Transplantation Laboratory

Haartman Institute, Faculty of Medicine

University of Helsinki

MASS SPECTROMETRY IN CLINICAL PROTEIN BIOMARKER DISCOVERY

Ville Parviainen

ACEDEMIC DISSERTATION

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“It´s a magical world, Hobbes,
ol´ buddy…

…Let´s go exploring!”

- Calvin

Contents

LIST OF ORIGINAL PUBLICATIONS

This thesis work is based on the following articles. Each article is referred in the text by the respective Roman numeral:

I **Parviainen V, Joenväärä S, Peltoniemi H, Mattila P, Renkonen R**

A combined database related and *de novo* MS-identification of yeast mannose-1-phosphate guanyltransferase PSA1 interaction partners at different phases of batch cultivation. *International J Mass Spectrom 2009, 281(3):126-133.*

- VP participated in the protein complex isolation, prepared the samples for mass spectrometry (MS), analyzed the data and wrote the manuscript

II **Parviainen V, Joenväärä S, Tukiainen E, Ilmakunnas M, Isoniemi H, Renkonen R**

Relative quantification of several plasma proteins during liver transplantation surgery. *BioMed Research International 2011, Article ID 248613.*

- VP prepared the samples, performed the MS runs and data analysis and wrote the manuscript

III **Parviainen V, Joenväärä S, Tohmola N, Renkonen R**

Label-free mass spectrometry proteome quantification of human embryonic kidney cells following 24 hours of sialic acid overproduction.
Proteome science 2013, 11:38.

- VP prepared the samples for MS, did the MS and data analysis and wrote the manuscript

IV **Tamminen J, Parviainen V, Rönty M, Wohl A, Murray L, Joenväärä S, Varjosalo M, Leppäranta O, Ritvos O, Sengle G, Renkonen R, Myllärniemi M, Koli K**

Gremlin-1 associates with fibrillin microfibrils in vivo and regulates mesothelioma cell survival through transcription factor slug.
Oncogenesis 2, e66 (2013).

- VP prepared the samples for mass spectrometer, performed the MS runs and data handling, analysis and interpretation. VP also wrote parts of the manuscripts related to MS data.

ABBREVIATIONS

1D	One dimensional
2-DE	Two-dimensional gel electrophoresis
AMRT	Accurate mass and retention time
AP-MS	Affinity purification mass spectrometry
AQUA	Absolute quantification using stable isotope labeled synthetic peptides
AUC	Area under the curve
BCA	Bicinchoninic acid assay for proteins concentration measurements
BLAST	Basic local alignment search tool
CID	Collision induced dissociation
CML	Chronic myeloid leukemia
CMP	Cytidine 5-monophosphate
Co-IP	Co-immunoprecipitation
CSF	Cerebrospinal fluid
DDA	Data directed acquisition
DIA	Data independent acquisition
emPAI	Exponentially modified PAI
ESCC	Esophageal squamous cell carcinoma
ESI	Electrospray ionization
ETD	Electron transfer dissociation
FT-ICR	Fourier Transform – ion Cyclotron Resonance
GDP	Guanosine diphosphate
GO	Gene ontology
HCC	Hepatocellular carcinoma
HCl	Hydrogen chloride
HIV	Human immunodeficiency virus
ICAT	Isotope coded affinity tags
IMAC	Immobilized metal affinity chromatography
IMS	Ion mobility separation
IPA	Ingenuity Pathway Analysis
iTRAQ	Isobaric tags for relative and absolute quantification
KCl	Potassium chloride
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC	Liquid chromatography
LC-MS	Liquid chromatography mass spectrometry
m/z	mass / charge- ratio
MALDI	Matrix Assisted Laser Desorption/Ionization
ManNAc	N-Acetylmannosamine
MCP	Micro-channel Plate
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS^E	Data independent mass spectrometry acquisition method
MS/MS	Tandem mass spectrometry

MudPIT	Multidimensional protein identification technique
nanoESI	Nanoflow rate electrospray ionization
Neu5Ac	N-Acetyl neuraminic acid
OD600	Optical density at 600nm wavelength
PAI	Protein abundance index
PE	Preeclampsia
POAG	Primary open end glaucoma
ppm	Parts per million
PTM	Post-translational modification
Q-TOF	Quadrupole - time-of-flight
QconQAT	Quantification concatamer
QQQ	Triple quadrupole
RP	Reverse phase
SCX	Strong cation exchange
SGD	Saccharomyces Genome Database
SILAC	Stable isotope labeling by amino acids in cell culture
TAP	Tandem affinity purification
TCA	Trichloroacetic acid
TEV	Tobacco etch virus
TMT	Tandem mass tags
TOF	Time-of-flight
WB	Western blot

ABSTRACT

The field of biological sciences has expanded enormously within the last few decades. Developments in techniques and instrumentation have allowed biologist to explore biological mechanisms in an unprecedented detail. One of the most evolved disciplines is the field of proteomics. In general, proteins function in many different biological roles. They serve as structural molecules, in signaling routes mediating information in the cell, in intra- and extracellular transport and trafficking as well as in numerous other cellular functions. The area of protein research entails the study of all things relating to proteins and their functions. These include cellular protein composition, expression changes, protein structure, post-translational modifications and protein-protein interactions.

Mass spectrometry (MS) has become one of the key technologies in proteomic research. The relative ease of sample handling and automated MS machinery has made proteomic analysis relatively straightforward. Mass spectrometers work by measuring the weight of intact proteins or protein-derived peptides. Proteomic MS identification is usually done by fragmenting the proteins or peptides in the mass spectrometer and using the resulting mass spectral information in identification of peptide sequence. There are two main strategies of peptide sequence identification: database dependent and *de novo* identification. Database dependent algorithms utilize known sequence information stored in databases to decipher the peptide amino acid sequence of the MS-observed spectra and use that information to predict the protein from which the peptide is derived from. On the other hand *de novo* methods try to construct the peptide sequence solely based on the fragmentation patterns of the peptide. The completeness of sequence databases of many species and the speed and efficiency of the search engines have made the database dependent search as the main method in peptide and protein identification.

The modern high resolution mass spectrometers along with ultra-performance liquid chromatography have enabled the detection of thousands of protein in one single MS run. This, together with advances in MS-based protein quantification has extended the use of mass spectrometers in discovery type biomarker search. Mass spectrometers are able to produce a large amount of data on numerous proteins that can be used to detect and quantify differences in patient and control samples. This in turn can be used as starting point for more focused validation studies on the acquired data and ultimately lead to useful clinical biomarkers.

The focus of this study was to utilize and learn mass spectrometric methodologies and to analyze different proteomic processes in sample types. We analyzed the protein-protein interactions in Baker´s yeast PSA1 protein in various points of batch cultivation using database dependent and *de novo* protein identification methods. We showed that the interactome of PSA1 is very dynamic depending on the phase of the cultivation. We also showed the limitations and benefits of *de novo* identification and the combined use of both search strategies in improving the confidence of the identifications. In another study using affinity purification and mass spectrometry we identified Fibrillin-2 as the binding partner of lung cancer associated Gremlin-1 protein. This finding elucidates functions and mechanisms of Gremlin-1 and Fibrillin-2 in malignant tissues. In two mass spectrometry-based protein quantification studies we characterized the protein concentration changes in human plasma during liver transplantation surgery as well as the effect of excess sialic acid production in HEK293 model cell line. In the liver transplantation plasma project we identified protein concentration changes in liver in response to the trauma caused by the surgery using label-based iTRAQ method. We showed consumption and secretion of several coagulation related proteins within the liver suggesting activation of coagulation cascade in the very early phases of the craft reperfusion. In the

study of excess sialic acid production we first verified the amounts of sialic acid using mass spectrometry-based multiple reaction monitoring method. We were able to induce the production of sialic acid to almost 70-fold compared to control cells. We also monitored the protein abundance changes in sialic acid producing cells using label free proteins quantification method identifying 105 changed proteins. We analyzed those proteins with several functional enrichment tools revealing modifications in cellular protein transport, metabolic and signaling pathways and in remodeling of cellular adherens junctions. Such large scale MS-analyses using ontology-based tools can significantly aid in deciphering the effect of perturbations to complex systems but also reveal novel functional targets for biomarker discovery.

The results obtained from targeted interaction experiments as well as large scale quantification studies can be used as basis for more rigorous investigations on the various subjects in search for potential biomarkers for clinical use. The techniques and methods used in the studies also demonstrate the many uses of mass spectrometric techniques in several fields of proteomic and biological research.

REVIEW OF LITERATURE

Introduction

In the past two decades mass spectrometry (MS) has become a major workhorse in biological research. Especially the field of proteomics, or the characterization of entire protein content of biological samples, has benefitted from the increasing sensitivity and resolution of rapidly evolving mass spectrometers[1,2]. The capability of modern mass spectrometers to identify and characterize hundreds of proteins in a single MS run has widely expanded our knowledge of the functional organization of the cellular proteomes[3]. The concurrent developments in liquid chromatography (LC) and mass spectrometer technology combined with highly sophisticated bioinformatics methods have also increased the use of MS machinery in proteomic quantification. This has led to an increase in the usability of mass spectrometers in proteomic biomarker discovery and in clinical proteomics[4,5]. In this Ph.D. project modern mass spectrometric methods were utilized in examination of general proteomic events in biological systems and in research for potential biomarkers for clinical samples.

1. Proteomic research

The term proteome was first coined by Wasinger et al.[6] in their study of protein content of Mycoplasma genitalium. Thereafter the term proteomics has expanded to include a multitude of protein-related subjects such as post-translational modifications (PTM), protein-protein- interactions and cellular protein quantities (Figure 1.). The importance of proteomic research has been emphasized by the increasing knowledge of the complexity of biological systems and the emergence of system biology view of biological events[7,8]. The classical view of Genes-to-RNA-to-Protein-to-Function has been replaced by an intricate network of space and time dependent interactions and regulation of different parts of cellular systems[9].

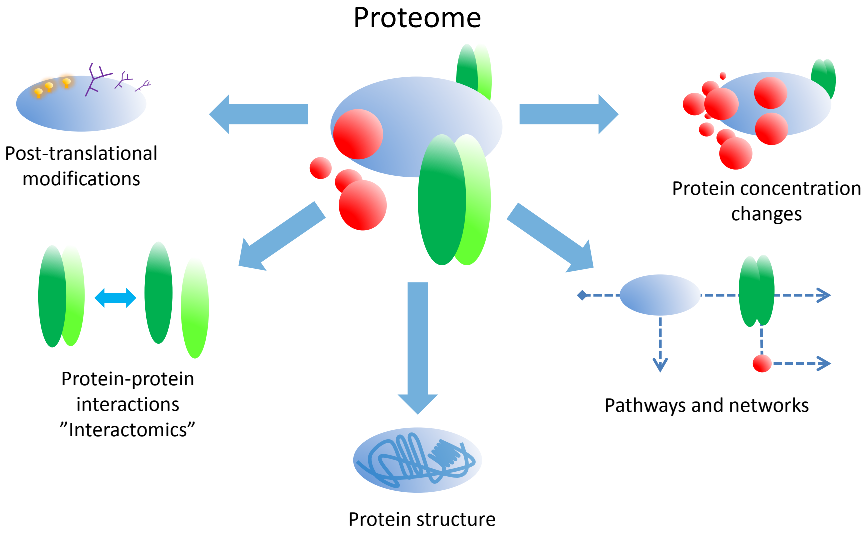

Proteome
Post-translational modifications
Protein concentration changes
Protein-protein interactions ”Interactomics”
Pathways and networks
Protein structure

Figure 1. Different types of proteomic research

Proteomic research can be divided into several different subcategories. These include analysis of post-translational modifications, protein-protein interactions, proteins structure determination, changes in protein abundances as well as functional proteomic network and pathway analysis

The complexity of cellular and humoral proteome is immense. In human plasma the protein quantities extend over ten orders of magnitude with low levels of cellular leakage proteins to serum albumin constituting over 50 percent of the entire serum protein content[10,11]. Similarly, the intracellular proteome can range several orders of magnitude depending on the cellular state. Some proteins are expressed transiently at low levels in response to perturbations or at distinct points during cell cycle as others, mainly housekeeping proteins, are constantly expressed at high abundance[12].

Post-translational modifications of proteins (PTM) create an additional layer of complexity to biological systems. So far more than 160 different mammalian post-translational protein modifications have been characterized in over 45000 different sites (24.4.2013)[13]. Most common of these include phosphorylation of serine and threonine, N-glycosylation of asparagine and acetylation of lysine. PTMs modulate cellular processes such as signaling cascades, protein-protein interactions, subcellular localization and protein degradation[14]. The PTM status of individual proteins depends on the cellular state. For example, external stimuli may activate specific and local signaling routes by phosphorylation a subset of proteins and consequent response to the stimuli. After response the phosphoryl groups are removed by phosphatases and the signaling route deactivated[15]. The dynamic nature of different PTMs creates subproteomes of differentially modified proteins that further complicate proteomic analysis.

Proteomic research entails also the study of protein-protein interactions. Similarly to PTMs, physical interactions modulate the functions of most proteins. Some proteins are direct constituents of larger proteomic machines, such as ribosomal, spliceosome and proteasome proteins, while others exhibit transient interactions that briefly regulate the activity of the interacting proteins[16,17]. The study of interactomics tries to elucidate the networks of the interacting proteins and characterize the systemic changes occurring in response to perturbations or in diseased states.

1.1 Principles of proteomic mass spectrometry

Mass spectrometric analysis of proteins means the characterization of proteins or peptides based on their respective masses. There are two main types of analysis in proteomic MS research: top-down and bottom-up MS analysis. In top-down MS analysis[18] intact proteins can used to identify the proteins[19], examine the post-translational modifications[20] of individual proteins or to characterize intact protein complexes[21]. However, the most classical type of analysis is still the analysis of protein derived peptides, or bottom-up proteomics[22] (Figure 2.). In shotgun protein characterization the proteins are digested to short fragments of few kilodalton using proteolytic enzymes. Combined with prefractionation and liquid chromatography analysis, shotgun proteomics can identify thousands of proteins from very complex mixtures. The applicability of shotgun proteomics has been demonstrated in a wide array of complex proteomic experiments including identification and quantification of proteins[23] but also in verification of novel genes and splice forms[24].

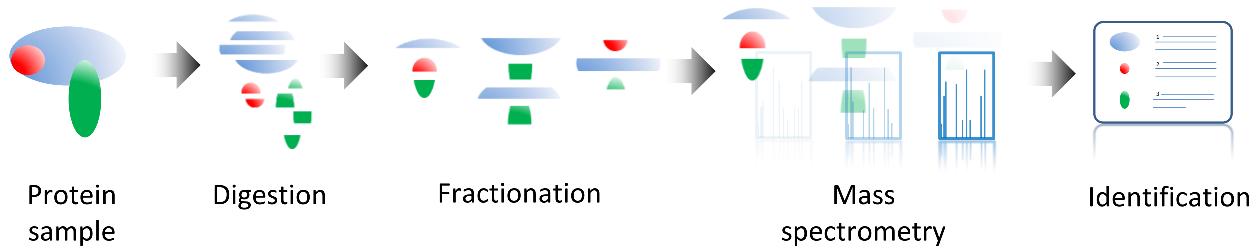


Figure 2. Bottom-up proteomic MS workflow

The protein sample is first digested with proteolytic enzyme, usually trypsin. Then the sample is fractionated using online reverse phase chromatography before analysis with mass spectrometer. The resulting MS spectrum is processed and the proteins are identified with search engines.

In shotgun LC-MS proteomics the peptides are introduced to mass spectrometer by direct coupling of liquid chromatography system to the mass spectrometer. The LC system is on-line with MS so that eluting peptides are directly injected to MS using electrospray ionization (ESI)[25]. In the LC-ESI interface the peptides are ionized and transferred from liquid to gas phase. After ionization they are transferred through the mass spectrometer with ion optics that uses magnetic fields and radiofrequency lenses to guide and select molecules in the mass spectrometer. Peptide can also be fragmented in the mass analyzer. The MS fragmentation produces a series of short peptides that contain the sequence information needed for identification of the peptide. At the end of the mass spectrometer the ions are detected by mass detectors that scan and record the mass spectra of the peptides. The result is a peak list of all the detected masses represented as a ratio of the ion mass and its accompanying ionization charge (mass/charge = m/z). This peak list of m/z-values can then be imported to different bioinformatics search tools that compare the detected peptide masses to computer generated list of all possible masses derived from predefined protein database. By matching the detected peptide mass to theoretical mass the search programs can reliably and efficiently identify the proteins that are present in the analyzed sample.

1.2 Proteomic sample preparation

1.2.1 Enzyme digestion

The modern high end mass spectrometers are able to reliably identify very large peptides or intact proteins, but in general the limitations in resolution of most mass spectrometers require that the analyzed molecule is not too large and contains adequate number of charges. Therefore prior to shotgun MS analysis the proteins must be digested with proteolytic enzymes to yield shorter peptides. This can be done directly to sample proteins (in-solution digestion) or after separation steps (one- or two-dimensional gels). Most of the commonly used digestion enzymes produce peptides with suitable m/z range and charge for MS analysis. There are several different enzymes available for protein digestion for mass spectrometry that have different specificities on the digestion site of the protein[26]. These include Asp-N that cleaves from amino sides of asparagine and cysteine, Glu-C cleaving carboxyl side of glutamine and asparagine, and Lys-C and Arg-C that cleave from carboxyl sides of lysine and arginine respectively. In order to allow the identification of peptides participating in sulfur bridges, the sulfur bonds are usually reduced after digestion and the resulting free cysteines alkylated. The most common digestion enzyme used in proteomic research is trypsin. Trypsin cleaves proteins after lysine or arginine provided that there are no prolines adjacent to the site[27]. At acidic pH the peptides normally retain one positive charge at the amino group of N-terminus. The addition of potential secondary ionization site at the C-terminus by trypsin increases the coverage of mass spectrometric fragmentation in MS and subsequent identification of peptides. Trypsin also has the advantage of tolerating quite high salt or detergent conditions and is able to penetrate SDS-PAGE matrix in in-gel digestion. Additionally there are few commercial trypsins that have been modified to resist autodigestion thus simplifying the MS data-analysis.

1.2.2 Prefractionation of proteomic samples

Due to the complexity of biological samples the characterization of complex proteomes often requires sample fractionation. Even though modern mass spectrometers have a high sensitivity with detection limits up to low femto- or even attomolar range, the dynamic range of the MS machinery is limited by ion suppression and other matrix effects caused by different physicochemical properties of molecules[28]. MS dynamic range refers to the range of lowest and highest analyte that can be detected by mass spectrometer. Current MS instrumentation has a dynamic range approximately 3-4 orders of magnitude depending on the used technology[29]. Another bottleneck in MS analysis of complex mixtures is the duty cycle of MS instrumentation. Due to the inherent constraints of the MS machinery, a large proportion of eluting peptides may go undetected by the mass detector[30]. In order to avoid the constraints in the MS duty cycle and ion suppression and to enhance the dynamic range of MS acquisitions, several methods have been developed for prefractionate the sample prior to MS analysis.

1.2.2.1 SDS-PAGE

Protein separation with SDS-polyacrylamide gels has been one of the main tools in biological research for over four decades[31]. One dimensional gel (1D SDS-PAGE) separates proteins based solely on the respective size of the protein. Two dimensional (2-DE) gels separate the proteins first by their isoelectric point and then orthogonally by their size[32]. In MS analysis the gel-separated proteins are excised from the gel, digested enzymatically and analyzed with LC-MS. In spite of being one of the most popular preparation methods in proteomics, there are some drawbacks using gel-based system in mass spectrometric analysis. These include sample loss from processing steps, issues with dynamic range of the sample and compatibility of SDS-PAGE protein detection methods with MS analysis[33].

1.2.2.2 Liquid chromatography methods

Liquid chromatography fractionation is generally used in conjunction with mass spectrometers. LC systems offer an unparalleled separation dimension to mass spectrometers by reducing the complexity of the sample and by concentrating the individual peptides during elution. Current ultra-pressure LC instrumentation[34,35] with nanoliter flow rates and modern chromatography materials have made it possible to identify several hundreds of proteins in one single LC-MS run[36].

Reverse phase (RP) column chromatography is currently the main method of on-line separation of peptides in LC-MS analysis. In RP separation the peptides are separated based on their interactions with hydrophilic stationary phase of the column. Elution is done by gradients of increasing concentration of non-polar, MS compatible solvents in the mobile phase[37]. Another widely used technology is ion exchange chromatography such as strong cation (SCX) and anion (AEX) chromatography. Ion exchange separates molecules by interaction between charged side chains of peptides and charged stationary phase. Elution can be done using increasing amounts of salts or modifying the pH of the mobile phase. However large concentrations of salts interfere with mass spectrometry analysis so SCX is used either off-line or in two dimensional on-line separations[38,39].

Similarly to 2-DE gels, LC methods can be used on-line in two dimensions as orthogonal technologies in order increase sample fractionation efficiency and separation[40]. Most commonly used 2D-LC method is multidimensional protein identification technique (MudPIT)[41]. In MudPIT chromatography the peptides are first fractionated stepwise with SCX. Eluted peptides are then introduced to RP column where they are retained while the salt-containing eluent is washed away. Normal RP separation is then applied to the peptides on-line with HPLC. 2D-LC separation can significantly improve the identification efficiency and the amount if identified peptides from complex matrices[42]. Several other methods can be used instead of SCX-RP[43]. For example, tandem RP-RP with different pH in both steps has been shown to be a comparable method to SCX-RP[44].

1.2.3 Enrichment methods

RP and SCX are used in global separation of peptides based on the general properties of the amino acid side chains. However, there are methods that can be used to enrich a specific subproteomes of complex samples. These include post-translational modifications such as phosphorylation and glycans as well as enrichment of different organelles.

1.2.3.1 Phosphorylation enrichment

The most common post-translational modification is protein phosphorylation[13]. A variety of protein functions are modulated by addition of phosphate group to serine, threonine or tyrosine side chains. Phosphorylation is used in many signaling routes and other processes that are dynamically activated and deactivated and may be found in low stoichiometric amounts. For his reason the MS identification requires that the low abundance phosphopeptides are enriched and purified from the non-phosphorylated material. The phosphate group possesses a high negative charge which can be utilized to enrich the phosphoproteome of samples (Figure 3.). Currently the most common and sensitive phosphopeptide enrichment material is immobilized titanium dioxide (TiO_2). Along with modifiers that inhibit binding of nonphosphorylated and acidic peptides, TiO_2 can be used to enrich phosphopeptides to high degree[45]. Additionally, TiO_2 can be used in on-line 2D-LC-MS where phosphopeptides are first enriched with TiO_2 column and then fractionated with RP that is directly coupled to ESI-MS[46]. Alternative methods in phosphoproteome enrichment include immobilized metal affinity chromatography (IMAC) that captures phosphopeptides by binding of the negatively charged phosphates to ferric iron[47] or immunopurification of phosphopeptides with antibodies recognizing phosphorylated amino acids.

1.2.3.2 Glycosylation enrichment

Glycosylation involves addition of distinct glycan structures to mostly cell surface and secreted proteins[48]. The glycosylation pattern is highly versatile with thousands of different glycan structures. Carbohydrate binding proteins called lectins recognize and bind specific glycan structures[49,50]. By attaching the lectin to immobilized support the specific glycan containing proteins or peptides can selectively be isolated from non-glycosylated or uninteresting material (Figure 3.). Additional glycopeptide and glycoprotein enrichment methods include size exclusion that separates larger glycan containing peptides from smaller non-glycosylated material[51] and chemical derivatization of glycopeptides[52]

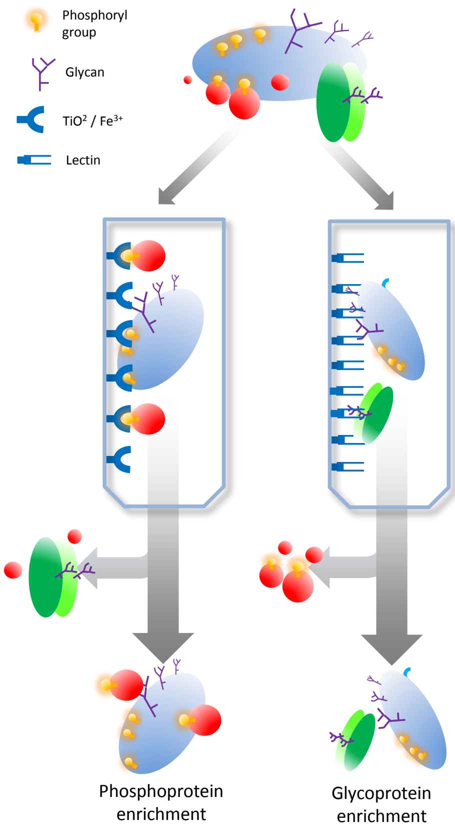


Figure 3. Phosphoprotein and glycoprotein enrichment methods

Proteins or peptides can be enriched using specific material that binds and retains the desired proteins in the support material while the unwanted proteins are washed away. Phosphoproteins can be enriched using ferric iron (IMAC) or titanium dioxide (TiO_2). Specific proteins called lectins that bind certain glycan moieties are used in glycoprotein enrichment.

1.2.3.3 Organelle enrichment

In some cases the interesting biological phenomena occur in organelles and other subcellular structures[53]. When analyzing tissues or other cell samples the material is usually lysed by mechanical or chemical means to free the contents of the cells. This mixes the contents of the organelles to cytosolic and other proteins making the analysis of the organelle impossible[54]. In order to examine the different subproteomes of these structures, they can be enriched prior to analysis. For example, several organelles can be fractionated after gentle disruption of plasma membrane and by centrifugation of the lysate in gradients mediums that separate the organelles by their density[55]. Additional enrichment methods such as affinity purification and electrophoretic and mechanistic sorting have also been successfully employed in organelle enrichment[56].

1.2.4 Protein complex affinity purification

The field of interactomics or protein-protein- interaction research has emerged as an important aspect of proteomic research[57]. In order to study the interactions of individual proteins with mass spectrometry, the interacting proteins must be purified with their interacting proteins. The strength of physical interactions between proteins vary from transient and low affinity to extremely stable. For this reason the optimization of the isolation protocol is extremely important[58]. Inadequate or weak handling may result in extremely high unspecific binding masking the true interactors. On the other hand too harsh conditions can remove the low affinity partners reducing sensitivity of the protocol.

The main method in interactomics has been co-immunoprecipitation of proteins (Co-IP) (Figure 4.1). In Co-IP the proteins, along with their interacting partners are purified using antibodies against the protein of interest coupled to a solid support. The main benefit of Co-IP is that it can be used on all biological material and proteins with suitable antibodies. However, some antibodies have a tendency for to bind unspecific proteins creating a high background of false positives in Co-IP[59].

An alternative method for Co-IP is affinity-purification mass spectrometry (AP-MS)[60]. AP-MS uses biochemical tags that are genetically inserted to the protein of interest. The tag is chosen so that the tag has a high affinity binding partner. By immobilization of the tags binding partner to solid matrix and the introduction of sample to the bound matrix, the tagged protein along with its bound interactions partners can be isolated and purified[61]. After elution from the matrix the purified complexes can be easily digested in solution and analyzed with MS. Several methods have been developed which use two different affinity tags[62,63] (Figure 4.2). These tandem affinity purification tags (TAP) enable two-staged purification with different washing steps removing most of the contaminating unspecific interactions from the sample[64]. The downside of using two washing steps is the potential loss of low affinity and transient binding partners that may be removed by the extra sample handling steps[62]. The power of tandem affinity tags and AP-MS has been proven by thorough investigation of the interactomes of yeast[65], *E. coli*[66] and fruit fly[67]. The main drawback of AP-MS purifications is that it often requires genetic manipulation of the organism under investigation. Even though it has been successfully used in interactomic studies of genetically modified fruit fly[68] and mouse[69] the applicability of genetically engineered AP-MS cannot be extended to human studies due to obvious ethical reasons.

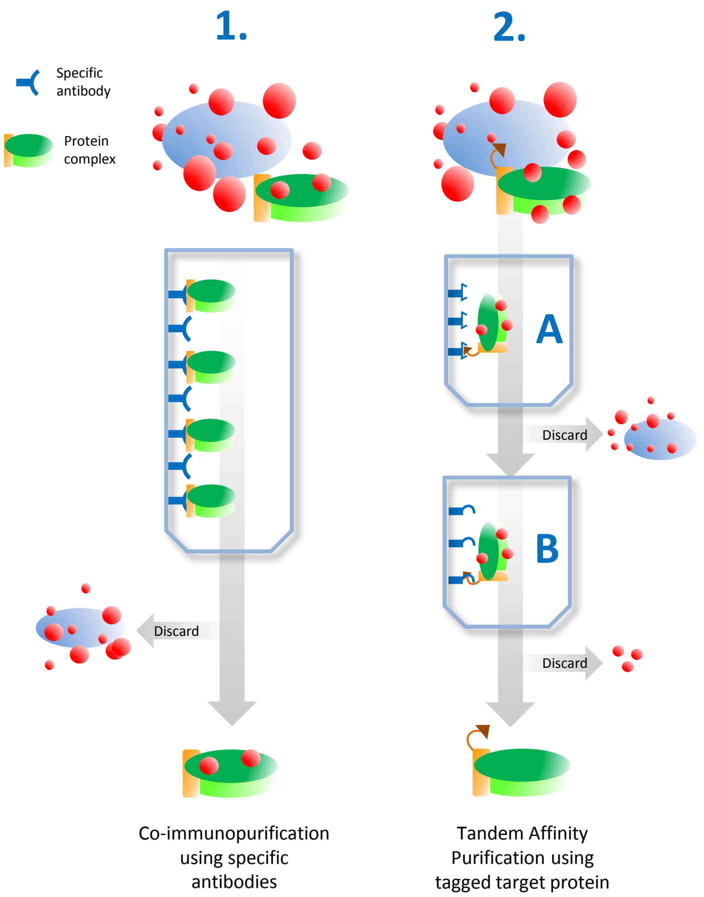

1.
2.
Specific antibody
Protein complex
A
Discard
B
Discard
Discard
Co-immunopurification using specific antibodies
Tandem Affinity Purification using tagged target protein

Figure 4. Protein affinity purification

1. Co-immunopurification (Co-IP) enrichment is done using specific antibodies that recognize and bind the proteins of interest. The protein mixture is applied to immobile antibodies. The unbound proteins are discarded, the bound proteins along with the interacting partners is washed and then eluted. Co-IP can produce a high nonspecifically binding protein background.

2. In protein complex isolation using tandem affinity purification (TAP), the protein of interest is tagged by genetic or chemical means. In the first step (A) sample is applied to first immobilized affinity material which binds the tagged protein from the first part of the affinity tag. The majority on nonbinding and unspecifically bound proteins are washed away and discarded. The tagged protein is eluted and applied to the second immobilized affinity material (B) that recognizes the second part of the affinity tag. Additional washes are used to remove remaining nonspecific proteins and finally the tagged protein along with its binding partners is eluted. The two step washing usually removes most nonspecifically binding proteins.

1.2.5 Plasma depletion

Blood plasma is a valuable diagnostic source for disease biomarkers and is commonly used in clinical diagnosis and research. The wide dynamic range of plasma protein abundances poses a challenge for MS-based research. Differences in in protein abundance range from serum albumin with approximately 50 mg/ml to interleukins with approximate concentration of less than 5 pg/ml covering a dynamic range of over ten orders of magnitude[70]. Usually the biomarkers for cancers and other maladies are mainly found in low-abundance protein category[10]. For this reason the low-abundance fraction of plasma proteins must be separated prior to MS analysis by depletion of the most abundant proteins. Clotting factors can be removed from plasma by normal clotting procedures to create blood serum. However, this is not enough to limit the dynamic range to MS tolerable level or to enable the detection of the low abundance protein fraction[71]. For depletion of plasma from the high and medium abundance constituents, several affinity purification methods have been developed[72]. Currently the most advanced depletion systems are able to remove fourteen of high abundance and further 45 medium abundance proteins from plasma or serum increasing the detection limits of low abundance proteins to less than one nanogram per milliliter levels[73]. However, a notable disadvantage in protein depletion is the removal of interesting proteins that are bound to albumin or other high abundance proteins[74]. Several peptides and proteins have been reported to be carried by albumin[75] in the circulation so the removal of these high abundance components also eliminates the bound proteins from analysis.

2. Mass spectrometric analysis

2.1 Ionization

The mass spectrometers utilize electric fields to guide the molecules within the MS instrument (Figure 5.). In order for the molecules respond to electric fields, they must be charged. Several different methods are used to ionize the analytes such as chemical or thermal techniques; however, in proteomic MS the peptides are most commonly ionized by soft ionization methods MALDI (Matrix Assisted Laser Desorption/Ionization)[76] or ESI[25]. Soft ionization method means that the ionization procedure is gentle enough so that it doesn't fragment the peptides upon ionization. In MALDI the analyzed material, such as peptides or proteins, are deposited to solid surface along with matrix in water-solvent mixture and then allowed to dry. This causes the matrix and the analytes to co-crystallize to the surface. A short laser burst is then applied to the spot resulting in ablation of the matrix and the analyte to the gas phase. This leads to ionization of the analytes and entry to the mass spectrometer for analysis.

Along with MALDI, electrospray ionization or ESI, is another widely used soft ionization technique. In ESI the solution with eluting peptides is pushed through an orifice with applied voltage. Due to the high pressure and the voltage, the eluting solution is dispersed into small droplets. These droplets undergo solvent evaporation with the aide of high temperature and gas flow on the ESI source. The evaporation concentrates the molecules and the charges in the droplets until the charge repulsion causes the droplets to fragment into smaller and smaller pieces in a series of Coulomb fissions. The pH of the ESI solvent also results in the charging of the analytes in the liquid phase. After several rounds of Coulomb fission and solvent evaporation, only the charged peptides are left in the gas phase and can then enter the mass spectrometer[77].

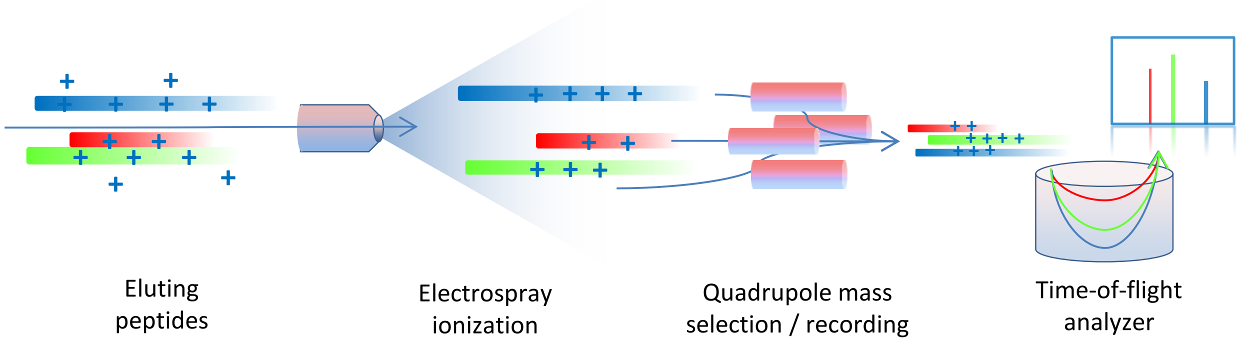


Figure 5. Overview of ESI ionization and hybrid quadrupole-time-of-flight (Q-TOF) mass analysis

The peptides are first ionized by electrospray ionization. The charged peptides are guided by quadrupole mass selectors before entering time-of-flight (TOF) mass analyzer. Based on the flight time in the TOF analyzer the mass spectrometer is able to calculate the masses of each peptide.

Using nanoliter flow rates in the nanoESI ionization the sensitivity and the ionization efficiency can be significantly improved allowing the detection of very low peptide quantities[78]. ESI is usually used with liquid chromatography separation due to the compatibility of the LC-solvents with ESI and MS. The LC-ESI interface is usually used on-line with MS machinery to inject the eluting analytes straight to the MS. This allows the automation of the analysis but also the characterization of the retention times of the analytes. One of the main benefits of ESI ionization is that it is capable of producing multiply charged peptides. This is a key factor in the analysis of large molecules but also aides in the fragmentation of peptides in MS/MS analysis[79]. The propensity of ESI to generate multiply charged peptides can also be a disadvantage. The same peptide species can be present in few different charge states that complicate the data analysis.

2.2 Common mass analyzers

Mass analyzers are the core of mass spectrometers. They are used to concentrate, store, guide and separate the ion travelling within the mass spectrometer. Different types of mass analyzers have been developed with different benefits and drawbacks (Table 1.). Quadrupole mass analyzers use radiofrequency fields and electric currents to alter the flight paths of charged particles within the electric field. In the simplest case the quadrupoles pass all ions that enter the mass analyzer producing MS spectra. MS spectra contain all the ions that are detected at a given time and can be used to characterize the entire m/z range of the sample. By modulation of the electric fields, quadrupoles are also able to select molecules with specific m/z values to pass to the mass detector while discarding the rest[80]. Ion trap analyzers operate as quadrupoles but can trap and concentrate the ions for a brief period of time. This allows for higher sensitivity as the ion scan be concentrated prior to ejecting to detector. As with quadrupoles, the ion trap can be used to pass and detect all ions that are present or just a single ion with specified m/z.

Table 1. *Common mass analyzers and respective advantages and disadvantages*

Analyzer	Advantage	Disadvantage
Quadrupole (single and triple)	Relatively cheap, MRM capabilities (triple quadrupoles)	Limited mass resolution and range
Ion trap	Good mass range, sensitivity, fast scan rate, low cost	Limited mass resolution and dynamic range
Time-of-flight	High mass range, fast scan speed, good resolution and accuracy	Limited dynamic range
Orbitrap	High resolution and mass accuracy	Moderate scan speed
FT-Ion cyclotron resonance	Very high resolution and mass accuracy	Very expensive, limited dynamic range

The Fourier Transform – ion Cyclotron Resonance (FT-ICR) uses strong magnetic fields to trap ions. This is followed by frequency excitation causes the ions to move in a circular path to the detector which records an image current of the ions movement. This is transformed to mass spectrum using Fourier transformation algorithms. The FT-ICR mass spectrometers are highly accurate and have very high resolution but also come with a very high prize and size. Another mass analyzer that uses Fourier transformation algorithms to determine ion mass is the Orbitrap analyzer. Orbitrap captures ions around central spindle of the trap using electrostatic forces rather than magnets. The ions begin to orbit the central spindle but also begin to oscillate axially based on the m/z value. The image current of this m/z-dependent oscillation is detected and transformed to mass spectra[81]. Within the last decade, the high resolution and mass accuracy in combination with moderate price of Orbitraps have made them one of the most important MS tools in proteomics.

In addition to Orbitraps Time-of-flight or (TOF) analyzers[82] are frequently used in proteomic MS. TOF detectors work by measuring the time of ions travelling in the analyzer. When a group of ions arrive to the TOF mass analyzer they are first injected to the detector by small electric pulse by the pusher in to the constant electric field of the TOF analyzer where they are accelerated by constant electric field. The velocity of each individual ion depends on the m/z of the ion so that smaller m/z ions arrive to the ion detector faster. By measuring the time of arrival after the initial pulse, the exact m/z of each ion can be recorded and calculated[83]. The differences in the original kinetic energies of the ions with same m/z cause reduced of resolution in linear TOF analyzers. This can be corrected using reflectors in the TOF flight tube. Instead of linear flight path, the reflector curves the path of ions back towards the source in U-shaped trajectory. The depth that the ions enter the reflector depends on the velocity of the ion. Higher velocity ions penetrate deeper and stay in the reflector a longer time while the slower ions take a shorter path and less time. This balances the initial differences in the velocity and allows the ions to arrive to the detector. The ions are finally detected using micro-channel plate (MCP) detectors that detect and multiply the signals which are then converted to mass spectra.

The most efficient MS analysis is usually achieved using hybrid mass spectrometers. Hybrid MS equipment utilize several different types of mass analyzers. The use of various analyzers in conjunction can be used to bypass the inherent drawback of the analyzers thus increasing the resolution, sensitivity and mass accuracy of the MS analysis. Examples of hybrid MS instruments include Linear ion trap-Orbitrap, Quadrupole-ion trap as well as Quadrupole-TOF.

2.3 Ion mobility separation

In some cases the second fragmentation quadrupole can be replaced by ion tunnels that are used to trap and further separate the peptides. In one example the second quadrupole is replaced by the TriWave device that composes of ion trap, ion mobility separation (IMS) and transfer regions[84]. In IMS the ions travel through the IMS cell filled with gas. Instead of fragmenting the peptide, the IMS gas slows the velocities of the ions depending on their shape[85]. As a result the ions arrive to the detector at different times freeing the detector to scan the arriving ions with more precision and sensitivity. By separating the ion by their shapes, IMS creates and additional separation dimension for peptides with similar elution times in the liquid chromatography step prior to MS. The use of IMS separation step can increase the detection efficiency and sensitivity of peptides by almost 60% compared to normal separation allowing a much greater number of proteins to be identified in one single MS run[86]. IMS can also be used in separation of intact protein complexes[87] and peptides with similar mass but with different shapes. These include glycan isoforms[88] as well as analysis of structural variants of proteins[85].

2.4 Quadrupole tandem mass spectrometry

In tandem mass spectrometry the ions are fragmented to produce smaller fragments which are then analyzed and used in sequence or structure elucidation of the original ion. Tandem mass spectrometry can be done in ion trap, TOF-TOF as well as quadrupole analyzers. In quadrupole tandem mass spectrometry two quadrupoles are used back-to-back (Figure 6.). In data dependent acquisition (DDA) the mass analyzers selects and passes only one single species of ions (or m/z values), called precursor ion, for collision induced fragmentation (CID) in the second quadrupole[89]. Quadrupole also records the mass of the precursor so that that the product ions can be traced back to this precursor for peptide identification. After passing the first quadrupole the precursor ions enter the second quadrupole where a curtain of inert collision gas, such as argon or helium, is applied. The ions that enter the gas filled chamber collide with the gas molecules causing the peptides to fragment. In CID the peptide fragments mainly from the amide bonds of the peptide backbone but in different positions of the peptide sequence[90]. This semi-random dissociation produces two species of product ions: one derived from the N-terminus (denoted as the b-ion) and one from the C-terminus (the y-ion) of the peptide. During the fragmentation the charge of the precursor peptide is either divided between the fragments or stays on one fragment while the other fragment acquires a neutral charge. Since charge is prerequisite for mass characterization of the ion, the ability to create multiply charged ion species is an advantage. The benefit of using trypsin as digestion reagent is that it creates a positively charged C-terminus to the peptide as it cleaves after lysine or arginine. This enables one charge to remain in the y-ion after fragmentation as others may locate to the N-terminal b-fragments of the precursor. CID of large number of peptides with same sequence results in a series of product ions that differ in mass based on the site of the fragmentation. The mass differences between the product ions represent the differences in masses of individual amino acids of the peptide sequence. In ideal case the fragmentation would produce a series of product ion have been fragmented after each amino acid generating a full series of b- and y-ions. However, this is rarely the case as some amino acids are more prone to fragmentation than others so the ion series pattern is generally dependent on the peptide sequence[90].

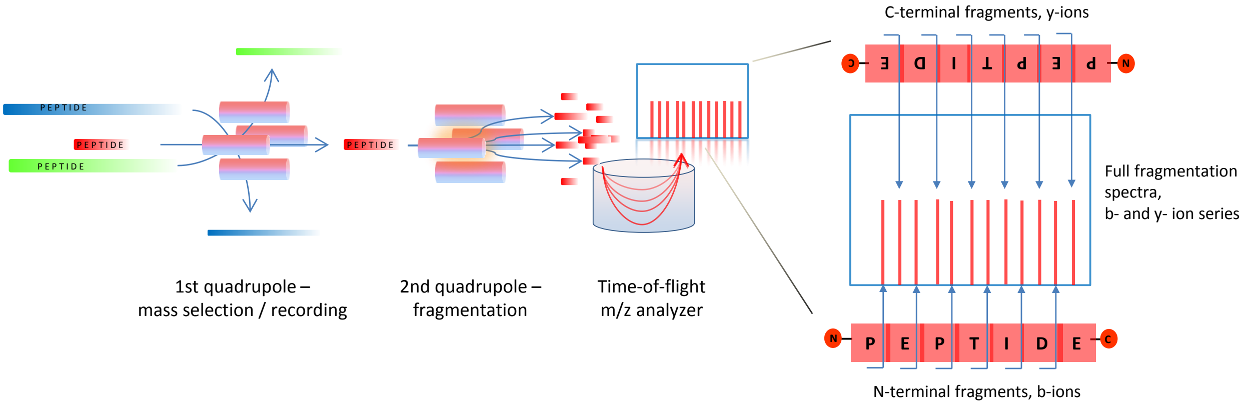

C-terminal fragments, y-ions
PEPTIDE
PEPTIDE
PEPTIDE
PEPTIDE
PEPTIDE
C E D I T P E P N
Full fragmentation spectra,
b- and y- ion series
1st quadrupole – mass selection / recording
2nd quadrupole – fragmentation
Time-of-flight m/z analyzer
N P E P T I D E C
N-terminal fragments, b-ions

Figure 6. Overview of Q-TOF type mass selection and fragmentation

The ionized peptides enter the first quadrupole which passes only one peptide for fragmentation and discards the rest. The second quadrupole fragments the selected peptide. The fragments enter the TOF detector that uses the fragment flight times to calculate the fragment masses. The fragmentation pattern of b- and y-ions of hypothetical peptide "PEPTIDE" is also shown.

2.5 MS^E tandem fragmentation

MS/MS identification is limited by the possibility of fragmenting only few precursor ions during one duty cycle. It is estimated that a full tryptic digest of all yeast approximately 5000-6000 proteins can produce up to 300 000 different types of peptides[91]. Even though quadrupole-TOF mass spectrometers can fragment up to eight precursor peptides in one duty cycle of one second or less, the majority of the 300 000 are not analyzed and the sequence information is lost[92]. To overcome this limitation a data independent acquisition (DIA) method called MS^E has been developed where all peptides enter the MS analyzer at the same time[92] (Figure 7.). The first pass of MS^E analysis scans only the precursor ions that are eluted from the LC at a given time. In the second pass the same set of precursor ions is fragmented to produce a complex set of product ions. By repeating this cycle of precursor/product scans through the entire LC gradient, the mass spectrometer generates a chromatogram of eluting precursors and also of their fragmentation products. In order to combine the fragmentation information to correct precursor ions, the chromatogram and the fragmentation spectra is processed with bioinformatics tools. In processing of MS^E data the program looks for the similar elution patterns of both the precursor and the fragment ions. When a perfect alignment is found between the retention times of precursor and product fragments the program links these product fragments to distinct precursors. Utilization of DIA methods such as MS^E has the potential to improve the identification efficiency of proteins dramatically. For example, MS^E study of tomato leaf proteome achieved over 350% increase in protein and almost 500% increase in peptide identifications compared to DDA method[93].

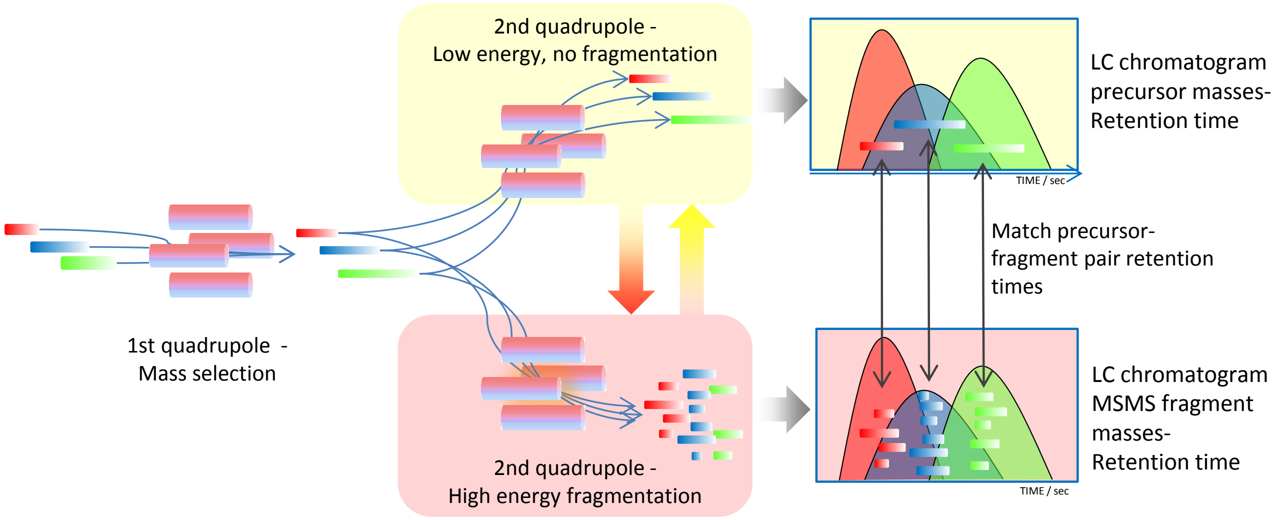


Figure 7. Principle of Q-TOF type MS^E identification

In MS^E identification the quadrupoles pass all peptides to second quadrupole. The second quadrupole cycles between low energy mode that keeps the peptides intact and high energy mode when all peptides are fragmented. The TOF records the masses of non-fragmented precursor ions as well as the fragment masses. The retention time for all masses is also saved. In the data processing step the algorithm matches the retention times so that the correct precursor-fragment pairs can be linked. This enables the detection of significantly larger number of peptides than in normal DDA method.

2.6 Data analysis of MS data

The mass detector produces a raw file of the detected m/z values. Before analysis and peptide identification this file must be processed. Several different algorithms have been developed to clear the data from noise and to extract the detected ions from the raw files[94-95]. The resulting list of detected ions, their charge, retention time and the intensity of the ions is finally compiled in to one peak list. This peak list is the basis of mass spectrometric identification.

2.6.1 Protein sequence databases

Database-related protein identification is one of the main methods in proteomic research. The sequencing of the genomes of several species has produced a library of genetic information that can be computationally translated in to protein sequence information[96,97]. Several different public databases have emerged that offer the sequence information of different proteins from different species to be downloaded and used in MS data-analysis[98]. One of the most notable proteomic databases is the UniProt/SwissProt database[99]. It contains protein sequences of almost 13 000 species and bacterial strains but also a reviewed database of manually validated protein sequences.

2.6.2 Peptide mass fingerprinting

The simplest way for peptide identification is peptide mass fingerprinting (PMF)[100]. In PMF the peptide masses are compared to a database of *in silico* calculated peptide masses of known protein sequences. If a match is found, the protein where the peptide ion originated can be identified with certain confidence. The problem with PMF is that there can be several different proteins that produce peptides with similar mass. This becomes an issue when the sample is complex or with low resolution mass spectrometers are used for PMF. For this reason PMF is used mainly in analysis pure protein solutions or of spots excised from 2-DE gels[101].

2.6.3 MS/MS identification

PMF identification relies just on the comparison of non-fragmented peptide masses to *in silico* digested masses but in complex samples additional round of search is needed[101]. In MS/MS search the second pass computationally fragments the *in silico* generated peptides to their respective fragment ions. When a match is found between the acquired precursor and computer generated ions, the fragmentation pattern on both are compared. If the patterns match, the program can statistically identify the peptide that has been fragmented and the protein where the peptide is derived from. The database-related search fails when an unknown or modified sequence is obtained. Mutations such as insertions, deletions or change of amino acids cause shifts in the precursor and fragment masses which cannot be identified when matching the observed spectra to the *in silico* generated mass list. Different search strategies[102-103] have been developed to address this issue however, they suffer from sensitivity issues and high false positive rates[104].

MS analysis of post-translational modifications can be done using optional search parameters in the MS/MS identification. When performing the *in silico* digestion and fragmentation, the program adds the mass of the modification to the precursor ion and on all the fragments. This can then be compared to the observed mass spectra of the peptide and if a match is found, the peptide and the modification can be identified. The MS identification of PTMs requires that the exact mass of the modification is known and that the amino acid where the identification is attached is specified[105].

2.6.4. *De novo* identification

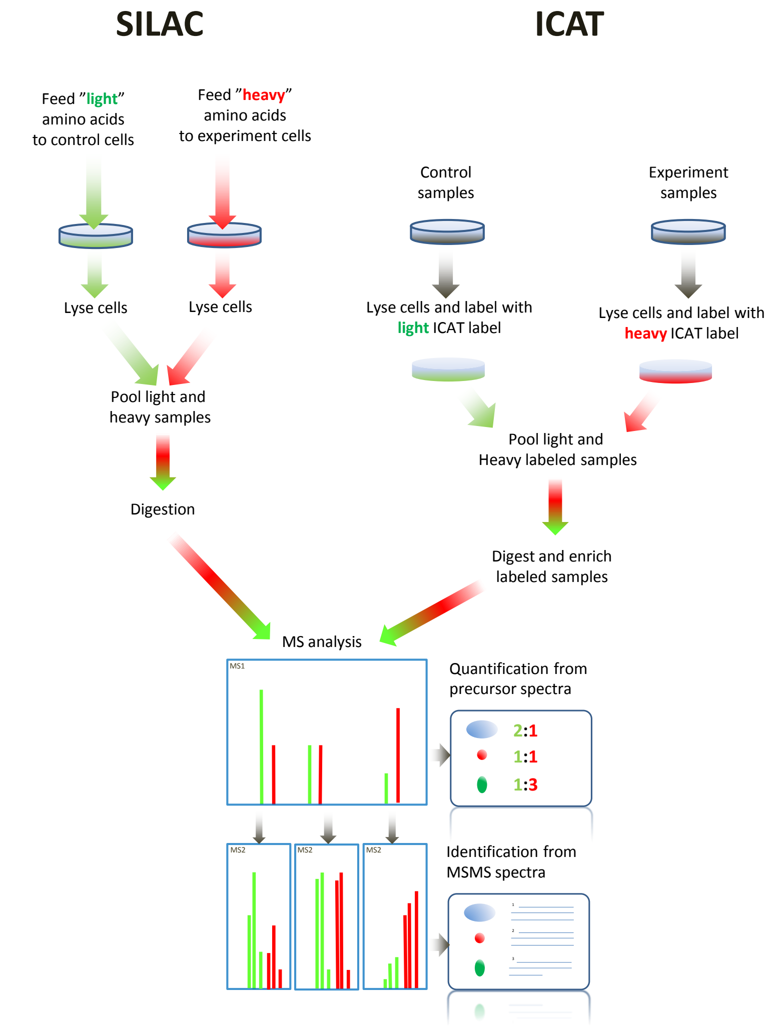
De novo identification is a complementary method of peptide identification that can be used to bypass the constraints posed by database-related searching[106]. In *de novo* identification, the peptide sequence is built from the fragmentation pattern of the precursor peptide. Instead of comparing the obtained fragmentation pattern to the database generated spectra, the sequence is deduced from the mass differences between the fragment peaks. Even if the spectra does not produce a full peptide sequence, short stretches of the deduced sequence can be used in BLAST searches to identify the original protein[107]. The problem with *de novo* sequencing is that it is computationally labor-intensive and it requires good quality spectra for correct identification[108].

2.7 MS-based protein quantification

One of the main improvements in the past few years in MS technology has been the extension of MS quantification capabilities. 2-DE gels have been the main method when comparing the expression differences of two samples but the limitations of the 2-DE system and in both detection and separation capabilities[109] have prompted researches to develop several MS-based solutions for global protein quantification[110]. These MS methods can be divided in to relative or absolute protein quantification. Relative methods are also further divided into labeled or label-free quantification. Absolute methods use added standard compounds to report the actual amount of peptides or proteins that are present in the sample while relative methods are used to compare two or more samples and to report the relative differences in quantities between them.

2.7.1 Label-based relative quantification methods

The labeling methods rely on the introduction of stable isotopes to the peptides to use in relative quantification. SILAC[111] (Stable isotope labeling by amino acids in cell culture) is a widely utilized method in proteomic quantification. In SILAC the quantification is done metabolically by feeding normal, or “light” amino acids to the control cell culture (Figure 8.). In contrast, the experimental sample is supplemented only with isotopically labeled “heavy” amino acid, usually arginine or lysine, which is incorporated to the proteome of the cell line. When performing SILAC experiments lysates from the light and heavy samples are pooled and analyzed with LC-MS. The incorporation of the heavy amino acids does not significantly alter the elution or other properties of the peptides. The only difference is the mass shift on the sample with heavy peptides caused by the isotope labeling and this mass difference can be used to separate the control from the experiment sample. The SILAC quantification is done by comparing the signal intensities of the precursor ions between the light and heavy labeled samples while the identification of the peptides is done from the fragmentation spectra of the respective precursor peptides. SILAC has been primarily used by labeling cell lines; however, the introduction of fully heavy labeled SILAC mouse[112] and recently developed super-SILAC method[113] have extended the use of SILAC to animal models and tissue samples.



SILAC
ICAT
Feed "light" amino acids to control cells
Feed "heavy" amino acids to experiment cells
Lyse cells
Lyse cells
Pool light and heavy samples
Digestion
Control samples
Experiment samples
Lyse cells and label with light ICAT label
Lyse cells and label with heavy ICAT label
Pool light and Heavy labeled samples
Digest and enrich labeled samples
MS analysis
MS1
Quantification from precursor spectra
2:1
1:1
1:3
MS2
MS2
MS2
Identification from MSMS spectra

Figure 8. SILAC and ICAT labeling protocol

SILAC quantification is performed by feeding isotope labeled, "heavy" amino acids to experiment cells. Control cells are fed with normal "light" amino acids. Both samples are prepared and then pooled and digested. In the MS analysis the light and heavy labeled peptides can be distinguished by the mass shift due to the heavy labeling. The SILAC quantification is made from the relative intensities of precursor ions and the identification from fragmentation spectra. In the ICAT method the samples are normally processed before being labeled. The control sample is labeled with "light" ICAT label while the experiment sample is labeled with "heavy" ICAT label. Similarly to the SILAC method, ICAT quantification is done using precursor intensities and identification from MS/MS spectra.

Similarly to SILAC, the ICAT[114] (Isotope Coded Affinity Tags) method uses two different isotope labels to distinguish between samples (Figure 8.). In contrast To SILAC the ICAT tags are attached to the proteins after lysis of the cells. The ICAT tag contains also a biotin component that is used in purification and enrichment of the tag containing peptides. The labeling is done by chemical attachment of the tags to cysteine. As with SILAC, the differentially labeled samples are pooled after digestion and the relative quantification is done based on the signal intensity differences of the two differentially labeled samples and identification from the product ion spectra.

The SILAC and ICAT methods use precursor spectra to quantify the peptides. Some methods employ the fragmentation spectra for both identification and quantification of the samples. As with ICAT, the iTRAQ[115] (Isobaric Tags for Relative and Absolute Quantification) and TMT[116] (Tandem Mass Tag) tags are chemically attached to amino acids of peptides before the samples are pooled (Figure 9.). However, the tagging is usually done to peptides after digestion instead of intact proteins. The TMT and iTRAQ tags consist of three separate regions: the reactive group for attachment to free amine groups of the lysines and amino termini of the peptides, a mass balancer region and the actual reporter region from which the quantification is made. The design of the tags is such that each reporter ion has a mass difference of one Dalton. This mass difference is compensated by the balancer region so that the overall masses and other properties of the tags are identical. After labeling, the samples are analyzed with LC-MS. In the MS/MS the peptide is normally fragmented to produce the spectra for peptide identification. At the same time the isobaric tag is also fragmented to free up the reporter and the mass balancer regions. The balancer is discarded but the reporter ions are detected by the mass spectrometer. The relative quantification between samples is then done from the ratios of the reporter ion intensities. The benefit of the isobaric labeling strategy is that the mass of the precursors derived from different samples is the same due to the balancer region. Compared to SILAC and ICAT, this simplifies the MS data analysis and does not reduce the precursor ion intensity. It also enhances the sensitivity of the identification as the pooling of the samples adds to the amount of each tagged peptide thus increasing the precursor peptide intensity. Additionally the incorporation of the cleavable isobaric tags enables multiplexing of samples[117]. Multiplexing allows the analysis of up to eight samples simultaneously thus reducing the time and costs of the analysis[118].

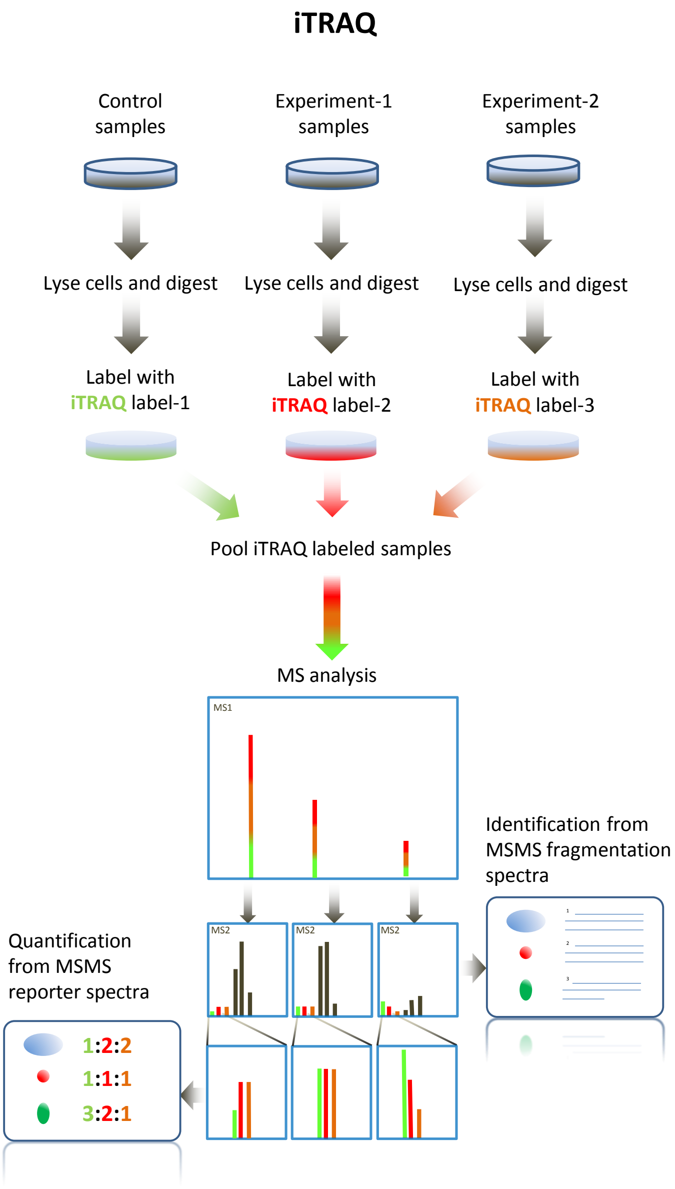


Figure 9. iTRAQ labeling protocol

In the iTRAQ quantification samples can be multiplexed using iTRAQ labels with different reporter masses. The Samples are processed individually and labeled using different iTRAQ labels. After labeling the samples are pooled and analyzed with MS. Since the iTRAQ labels have isobaric masses the precursor scan shows only one m/z for peptides compared to two same precursors (light and heavy labeled) in ICAT and SILAC. The identification of peptides is made from MS/MS fragmentation spectra. Quantification in iTRAQ is made by comparing the dissociated reporter ion intensities in the low part if MS/MS spectra. Each reporter ion of certain mass represents one sample and can be compared to rest of samples to produce relative quantification of peptides and proteins.

The problem with isotope labeling of peptides is that the labeling efficiency must be almost complete. Insufficient metabolic labeling of the heavy sample will show as an increase in the intensity of the light sample thus skewing the quantification. ICAT, iTRAQ and TMT may suffer from problems in the digestion and labeling efficiency of some samples that render the quantification unreliable. Additionally, iTRAQ and TMT can be contaminated by fragmentation of co-eluting peptides[119]. The mass window that passes the selected precursor to fragmentation for MS/MS may also pass other precursor ions with similar m/z. If two or more precursors are fragmented simultaneously, which may be the case with complex samples, the reliable quantification and identification is impossible.

2.7.2 Label-free relative quantification

Label-free quantification methods do not require derivatization of the samples prior to MS-analysis. Instead the quantification is done based on the amount of spectral matches to each protein or by the intensity of the eluting precursors[120] (Figure 10.). Spectral counting (SC) utilizes the principle that the more abundant the protein, the more spectral matches it has on the MS/MS[121]. Methods for SC include protein abundance index

(PAI)[122] and EmPAI[123] (exponentially modified PAI). In PAI the approximate quantities of proteins in the sample are calculated by simply dividing the number of observed spectra with the number of calculated observable spectra. emPAI quantification modifies the PAI score by using only unique peptides and refining the quantification algorithm.

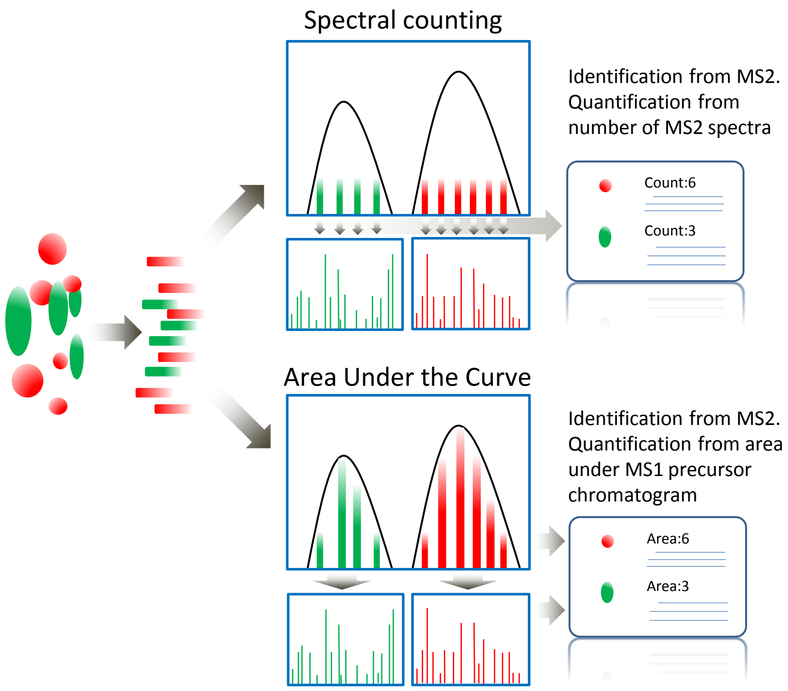

Spectral counting
Identification from MS2. Quantification from number of MS2 spectra
Count:6
Count:3
Area Under the Curve
Identification from MS2. Quantification from area under MS1 precursor chromatogram
Area:6
Area:3

Figure 10. Principles of spectral counting and area under the curve methods

In spectral counting the quantification is made by counting the number of MS/MS spectra for each peptide. In area under the curve methods the area of precursor chromatogram is integrated and quantification performed based on the total area under the chromatogram curve. In both methods the identification is made from the MS/MS fragmentation spectra.

The PAI method has some inherent problems that may distort the quantification. Different physicochemical properties of peptides cause differences in peptide ionization which result in skewed quantification[124]. Also, low spectral counts from low abundance proteins or the saturation of detector by high abundance proteins and undersampling resulting from complex mixtures interfere with quantification[125,121]. In order to overcome these issues other spectral counting software and algorithms have been developed[126,127] that have expanded the detection limits and accuracy of spectral counting quantification.

Alternative way for SC is the quantification based on integrating the ion areas of eluting peptides or AUC (Area Under the Curve) methods[128,129]. AUC relies on the discovery that in ESI the signal intensity is proportional to the molar concentration of the analyte in the sample[128]. In AUC methods the peptide quantification is made by integrating the peak area of each eluting m/z and the identification is made simultaneously from the MS/MS fragmentation of the precursor. Quantification using AUC methods has been shown to be linear within the range of 10-1000 pmol and with dynamic range of four orders of magnitude[129]. The requirement of both quantification of precursor ions and identification from the MS/MS fragments poses difficulties in DDA-based AUC quantification[130] in Q-TOF type MS instruments. An adequate number of precursor level data points must be gathered for reliable quantification but also the fragmentation spectra of the precursor must be good enough for identification of the peptide. Due to the limits in mass spectrometer duty cycle, the DDA methods often undersample complex data[131]. DIA methods can be used to circumvent the undersampling issues of DDA. As the precursor and product spectra are recorded simultaneously in DIA, enough data is collected for reliable quantification and identification. For example, MS^E-based quantification using accurate mass and retention time pairs (AMRT) was shown to identify and quantify serum spiked proteins to 100 fmol level with an average quantitative variation below 15%[130].

A drawback of the label-free methods is that multiplexing is not possible and the LC-MS analysis must be done one sample at a time. Quantification based on the comparison of different LC runs require also reliable chromatographic system that is able to deliver repeatable consecutive LC runs[132]. Current UPLC-level instrumentation can to produce good quality chromatographic separation in terms of separation, peak width and retention time stability to enable label-free quantification[133]. In addition to good quality chromatography, the bioinformatics tools must be able to process the data efficiently and with good precision. Adequate normalization of the data must be performed to account for the variations in injected sample amount or ionization. Also, the algorithms should include the different charge states of the same peptides for correct quantification[132].

Multiple reaction monitoring or MRM has been generally used in the quantification of metabolites but the developments in instrumentation and bioinformatics tools have allowed the method to be used also in the quantification of proteins[134]. MRM instrument is usually a triple quadrupole MS system (QQQ). Similarly to Q-TOF tandem mass spectrometer, two quadrupoles are placed back-to-back. The first selects the precursor ions which are then fragmented in the second quadrupole. In QQQ this is followed by a third quadrupole which is used to select certain fragments, known as transitions, which pass to the detector. The quantification is done by integrating the elution chromatogram for each selected precursor-transition pair. The main benefit of the MRM methods is its specificity and sensitivity[135]. As the precursor-transition masses are set beforehand with additional information of retention time and charge, the MS does not have to scan the entire mass range but only a small subset of the range. This frees up the duty cycle and allows the acquisition of more spectra per analyte. Also the requirement of identification of both precursor and transition masses allows for excellent specificity by eliminating majority of the unspecific precursors and fragmentation. The disadvantage of the MRM method is that the precursor-transition pairs must be known and set beforehand. In proteomics this requires the knowledge of the precursor peptide masses and the masses of the fragmentation products so analysis of unknown proteins that is not possible with MRM. The requirement of knowledge of precursor-product pairs before MRM analysis has been addressed by construction of proteotypic peptide libraries that contain the information of precursor masses of a variety of peptides and calculated fragmentation products[136].

2.7.3 Absolute MS quantification

MS-based quantification can be used also for quantification of the absolute amount of proteins in the sample. The AQUA method[137] relies on isotopically labeled synthetic peptides that correspond to the protein sequence that is quantified. A known amount of AQUA peptide is added to the digested sample and LC-MS is performed. The properties of the isotope labeled AQUA peptide and the normal peptide are the same, only difference is the mass shift from the labeling. Absolute quantification is done by comparing the ion area of the known amount of the AQUA peptide to the normal peptide. The problem with AQUA quantification is that it requires the synthesis of specific peptides so the quantification is feasible only to one or few proteins of interest. QconCAT[138] is an extension of AQUA strategy. QconCAT is made using an artificial gene that contains the sequence of a number of peptides from different proteins that are under investigation. By adding a known amount of the isotopically labeled QconCAT protein to the sample prior to digestion and analysis, the absolute amount of proteins with corresponding sequences in QconCAT can be deduced.

Label free methods can also be utilized in absolute quantification. Hi3 method is based on the observation that the top three most intense peptides per proteins reflect the absolute amount of each protein to a high degree[139]. Addition of a set of known internal standard peptides can be used to create a universal signal response factor which can be set as a reference which to compare the rest of the peptide intensities. This method has been successfully used to quantify proteins in complex mixtures with a sensitivity of less than 5 pmol/ml[139].

2.8 From MS data to biological interpretation

Mass spectrometers are able to produce a wealth of information in terms of identification and quantification. Ultimately this experimental data must be put to context and translated to biological information. For this reason several ontologies describing biological processes and connections have been developed[140]. Most notable of these is the Gene Ontology (GO)[141]. GO is used to annotate each protein to a set of descriptions that best characterize the properties of the protein. The GO uses a preset terms as descriptions so that the same term can be annotated to several proteins that share a common property. In addition, the terms are hierarchically ordered so they can be classified in the terms of their more general parent terms. This general annotation and the hierarchical structure can be used in enriching a set of interesting proteins. If experientially acquired set of proteins enriches statistically to a certain GO term, a more general view of the biological phenomena can be acquired than just examining the proteins individually. Many ontologies have been developed that use different terms based on the context of the ontology[142]. For example disease ontologies are used to specify that set of proteins that have been experimentally linked to certain diseases[143,144]. On the other hand phenotypic ontologies can be used to associate genes and proteins to different phenotypic and anatomical traits[145,146].

To help the researcher to utilize the ontologies and to combine data from different disciplines in biology, several bioinformatics tools have been devised for enrichment and analysis. Simple gene enrichments to Gene Ontology can be done using Amigo tool[147]. For more in-depth analysis researchers can use DAVID Functional annotation tool[148] or Ingenuity Pathway Analysis[149] platform for functional enrichment and analysis of proteins in several different categories and ontologies. In addition to analysis, visualization of large amounts of data has proven to be invaluable for the interpretation of the data[150] and programs like Cytoscape[151]can be used in construction, analysis and visualization of large networks of multifaceted biological data.

3. Biomarker discovery

One of the main goals of medical research is to transfer the acquired knowledge into clinical applications and biomarkers for known diseases. The term biomarker refers to measurable indicator of risk, existence, stage or response to treatment of specific diseases[152,153]. Biomarker can be almost any biological material that is found in the human body. Numerous genetic panels that test DNA sequences of known genes associated with diseases are commonly used in clinical diagnosis to evaluate the likelihood of acquiring the disease[154]. Metabolites and their intermediates are used in standard blood tests but also in diagnosis of cancers[155] and congenital disorders[156].

In recent years the promise of new clinical protein markers for diseases has been great. Numerous early findings are characterized as “potential biomarkers”, however this promise has not been fully realized in terms of clinical practise[157]. Even though several protein biomarkers have been introduced to be used to test for diseases such as prostate[158] and ovarian cancer[159] the general amount of new clinical protein biomarkers has been low within the recent years[157]. The reasons for this may be due to lack of access to clinical patient material or the costs and effort required to transform initial discovery to validated clinical solutions[153].

3.1 Biomarker categories

Biomarkers can be divided into different categories based on the use of the biomarker[152,160]. Diagnostic biomarkers are used in screening and assessment of predisposition to certain diseases. The knowledge obtained from diagnostic biomarkers can be used in preventative medication or counseling in life style or nutrition. In most diseases and especially in cancer, the early diagnosis is of utmost importance[161]. Stage specific biomarkers that signal the early phases of cancer development before it has metastasized or matured into more malignant species can significantly improve the life expectancy of patients as therapy can be started in time. Additionally, progression of the disease can be assessed by analysis of stage specific biomarkers[162] and the treatment adjusted accordingly. Special biomarkers related to drug response can be used to follow the effect of the treatment. Due to individual variations in drug metabolism same drug may not be suitable for all patients. In addition, heterogeneity of some diseases, such as cancer, may render drugs inefficient or cause adverse responses[163] so the identification of biomarkers signaling drug resistance could be used to rectify the medication and improve prognosis. Finally, outcome biomarkers can be used to evaluate the result of the treatments but also to monitor the long term recurrence of the disease.

3.2 From discovery to validation

In biomarker research there are several different phases that need to be passed before marker can be declared clinically relevant and useful[153]. The search for biomarkers starts from discovery phase where control samples are compared to diseased samples. The discovery phase can be done in model systems such as cell lines and model organisms. From this comparison of control and diseased samples a list of differentially expressed candidate markers is obtained. However, this list still contains a large portion of false positives which must be distinguished from the true positive markers. The verification step narrows and confirms the list of candidates by more comprehensive analysis of markers by orthogonal verification of the findings. Verification is conducted using methods and material more resembling the final analysis and with more samples derived from a larger population to account for individual variation. After discovery and verification the biomarker enters the validation phase. In validation the found biomarkers and the developed methodology undergo a rigorous testing for sensitivity, specificity, repeatability, reproducibility, costs and a large variety of other parameters that must be passed for biomarker and method to qualify for clinical use[164].

3.3 Protein biomarkers

Finding proteomic biomarkers for cancers and other diseases has been the goal of numerous experiments and studies[152]. The use of proteins as biomarkers is based on the idea that as the major workhorses of biological systems, diseases and other biological malfunctions may be reflected the proteomic level. For example, mutations in DNA can cause impairments in protein function or amount leading to cancer and other malignancies. Additionally, biological responses to diseases or injuries manifest often on the proteomic level so the discovery of biomarkers that signal these specific events would be extremely valuable (Figure 11.).

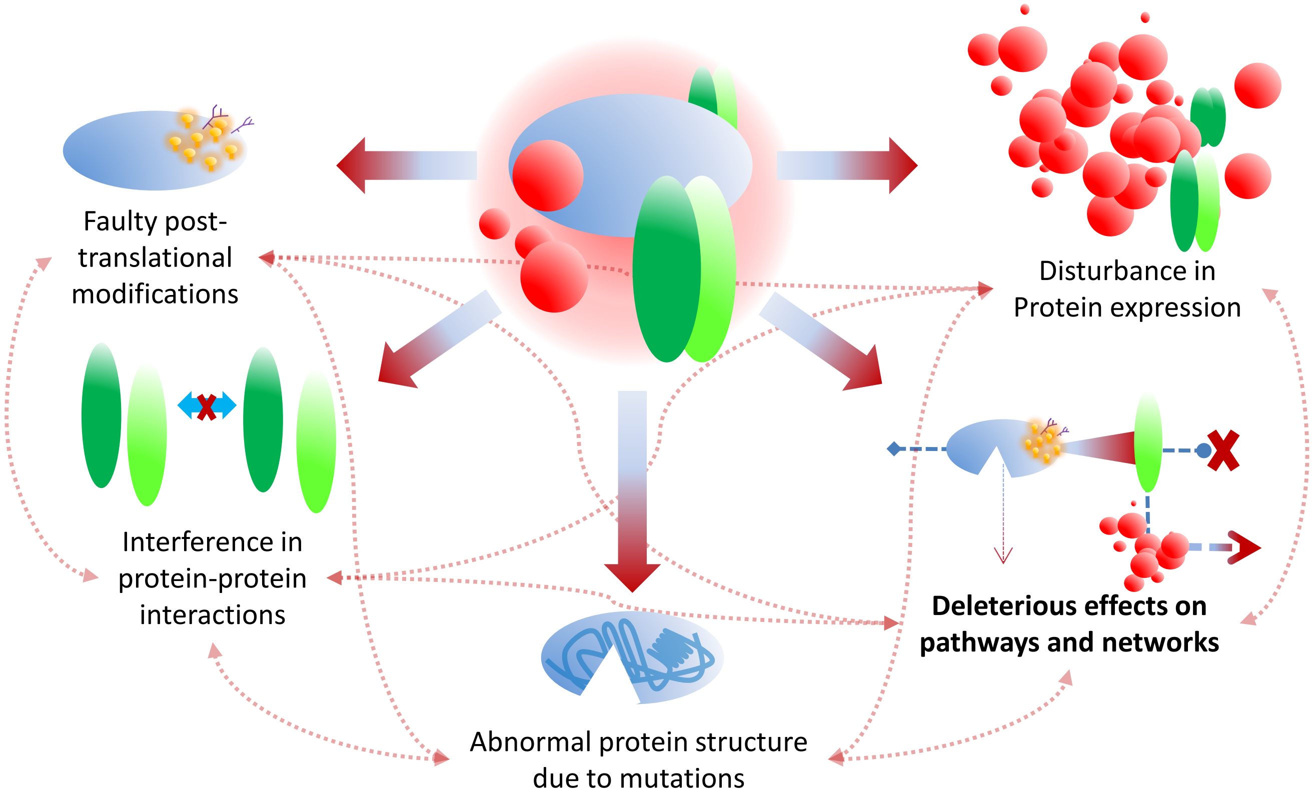

Faulty post-translational modifications
Disturbance in Protein expression
Interference in protein-protein interactions
Deleterious effects on pathways and networks
Abnormal protein structure due to mutations

Figure 11. Effects of disturbances in proteome

The disturbances in proteome may be manifested in several ways. Mutations may cause changes to protein structure, abnormal post- translational modifications, disturbed protein-protein interactions and unnatural changes in protein expression. All of these can have a significant effect in many cellular pathways and networks. Unnatural proteomic changes may also contribute to the emergence of disturbances in other proteomic areas further complicating the search for suitable biomarkers for diseases.

3.4 Source of protein biomarkers

One prerequisite of clinical biomarker is that it can be readily acquired and analyzed. Blood is considered as a good source of proteomic biomarkers as it easily sampled and contains a huge variety of proteins ranging from normal circulatory proteins as well as condition specific transient proteins. Diseased and abnormal tissues shed and release proteins that normally would not be present in blood and these can be used as biomarkers for the diagnosis of the disease[157]. Analysis of protein biomarkers from blood suffers from high dynamic range of plasma proteins and the low concentration of the analyte biomarker. Even though the local concentration of the biomarker in close proximity of the diseased tissue is high, it is significantly diluted as it enters the bloodstream. Direct analysis of proximal fluids has been shown to be more sensitive than analysis from normal blood sample[165], however the acquisition of the sample from proximal or interstitial[166] fluids is more difficult that from normal blood sample taken from forearm. Other easily obtained fluids can also be used in biomarker detection. These include urine, nipple aspirate, cerebrospinal fluid and saliva.

3.5 Mass spectrometry based proteomics in candidate biomarker discovery

The role of mass spectrometry in proteomic biomarker discovery has been growing in recent decades. The ability to analyze samples globally and to create comprehensive lists of possible candidate biomarkers makes mass spectrometers excellent tools for the discovery phase of biomarker research[167]. Quantitative MS methods allow rapid identification but also allow comparative quantification of several hundreds of proteins simultaneously. Multiplexing methods also allow the analysis of several different samples during a single MS run reducing the time, costs and inter-analysis variability[110].

3.5.1 Label-based MS quantification in clinical protein biomarker research

Several studies on discovery-based proteomics have been conducted using protein labeling and MS-based quantification. SILAC method has been employed in studies of cancerous cell lines that have identified several potential biomarkers to distinguish cancerous tissue from healthy. Everley et al.[168] compared the microsomal proteomes of two human prostate cancer cell lines with different metastatic potential using SILAC. Of the nearly 1000 identified proteins, 444 were quantified and of those 82 were shown to be significantly different between the cell lines. Another study by Kashyap et al.[169] examined the differences between the secreted proteins of esophageal squamous cell carcinoma (ESCC) cell line and normal esophageal squamous epithelial cells with SILAC-based quantification and found 120 up- or downregulated proteins including several previously identified proteins with elevated abundance in ESCC cells. SILAC methodology can be applied to tissue samples by the recently introduced super-SILAC system[113]. In a study of cell cultures done from different phases of breast cancer tumor Geiger et al.[170] used super-SILAC quantification to identify candidate biomarkers. These were then confirmed using super-SILAC on breast cancer tissue samples and based on the finding the team proposed six new candidate biomarkers for advanced breast cancer.

The SILAC method requires direct labeling of living cells and cannot be applied to human subjects. However, labeling methods such as ICAT and iTRAQ are compatible for human studies and have been used to characterize the biomarkers in human serum. Kang et al. used ICAT to search for biomarkers of hepatocellular carcinoma (HCC) from the serum of liver cirrhosis patients[171]. They identified and showed the discriminatory power of alpha-1 acid glycoprotein as a marker for HCC in cirrhosis patients. A similar study was conducted from the urine of bladder cancer patients by Chen et al.[172] identifying 55 differentially regulated proteins in cancer samples using iTRAQ. The accuracy of iTRAQ was further validated by analyzing Apolipoprotein A-1 (APOA1) on ELISA demonstrating specificity and sensitivity of over 90%. The use of iTRAQ in tissue samples was exemplified by the analysis by Ralhan et al.[173]. They examined differences in tissue samples from head and neck carcinomas and healthy control tissues taken from a same patient. Several distinguishing proteins were identified and a panel of three proteins was shown to be able to separate the carcinoma from healthy samples with good sensitivity. Cerebrospinal fluid (CSF) is a rich source of biomarkers of diseases affecting the neurological system. In a study conducted by Lehnert et al.[174] two proteins signaling the risk of Parkinson related dementia were identified with iTRAQ from CSF and then verified with MRM. Similarly, Kolla et al.[175] used plasma from pregnant women to search for candidate biomarkers for preeclampsia (PE). iTRAQ analysis was done from plasma taken from women with normal and plasma from women who developed PE. Quantification analysis revealed a list of altered proteins, including several known protein markers, which could serve as potential biomarkers for early stage PE.

3.5.2 Label free methods in MS quantification of clinical protein biomarkers

Even though the MS-based labeling methods have been proven useful in biomarker discovery the label free methods offer an alternative method of quantitative MS-analysis. Type-2 diabetes has been one of the most rapidly increasing metabolic diseases in the western world. Early diagnosis has the potential to reduce the costs and mortality of diabetes; however, an efficient and easy diagnosis method is still lacking. Saliva is an attractive source of biomarkers due to the availability and ease of procurement. To this end, Paturi et al.[176] used spectral counting to differentiate the proteomes of type-2 diabetic patients from healthy individuals. 65 proteins were identified to be differentially regulated in diabetic patients. Category enrichment revealed several metabolism and immune response related proteins among the regulated proteins. These categories have been previously reported to be associated with diabetes. In addition to identifying and verifying differentially regulated proteins, Paturi also demonstrated the increasing abundance of several identified proteins during the progression of diabetes highlighting the usefulness of saliva derived biomarkers in diabetes diagnosis and monitoring.

The advent of high definition UPLC-MS^E quantification has emerged as a promising technology in proteomic biomarker research. In an exemplary study of the Fabry disease Manwaring et al.[177] identified urine Prosaposin (PSAP) as a candidate marker for Fabry disease using MS^E-based quantification. The group also showed that the amount of PSAP decreased after 12 months of enzyme replacement therapy used to treat Fabry demonstrating the validity of PSAP in treatment monitoring. The added sensitivity and larger scale of shotgun type MS analysis has made it possible to characterize pathway-wide changes occurring in diseases and to assess the utility of different pathways as biomarkers. In a study conducted by Pizzatti et al. on chronic myeloid leukemia (CML)[178] the proteomes of plasma from treatment responsive and resistant patients was quantified using MS^E-based quantification. Ingenuity Pathway Analysis revealed altered lipid metabolism and Wnt pathway in the resistant patients. The identification of two interconnected pathways in treatment resistant CML can aide in the development of novel biomarker panels to stratify CML patients and to target the treatment more efficiently. Analogous study on primary open end glaucoma (POAG) was done by Pieragostine et al.[179]. They identified 27 differentially expressed proteins in tear fluid of POAG patients that could be used as predictive biomarkers. Pathway enrichment of these proteins indicated several inflammation- related proteins in the set of differentially expressed proteins confirming the earlier reports of the role of inflammation in glaucoma.

3.5.3 MS detection of PTM biomarkers

Differential post-translational modifications are also tempting targets in biomarker research. Some diseases may manifest in altered abundances of modified proteins that may be used as biomarkers. Potential disease PTM´s include phosphorylation, glycosylation, methylation[180] as wells as acetylation[181].

Protein phosphorylation is the most common PTM in mammalian cells and controls several signaling cascades within the cell. As the cell signaling processes are often altered in cancer, the identification of abnormal phosphorylation in cancerous patients could be highly valuable biomarkers in diagnosis and treatment. The role of phosphorylation as biomarker is limited mainly to tissue samples or other cellular samples as it not generally found in secreted fluids such as plasma. In a study of the phosphoproteome of breast cancer cells using SILAC, Oyama et al.[182] characterized the differentially modified proteins in control and resistant cells after stimulation. In addition, using a combined analysis of the phosphoproteomic and gene expression data the team identified a candidate phosphorylated biomarker that could be used to predict the relapse and survival of breast cancer patients. MS analysis of phosphoproteomics has been utilized also to search for biomarkers in early stages of Anthrax infection[183]. Manes et al. isolated the phosphopeptides from the spleen of mice in order to examine the early effects of *Bacillus anthrax* infection. 26 phosphopeptides were shown to be differentially regulated after 24 hours of anthrax exposure. If released in the circulation these phosphopeptides could serve as an early marker of anthrax exposure and subsequently as a sign for pre-emptive medication.

Studies have shown that cancer can have a major impact on the glycosylation pattern of several proteins[184]. Characterization of the altered glycoproteome has the potential to be a rich source of biomarkers in cancer diagnosis and treatment[185]. Glycosylation of Kallikrein 6 (KLK6) is altered in ovarian cancer and the can be used as biomarker for prognosis. However, the cancer produced KLK6 is masked by the normal production in the central nervous system. In order to distinguish the normally found KLK6 from cancer derived protein, Kuzmanov et al.[186] used mass spectrometry to identify differentially sialylated KLK6 that is exclusively derived from cancerous tissue. The differential glycosylation can be used to distinguish the cancer-related KLK6 from normally expressed protein and utilized as a prognostic biomarker of ovarian cancer. Characterization of global glycosylation patterns can also be used identify biomarkers. In a large study of eleven different breast cancer cell lines Boersema et al.[187] identified 1398 unique glycosylation sites from the secretome of the cells. The team used lectin affinity enrichment and MS-based super-SILAC method to compare five different stages of breast cancer progression represented by the different cells lines. The team demonstrated that the amount of differentially glycosylated proteins changes during cancer development. They also demonstrated the usability of super-SILAC in human studies by comparing the cell line derived super-SILAC mixture to plasma from human subjects resulting in identification of several common peptides.

3.5.4 MS identification of protein-protein interactions in clinical research

Abnormal changes in protein-protein interactions are usually a symptom of a disease. For example viral infections can take over the cellular machinery for their own replicative purposes. Gene mutations may lead to alterations in physical interaction sites of proteins resulting in abnormal protein activity and harmful phenotypes[188]. Elucidation of these altered interactions may provide new insights into the mechanism of the disease but also in the search for candidate biomarkers and targets for drug intervention.

HIV is one of the most devastating viral epidemics that have affected the world in the past decades. Even though HIV has been studied extensively, a cure to the disease is yet to be found. To expand the information of HIV in general and to identify candidate drug targets, several MS studies on the interactions of HIV proteins on host proteome have been conducted. Gautier et al.[189] used immobilized HIV nuclear regulatory protein Tat as bait to capture the interacting proteins from T-cell nuclear protein fraction. They identified 129 different Tat- interacting proteins that can be organized into different functional modules pertaining transcription activator and suppressors, chromosome organization factors and nuclear structure components. In a similar large scale experiment Jäger et al.[190] utilized AP-MS to characterize the entire virus-host interactome of HIV protein in Jurkat and HEK293 cells. In total 497 interactions were identified in several biological processes and cellular compartments. Of the 497 identified interactions only 19 had been previously characterized demonstrating the need for further characterization of HIV interactome.

Oncogenes are a set of proteins that have the propensity to induce cancer[191]. Usually oncogenes are a product of gene mutation or alteration in the gene expression regulating mechanisms. The oncogene expression may manifest in altered protein-protein interactions resulting in rampant cell growth and cancer progression. Identification of these altered interactions can lead to potential drug targets and cancer treatment. To this end AP-MS experiments have been conducted using oncogenes as interaction baits. In order to characterize the interaction of cancer associated histone deacetylase HDAC1 in hepatocellular carcinoma cell line, Farooq et al.[192] tagged HDAC1 with double affinity tag. 41 binding partners were identified of which 27 were novel interactors of HDAC1. Among the identified proteins were several members of cellular TCP1 and prefoldin chaperonin complexes. Similarly, Song et al.[193] probed the binding partners of colorectal cancer oncogene APC using FLAG-affinity tagged APC. The team expressed tagged protein at endogenous levels identifying several known and novel interacting proteins.

AIMS OF THE STUDY

Mass spectrometric methods and instrumentation have evolved significantly within the last decade. Novel techniques have been developed to tackle several different proteomic challenges. The goal of this work was to learn new MS methodologies and to use them to elucidate biological and clinical phenomena. The specific aims of were:

- To use different mass spectrometric identification and quantification methods to elucidate the changes in biological processes after perturbation

- To analyze different types of mass spectrometry generated proteomic data using various functional analysis tools and to generate meaningful biological and medical interpretations from the derived data and analysis results

- To use the acquired knowledge of mass spectrometric methods and biological data analysis in order to search for novel biomarkers in clinical cases

MATERIALS AND METHODS

The materials and methods are briefly displayed below. Full methodology and materials, including manufacturers and exact MS parameters can be found in the original publication or the accompanying supplementary information.

Cell lines and strains

The yeast cell line used in study I. was C-terminally TAP-tagged PSA1 (YDL055C) in W303 host strain. The HEK293 cell line for studies III. and IV. was FlipIn-293 that was transfected either with GREM1 expression vector (study IV.) or empty expression vector (study III.).

Cell cultivations

The yeast batch cultivation (study I.) was done in 30 l Braun Bioreactor at +30°C, agitation speed 800 rpm, airflow 1 l/min, and pH at 5.0. Verduyn (2X) was used as cultivation medium. Five liter samples were taken at 4, 8, 12 and 24 hours after beginning of cultivation. The cell density was calculated using OD600 measurements. The mammalian cell lines (studies III. and IV.) were done on cell cultivation plates. In study III. the cells were treated by adding 30 mM N-acetylmannosamine to induce the production of sialic acid. Control cells were treated with similar volume of PBS. Induction was performed for 24 hour before sample processing. The GREM1 expression in study IV. was induced by adding 25 ng/ml of tetracycline to cells and then incubated for 24 hours.

Plasma sampling

Three patient plasma were sampled for study II. All patients were undergoing liver transplantation surgery for primary sclerosing cholangitis. 10 ml samples were drawn simultaneously during surgery from hepatic vein (hepatic sample) immediately after flush with portal blood before connecting the craft to the normal circulation, from portal vein (portal sample) and radial artery (arterial sample). Plasma was then separated from samples and then stored at -70°C.

Protein preparation techniques

The cells in study I. were lysed using mechanically by disruption of cells in bead beater in lysis buffer. Cell suspension was cleared using ultracentrifugation. The cells for protein quantification in study III. were lysed by adding cold lysis buffer to cells, incubating for 30 min on ice and snap frozen in liquid nitrogen. The cell samples for MRM-MS were lysed by incubation in 50% acetonitrile with added internal standard for ten minutes on ice followed by centrifugation to remove debris and then dried in Speedvac concentrator. Affinity purification samples for study IV. were lysed by incubation in lysis buffer.

Protein concentration measurements were done using BCA method using Bovine albumin as standard. Plasma depletion for study II. was done using Agilent Multiple Affinity Removal column. The samples were injected to column and the flowthru collected. Three injections were made from each patient.

Affinity purification for study I. was done by applying the cleared lysate to IgG sepharose. The sepharose was washed and the protein complexes eluted by incubation with TEV protease at +16°C overnight. The flowthru was then applied to Calmodulin agarose for one hour at +4°C. The complexes were eluted using EDTA containing buffer. The purification for study IV. was done by passing the lysate through Strep-Tactin column, washing of the column and elution with biotin. The eluted GREM1 complexes were then applied to anti-HA agarose, washed and eluted using 0.2 M glycine.

The proteins in studies I. II. and III were precipitated with TCA precipitation. The TCA concentration was adjusted to 25% and proteins were precipitated for 30 min on ice. Proteins were pelleted by centrifugation at +4°C for 30 minutes before washing with ice cold 0.1 M HCl in acetone using centrifugation. The washing step was repeated with ice cold acetone and similar centrifugation. The samples were briefly air dried and stored in freezer until analysis. The samples for proteomic quantification in study III. were done using commercial detergent removal resin. The samples were incubated in the resin for two minutes and eluted with brief centrifugation.

The Western Blot validation in study III. was done using equal amount of samples in SDS-PAGE. Three control and three induced biological replicates were used. The proteins were first separated with SDS-PAGE and then transferred to PVDF membrane using semi-dry blotting. The membranes were blocked overnight with 2% BSA in PBS + 1% Tween20 at +4°C. Primary antibodies were anti ATIC (dilution 1:500), anti-NME2 (1:500) and anti-RAB5 (1:1000). Anti-beta Actin antibody (dilution 1:2000) was used as loading control. All antibody dilutions were done on PBS + 1% Tween20. Membranes were incubated in primary antibodies for 90 minutes at room temperature and then washed briefly three time with PBS + 1% Tween20 followed by three 10 minute washes in same buffer. Polyclonal secondary antibodies were HRP-conjugated goat anti-rabbit (RAB5 and beta Actin) and rabbit anti-mouse (ATIC and NME2). Washes for secondary antibodies were similar to first antibodies. The detection was performed using ECL Plus system and fluorescence detector. Quantification of proteins was done using ImageQuant TL and Excel software.

Sample processing for MS

Trypsin digestion in studies (II. III. and IV.) was performed by reducing the samples followed by alkylation of the free cysteines. The reducing and alkylation step was omitted in study I. Trypsin digestion was performed by digesting all samples overnight at +37°C.

In study II. the peptides were labeled with iTRAQ 8plex kit according to manufacturer's protocol. Labeling was performed for two hours followed by quenching with 10 µM ammonium bicarbonate for 30 minute at room temperature. The samples were then pooled and purified with SCX fractionation using cation-exchange cartridge. The bound peptides were washed and eluted using increasing KCl concentrations (5mM, 10mM, 15mM, 25mM, 35mM, 50mM, 75 mM, 100 mM, 150 mM, 200 mM, and 350mM). The samples were dried in SpeedVac concentrator and stored in -20°C.

The digested samples for studies III. and IV. were purified using C18 spin columns. The samples were applied to conditioned columns and washed. Elution was done using acetonitrile. The samples were then dried using Speedvac.

LC-MS

The peptides in study I. were analyzed with Waters Micromass nanoLC CapLC on-line with QTOF Ultima Global mass spectrometer. The used trapping column was Waters Symmetry 300 (C18, 5 µm, 300Å, i.d. 0.18mm × 23mm) and analytical column LC Packings PepMap100 (C18, 5 µm, 100Å, 75 µm i.d. × 25 cm). The gradient was done using 0.1% formic acid in acetonitrile as mobile phase A and .1% formic acid in 95% acetonitrile as mobile phase B. The gradient was from 95% mobile phase A to 5% in 600 minutes using variable flow technique at positive ion mode and data dependent acquisition. The LC-MS analysis for study II. was done using same LC set-up as study I. The gradient in study II was 95% of mobile phase A to 5% in 90 minutes. The data was acquired at positive mode using data dependent acquisition. The LC-MS analysis in study III. was performed using Waters nanoACQUITY UPLC coupled to Synapt G2-S HDMS mass spectrometer. LC gradient was run from 3% phase B (0.1% formic acid in acetonitrile) to 30% over 140 minutes. 0.1 % Formic acid in H_2O was used as mobile phase A. The used trap column was nanoACQUITY UPLC Symmetry C18 (180 µm × 20mm, 5 µm) and nanoACQUITY UPLC BEH130 C18 (75 µm × 150 mm, 1.7 µm) as analytical column. Gradient was run at +30°C. Data was collected at data independent MS^E acquisition method with positive polarity using IMS separation at 900 m/s. The LC-MS analysis in study IV. was done with nanoAcquity UPLC on-line with a Waters Synapt G2 mass spectrometer. Columns were identical to study III. and the gradient was from 3% to 40% mobile phase B in 90 minutes. Data was collected at data-dependent acquisition manner fragmenting eight peptides simultaneously using positive polarity.

The LC-MS in the MRM-MS analysis in study III. was done using Waters 626 LC system on-line with Quattro Micro mass spectrometer. The used column was Synergi Fusion-RP 80A (250 mm × 2 mm, 4 µm) with flow rate 170 µl/min. The mobile phases were 0.1 % formic acid in H_2O as phase A and 0.1 % formic acid in methanol as phase B. Gradient was run on stepwise manner for six minutes. Neu5Ac, ManNAc and labeled fructose were analyzed using negative mode. The MRM transitions for analytes were m/z 308.0 → 86.9 for Neu5Ac using collision energy 17 V; m/z 220.0 → 58.8 for ManNAc with collision energy 16 V and m/z 185.0 → 92.0 for labeled fructose with collision energy 9 V.

MS Data analysis

The raw data in studies I. II. and IV. was processed using Mascot Distiller software. In study II. the proteins were identified using X!Tandem and Lutefisk search engines. The identifications in study II. was done using Mascot search engine. The reliably identified peptide m/z-values were exported and used in MS runs of the same sample as exclusion lists to increase the number of peptide identifications. Mascot was also used to identify proteins in study IV. along with X!Tandem search engine. The protein identifications in study III. was done using Waters ProteinLynx Global Server search engine and protein quantification using Expression-E software. MRM quantification in study III. was performed with QuanLynx software and Excel.

Functional analysis

Gene ontology and KEGG analysis were used in study I. DAVID Functional tool was used in study III. using KEGG, Reactome, and Panther as limiting ontologies. Ingenuity Pathway Analysis was also used in study III. to characterize the functional changes.

RESULTS

Study I. A combined database related and *de novo* MS-identification of yeast mannose-1-phosphate guanyltransferase PSA1 interaction partners at different phases of batch cultivation

Model organisms are generally used as a starting point in many biological experiments. The ease of cultivation and manipulation enable a wide array of experiments that would not be possible using live animals or human subjects. In order to examine the interactomic changes as well as effects of enzyme limitation in database related MS identification and compare that to *de novo* identification, we analyzed the interactome of popular model organism Baker´s yeast *Saccharomyces cerevisiae* PSA1 protein during batch cultivation.

PSA1 participates in yeast cell wall synthesis by catalyzing the conversion from mannose-1-phosphate to GDP-mannose. We chose to examine the effects of different cultivation conditions on the PSA1 interactome by tandem affinity purification from different points in batch cultivation. Time points (Figure 12.) were chosen to represent the end of initial lag phase and beginning of logarithmic growth (4h), mid-logarithmic growth (8h), end of logarithmic phase and beginning of nutrient limited growth (12h) and final time point representing starvation and very low growth (24h).

The growth rate was monitored by measuring the optical density (OD600) during cultivation and calculating the growth rate (μ). The observed growth followed the expected sigmoidal curve of batch cultivated yeast population (Figure 12.). The growth curve also confirmed that the chosen time points represented the expected characteristics of population growth.

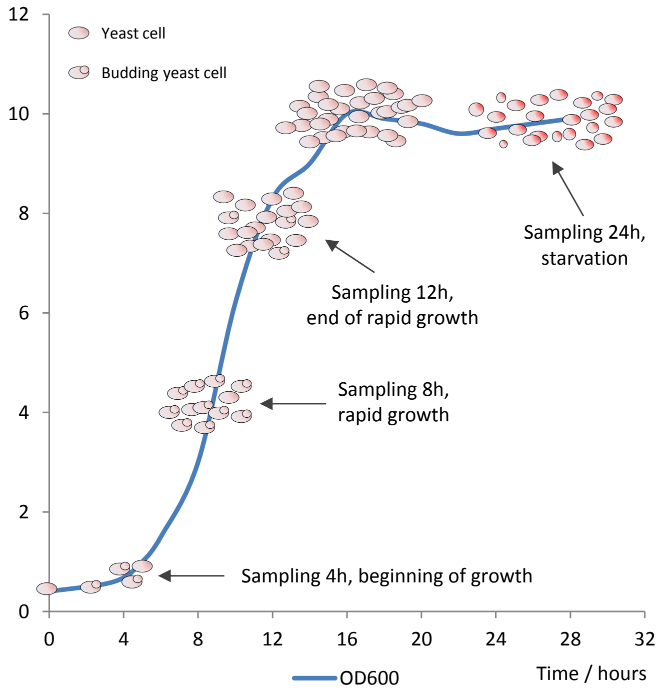


Figure 12. Yeast batch cultivation growth curve

The growth curve showed predictable characteristics of batch cultivation. At four hour sampling point the cell are beginning the rapid growth phase. At eight hours the cell are rapidly dividing and the growth rate is high. After 12 hours the lack of nutrient begins to limit the growth and the cell must adapt to new situation. After 24 hours the nutrients are scarce and the cell have adapted to starving conditions.

The protein identifications were made using database-related X!Tandem search engine[194,195] with trypsin and no-enzyme as search restrictions and Lutefisk *de novo* search algorithm combined with BLAST sequence alignment. Using all three methods and time points we identified 235 distinct proteins with at least one peptide. All three identification methods performed similarly as X!Tandem-trypsin limited identified 106, X!Tandem with no enzyme 108 and Lutefisk-BLAST 112 different proteins (Table 2.). Out of the 235 distinct proteins 31 were identified with all methods. We then limited the search based on the amount of detected peptides. Using two peptides as detection limit, the overall number of all detected proteins fell to 74 with 58 identified with X!Tandem-trypsin, 35 with X!Tandem no-enzyme and 24 with Lutefisk-BLAST. Three peptide limit further reduced the overall number of detected proteins to 43 with 38 found with X!Tandem-trypsin, 29 with X!tandem no-enzyme and only seven with Lutefisk-BLAST.

We then analyzed the proteins that were identified with at least two peptides with Gene Ontology, KEGG Pathways and Intact Protein Complex database. Largest detected category was ribosomal proteins. Of the 92 identified proteins 62 were ribosomal. Non-ribosomal proteins contained proteins involved in energy producing pathways, amino acid and lipid biosynthesis, cell signaling and cell wall morphology. Even though PSA1 is a cytoplasmic enzyme, several mitochondrial and nuclear proteins were also identified.

Comparison of identifications between time points revealed that 21 unique ribosomal proteins were identified in the four and eight hour time points. At 12 and 24 hour time points the number of ribosomal interaction partners for PSA1 fell to 11 (12h) and 10 (24 hours). In non-ribosomal proteins most interaction partners were found at eight hour time point with 21 identifications. Non-ribosomal PSA1 interacting proteins for 4 hour, 12 hour and 24 hour time points were 12, 12 and 16 proteins respectively.

We finally assessed the sensitivity of our study by comparing our data with known interaction partners for PSA1 obtained from Saccharomyces Genome Database (SGD) (Table 2.). Out of all 74 known PSA1 interaction partners we were able to identify 18 in our set of one peptide limit protein hits. Out of these nine were found with all methods, 16 with X!Tandem trypsin, 15 with X!Tandem no-enzyme and 11 with Lutefisk-BLAST. When the peptide limit was set to two, X!Tandem-trypsin identified 13, X!Tandem no-enzyme nine and Lutefisk-BLAST six SGD reference proteins. In the most stringent analysis of three or more proteins the number of Lutefisk-BLAST reference identification fell to only three proteins, while the X!Tandem no-enzyme identified seven and X!Tandem trypsin ten proteins.

Table 2. *Number of protein identifications of PSA1 binding partners in peptide limited sets by different search methods and comparison to reference set of known 74 PSA1 protein interactions.*

Identifications	***X!Tandem-trypsin***	***X!Tandem-no enzyme***	***Lutefisk***	***Total number of identifications***
One peptide limit	106	108	112	236
Two peptide limit	58	35	24	74
Three peptide limit	38	29	7	43

Number of known interactions	***X!Tandem-trypsin***	***X!Tandem-no enzyme***	***Lutefisk***
One peptide limit	16/74	15/74	11/74
Two peptide limit	13/74	9/74	6/74
Three peptide limit	10/74	7/74	2/74

Study II. Relative quantification of several plasma proteins during liver transplantation surgery.

Organ transplantation involves many phases that can influence the success of the operation. The procurement, storage, actual transplantation and following reperfusion can cause damage to the craft that may eventually lead to malfunction and loss of the craft and patient. The prognosis and treatment would significantly benefit if early markers of poor outcome could be identified. In order to characterize the proteomic changes occurring during transplantation surgery we analyzed the plasma proteomes of three individuals using iTRAQ labeling method and mass spectrometry. The plasma samples were drawn from arterial systemic circulation, portal vein that supplies blood to the liver and hepatic vein that carries the blood from the liver (Figure 13.). Sampling was performed early during the reperfusion to represent the initial phases of reperfusion-related plasma changes.

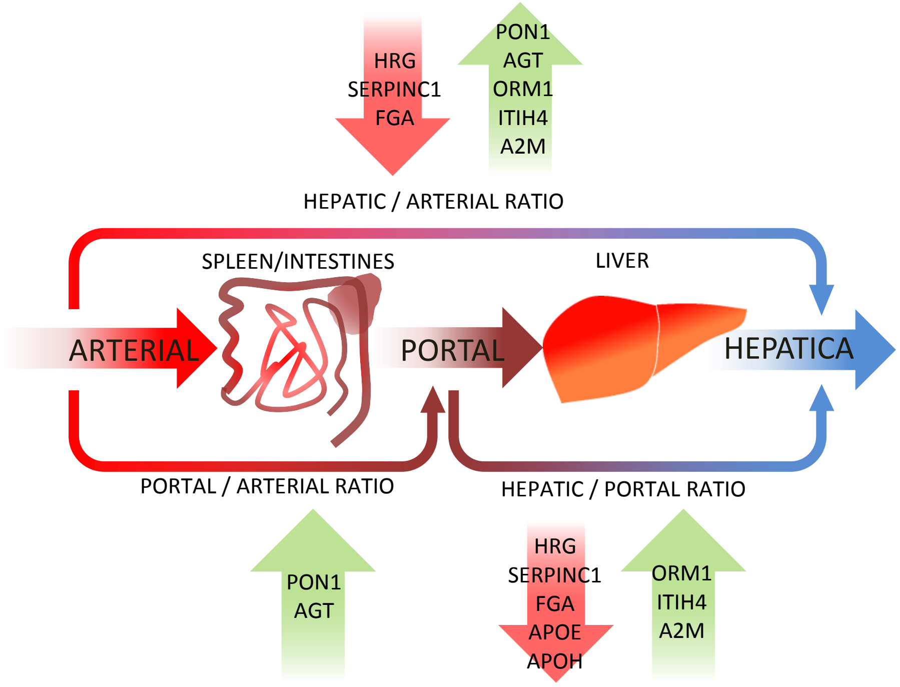


Figure 13. Plasma sampling and significantly changed proteins.

Hepatic/arterial ratio represents differences between arterial and hepatic proteins, portal/arterial represents change across intestines and hepatic/portal differences occurring within the liver

Generally the plasma samples taken from hepatic vein had approximately 30% lower protein concentration, average 53.6 mg/ml, than samples from portal vein or artery, average 76.4 mg/ml and 74.5 mg/ml respectively. In order to reduce the wide dynamic range of the plasma we removed six high abundance plasma proteins (albumin, IgG, IgA, transferrin, haptoglobin and antitrypsin). The depletion step removed on average 70% of the protein content from the samples.

Remaining plasma proteins were then digested and labeled with iTRAQ labeling kit. In order to reduce the sample complexity, the labeled samples were fractionated off-line by strong cation exchange (SCX). An exhaustive MS analysis was done on all the fractions by running each sample twice. After identification, an exclusion list was created from all the identified m/z values and new MS run was performed on the same samples. By excluding the identified peptides we were able to expand the number of identified peptides significantly as the first exclusion run identified on average 28% novel peptides compared to the no-exclusion run. The second exclusion produced an additional 10% increase in peptide identification results proving the benefit of sample mining through repeated exclusion runs.

The total number of identified proteins in all patient samples and repeat MS runs was 72. 53 of the identified proteins are found in the list of top 150 of most abundant plasma proteins[196] highlighting the limitations in sensitivity of the used methods and MS machinery. Of the 72 identified proteins 31 could be also quantified. The overall change in abundance was generally rather low as only ten proteins passed the 10% significance limit.

The examination of the over ten percent changed proteins in the portal to hepatic comparison revealed five proteins that had reduced in abundance within the liver (Figure 13). These included Histidine-rich glycoprotein (HRG), Antithrombin-III (SERPINC1), Fibrinogen alpha chain (FGA), Apolipoprotein E (APOE) and Beta-2-glycoprotein 1 (APOH). The largest reduction was observed with HRG as the amount decreased by almost 30% within the liver. Three proteins were found to increase over ten percent. Alpha-1-acid glycoprotein 1 (ORM1) was increased by 15%, Inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4) increased by 20% and Alpha-2-macroglobulin by almost 25%. The comparison of hepatic plasma to arterial plasma showed very similar changes to the hepatic to portal sample. Eight out of ten significantly increased proteins were common to both, only APOE and APOH displayed smaller than ten percent change (approximately -4% in APOE and ~-8% APOH). The changes observed in portal to arterial plasma was less pronounced as only two proteins, Serum paraoxonase/arylesterase 1 (PON1, +16%) and Angiotensinogen (AGT, +12%) were found to change more than ten percent.

Study III. Label-free mass spectrometry proteome quantification of human embryonic kidney cells following 24 hours of sialic acid overproduction

The main benefit of label-free quantification is that it does not require sample derivatization prior to MS runs. This eliminates the inevitable loss of sample during processing steps. In order to view the functional changes caused by extensive sialic acid, N-Acetylneuraminic acid (Neu5Ac), production in mammalian cells, we utilized high resolution mass spectrometry and label-free protein quantification in proteome analysis of in embryonic kidney cells. The peptides were analyzed using ion mobility separation, MS^E fragmentation and then quantified with Expression-E label-free quantification method. We also monitored the levels of Neu5Ac and ManNAc by multiple reaction monitoring MS (MRM-MS).

We induced the production of Neu5Ac by adding a 30mM ManNAc to the cell culture media. The ManNAc and Neu5Ac levels were measured prior to induction, 15 and 30 minutes after and hourly for the first six hours. Final measurement was done 24 hours after ManNAc addition. We saw no change in the levels of Neu5Ac or ManNAc in the non-induced control samples. However, the ManNAc level began to rise 15 minutes after induction. Intracellular ManNAc stabilized after one hour to approximately 2.7 times that of the zero-hour sample. After 24 hours the ManNAc level had increased to approximately 4.8 times higher than the original zero-hour sample. The Neu5Ac level did not show significant increase in the first 30 minutes but began to rise after one hour. The levels continued to rise almost linearly and were had risen approximately 70-fold after 24 hours in induction.

The proteomic changes occurring after 24 hours of induction were examined using label-free MS quantification. All samples were done using three biological replicates and all samples were run three times to ensure good technical reproducibility. Prior to quantification the quality of MS-data was assessed. The mass accuracy of runs was approximately 1 ppm. The retention time and intensity errors in detected ions between runs were on average 5.1% and 0.8% respectively. The number of identified peptides that were found in three technical replicates was on average 58% and 72% on at least two replicates. Overall the quality of data was judged good enough for label-free quantification.

In all the biological and technical replicates we were able to identify altogether 1802 distinct peptides with at least one good quality peptide. Out of these we were able to quantify 1193 in at least two biological replicates. 105 proteins were shown to be significantly up- or down-regulated (>1.3 fold regulation). Out of these only seven were up- and 98 were down-regulated. The MS quantification results were verified with Western Blot analysis of three representative proteins.

We then performed functional analysis of the set of 105 changed proteins using Gene Ontology enrichment, DAVID functional analysis and Ingenuity Pathway Analysis tool (IPA). Several categories were identified in the set of changed proteins. These included Protein transport, Plasma membrane, Signal transduction, Small GTPase, Golgi apparatus, Metabolic pathways of S-adenosylmethionine as well as Purine and Pyrimidine biosynthesis pathways and Remodeling of cellular adherens junctions. IPA analysis also revealed two interconnecting pathway clusters among the significantly changed proteins. One network contained several signaling pathways related to cytoskeletal organization, cell-cell contact and Remodeling of adherens junctions. Other interconnected network contained cell cycle, apoptosis and protein synthesis- associated categories.

In order to characterize the protein-protein interactions among the changed proteins, we downloaded all the interactions of all the 105 significantly changed proteins from PINA database[197,198]. This resulted in a list of 2421 interacting proteins and 4539 interactions. This list was filtered to include only those interactions occurring between the changed set. This resulted in 40 proteins and 47 interactions including proteins sharing functional similarities such as ribosomal, proteasome and spliceosome proteins.

Study IV. Gremlin-1 associates with fibrillin microfibrils in vivo and regulates mesothelioma cell survival through transcription factor slug

Analysis of model organisms is usually one of the first steps in biomarker discovery. Cell lines derived from diseased tissues can be examined to elucidate the mechanism behind the disease. Normal cell lines can also be manipulated in order to investigate the effects in cellular functions. In affinity purification the interaction partners of specific proteins are studied by expressing the affinity tagged protein in cell lines. The acquired interactomes of proteins can reveal interesting new biological functions but also the role of the proteins in diseases and other abnormal states. In a study of Gremlin-1 (GREM1), a cytokine inhibiting the functions of bone morphogenic factors, the interactome of GREM1 was investigated using affinity purification mass spectrometry. The findings were then validated in primary cell cultures from aggressive mesothelioma cell line and patient derived tumor tissue samples.

The interactome of GREM1 was purified using tandem affinity purification method and analyzed with high-resolution mass spectrometer. The interacting proteins were identified using Mascot and X!Tandem search engines. The use of two search strategies improves the reliability of the identifications as the algorithms are different and can be considered as orthogonal methods. The final list of GREM1 interactions composed of twelve proteins that were found in all three biological replicates and using both search engines.

The tagged GREM1 was transfected to the FlipIn HEK293 cell line using stable insertion to genome with an inducible tetracycline promoter. However, even with small induction, the amount of tagged GREM1 may be considerably higher than the native form resulting in unspecific interactions. Additionally, the tag itself or purification material may bind some proteins that will produce false positives in the results. For this reason we filtered the list of acquired interactions with those proteins that were identified from mock purification done using the cell line that was transfected with the tag-containing expression vector. 377 distinct proteins were found to bind the tag or purification material. These were removed from the identified GREM1 interactions resulting in a list of four different interacting proteins. These included Cytokeratin-9 (KTR9), Cytokeratin 2a (KRT2), APOBEC1-binding protein 2 (DNAJB11) and Fibrillin-2 (FBN2). Fibrillin-2 was identified in all three replicate experiments and at least with two peptides in two replicates.

The interaction between GREM1 and FBN2 was further investigated with surface plasmon resonance technology suggesting a strong interaction between the proteins. Additional colocalization and -expression assays in primary mesothelioma cell lines and tumor tissues also confirmed association with GREM1 and FBN2.

DISCUSSION

Database dependent search engines have become the default protein identification methods in proteomic mass spectrometry[199]. Several different algorithms have been developed to identify the peptides and their respective proteins from complex mixtures. In this work we utilized several database dependent search strategies, including Mascot[94], X!Tandem[194,195] and Waters ProteinLynx Global Server (PLGS) to identify proteins from mammalian and yeast cells as well as human plasma. In general the database dependent methods are quite straightforward. The proteins are purified, digested with trypsin and then analyzed with MS^2. However, some samples require extensive sample handling prior to MS analysis. The dynamic range of plasma proteins and the especially the overrepresentation of albumin in plasma can significantly reduce the number of identification by charge competition and other ion suppression effects[28]. In a study of proteomic changes in plasma during liver transplantation surgery we depleted six high abundance proteins from plasma samples removing approximately 70% of the proteins content. In order to further reduce the complexity of the samples prior to the MS we fractionated the digested peptides using strong cation exchange to eleven fractions. Samples were then analyzed with LC-MS/MS and Mascot search engine to identify the proteins. Overall we were able to identify 72 proteins in all patients, which is a rather low number of identifications considering the complexity of plasma[10]. One reason for the lack of proteins identification could be that the original depletion step was not adequate enough to reduce to number of high abundance proteins that mask the lower abundance peptides in the MS. Additionally, the limitations in detection sensitivity of the used, limited performance mass spectrometer has the potential to limit the detection to only the high abundance fraction of the plasma proteome.

A complementary way of database related identification is *de novo* peptide sequencing. Deciphering of peptide sequence from spectra can be used to bypass the dependence of known sequences in database related protein search[106] but also to add confidence to the data if used in conjunction with other identification methods. We used database–related search engine X!Tandem and Lutefisk *de novo* sequencing algorithm to investigate the changes in yeast PSA1 protein during various points of batch cultivation. We tested the effect of enzyme limitation to protein identifications by setting the search parameters to trypsin digested peptides and also setting the parameter to all possible peptides with no specified digestion enzyme (Table 2.). The quality of the PSA1 binding partner identifications was also examined by observing the effects of limiting the number of detected peptides in protein identification. The identification results when the identification was limited to only one detected peptide, produced almost equal number of proteins identifications in X!Tandem trypsin- limited, X!Tandem no-enzyme limitations and Lutefisk searches. However, as the peptide limit was raised to two or three the number of the quality differences between the methods became more evident. X!Tandem trypsin identified the highest number of proteins in both sets while the number of Lutefisk identified proteins fell almost 80% in two peptide limited search and 93% in three peptide limited set. X!Tandem no-enzyme identified approximately 30% less proteins in both peptide limit sets than X!Tandem trypsin. Such comparison illustrates that the traditional database-related methods still exceed *de novo* identification methods in the identification efficiency. Also, the use of specific enzyme limitation is extremely beneficial as it limits the search space of the search thus reducing the processing time but also improves the identification efficiency[199]. However, the use of *de novo* method is not without merit. In the one peptide limited search we could identify 31 proteins with all three methods. Such combined use of two totally different search algorithms can increase the confidence in the protein identification since it is unlikely that the both algorithms will identify the same random peptide. This becomes valuable especially in the low abundance proteins that are often found by only one peptide.

The added confidence in identification can be achieved also by using two database-related search engines[200]. Even if the basic principle of protein identifications is the same, the algorithms that are used to detect and score the identified proteins are different so the two search engines can be considered as complementary methods[201]. The need for more rigorous filtering criteria and validation is particularly evident in interaction studies as they have a tendency to contain a number of false positive results arising from nonspecific binding either to the proteins themselves or the material that is used in the complex isolation[58]. Therefore the validation of identifications and removal of false positives is crucial especially in cases where specific biomarkers for diseases are being examined. In the study of biological functions of lung cancer related[202,203] GREM1 in mesothelioma, we examined the interactome of GREM1 using AP-MS in HEK293 model cell line using two database-related search engines, Mascot and X!Tandem. We used stringent criteria and background filtering scheme to limit the identification to true positives. The criteria included detection in all biological replicates with good peptide hits on both search engines. We also performed a background protein subtraction using a list of proteins that were identified in AP-MS analysis of non-tagged cell line. Four proteins passed our criteria, including Fibrillin-2 (FBN2). FBN2 is a structural part of extracellular microfibrils that regulate the bioavailability of several growth and morphogenic factors such as BMP- 2, -4 and -7[204,205] which are also shown to bind and be inhibited by GREM1[206,207]. Even though we used stringent filtering criteria with the MS results to limit the results, the interaction between GREM1 and FBN2 was further examined using surface plasmon resonance technology. The results confirmed the strong physical interaction between GREM1 and the N-terminal peptide of FBN2. In order to examine the interactions *in vivo*, the expression and localization of GREM1 and FBN2 was monitored in cultured primary mesothelioma cells and in tumor tissue. In mesothelioma tumor biopsies the GREM1 and FBN2 staining patterns were very similar suggesting strong colocalization. Additionally, gene expression analysis of primary mesothelioma cell line revealed strong expression of both GREM1 and FBN2 further confirming the association between these two proteins. This work clearly illustrates the utility of AP-MS in targeted discovery type biomarker research where novel targets of diseases are being studied. Even though the exact mechanism and functions of GREM1 and FBN2 in mesothelioma need further elucidation, information obtained from this interaction experiment may greatly aide in future mesothelioma biomarkers search.

Database-related search engines identify the peptides by comparing the observed spectra to theoretical *in silico* digested spectra and calculates the probabilities of the detection[95]. In high background samples or low intensity spectra the identifications may be incorrectly assigned producing a false positive identification or rejection of true positives. In the liver transplantation plasma quantification experiment we used iterative strategy of excluding detected peptides from subsequent searches to increase the number of detected peptides. Based on the first MS run we assigned those m/z values with good peptide identification to exclusion lists. The same sample was then run again with MS/MS exclusion list, this time fragmenting only those peptides that were not identified in the first run. By using several rounds of this run and exclude-strategy and combining all of the peak lists in the final Mascot search, we were able to significantly expand the number of identified peptides and add confidence to our protein identifications.

One of the major goals of this work was to learn and utilize mass spectrometric quantification in biological samples and to examine that data in biological and clinical context. To this end we used iTRAQ labeling method[115] and label-free quantification[130] to assess the proteomic changes in human plasma and cellular lysate. In an experiment to learn more about the changes occurring in liver during liver transplantation, we labeled depleted plasma samples from three sampling points representing blood flow to and from liver and gut with iTRAQ (Figure 13.). Even though we used extensive sample fractionation and several MS runs including exclusion lists, we were able to identify altogether only 72 distinct, mostly high abundance proteins[196] and quantify 31 of those with confidence in all samples. Of the 31 quantified proteins only ten showed changes larger than 10%. Of the ten proteins that were changed more than ten percent five were reduced in abundance within liver. Four of these, (Histidine-rich glycoprotein HRG, Antithrombin-III SERPINC1, Fibrinogen alpha chain FGA, Beta-2-glycoprotein-1 APOH) are related to blood coagulation[208-209]. Additionally Beta-2-glycoprotein (APOE) was found to be consumed by the liver. APOE functions as a part of lipoprotein particles mediating their removal by hepatocytes so the clearance in the liver represents the normal liver functions[210]. Three proteins increased in abundance within liver. These included Alpha-1-acid glycoprotein (ORM1), Alpha-2-macroglobulin (A2M) and Inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4). ORM1 and ITIH4 are both acute phase, liver produced proteins[211,212]. ORM1 functions as a general transporter protein in the plasma but also as a modulator of immune system during acute-phase[213]. ITIH4 has been linked to acute phase response in trauma and has been shown to increase during acute ischemic stroke and surgery[214,211]. A2M is a high abundance protein that functions as general plasma proteinase inhibitor[215]. In general our data suggests that the coagulation cascade is activated within the craft in the very early phases of reperfusion. Simultaneously acute phase proteins are released from the liver in response to the trauma from the surgery. The quite small number of changed proteins could be due to the fact that the samples were taken from the very first rinse of the portal blood in the craft and the full proteomic effects have not had the time to occur. Alternatively, the reason could be that the levels of high abundance proteins are very unlikely to show major fold changes. A massive increase or reduction in the levels of several high abundance proteins could alter the osmotic balance of the blood leading to disruptions in humoral homeostasis and functions. Conversely, the low abundance proteins could exhibit much larger changes in response to trauma without affecting the major humoral proteomic status. However, due the sensitivity limitations of the used MS instrument and possibly insufficient sample fractionation[216,119] we were unable to characterize the low abundance fraction of plasma

One problem with iTRAQ is that the labeling protocol requires several handling and fractionation steps that may cause sample loss and unreliable quantification. Label-free quantification offers an alternative, more straightforward way of MS-based protein quantification. We utilized label free protein quantification to examine the effects of excess sialic acid production in N-Acetylmannosamine (ManNAc) induced HEK293 cells[217] (Figure 14.). Exogenous ManNAc has been shown to induce the production of sialic acid in mammalian cells[218], sialic acid in turn is generally used as terminal glycan in many glycoproteins[48]. The excessive production of sialic acid was quantified and verified using multiple reaction monitoring mass spectrometry (MRM-MS). With the added sensitivity of UPLC level separation, MS^E-based fragmentation and ion mobility separation we were able to identify over 1800 distinct proteins from HEK293 cells. We quantified the proteins using label-free, area under the curve Expression-E method[116]. Altogether we quantified the relative changes of 1193 different proteins in at least two biological repeats. The added sensitivity and resolution of the newer MS instrumentation can be clearly illustrated by comparing the iTRAQ results and Expression-E quantification. The iTRAQ experiment was performed on a limited resolution and sensitivity LC-MS system limiting the identification and quantifications to tens of proteins. On the other hand, the Expression-E experiment was done using state of the art MS instrument with high precision liquid chromatography. The effect of high pressure liquid chromatography has been shown to significantly increase the peptide separation and subsequent MS identification efficiency[133]. Additionally, in Expression-E experiment we utilized IMS separation[84] that further separates the ions within mass spectrometer thus allowing more peptide to be detected and identified. Fragmentation using DDA methods can significantly limit the number of identifications[219] but also bias the identifications to the high abundance proteins of the sample[220,3]. In the plasma work where identification was limited to high abundance proteins, the fragmentation was done using DDA. In contrast, the Expression-E experiment used MS^E method to fragment all eluting peptides thus expanding the dynamic range and number of detected proteins.

The biological data obtained using MS can be divided in to two categories. Global proteomic experiments characterize the behavior of multitude of proteins in different states as targeted experiments focus more on the characteristics of individual or a selected group of proteins. A part of the targeted proteomic research is the study of protein-protein interactions. In the study of the interaction partners of yeast GDP-mannose producing enzyme PSA1[221] in various points of batch cultivation (Figure 12), we identified major changes in the amount of ribosomal proteins binding to PSA1. The first two sampling points, representing time of high growth, showed the highest number of ribosomal protein binding totaling of 21 unique proteins. After reaching the limits of growth due to limitation in nutrients in the final two time points the number of ribosomal PSA1 binding proteins halved. Non-ribosomal proteins were seen to vary also throughout the cultivation. We identified proteins of plasma membrane functions, biosynthesis machinery, cellular signaling and energy production- related categories. Based on our data the interaction landscape of the PSA1 protein is quite dynamic. During the early phases of cultivation and time of rapid growth, the biosynthesis of PSA1 is high due to the high demand of proteins of cell wall synthesis of rapidly growing and budding cells. This may be reflected on the number of ribosomal proteins binding to newly synthesized PSA1. In the latter time points the growth is slow so the synthesis of PSA1 is diminished leading to reduced number of interacting ribosomal proteins. Similarly the interactions to non-ribosomal proteins vary based on the cultivation phase. As a key player in the cell wall synthesis machinery[221,222] PSA1 could influence the rate of cell wall synthesis by altering the direct interactions to other proteins participating in cell wall synthesis. It may also modulate other systems indirectly by binding and affecting signaling proteins or directly with interactions to proteins of biosynthetic processes and energy production. The dynamic nature of PSA1 interactome may be the reason for the rather low sensitivity when comparing to known PSA1 interactors. Only 18 out of 74 known PSA1 binding proteins were identified in the experiment. This low number could be due to false negatives resulting from missed MS detection of low stoichiometry proteins or loss of binding partners in sample processing steps. Additionally, the differences may be a result of different cultivation conditions in the reference set as we showed that the interactions are strongly dependent on the state of the cultivation and possibly other environmental factors. Even with the limitations in sensitivity, our experiment showed that AP-MS-identification and interaction analysis of samples can bring new information about the behavior of proteins in different phases of cultivation.

The global proteomic experiments produce a wealth of data of numerous proteins. Examination of each individual protein is not enough to create a full systemic picture of cellular events so analysis is often done using different ontologies[140]. When examining the effects of sialic acid overproduction in HEK293 cells we analyzed the data using several different ontologies. In our study we identified 105 proteins that were found significantly altered after induction of sialic acid production with N-Acetylmannosamine. Interestingly 100 of those proteins showed down-regulation and only seven up-regulation. The reason for such bias towards down-regulated proteins may be that cells adapt to the stress resulting from increased sialic acid concentration by down-regulating certain cellular systems rather than initiating new processes by protein up-regulation. To view the exact processes that were affected we analyzed the resulting list of 105 changed proteins using ontologies such as Gene Ontology, DAVID functional analysis tool and Ingenuity Pathway Analysis. Several enriched categories were identified. One of the main findings was that the protein transport category was clearly enriched as we identified 16 proteins in that category. Among the proteins annotated to protein transport category were several small GTPase proteins. Small GTPases are used in targeting cellular transport vesicle and also in organelle identification purposes[223-224]. A set of these small GTPases are also associated with Golgi apparatus. Golgi contains the glycosylation machinery and is the ultimate destination of CMP-activated sialic acid so the proteomic changes relating Golgi apparatus are not unexpected. Another strongly enriched category was Remodeling of Adherens Junction category. Plasma membrane bound adherens junctions mediate cellular contacts between adjacent cells using E-cadherin protein[225]. E-cadherin has been shown to contain sialic acid[226] so excess cellular sialic acid could alter the sialylation state of E-cadherin resulting in remodeling of the junction to adapt to the new situation. We also identified select metabolic and cellular proliferation related categories in the changed set of proteins. The physical interactions between the changed proteins were examined by downloading all known interaction from PINA database[198,197]. 47 interactions were identified between 40 of the changed proteins including several proteasomic, spliceosomic and ribosomal proteins. Taken together our data suggests that overproduction of sialic acid alters the transport of cellular cargo within the cell (Figure 14.). Increased flux through glycosylation machinery has been shown to affect the cell surface protein glycosylation pattern[227,228] so the changes observed in the protein transport machinery could be the result of this altered trafficking to the cell surface. One possible target of the differentially glycosylated proteins is the cellular adherens junction and its resident E-cadherin. Excess sialic acid in E-cadherin could result in disturbed cellular contacts and subsequent remodeling of the adherens junction contact points. Simultaneously the cell proliferation and metabolic processes are regulated to adapt the stress caused by abnormal sialic acid levels. Additional studies are still warranted to verify and further characterize the results; however, our experiment demonstrates the usability of global MS protein quantification and ontology-based analysis in deciphering complex cellular processes.

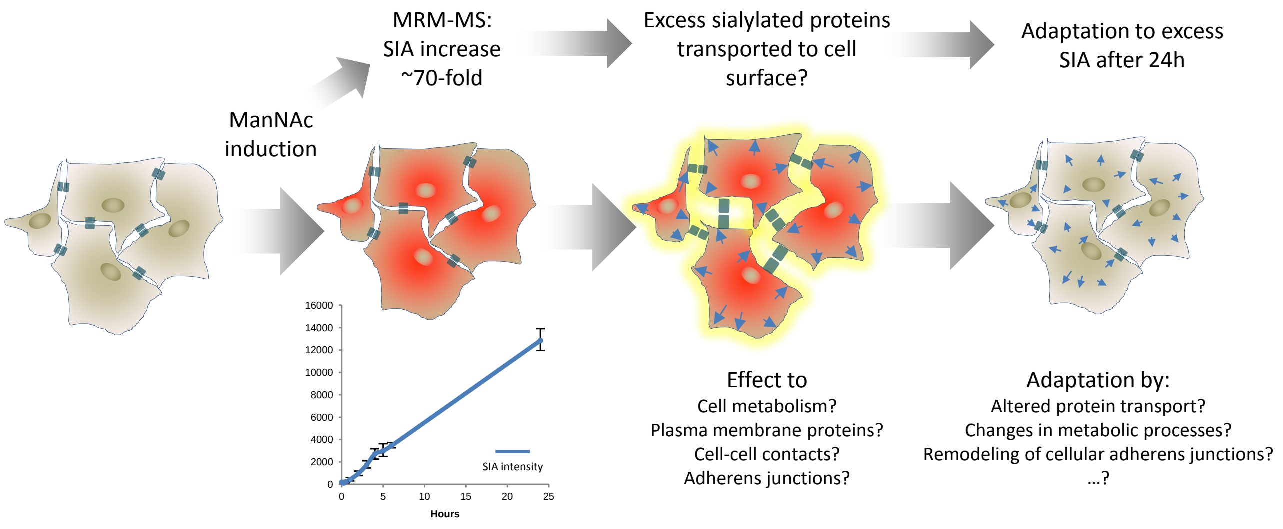


Figure 14. Effect of ManNac induction and sialic acid overproduction.

After induction with 30mM N-Acetylmannosamine the cellular sialic acid levels increased over 70-fold. This may lead to accumulation of excess sialic acid and subsequent incorporation to glycoproteins. The transport of these proteins to cell surface leads to changes in cell-cell contacts. The cells then try to adapt to the new situation by changing the cellular tranport routes and cellular adherens junctions. Additionally select metabolic routes are affected by the increase in sialic acid content.

CONCLUDING REMARKS AND FUTURE PROSPECTS

In this project we utilized mass spectrometry methods in proteomic research. We analyzed the global proteomic patterns of human plasma and model cell line as well as characterized the proteomic changes occurring after perturbations using two different mass spectrometric quantification methods. We also used several protein identification algorithms to identify protein-protein interactions from mammalian cells and Baker´s yeast. In the interaction experiment involving yeast PSA1 proteins we showed that the physical protein-protein interactions vary considerably depending on the stage of the batch cultivation. We also demonstrated the applicably of combined use database dependent and *de novo* protein identification methods in mass spectrometric proteome research. Similarly, in the study to decipher the interaction partners of lung cancer related GREM1 protein, we used two database dependent search algorithms to limit the results to true positives. One of the identified proteins was FBN2. The interaction with GREM1 and FBN2 *in vitro* was confirmed by surface plasmon resonance. *In vivo* experiments also showed strong co-localization and co-expression of GREM1 and FBN2 in primary cell lines and tumor tissue. This type of AP-MS identification of novel binding partners clearly demonstrates the benefit of targeted MS analysis of disease related proteins in basic functional characterization of these proteins but also in search for novel disease biomarkers.

The developments in quantification methodology of mass spectrometers have had a significant effect in large scale proteomic research. In this work we utilized label-based iTRAQ quantification to elucidate the changes that occur in human liver during transplantation. We employed rigorous fractionation of samples and multiple rounds of exclusion MS runs to identify 72 distinct plasma proteins. Ten of these showed abundance changes of more than ten percent. The results indicate that coagulation cascade is activated within the transplanted craft immediately upon reperfusion. The liver consumes but also secretes several proteins related to coagulation and acute phase response. The data obtained could be used as a starting point for expanded studies to find biomarkers for craft rejection as well as in patient prognosis. In addition to label-based methods we used high resolution mass spectrometry and label free MS quantification to examine the proteomic effects of excess sialic acid production in mammalian cells. After 24 hours of sialic acid overproduction we were able to show significant abundance changes of 105 proteins. Functional ontology analysis of the changed proteins showed reduction in cellular protein transport, select metabolic and signaling pathways and in the organization of cellular adherence junctions. The analysis of this type demonstrates the capabilities of simple, large scale quantitative MS analysis in decoding the larger functional processes in model biological systems but also the possibilities of such MS-analysis in novel biomarker discovery in clinical cases. In combination with external data such as interaction databases, gene expression repositories and other data sources the obtained MS data can significantly improve the analysis of cellular systems and generate information that could not have been done without the use of these high throughput methods.

This work was carried out during a time when the developments in mass spectrometric instrumentation and data processing tools have increased the amount of biological information tremendously. The ever increasing sensitivity and resolution of mass spectrometers and evolving bioinformatic solutions will surely continue to add to the already vast amount of data available. At the same time the gene expression, genetic sequence, metabolic, phenotype and other -omics data has seen a similar increase in abundance creating a wealth of data for public use. Combining all this data will provide deeper insight in the biological processes under investigation but also a deeper understanding of the complexity of entire biological systems. The challenge for future biologist is in how to decipher the multitude of data resulting from these systems biology analyses and in how to translate that in to relevant clinical solutions.

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-Ville

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