of error. Indications for this was revised for within the results and imply that this is unlikely due to the following: 1.) Determination of isoforms were also of high concentrations indicating high concentration of total ProGRP, and, 2.) The IS NLLGLIEA[K_$^{13}C_6{}^{15}N_2$] is assumed to have the same ionization properties as NLLGLIEAK, and this had normal signal (no suspicious signal deviations) from the rest of the samples. This will not be discussed further as a study with a larger number of samples needs to be conducted to investigate if these differences are significant of just an artifact.

3.4.2.2 NSE

Both the multiplexing method and the NSE IRMA were used for six patient samples (the same samples as in section 3.4.2.1, *Paper IV*) and they both determined γ-enolase from NSE which can be compared (see Table 3.8). The results show that the MS method systematically gave absolute values of approximately two folds of that from the IRMA, and the correlation

between the very few samples was very good (R^2=0.997, calculated from the patient values in Table 3.8). Again, systematic differences in the measured absolute values between MS method and assay are observed (as for ProGRP in section 3.4.2.1), and different calibrators and different method designs may be the cause for this. The difference in values does, however, not imply a difference in clinical specificity, and systematical differences may also lead to comparable results when taken into account[193].

3.4.3 Establishment of simultaneous measurement: proof of principle

The ultimate goal was to measure both markers; ProGRP and NSE, and their isovariants in patient samples to demonstrate the combination of immunocapture and SRM as a suited approach for clinical relevant multiplexing. The combined method was used to quantify the levels of the SCLC markers, where concentrations of the selected variants of ProGRP and NSE was simultaneously determined, as shown by the Table 3.8 and illustrated by the chromatogram to the right in Figure 3.10. The table and figure depict the success of the tailoring of an immunocapture MS method for quantification of two valuable SCLC markers, with the feature of simultaneous determination of the different isoforms and isoenzymes of both markers.

Table 3.8: Results from analyses by conventional assays and the combined immunocapture MS method. A number of six patient serum samples and four serum samples from healthy donors were analysed with the combined immunocapture methods, and the ProGRP TR-IFMA and the NSE IRMA. The two boxed ProGRP values are considerably different, which deviate from what was the trend of deviation between the two methods.

	TR-IFMA	Immunocapture LC-MS					IRMA
Serum sample Identity	Total ProGRP (pM)	Total ProGRP (pM)	ProGRP isoform 1 (pM)	ProGRP isoform 3 (pM)	NSE α-subunit (ng/mL)	NSE γ-subunit (ng/mL)	NSE γ-subunit (ng/mL)
Patient A	173	156	13	89	131	161	79
Patient B	7	NF	NF	NF	39	56	25
Patient C	5	116	19	66	123	160	66
Patient D	1810	872	481	578	74	101	43
Patient E	699	344	174	133	32	41	24
Patient F	1767	15893[a]	1618	4881	458	742	451
Donor 1	NF	NF	NF	NF	17	20	NA
Donor 2	NF	NF	NF	NF	10	12	NA
Donor 3	NF	NF	NF	NF	15	19	NA
Donor 4	NF	NF	NF	NF	11	16	NA

Symbol explanation: NF: peak not found, NA: not analyzed, [a] outside the calibrated range.

3.5 FUTURE PERSPECTIVES

The use of LC-MS has so far mainly been applied for smaller molecules than peptides and proteins, however, in the last decades clinical laboratories has expanded greatly into targeted peptide and protein detection and clinical proteomics[194]. This involves shift towards interest in absolute quantification strategies for proteomics which has both been linked to and further stimulated to advances in LC-MS. Specificity is one of these methods greatest strengths, which is superior to the widely used immunometric assays. Throughput can often be a limitation caused by manual workflows and complexity of operation, but may be relieved by the multiplexing potential which is derived from both the MS, SID and the dimension(s) of LC separation. Regarding sensitivity and capacity limitations the system generally relies on extensive sample preparation due to restricted compatibility with matrixes.

The presented immunocapture MS methods for targeted quantitative determination of ProGRP and NSE have demonstrated success. However, this specific analytical approach does have room for improvements related to recent advances that requires resources in terms of new equipment and chemicals. More specifically; the methods would benefit from lowering the quantification limits (LLOQs) for the isoforms of ProGRP, and from reduction of both operation time and resource use by both automation of the immunocapture process and by speeding up the LC-MS analysis.

As the methods are now, they enable measurements of total ProGRP and NSE at reference levels. However, to fulfill their potentials, the sensitivity for ProGRP isoforms should be improved to also enable determination at their low levels. Additionally, sensitivity improvement will be a further necessity if use of lower sample volumes should be strived. This measure would improve the use of the method due to the often limited patient samples.

Both sensitivity and time of analysis are features that can be affected by upgrading and modifying the LC-MS system. Miniaturization, from microflow to nanoflow LC-ESI-MS, is one possible adjustment to improve the MS sensitivity. The chromatography can also be improved, to achieve more narrow and well-separated peaks, by reducing band broadening and plate height, and by increasing column efficiency, which could affect both sensitivity and LC-MS analysis time. A disadvantage of such hyphenated LC-MS set-ups may be an increased demand for advanced operator skills and decreased robustness of the system which could limit applicability and use of the method.

Further sample clean up may be of necessity if miniaturized LC-MS systems are to be used. This part of the method is perhaps already the most labour and time consuming part of the approach, but the sample preparation could be automated to a much larger extent to reduce resource use.

Multiplexing by simultaneous extraction and quantification two markers and different isovariants has been demonstrated to reduce the total time of analysis, reduce the analytical variance, and better utilize the sample. The two chosen markers, ProGRP and NSE, are clinical complementary SCLC markers used to exemplify and demonstrate the multiplexing properties of this approach. This multiplexing method can be used to build a larger diagnostic panel by adding more markers for neuroendocrine tumours or, for differentiation purposes; to add markers to differentiate between diseases such as SCLC and NSCLC without neuroendocrine characteristics. CEA, SCC, CA-125 and CYFRA-21 are such highly relevant lung cancer markers that are possible candidates for this purpose[27, 32, 99, 101, 195-197]. Alternatively, markers for other carcinomas or diseases could be added to broaden the clinical usefulness of such an IA extraction MS tool.

The clinical value of combining ProGRP and NSE measurement is already been reported by others, however, the additional clinical value of differentiation between variations of the markers is still unknown, and with this tool it should be further explored in a larger patient study.

4 CONCLUDING REMARKS

This thesis has elucidated potentials of immunocapture MS method design for protein biomarker measurement. The chosen model markers were SCLC-markers ProGRP and NSE, which are clinically complementary markers occurring in various isoforms and isoenzymes in serum. Novel information on their endogenous expression was assessed by a targeted bottom-up SID approach, through selective purification and enrichment by immunocapture and in combination with MS detection of signature peptides. This was performed by use of external standards for calibration, AQUA peptides for SID and specific and sensitive SRM-MS determination of the proteotypic surrogates in place of their macromolecules.

The quantification of pico- and femtomol levels of model marker isovariants in patient serum was performed by using two validated methods to individually determine ProGRP (ProGRP isoform 1, ProGRP isoform 3, total ProGRP) and NSE (α- and γ-enolase from NSE). Finally, simultaneous extraction and quantification of both markers' isovariants was enabled in a multiplexing method to reduce the total time of analysis, reduce the analytical variance, and better utilize the sample.

The feasibility of these immunocapture MS methods was proven through determination and variant differentiation of these markers in serum samples from healthy subjects and SCLC suffering patients, and all methods provided additional qualitative and quantitative information on the selected low abundant markers compared to that from the conventional clinical assays. The potential of implementing this technology in biomarker monitoring has thus been demonstrated for SCLC, and may in its flexibility also be suited for other biomarkers and biomarker combinations.

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