From DEPARTMENT OF MEDICINE

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MEMBRANE PROTEINS AND PROTEOMICS – UN AMOUR POSSIBLE?

Hanna Eriksson

Karolinska Institutet

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”Dä ä ba en liten väst...”

”Research is what I’m doing when I don’t know what I’m doing”

Wernher von Braun (1912-1977)

ABSTRACT

Membrane proteins constitute 20-30 % of the human genome and make up 60 % of all drug targets. They play important roles in key cellular functions such as small molecules transport, cell-cell interactions and cell signaling. Membrane proteins and proteomics have been notoriously difficult to combine. Most proteomic methods commonly used for analysis of soluble proteins cannot be used for membrane proteins, mainly due to their amphipathic nature. In this thesis, a method is described for quantitative proteomic analysis of membrane protein enriched samples. In **paper I** the method is applied to small cell lung cancer cell lines to elucidate Doxorubicin resistance mechanisms. We demonstrate that the microsomal preparation and iTRAQ labeling is reproducible regarding protein content and composition. The rationale using narrow range peptide isoelectric focusing separation is demonstrated by its ability to: i) lowering the complexity of the sample by two thirds while keeping a high proteome coverage (96%), ii) providing high separation efficiency and iii) allowing for peptide validation and possibly identifications of post transcriptional modifications. The work from paper I encouraged us to further explore the properties of narrow range peptide IEF as a separation strategy. We and others have shown that using IEF as the first dimension of separation is a highly suitable method when analyzing complex samples. It provides an orthogonal separation strategy to reversed phase chromatography as well as giving the opportunity to reduce false positives and false negatives generated in the database search, using the p*I* values of the peptides. In **paper II**, we wanted to explore the possibilities of using a combination of narrow range peptide IEF and reversed phase chromatography to increase the information content of a proteomic analysis. In the final two papers of this thesis, the method described in paper I is subsequently applied to clinical material. In **paper III**, the membrane protein fractions of benign and malignant adrenocortical tumors are compared. The mitochondrial membrane protein GRIM-19, a negative regulator of STAT3, is identified as down-regulated in the malignant tissue. The possible role of GRIM-19 down-regulation in the tumorigenesis is discussed. In **paper IV**, the membrane-associated protein population of alveolar macrophages isolated from Sarcoidosis patients is compared with healthy controls. Affected pathways are described and discussed.

LIST OF PUBLICATIONS

I. **Eriksson, H**.; Lengqvist, J.; Hedlund, J.; Uhlén, K.; Orre, L. M.; Bjellqvist, B.; Persson, B.; Lehtiö, J.; Jakobsson, P-J., Quantitative membrane proteomics applying narrow range peptide isoelectric focusing for studies of small cell lung cancer resistance mechanisms. *Proteomics* 2008, 8(15):3008-18.

II. Lengqvist, J.; **Eriksson, H**.; Gry, M.; Uhlén, K.; Björklund, C.; Bjellqvist, B.; Jakobsson, P-J.; Lehtiö, J., Observed peptide pI and retention time shifts as a result of post-translational modifications in multidimensional separations using narrow-range IPG-IEF. *Amino Acids* 2010, 40(2):697-711.

III. **Eriksson, H**.; Johansson, H.; Höög, A., Lehtiö, J.; Kjellman, M.; Jakobsson, P-J., Membrane proteomics analysis of adrenocortical tumors identifies a down-regulation of the tumor suppressor protein GRIM-19 in malignant tissue compared to benign. *Manuscript*

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Pernemalm, M.; De Petris, L.; **Eriksson, H**.; Brandén, E.; Koyi, H.; Kanter, L.; Lewensohn, R.; Lehtiö, J., Use of narrow-range peptide IEF to improve detection of lung adenocarcinoma markers in plasma and pleural effusion. *Proteomics* 2009, Jul;9(13):3414-24.

Silva, E.; Bourin, S.; Sabounchi-Schütt, F.; Laurin, Y.; Barker, E.; Newman, L.; **Eriksson, H**.; Eklund, A.; Grunewald, J., A quantitative proteomic analysis of soluble bronchoalveolar fluid proteins from patients with sarcoidosis and chronic beryllium disease. *Sarcoidosis Vasc Diffuse Lung Dis* 2007, Mar;24(1):24-32.

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LIST OF ABBREVIATIONS

2-DE	Two-dimensional gel electrophoresis
16-BAC	16-benzyldimethyl-*n*-hexadecylammonium chloride
ACA	Adrenocortical adenoma
ACC	Adrenocortical carcinoma
AM	Alveolar macrophage
BN	Blue native polyacrylamide gel electrophoresis
CHCA	*alpha*-cyano-4-hydroxycinnamic acid
DNA	Deoxyribonucleic acid
ESI	Electrospray ionization
FA	Formic acid
FDR	False discovery rate
FTICR	Fourier transform ion cyclotron resonance
GRIM-19	Gene associated with Retinoic- and Interferon-induced Mortality-19
IEF	Isoelectric focusing
IHC	Immunohistochemistry
IMP	Integral membrane protein
IPG	Immobilized pH gradient
iTRAQ	Isobaric tags for relative and absolute quantification
LC	Liquid chromatography
MALDI	Matrix-assisted laser desorption/ionization
MAP	Membrane-associated protein
mRNA	Messenger RNA
MS	Mass spectrometry
MudPIT	Multidimensional protein identification technology
m/z	Mass-to-charge ratio
PAGE	Polyacrylamide gel electrophoresis
pI	Isoelectric point
RP	Reversed phase
RNA	Ribonucleic acid
SAM	Significance analysis of microarrays
SCX	Strong cation exchange
SCLC	Small cell lung cancer
SDS	Sodium dodecyl sulfate
SRM	Selected reaction monitoring
TCA	Trifluoroacetic acid
TMD	Transmembrane domain
TOF	Time of flight

1 BACKGROUND

1.1 PROTEOMICS

The *genome* is the entirety of an organism's hereditary information. The information is coded in the DNA, which consists of both coding and non-coding sequences. The human genome was mapped in 2001[1-2] and is estimated to contain approximately 20 000 protein-coding sequences, or *genes*[3-4]. A *gene* is a series of nucleotides, or simplified, letters, and the order of these letters decide into what kind of *protein* the gene will be coded into. The proteins are the true work horses of an organism; each protein has specific functions within or outside the cell. A protein can have different functions or characteristics depending on for instance certain structural modifications or cellular location. Also, a gene can have alternative splicing variants and become differentially edited at RNA level, and consequently give rise to different proteins. This means that the number of human proteins greatly exceeds the number of human genes. The term *proteome* (in analogy to genome) was first introduced by Marc Wilkins in 1994 an was subsequently published in 1995[5]. Wilkins defined the proteome as the entire protein complement of a genome, a cell, a tissue or an organism. The human proteome has not been mapped but a single human cell has been estimated to contain ~100 000 proteins[6].

Proteomics is the common name for the methods used to study the proteome. Several "omics" research areas have emerged in the years following the mapping of the human genome, e.g. genomics (study of the genes), transcriptomics (study of the transcripts, mRNAs) and metabolomics (study of the metabolites), and new omics fields regularly pop up. The overall aim of these technologies is that by studying several molecules at the same time instead of one at the time, patterns can be recognized and previously unknown molecules may be identified of potential interest for the question at hand. In an ordinary research project, the researcher usually has a hypothesis and experiments are then performed to prove this hypothesis. In omics research, the experiments are instead hypothesis driving; you go in the direction the experiments point you.

In a proteomic experiment, either a *top-down* or a *bottom-up* approach may be employed (figure 1). In a top-down approach, focus lies on separating intact proteins, either by two-dimensional electrophoresis (2-DE) or by mass spectrometry (MALDI-TOF MS or SELDI-TOF MS). 2-DE[7] was for long the standard method in a proteomic experiment. The basic principles consist of first separating the proteins by their isoelectric point (p*I*) in an immobilized pH gradient, after which the proteins are separated by size by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The generated gels are then stained, the spots are excised and the proteins identified using mass spectrometry. On a single gel, thousand(s) of proteins can be visualized, however far from all of them are assigned with identities. Using mass spectrometry to separate intact proteins usually generates protein profiles that are compared between samples, and an alternative technique is then employed to identify the proteins. In a bottom-up approach (or shotgun approach), the proteins are first digested into peptides and focus lies on separating the peptides before mass spectrometry analyses. This approach has been used throughout the studies comprising this thesis and will be further presented in the coming sections.

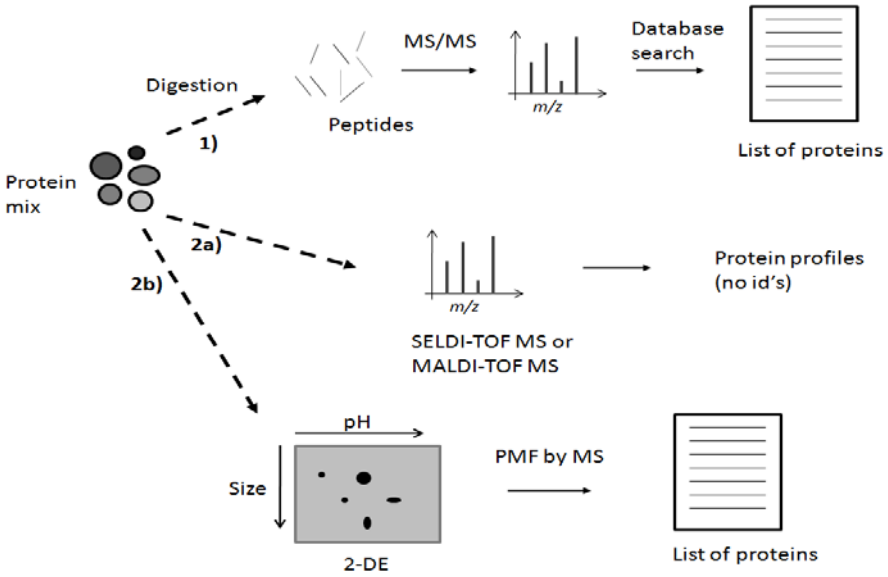

Figure 1. Proteomic experiments can have either a bottom-up approach (1) or a top-down approach (2a and 2b).

1.1.1 Fractionation

The dynamics of the proteome, as opposed to the genome, makes it more challenging to study. Not only is it greater in number, as discussed above, but the proteome constantly changes by responding to environmental parameters. Proteins are also chemically more heterogeneous as a group than DNA or RNA; they differ largely in size, solubility and p*I*. Additionally, some proteins only exist in a few copies per cell, whereas others can be high-abundant; this is often referred to as the high dynamic range problem. This presents an analytical challenge, since mass spectrometers have limited dynamic range. The complex nature of a proteome sample calls for strategies to reduce the complexity

in order to aid identification of as many proteins as possible. To do this, the sample is usually *fractionated* and this can be done either on protein level or on peptide level or by using a combination of the two.

Fractionation on a protein level can be performed either by using chemical characteristics of the proteins or their cellular location (section **1.2.1**). Liquid chromatography methods can be used to separate proteins based on hydrophobicity, size or charge[8]. There are also methods targeting specific sub-groups of proteins, e.g. glycosylated proteins[9] or high-abundant proteins[10]. One-dimensional gel electrophoresis followed by in-gel digestion of protein bands has been employed to separate proteins according to size[11].

When using a bottom-up approach, fractionation at peptide level is crucial since the complexity of the sample is further increased due to enzymatic cleavage. In analogy to protein separation, liquid chromatography can be used to separate peptides based on hydrophobicity or charge. One of the most commonly used techniques is the MudPIT (multidimensional protein identification technology) approach, where the peptides are separated both by strong anion exchange (SCX) and reversed phase (RP) [12-13]. Isoelectric focusing is a technique that has gained increasingly more interest[14-15]. It can be performed both in-gel and in-solution[16] and different pH ranges can be utilized, depending on the main purpose of the strategy. Usually, the peptides are first separated by p*I* after which a standard reversed phase separation is performed. Narrow range isoelectric focusing has been employed in all four papers included in this thesis (**papers I-IV**). In this technique, peptides are separated in an immobilized pH gradient, which spans a little over 1 pH unit. This way, the sample complexity is reduced by two thirds, while high proteome coverage is maintained (see section **2.3.6** and **paper I**). Reduced sample complexity increases the chances of identifying low-abundant proteins. Another advantage of using IEF as the first dimension separation is that the added information about the peptide p*I* can be used to validate the identifications. Several algorithms to calculate the p*I*s have been developed[17-18]; this is further explored in **paper II**.

1.1.2 Mass spectrometry

To obtain the identity of the proteins studied, mass spectrometry is by far the most widely used method today. The basic principle of mass spectrometry consists of ionizing chemical compounds to generate charged molecules or molecule fragments and then measuring their mass-to-charge (*m/z*) ratios. A mass spectrometer is built up by three modules; an ion source, a mass analyzer and a detector.

The analytes of interest are ionized and brought to gas-phase in the **ion source**. There are different types of ion sources; the two most commonly used in proteomics are MALDI (matrix-assisted laser desorption/ionization) and ESI (electrospray ionization). MALDI[19] is based on the co-crystallization of sample molecules with an energy-absorbing matrix. When a laser beam hits the crystals, a matrix plume is formed that expands into the gas-phase and the sample molecules are ionized, forming predominately singly charged ions. In ESI[20], ionization *per se* is not occurring but rather pre-existent ions are brought from solution into the gas-phase. It is a soft ionization technique giving rise to little or no fragmentation of the molecules. Since the

ESI process is starting from droplets, it is easily coupled to on-line liquid chromatography (LC).

The next step takes place when the ions enter the **mass analyzer**. Here the ions are separated based on their mass-to-charge ratios. *Quadrupoles* are the most common type of mass analyzers. Briefly, it consists of four circular rods and ions are separated based on the stability of their trajectories in the oscillating electric fields that are applied to the rods. Often three quadrupoles are placed in series; the first one is used to scan across a preset m/z range and select an ion of interest, the second quadrupole is the collision cell and the third analyzes the fragment ions generated in the collision cell[21-22]. In the *quadrupole ion trap* analyzers, ions are introduced in a pulsing mode, as opposed to normal quadrupoles where they enter in a continuous mode. The ions are detained in the ion trap, and how stably trapped the ion will be depends on the mass-to-charge ratio[22]. A *linear quadrupole ion trap* (LTQ) is similar to a quadrupole ion trap, but it has an extended volume in the trap to increase the sensitivity. In the *time-of-flight* analyzer, ions are accelerated by an electric field of known strength where the velocity of the ions depends on the mass-to-charge ratio. The time that it takes for the ion to reach the detector is measured and this time depends on the mass-to-charge ratio; smaller ions travel faster[23]. The *fourier transform ion cyclotron resonance (FTICR)* analyzer measures mass by detecting the image current produced by ions cyclotroning in a magnetic field[24]. The ions that are affected by a magnetic field move at a given cyclotron frequency depending on their *m/z* and this is subsequently measured. By using Fourier transformation the frequency is converted to a mass-to-charge value. The *Orbitrap* analyzer is similar to the FTICR, with the difference that it uses an electrostatic field instead of a magnetic field to separate the masses[25-28]. A linear ion trap is often used as a front end of the Orbitrap (LTQ-Orbitraps), thereby combining the benefits of an ion trap (speed, large trapping capacity, MS^n capability and versatility) with the benefits of an FTICR instrument (high mass accuracy, high resolving power, high sensitivity and high dynamic range)[29].

The last module, the **detector**, registers the number of ions at any given *m/z* value. Typically, some type of electron multiplier is used; the most commonly used in modern instruments is the microchannel plate detector[30]. In FTICR and Orbitrap instruments, the detector consists of a pair of metal surfaces within the mass analyzer/ion trap region which the ions only pass near as they oscillate. In all detectors, the signal is converted to a mass spectrum with *m/z* on the x-axis and ion count/intensity on the y-axis.

1.2 MEMBRANE PROTEINS

Membrane proteins are part of the interface between the outside and the inside of the cell, as well as the interface between intracellular compartments. As such, they play important roles in key cellular functions such as small molecules transport, cell-cell interactions and cell signaling. It is estimated that approximately 20-30 % of all human proteins are membrane proteins[31-32]. The fact that ~60 % of all drug targets are directed towards membrane proteins reflects their importance[33].

The membranes of living cells are composed by a lipid bilayer, which serves both to separate different compartments of the cell and to separate the cell from the surrounding environment. The lipids in the membrane consist of hydrocarbon chains, which are hydrophobic, attached to polar phosphate head groups, which are water soluble. The name bilayer stems from the fact that the hydrocarbon chains of two monolayers of lipids are fused together to form a single sheet with the polar head groups on both sides. This elegant solution makes the membrane *soluble*, but not *permeable*.

There are different classes of membrane proteins. A first distinction must be made between integral membrane proteins (IMP) and membrane-associated proteins (MAP). MAPs are attached to the surface of the membrane whereas the protein in itself is a soluble protein. IMPs on the other hand, span the lipid bilayer. The driving force behind IMP structure is the hydrophobic core of this bilayer. Since peptide bonds are highly polar, proteins must adopt secondary structures that shield the backbone from the lipid core. This is achieved by allowing extensive hydrogen bonding between backbone amides and carbonyls. IMPs are further divided into *α-helical bundle proteins* and *β-barrel proteins*.

β-Barrel proteins mainly exist in the outer membranes of Gram-negative bacteria, chloroplasts and mitochondria, where they regulate membrane integrity and allow for passive influx/efflux of small molecules[34]. The membrane-spanning segments consist of β-strands organized in a barrel where the interior forms a pore with a polar environment. β-Barrel proteins have an overall hydropathy that is similar to soluble proteins[35].

α-Helical bundle proteins are abundant and found in all membrane types, except for the outer membrane of Gram-negative bacteria. These proteins are built up from α-helices that span the lipid bilayer, connected by loop regions that extend into the cyto- or periplasm. They can be divided into *bitopic* proteins (figure 2a), which have one transmembrane domain (TMD) or *polytopic* (figure 2b), which have multiple TMDs. Bitopic proteins are often cell surface markers, receptors or adhesion factors. The 7-TMD GPCRs (G-protein coupled receptors) make out a large part of the polytopic proteins in mammals. α-Helical bundle proteins have been the focus in the experiments in this thesis, and will hereafter simply be referred to as membrane proteins.

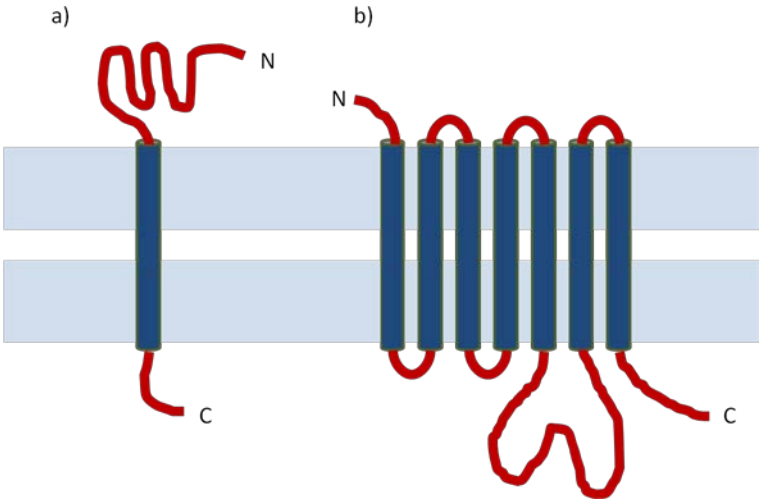

Figure 2. a) Bitopic integral membrane protein. b) Polytopic integral membrane protein.

1.2.1 Enrichment strategies

The most straightforward approach to fractionate the proteome involves cell lysis followed by sequential centrifugation steps to remove cell debris and isolate the membrane from the soluble protein fraction[36]. Further purification can be accomplished by subcellular fractionation, a concept that was pioneered by De Duve and colleagues in 1955 using rat liver tissue[37]. The procedure involves cell disruption, aiming at maintaining structural and functional integrity of the intracellular organelles, followed by a series of centrifugation steps to separate different populations of cellular compartments or organelles based on their mass and/or density[38]. One of the most widely used techniques for subcellular fractionation is density gradient centrifugation[39-40], using e.g. sucrose, sorbitol, Ficoll™ or Percoll™. Here different membranes partition into different fractions depending on the density of the membrane. This technique has been used to isolate numerous subcellular structures; e.g. plasma membranes[41], mitochondria[42], Golgi[43], synaptic vesicles[44] and platelet membranes[45]. Other techniques for subcellular fractionation include free-flow electrophoresis[46] and immunoaffinity purification[44].

The plasma membrane has gained much attention, being the physical barrier between a eukaryotic cell and its environment. Plasma membrane proteins carry out many important biological functions like intercellular communication and signal transduction. Therefore, efforts have been made to isolate only the plasma membrane, with as little contamination from other membranes as possible. Jacobson and colleagues developed a method where they coated cells with colloidal silica, after which the silica-membrane complexes were cross-linked using an anionic polymer[47]. After cell lysis, the plasma membrane sheets could be readily isolated by centrifugation owing to their increased density. The method has been applied to cells[48-49] as well as tissue[50-51]. Another method for isolation of the plasma membrane is aqueous-polymer two-phase partitioning[52]. Here, two structurally distinct water-soluble polymers are mixed in an aqueous solution and above a critical concentration the polymers will separate into two distinct phases,

with plasma membranes preferentially segregating into the more hydrophobic phase. Common polymers used in this method are polyethylene glycol (PEG) and dextran.

1.2.1.1 Enriching for integral membrane proteins

To further enrich for integral membrane proteins, there are different strategies for removal of membrane-associated proteins (MAPs) and lipids, the two most common "contaminators". MAPs can be quite readily removed from the membrane protein fraction by breaking their interactions with the polar head groups of the membranes or with the IMPs using high pH or high-ionic-strength washes. The most widely used high pH buffers are sodium carbonate and sodium hydroxide, see e.g.[53]. For ionic dissociation, typical salts include sodium chloride, potassium chloride, sodium bromide, and potassium bromide. Lipids can interfere with downstream analyses (e.g. enzymatic digestion and liquid chromatography) and the most widely used method for removal involves protein precipitation by adding methanol/chloroform[54]. Lipids partition into the chloroform layer and proteins precipitate at the chloroform/aqueous methanol interface. Other methods include cold acetone[55] (alone or in combination with other organic solvents) precipitation, TCA[56] (trichloroacetic acid) precipitation followed by acetone wash or ethanol treatment[57].

1.2.2 Prediction of transmembrane domains (TMDs)

The properties of the lipid bilayer limit the number of possible conformations of a membrane protein. Hence, there are large similarities between membrane proteins of different function and size, even if they originate from different types of membranes or different organisms. Using these similarities, important structural features can be predicted from the amino acid sequence alone. The most obvious feature that can be predicted is the number of transmembrane domains (TMDs) and where in the protein they are located. Several algorithms for prediction of TMDs have been developed; in this thesis the TMHMM[58-59] algorithm was used. This is a method based on a Hidden Markov Model. Briefly, it consists of modeling different regions of a membrane protein by choosing possible states and picking the most likely topology by comparison with experimentally derived models. Other prediction algorithms include TopPred[60], MEMSAT[61], PHDhtm[62], and HMMTOP[63].

1.3 PROTEOMICS OF MEMBRANE PROTEINS

Proteomics of integral membrane proteins has over the years proven to be challenging. The first paper dealing with a proteomic analysis of membrane proteins came as early as 1976[64]. However, it was a two-dimensional gel electrophoresis experiment and at that time there were no methods to couple gel electrophoresis with identification of the proteins. Improved analysis was claimed by changing the experimental conditions and by that showing more protein spots. Hence, there was no way to know if the proteins detected were in fact membrane proteins or not.

Membrane proteins are difficult to study mainly due to two reasons; their general *low-abundance* and their *hydrophobicity*, which resists interaction with aqueous buffers. Major challenges in any proteomic experiment are i) the sample complexity and ii) the dynamic range problem, i.e. the wide concentration range of proteins within a sample from e.g. a cell in face of the limited dynamic range of the mass spectrometer. In the light of these facts, there is no surprise that membrane proteins are under-represented in proteomic investigations.

1.3.1 Gel-based methods

Conventional 2-DE is less suited for membrane proteins, since they are often basic proteins (high p*I*) at which 2D gels have reduced resolving power. Also, at their p*I*, most proteins are less soluble and membrane proteins tend to precipitate. However, a few gel-based methods suitable for hydrophobic proteins have been developed, including 16-benzyldimethyl-*n*-hexadecylammonium chloride (BAC)/SDS-PAGE[65-66], blue native (BN)-PAGE[67] and doubled SDS-PAGE[68].

In a 16-BAC/SDS-PAGE analysis, the proteins are separated according to molecular mass in a discontinuous acidic gradient (pH 4.0-1.5) using the cationic detergent 16-BAC in the first dimension and standard SDS-PAGE in the second dimension. Although the proteins are separated my mass in both dimensions, the two detergents (16-BAC and SDS) have different binding properties, which allows for separation of proteins of similar molecular weight. The method has been used to identify mitochondrial proteins, human platelet membrane proteins and synaptic vesicle proteins. 16-BAC seems to be more efficient than conventional 2-DE at transfer into the second dimension[69], but still remains the problem of inefficient in-gel digestion and/or extraction, which hinder protein identification[44].

BN-PAGE was developed for determination of the mass and oligomeric state of mitochondrial membrane protein complexes[67,70]. The method maintains enzyme activity and native protein-protein interactions due to use of mild detergents and a running pH of 7.5. The proteins are first solubilized in a mild, nonionic detergent (e.g. digitonin or Triton X-100) after which the anionic dye Coomassie brilliant blue G-250 is added. The Coomassie dye has a relatively poor solubility in water and therefore binds to the hydrophobic surfaces of the proteins. The net negative charge of the protein-dye complexes confers electrophoretic mobility, aqueous solubility and reduced aggregation. To further resolve the individual components of the protein complexes, a second dimension SDS-PAGE is added. BN-PAGE has been quite successful in the identification of integral membrane proteins from E. coli[71] and rat mitochondria[72].

In a doubled SDS-PAGE analysis, a low percentage (10 %) acrylamide gel is used for the first dimension and a high percentage one (16 %) is used for the second dimension. Hydrophobic proteins tend to migrate faster in the first dimension due to the binding of more SDS but approach normal migration rates in the higher concentration of the second dimension, resulting in a different migration pattern than hydrophilic proteins[68]. This facilitates spot picking for subsequent identification analysis. The method has been used to identify proteins from synaptic vesicles[73] and mitochondria[42].

Even though improvements have been made in the field of gel-based methods for detection of membrane proteins, problems still reside concerning labor-intensive and tedious workflow, limited number of proteins resolved (usually in the range of 100s) and inefficient protein identification.

1.3.2 Shotgun methods

It has become increasingly popular to use shotgun approaches where the proteins are digested into peptides followed by mass spectrometry based identification and quantification[12,74] (figure 3).

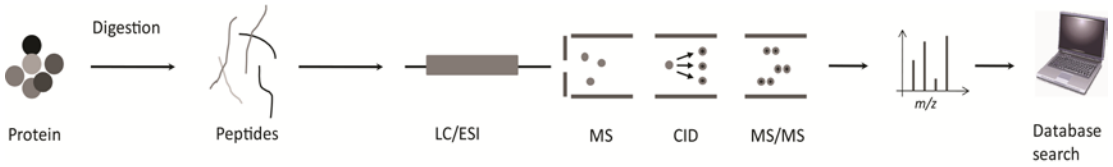

Figure 3. Shotgun proteomic workflow.

One of the advantages of using the shotgun approach is that peptides are generally more soluble than proteins in aqueous environments. This is especially an advantage when analyzing membrane proteins. However, sample complexity becomes an even greater issue, when thousands of digested proteins yield several hundreds of thousands of peptides. In 2-DE, validation of protein identifications can be carried out by using p*I* and molecular weight, and this parameter is lost in shotgun experiments. However, methods exist to estimate the false discovery rates of the database searches[75].

Shotgun proteomics methods have successfully been used for the study of membrane proteins[43,76-77].

1.3.2.1 Membrane protein solubilization

The main issue concerning membrane proteins in shotgun approaches is protein solubilization. Keeping the proteins in solution without aggregation is essential for efficient enzymatic cleavage. The hydrophobic domains of membrane proteins resist exposure to aqueous solvents, causing aggregation, adsorption and precipitation which could lead to sample loss and incomplete digestion. Thus, one must choose reagents that maintain membrane protein solubility, without interfering with downstream analysis.

Solubilizing agents can be divided into *chaotropes*, *detergents*, *organic solvents* and *organic acids*. Chaotropes are strong denaturing agents that stabilize unfolded proteins via hydrogen bonds and electrostatic interactions[78-79]. The most common ones are urea, thiourea and guanidium chloride. Thiourea is better than urea at breaking hydrophobic interactions why it is preferred for membrane protein solubilization, however it can inhibit proteases[80]. Chaotropes do not interfere with standard LC-MS/MS analyses. Detergents are a class of amphipathic molecules containing both hydrophilic and hydrophobic domains. They can self-associate into *micelles* and bind to hydrophobic surfaces such as the TMDs of membrane proteins. Detergents are classified into four groups; linear-chain ionic, nonionic, bile acid and zwitterionic. Ionic detergents have a cationic or anionic head group attached to a hydrocarbon chain. The classic example of an anionic detergent is SDS, which is extremely efficient at solubilizing and denaturing proteins[81]. However, SDS can interfere with enzymatic digestion (by steric hindrance) and can be difficult to remove[82]. SDS may also interfere with LC analyses and suppress ionization by MALDI and ESI. Nonionic detergents have a polar head group and a hydrophobic tail. They disrupt lipid-lipid interactions and lipid-protein interactions, but are generally less efficient at disrupting protein-protein interactions, and because of this nonionic detergents are considered relatively mild detergents. Examples are Triton X-100, NP-40 and *n*-octyl glucoside (OG). In general, nonionic detergents at low concentrations are compatible with downstream analyses; however Triton X-100 tends to be problematic for MALDI and ESI. Bile acid salts are ionic detergents, but their conformation differs from the linear-chain ionic detergents. They are instead steroidal compounds, with a polar and apolar face, and they have less solubilizing capacities. However, an advantage of bile acids is that they precipitate at low pH and thus can be readily removed from solution prior to downstream analyses. Examples are sodium deoxycholate (SDC) and sodium cholate. Zwitterionic (both positively and negatively charged) detergents have intermediate properties with better solubilizing capacities than bile acids/nonionic detergents but not as good as the linear-chain ionic detergents. They do not interfere with trypsin digestion or ESI, but can suppress ionization by MALDI.

As already mentioned, many of the most powerful solubilizing agents are not compatible with downstream analyses[83]. To circumvent this problem, MS-compatible detergents have been introduced. These include RapiGest (Waters)[84], PPS (Protein Discovery)[85] and Invitrosol (Invitrogen). RapiGest and PPS are acid-labile reagents which break down into non-interfering byproducts upon hydrolysis. Invitrosol exist in two variants, one MALDI-compatible and one LC/MS-compatible, where the first one do not interfere with peptide/protein analysis and the second one have orthogonal LC elution to most peptides. The MS-compatible detergents seem to perform relatively well at solubilizing membrane proteins in comparison with standard detergents.

Organic solvents are an alternative to detergents to facilitate enzymatic digestion. Due to their increased hydrophobicity relative water, they have stabilizing effects on nonpolar proteins that are unfolded. Organic solvents also have disruptive effects on the membrane and especially methanol has been proven to be efficient in aiding tryptic digestion of membrane proteins[86-88]. Organic solvents are directly compatible with downstream analyses and can be removed by evaporation, reducing potential sample loss.

Organic acids, e.g. formic acid (FA) or trifluoroacetic acid (TFA) can also disrupt membranes and solubilize membrane proteins. Usually, the membrane fraction is solubilized in 80-90 % organic acid and before digestion the sample is diluted to a more appropriate concentration for trypsin[89]. Alternatively, other proteases are used for cleavage, e.g. cyanogen bromide (CNBr)[12-13].

1.4 QUANTIFICATION AND DATA ANALYSIS

Different methods have been developed to obtain the abundances of the proteins that have been identified in a proteomic experiment. In a 2-DE experiment, the staining intensities of the gel spots are used for a relative comparison. In shotgun experiments, the methods for quantification are usually relative, meaning a comparison of peptides or proteins between experiments. Absolute measurement of protein concentrations by mass spectrometry is also possible by e.g. SRM (selected reaction monitoring).

Proteomic experiments often generate long lists of identified proteins and it can be very time-consuming to analyze the data. Therefore, programs and tools have been developed that enable visualization of the data in order to identify general trends and patterns. This kind of research is performed within the *bioinformatic* field. Bioinformatics is becoming increasingly important in aiding the proteomic investigators to extract the biological meanings of the experiments.

1.4.1 Quantification

In top-down proteomics, using e.g. MALDI-TOF MS, the quantification is based on the intensity of the peaks representing the proteins. The higher intensity, the more of the protein is expressed in that particular sample.

In bottom-up proteomic experiments, where the proteins have been digested into peptides, quantification is instead performed on a peptide level. This can be done in a label-free manor or by using some kind of labeling of the peptides. Label-free quantification is performed by either measuring the intensities of the peptide peaks generated in the chromatography step or by spectral counting in the MS step (see e.g.[90]). Peptide labeling for quantification can be performed either *in vivo* by metabolic labeling during cell growth or *in vitro* after cell lysis. The *in vitro* labeling can be further divided into *enzymatic* or *chemical modification* labeling.

The most common type of metabolic labeling is SILAC (stable isotope labeling by amino acids in cell culture)[91]. In brief, two populations of cell culture are grown separately, one with growth medium containing normal amino acids and the other with heavy isotope labeled amino acids. The medium can for example contain arginine labeled with ^{13}C instead of normal ^{12}C. This labeled arginine is subsequently incorporated into all the proteins in those cells. The cells are then combined and analyzed together and relative quantification is performed by comparing the intensity of labeled and non-labeled peptides in the MS spectrum.

In enzymatic *in vitro* labeling, enzymes (Glu-C or trypsin) are used to incorporate ^{18}O during protein digestion[92-93]. One drawback of this method is that not all peptides are labeled and hence the quantification becomes less reliable.

The most common chemical modification labeling techniques are ICAT[94] (isotope-coded affinity tag) and iTRAQ[95] (isobaric tags for relative and absolute quantification). In ICAT labeling, the cysteine residues are labeled with a mass tag containing either eight hydrogens (light reagent) or eight deuteriums (heavy reagent). After mixing and digestion, the cysteine-containing peptides are captured using avidin affinity

chromatography. Since not all peptides contain cysteines, this will also reduce the sample complexity. iTRAQ labeling targets primary amines in the peptides; hence all tryptic peptides will contain at least one iTRAQ label at the N-terminus. However, since trypsin cleaves after arginine and lysine, both having a primary amine, most peptides will have an additional label. The iTRAQ labels are isobaric, i.e. they have the same mass, and they also have the same chromatographic properties. As a result, peptides labeled with different tags will co-elute in the LC step as well as give rise to one single peak in the first MS part. What distinguish the individual tags are their fragmentation patterns in the MS/MS part, giving rise to reporter ions of different masses that subsequently can be quantified (figure 4). At present, up to eight samples can be labeled and quantified in parallel using iTRAQ labels.

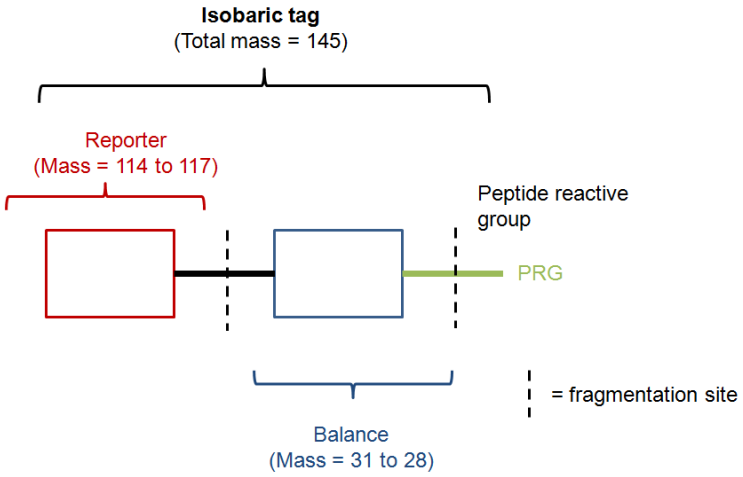

Figure 4. The iTRAQ isobaric tag consists of three groups; the peptide reactive group, the balance group and the reporter group. The reporter ions are used for quantification.

1.4.2 Statistics and Biological interpretation

The output from a proteomic experiment is not unusually a very long list of proteins with corresponding abundance data. To assist the data analysis, several statistical and bioinformatic tools are available.

To get an overview of the data and identify trends, groups or outliers, an *unsupervised* principal component analysis (PCA)[96] can be performed. In **papers III** and **IV**, this was done using Simca-P+ (Umetrics AB). In contrast, OPLS[97] (orthogonal projections to latent structures) is a *supervised* prediction method suitable when the number of variables (proteins) greatly exceeds the number of observations (samples). OPLS maximizes the covariance and correlation between x and y data (e.g. x being the protein id and quantity and y the disease state) and removes structured noise in the x data that is orthogonal (unrelated) to the response, y. Using OPLS, prediction models can be created and proteins responsible for separating two groups can be identified.

There are several software tools for functional analysis of proteomic data. Ingenuity pathway analysis (Ingenuity Systems), which was used in **papers III** and **IV**, can be used to organize proteins into networks and pathways. Other similar tools are DAVID[98], PANTHER[99], ProteinCenter (Proxeon) and FunCoup[100], where proteins are organized into groups of molecular functions, protein families, biological processes and pathways.

1.5 VALIDATION

In proteomic experiments, instrument time and sometimes reagents can be quite costly, why a limited number of samples can be included. This can be problematic from a statistical point of view and therefore validation of the findings is an important aspect. Usually a different technical platform is chosen and if possible more samples are included. Common techniques for validation are different immunoblotting methods, e.g. *western blotting* and *immunohistochemistry* (IHC). Western blotting is a well-established technique where the samples are first separated in a normal SDS-PAGE system after which the proteins are transferred to a membrane (e.g. nitrocellulose). The protein of interest is then probed with a primary antibody, recognizing only that protein, after which a secondary antibody (linked to a reporter enzyme) towards the primary antibody is used to enhance and visualize the protein-antibody complex. Western blotting is regarded as a semi-quantitative method. IHC is another established immunoblotting method to determine protein expression in a tissue. Here, information on protein expression levels comes together with information about the localization of the protein in the cells; i.e. if it is expressed in the nucleus, the cytoplasm or the membrane, and in what type of cells. Briefly, tissue sections are prepared and incubated with a primary antibody directed against the protein of interest, and subsequently with a secondary antibody directed against the primary antibody. In analogy with western blotting, the secondary antibody is linked to a reporter enzyme, which enables visualization. *ELISA* (enzyme-linked immunosorbent assay) is another immunoblotting technique frequently used.

Selected reaction monitoring (SRM) is a method for targeted quantitative analysis of peptides in cells or fluids[101-103]. Usually a triple quadrupole mass spectrometer is utilized. In peptide SRM selected peptides are fragmented and specific fragments are used for quantification. In addition to validating the identification, an absolute measurement of the protein can be obtained by the use of stable isotope standards.

1.6 LUNG CANCER

Lung cancer is globally the most commonly diagnosed type of cancer and also the most common cause of cancer death[104]. There are two major types of lung cancer with distinct clinical characteristics and behavior, namely small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). SCLC, which is the type studied in **paper I**, represents approximately 15 % of lung cancer cases and is strongly induced by smoking. It is very aggressive and it has often already begun to spread to form distant metastases at the time of diagnosis. SCLC is highly sensitive to chemotherapy and radiotherapy, but after an initial response, resistant clones start growing and the disease rapidly progresses[105].

1.6.1 Chemotherapy and drug resistance

Cytotoxic drugs, or chemotherapy, usually targets DNA and are more or less unspecific in the sense that both cancer cells and normal cells are affected. It was the use of mustard gas and other chemical warfare agents during world wars I and II that initiated research on their possible therapeutic potentials. Soldiers exposed to these agents displayed decreased white blood cell counts and showed lymphotoxic symptoms[106]. Mustine, a mustard gas analogue, was subsequently used in treatment of leukemia, and it was suggested that its mechanism of action was due to alkylation. Since then several cytotoxic drugs have been developed targeting DNA or associated processes. Apart from alkylating agents, cytotoxic drugs include antimetabolites (interfere with nucleotide metabolism), topoisomerase inhibitors (interfere with DNA replication), antitumor antibiotics (inhibit replication and/or induce cleavage of DNA) and tubulin interacting agents (inhibit mitosis and induce apoptosis).

Doxorubicin, an antitumor antibiotic, was originally isolated from bacteria, and it seems to have several cytotoxic effects. Through intercalation (fitting between base pairs in DNA) doxorubicin inhibits replication, but it has also been shown to act as a topoisomerase II inhibitor[107].

A major limitation in chemotherapy is that many tumors are resistant to specific cytotoxic drugs (primary resistance), or develop resistance during treatment (secondary resistance). Typical reasons for resistance are increased drug efflux, decreased drug uptake, increased drug metabolism, alteration of the drug target or resistance to apoptosis. Doxorubicin resistance in SCLC has been shown to be due to up-regulation of MRP1[108] (multidrug resistance-associated protein 1), however resistance to anthracyclines (e.g. Doxorubicin) is generally considered multi-factorial[109].

1.7 ADRENAL GLAND

The adrenal glands are named for their location relative to the kidneys. The term *adrenal* comes from *ad-* (Latin, "near") and *renes* (Latin, "kidney"). Sitting on top of the kidneys, they are endocrine glands mainly responsible for releasing hormones in conjunction with stress through the synthesis of corticosteroids such as cortisol and adrenaline. Adrenal glands affect kidney function through secretion of aldosterone, a hormone involved in blood pressure regulation. Each adrenal gland is separated into two structures, the *cortex* and the *medulla*, both of which produce hormones. The cortex mainly produces the steroid hormones cortisol, aldosterone and androgens, while the medulla produces adrenaline and noradrenaline. The cortex comprises three distinct regions; *zona glomerulosa*, *zona fasciculata* and *zona reticularis*. The first region produces aldosterone, while the second and the third regions produce cortisol. The cells that make up these three regions contain lipid droplets containing cholesterol, which can be converted into the steroid hormones.

Aldosterone controls the salt content of the body and by that also the blood pressure. The amount of aldosterone produced is controlled, in part, by a protein from the kidney, renin. When the blood pressure falls, the amount of renin is increased. Renin converts angiotensinogen to angiotensin I, which in turn is converted to angiotensin II. This results in increased aldosterone production from the adrenal cortex, which acts on the kidneys to retain more salt. The salt is followed by water, which results in increased blood volume, hence the blood pressure increases. *Cortisol* acts on cells in different tissues in influencing general metabolism, blood pressure and appetite. The amount of cortisol is controlled by the hormone ACTH (adrenocorticotrophic hormone), which is secreted by the pituitary gland. This secretion is in turn controlled by other hormones from the hypothalamus. The major stimulus to this sequence of hormone secretions is stress, and cortisol is therefore a key component of the "fight or flight" reaction in a moment of crisis.

The adrenal medulla is essentially a part of the sympathetic division of the autonomic nervous system. The main product of the cells in the medulla is *adrenaline*, which is involved in the "fight or flight" reaction alongside cortisol. More adrenaline is produced under stress, by sympathetic nerve stimulation. *Noradrenaline* is a neurotransmitter, and is also secreted by the medulla but in much smaller amounts.[110]

1.7.1 Adrenocortical tumors

Adrenocortical tumors, the focus of **paper III**, have a relatively high prevalence in the general population of up to 9 % in autopsy studies[111]. However, malignancies are rare, with a yearly incidence of 2 per million inhabitants, but they have a poor prognosis[112]. Recent advances in the bioimaging field together with the more frequent use of computed tomography (CT) and magnetic resonance imaging (MRI) have increased the number of detected adrenocortical tumors[113]. These accidentally discovered tumors are called adrenal incidentalomas and the majority of them are benign and non-functioning adenomas[114]. Distinguishing between adrenocortical carcinomas (ACCs) and adrenocortical adenomas (ACAs) can be difficult. In the clinical decision making, the tumor size and the CT Hounsfield measurements are the most important features in

determining if the tissue alteration is benign or malignant. Masses less than 3 cm in diameter are usually benign; by contrast, if the mass is larger than 6 cm the probability of malignancy increases [115]. Masses measuring between 3 and 6 cm are uncertain and since early resection of ACCs is the best chance of survival, an accurate diagnosis of a small tumor is very important [115]. All tumors with a diameter larger than 4 cm are recommended to be resected [116]. There is a need for more sufficient diagnostic tools, both from a diagnostic point of view but also to avoid unnecessary abdominal surgery.

1.8 SARCOIDOSIS

Sarcoidosis, the disease studied in **paper IV**, commonly affects young and middle-aged adults and frequently presents with bilateral hilar lymphadenopathy, pulmonary infiltration, ocular and skin lesions. The liver, spleen, lymph nodes, salivary glands, heart, nervous system, muscles, bones and other organs may also be involved[117].

Sarcoidosis is a systemic granulomatous inflammatory disease where the lungs are involved in 90% of the patients[118]. It is a multi-factorial disease where genetic as well as environmental factors are relevant[119-120]. The clinical manifestations are heterogeneous and can be divided into two main groups. Patients with Löfgren's syndrome have an acute onset with erythema nodosum and/or ankle arthritis, fever and bilateral hilar lymphadenopathy, are often HLA-DRB1-03^{pos}, and usually resolve spontaneously[121]. On the other hand, non-Löfgren's patients, accounting for 65 % in Scandinavia, usually have an insidious onset of the disease with a prolonged disease course, especially when they are HLA-DRB1-14/15^{pos} [122].

Alveolar macrophages (AMs) are implicated in the pathogenesis of sarcoidosis through multiple functions. They act as antigen-presenting cells (APCs) and express co-stimulatory molecules such as CD40, CD80 and CD86 and interact with CD4 positive T-cells, stimulating adaptive responses (Th1)[123]. IFN-γ and TNF-α produced by activated T-cells are essential for the formation of non-caseating granulomas, which is the hallmark of sarcoidosis[124].

The etiology of sarcoidosis is unknown, however some antigen candidates have been proposed as the cause of the disease, such as *Mycobacterium tuberculosis* antigen mKatG[125] and *Propionibacterium acne*[126-127]. Also autoimmunity responses to vimentin has been observed in sarcoidosis patients[128].

2 THE PRESENT STUDY

2.1 AIMS

The overall aim of this thesis was to develop a high throughput and reproducible method for proteomics on membrane proteins. The method had to be applicable to different materials since a downstream aim was to use the method to analyze clinical tissue in order to identify potential biomarkers or new therapeutic targets.

The specific aims were the following:

Paper I: To develop a membrane proteomics method, using cell line material.

Paper II: Explore the possibilities of using peptide p*I* and retention time data to increase the information content of proteomic investigations.

Paper III: Use the method developed in **paper I** on adrenocortical tumor tissue in order to shed light on the biology behind malignancy and to find markers that differentiate benign tumor from malignant.

Paper IV: Characterize and describe altered pathways in alveolar macrophages from Sarcoidosis patients, by looking at the membrane-associated protein population.

2.2 MATERIALS AND METHODS

A detailed description of the methods used can be found in each paper (**I-IV**). A brief description and comments are presented below.

2.2.1 Cell and tissue samples

In **papers I-II** two human cell lines were used; the SCLC cell lines H69 and H69AR. H69AR cells were incubated with 0.8 μM Doxorubicin twice monthly in order to maintain the resistant phenotype. The cells were cultured in humidified air in incubators under controlled conditions with a temperature of 37°C and at 5 % CO_2. In **paper II**, two different samples were used for the investigations of retention time effects. One was a dog plasma sample consisting of the supernatant from acetonitrile precipitated plasma and the second sample was a commercial human liver microsome preparation. In **paper III**, adrenocortical tumor tissue samples were used. The tumor tissue analyzed contained more than 70% tumor cells. Diagnostic criteria for malignancy were vascular invasion, invasion of surrounding organs and/or presence of distant metastasis. In cases where these criteria were not fulfilled the histopathological criteria proposed by Weiss was used[129]. Cases with high nuclear grade, increased mitotic figures and tumor necrosis were suspected to be malignant. In **paper IV,** bronchoscopy was performed on eight sarcoidosis patients within three months after onset of symptoms, as part of the initial diagnostic routine investigation, and on six healthy controls, as previously described[130]. The diagnostic criteria for sarcoidosis patients were in accordance with the WASOG criteria[118]: in line with sarcoidosis were symptoms, chest radiographic changes and pulmonary function tests, as well as the presence of granulomas in lung biopsies, and an increased CD4/CD8 ratio >3.5 on BAL T-cells. Alveolar macrophages were isolated from BAL cells by Percoll™ two phase density gradient ρ_1= 1.062 g/ml and ρ_2= 1.08 g/ml.

2.2.2 Microsomal preparation

Microsomes were prepared from both cells (**papers I, II, IV**) and tissue (**paper III**). Briefly, cell- or tissue homogenates were sonicated to break the cells and after a low-speed centrifugation they were subjected to ultracentrifugation at 100 000 x g for 1 h at 4°C to pellet the microsomes. Membrane-associated proteins were separated from integral membrane proteins by a high salt wash (2.5 M NaBr). The microsomal fractions were precipitated according to the method described by Wessel and Flügge[54]. Proteins were then solubilized in a buffer containing SDS.

2.2.3 Digestion and iTRAQ labeling

Proteins were digested by trypsin after dilution of the SDS concentration. Peptides were then labeled with iTRAQ; in **papers I-II** a 4-plex kit was used and in **papers III-IV** an 8-plex kit was used. This means that either four or eight samples can be labeled and pooled into one sample. In **papers III-IV**, an internal standard was included to be able to compare samples across experiments. This was done by pooling aliquots from the individual samples to one pooled standard, which was iTRAQ labeled and included in each iTRAQ experiment.

2.2.4 Isoelectric focusing

In all papers (**I-IV**), the peptides were separated by narrow range isoelectric focusing (IEF). Tryptic peptide samples were dissolved in 8 M urea. Narrow range IPG-strips for peptide focusing (pH 3.4 - 4.8 or 3.7 - 4.9, 24 cm long) together with dry sample application gels were kindly supplied by GE Healthcare Bio-Sciences AB, Uppsala, Sweden. The application gels were rehydrated in sample over night while the strips were rehydrated over night in 8 M urea and 1 % Pharmalyte™ 2.5-5. The IPG strips were put in the focusing tray and the application gels containing the samples were placed on the anodic end of the IPG strips with filter paper between the application gels and the electrodes. The strips were covered with mineral oil and the focusing was performed until 100 kVh had been reached. After focusing, peptides were extracted from the strips by a prototype liquid handling robot, kindly supplied by GE Healthcare Bio-Sciences AB. A plastic device with 72 wells was put onto each strip and MQ water was added to each well. After 30 minutes incubation, the liquid was transferred to a 96 well plate and the extraction was repeated 2 more times. Samples were then freeze dried and kept at -20 ºC. Prior to analysis, each fraction was re-suspended in 8 µl 3% acetonitrile and 0.1% formic acid.

2.2.5 LC-MS/MS analyses

2.2.5.1 LC-MALDI-TOF/TOF

In **papers I-II**, the LC-MS/MS set-up was an off-line LC system coupled to a MALDI spotter. The samples were subsequently analyzed using an ABI 4800 MALDI-TOF/TOF. Briefly, fractions from the IEF analysis were further fractionated by nanoLC chromatography, using a monolithic column. Monolithic columns have lower back pressure than traditional C18 columns, hence higher flow-rates and shorter gradient times can be used. The LC system was coupled to a MALDI spotter onto which fractions were automatically spotted together with matrix solution (CHCA). The MALDI-TOF/TOF mass spectrometer was set to perform data acquisition in the mass range of 700-4000 *m/z*. In each MS spectrum, maximum 10-15 peptides were chosen (only peptides above a set S/N threshold, usually 80-100) for fragmentation, starting with the strongest peptide.

2.2.5.2 LC-Q-TOF MS

In **paper II**, retention time analyses were performed on a UPLC-QTOF Ultima Global instrument combination. UPLC (ultra performance LC) enables faster separations with maintained separation and resolution capabilities due to small particle size in the columns (<2 µm).

2.2.5.3 LC-Orbitrap MS

In **papers III-IV**, the set-up was a nanoLC-Orbitrap MS system. Fractions from the IEF separation was loaded onto an Agilent HPLC 1200 system for online reversed-phase nano-LC using a C18 column. Acquisition in the MS system proceeded in ~3.5 s scan cycles. The top 5 ions from the full scan MS were selected firstly for collision induced dissociation (CID) with MS/MS detection in the ion trap, and finally for high energy collision dissociation (HCD) with MS/MS detection in the Orbitrap.

2.2.6 Data analysis and statistics

In **papers I-II**, peptide identification was carried out using the Paragon algorithm [131] in the ProteinPilot 2.0 software package. The searches were performed against the IPI database or NCBI non-redundant, limited to human sequences. In **papers III-IV**, the MS/MS spectra were searched by Mascot 2.2 using Proteome Discoverer 1.1 against the IPI human target decoy protein sequence database.

2.2.6.1 Membrane protein database

To evaluate membrane protein content, an in-house database was built and searched against. This database was constructed by first extracting all translated human sequences from ENSEMBL and then tagging them according to number of transmembrane segments predicted using the TMHMM algorithm[58]. The sequences were tagged with *0 tms*, *1 tms* or *2+ tms*.

2.2.6.2 pI predictions

In **papers I-II**, the peptide pI values were predicted using an algorithm kindly provided by Stephenson *et al*[17]. It was previously observed that deamidation of Asn and Gln (i.e. conversion to Asp and Glu, respectively) renders peptides significantly more acidic and they thus show up as outliers in p*I*-plots[132]. Before calculating the theoretical p*I* values, an in-house script was used to convert the Asn → Asp and Gln → Glu, where appropriate.

2.2.6.3 Retention time predictions

In **paper II**, retention time predictions were performed using SSRCalc (available at http://hs2.proteome.ca/SSRCalc/SSRCalc33B.html). In order to use SSRCalc online, one has to trim the equation according to the LC-system and gradient used. This can be done in different ways, where one is to use a digest of a known protein (e.g. BSA), extract the experimental retention times of identified peptides and plot these against the hydrophobicities of the peptides (calculated in the SSRCalc). From this dependence, RT=A+B*(HP), the A and B parameters are determined where A is the gradient delay time and B is a value related to the slope of the acetonitrile gradient. The A and B parameters are then used in the SSRCalc program, together with information about column properties, to predict retention times of other identified peptides in any runs under the same conditions. Using 31 identified BSA peptides (Mascot scores above 30) we determined the A and B parameters for our system. Plotting observed retention times against predicted, we could see a linear correlation with a R^2 value of 0.93, indicating adequate predicting of the SSRCalc algorithm.

2.2.6.4 Statistics

Univariate data analysis (**papers III-IV**) was carried out using Significance Analysis of Microarrays (SAM) [133], version 3.09, as an Excel add-in. SAM uses permutations of the repeated measurements to estimate the percentage of genes/proteins identified by chance, the false discovery rate (FDR). Multivariate data analysis was carried out using Simca-P+ (see section **1.4.2**).

2.2.7 Western blot and immunohistochemistry

For validation of identified deregulated proteins (**papers I, III**), western blot and immunohistochemical (IHC) analyses were performed. For western blot, proteins were first separated on a SDS-PAGE, transferred to a nitrocellulose membrane after which the protein(s) of interest were probed with antibodies. IHC analyses in **paper III** were performed on tissue that was fixed routinely in 4% formaldehyde buffered solution and paraffin embedded. All adrenal tumors were studied by routine histochemical staining i.e. haematoxylin and eosin staining. To perform immunohistochemical analysis tissue sections were cut at 4 µm, deparaffinized and rehydrated. Antigen retrieval by heating in citrate buffer was performed to obtain a distinct signal without interfering background. All antibodies were tested at different dilution with and without antigen retrieval technique. As positive controls, tissue samples from pancreatic gland (NDUFA13/GRIM-19) and normal adrenal gland (STAT-3) were used. Negative controls were performed by replacing the primary antibody with phosphate buffer.

2.3 RESULTS AND DISCUSSION

2.3.1 Microsomal preparation

To develop a reproducible high-throughput method for maximal coverage of the membrane proteome, we aimed to keep the number of sample preparation steps to a minimum. Therefore, we used a microsomal fractionation technique that should also be applicable to different samples, e.g. cells and tissues. The experimental workflow is outlined in figure 5. In short, the sample at hand was first homogenized with appropriate method after which it was sonicated to break up the cells and the membranes. The membranes will then form vesicles, i.e. microsomes. The homogenate was first centrifuged at low speed to remove cell debris, after which an ultracentrifugation at 100 000 x g was performed to pellet the microsomes. The supernatant now contained soluble proteins (SP). The pelleted microsomes were then washed with high salt buffer (2.5 M NaBr) to remove loosely attached proteins (membrane-associated proteins, MAP). A second ultracentrifugation was performed to pellet the membrane (microsomal) proteins (MP). The microsomal fraction predominately contained ER membranes, and to some extent membranes from Golgi, mitochondria and plasma membrane.

2.3.2 Delipidation, solubilization and digestion

The next step in the preparation was to remove lipids, which can interfere with the enzymatic digestion. This was done by using chloroform/methanol precipitation[54], one of the most widely used methods, whereby lipids will partition into the chloroform layer and proteins will precipitate at the chloroform/methanol interface.

Before digestion, the microsomal proteins are solubilized in a buffer containing detergent. Sodium dodecyl sulfate (SDS), the detergent of our choice, is the classic example of a detergent. It is an anionic detergent, meaning it has an anionic head group attached to a hydrocarbon chain. SDS is extremely efficient at solubilizing proteins but it has drawbacks concerning downstream applications. It is not compatible with liquid chromatography (LC) analyses and it can suppress ionization by MALDI and ESI. In our case, however, this was not a problem since we digest and clean the sample before these analyses. The microsomal proteins were digested using trypsin. The SDS concentration was first reduced to 0.1 %, to keep trypsin in an active form.

2.3.3 iTRAQ labeling

The digested samples were labeled with iTRAQ. In **paper I**, the 4-plex kit was used; two samples from the sensitive cell line and two samples from the resistant cell line. In **papers III and IV**, the 8-plex kit was instead used. Since we had 14 samples in each of these studies, two iTRAQ labeling sets were used per study and in each one we included an internal standard. The internal standard samples were created by pooling equal amounts from each individual sample, and the iTRAQ reporter intensities were normalized using the intensity of the internal standard. This way it was possible to compare samples even if they were run in different iTRAQ labeled pools. The samples

were cleaned by SCX after labeling to remove iTRAQ reagents and this step also removed most of the SDS.

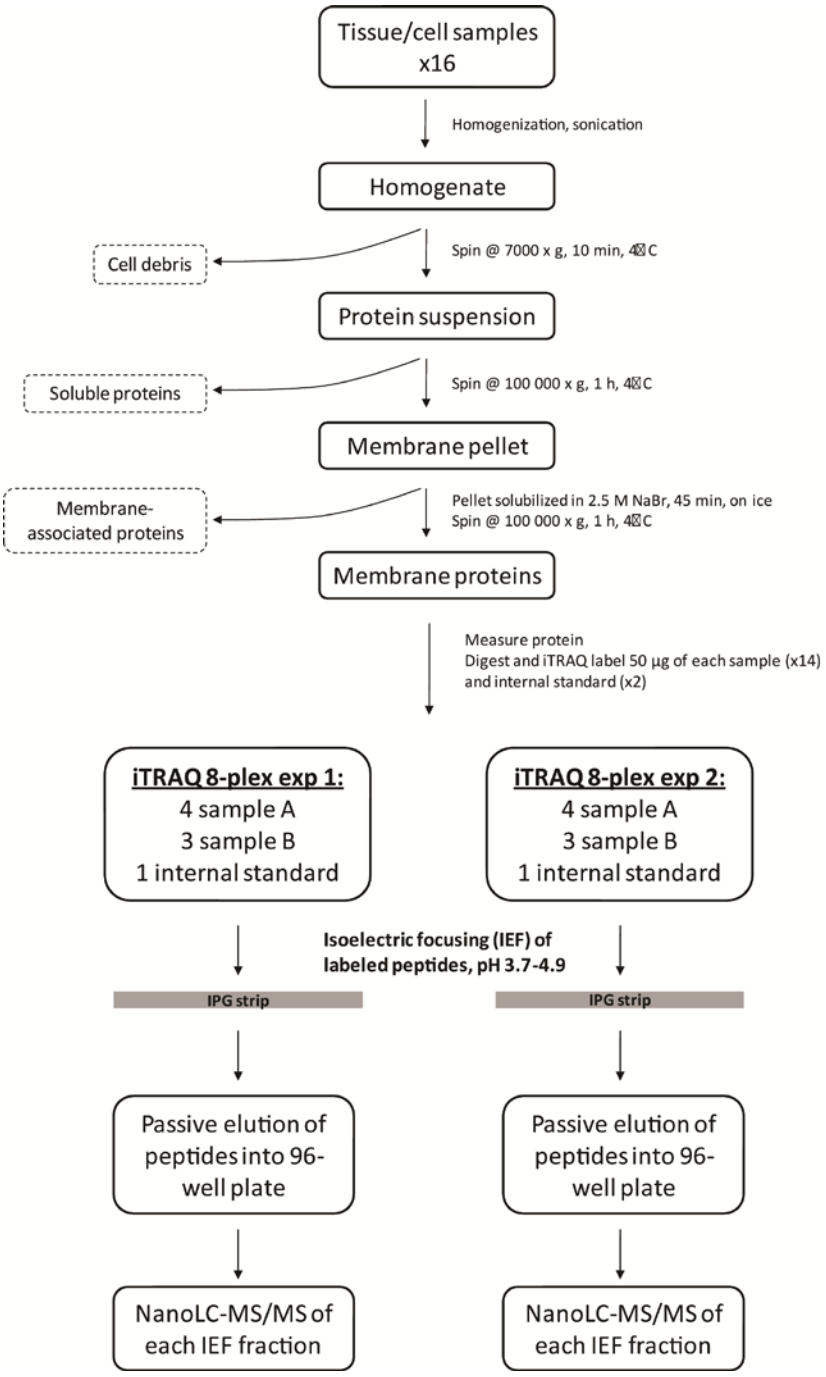

Figure 5. Experimental workflow.

2.3.4 Reproducibility of the microsomal preparation

To evaluate the technical reproducibility of the microsomal preparation, protein concentration in the resulting fractions was measured. This showed high reproducibility with low coefficients of variance (CVs); 5 % for the total protein fraction, 3 % for the soluble protein fraction, 13 % for the MAP fraction and 6 % for the microsomal protein fraction, see Fig 2 in **paper I**. The reproducibility concerning protein composition was also investigated. The majority of the CVs were below 30 % and CVs above 30 % predominantly came from ions of low intensity which may explain the larger variation. The reported variation originates from technical and experimental variation, including differences in iTRAQ-labeling efficiencies, previously reported to be approximately 12 % [134].

2.3.5 Membrane protein content

In **paper I** and **paper III**, the microsomal protein (MP) fractions were analyzed. In **paper IV**, we instead analyzed the membrane-associated protein (MAP) fraction. The proportion of predicted integral membrane proteins ranged from 15 % (**paper I**) to ~22 % (**paper III**). The predictions were based on searches against an in-house database which was constructed by first extracting all translated human sequences from ENSEMBL and then tagging them according to number of transmembrane segments predicted using the TMHMM algorithm[58]. The sequences were tagged with 0 tms (transmembrane segments), 1 tms or 2+ tms. The tags were divided into 1 and 2+ (two or more segments) to avoid assigning signal peptides as transmembrane segments. Proteins have a signal peptide in the beginning of their sequences directing them to the correct localization in the cell and this signal peptide is often of hydrophobic character. The proteins tagged with 1 tms were consequently considered as more uncertain membrane proteins. In **paper I** we also used ProteinCenter to analyze the data and then the reported proportion of membrane proteins were 48 %. ProteinCenter uses TMAP[135] in single sequence mode for prediction of membrane proteins. The original TMAP algorithm utilizes multiply aligned sequences for the predictions and when using TMAP on only a single sequence, the accuracy decreases, and it is therefore likely that the 48 % found is falsely too high. The total numbers of identified membrane proteins were 527 and 743 in **paper I** and **paper III**, respectively. Approximately 50 % of them contained more than two transmembrane segments. The proportion membrane proteins (~15-20 %) might seem low but one has to remember that these proteins are difficult to study for reasons discussed in the **Background** section. The preparation technique used here (microsomal enrichment) yields vesicles formed from all types of membranes in the cell (predominately ER membrane). These vesicles most probably contain trapped soluble proteins and since these are already in solution, they are easier to process. The membrane proteins need first to be solubilized for trypsin to get access for cleavage. By comparison, the predicted membrane protein content of an unfractionated sample was estimated to be 10 %; only ~3 % was predicted to contain more than 2 tms (data not shown), which means that by enrichment with our developed method, an increase of integral membrane proteins by ~300 % is reached.

The number of identified membrane proteins in **papers I and III** is comparable to other studies where similar approaches have been used. Scherl *et al* had, when using a similar membrane preparation technique on cultures of *Staphyococcus aureus,* a membrane protein content of 20 % [136]. Another study on vascular endothelial cells identified 43 % membrane-associated proteins [137]. This estimate was based on GO annotations, however it is difficult to decipher which GO terms have been used. Further, the term membrane-associated indicates that maybe not all proteins contained transmembrane segments per se, but can include peripheral membrane proteins as well. A characterization of the rat liver membrane proteome identified ~40 % predicted membrane proteins; approximately 50 % of these were predicted to contain more than two TMD's[88]. However, since there were fewer proteins identified (~1600), the number of membrane proteins was lower. Also, methanol-assisted digestion was employed, which hinders direct comparison of the methods.

2.3.6 Peptide fractionation

We employed narrow range (pH ~3.5 – ~4.9) peptide isoelectric focusing (IEF) as the first dimension separation step before a standard liquid chromatography (LC) separation step. We showed that by doing this, the sample complexity was reduced since only about a third of all theoretical tryptic peptides focus in this pH range (figure 5A). However, approximately 96 % of all human proteins were represented by at least one peptide. This reduction in complexity is a great advantage when working with low-abundant proteins like membrane proteins. Recently, Chick *et al* performed a study on membrane protein enriched samples from rat liver using a similar approach with isoelectric focusing as a first separation step[88]. They identified 626 proteins containing at least one transmembrane segment by using a pH 3 - 10 focusing strip sectioned into 24 fractions which were all analyzed by nano LC-MS/MS. The fact that approximately the same number of membrane proteins was identified by our approach (~500 in **paper I** and ~700 in **paper III**) using a much smaller pH interval (4.0 - 4.5) compared to their broad pH range supports the idea to use narrow pH range in the focusing step. Further, this raises the question if an even narrower pH range would enable a deeper analysis into the (membrane) proteome. For the human proteome, even a pH-range as small as 0.1 pH-units hold a considerable number of tryptic peptides (figure 5C). It must be stressed that plotted in figure 4 is a minimalistic estimation as the pI prediction was done using only fully tryptic, non-modified peptides. As recently shown, the complexity of proteome-wide tryptic digests may be far higher than theoretical enzyme cleavage predictions estimates[101,138]. Based on these facts, and given the high resolution demonstrated in **paper I**, it is clear that the use of pH-gradients <1.0 - 1.5 pH-units presents an important area of future research.

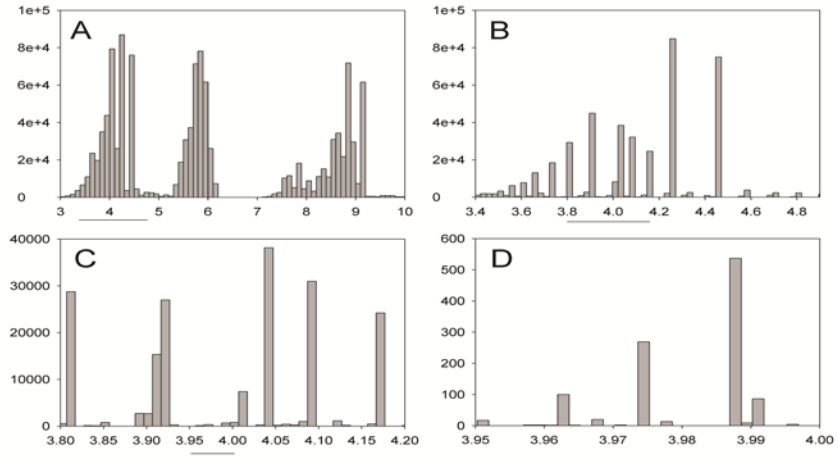

Figure 6. A plot of the predicted pI distribution among human tryptic peptides (included are peptides of lengths 4–60 amino acid residues, allowing for no missed cleavages). Approximately one third of the peptides have pI values between 3.5 and 4.5. (A) shows the distribution over 7 pH units, (B) over 1.5 pH units, (C) over 0.4 pH units and (D) over 0.05 pH units. X-axis defines the pI and on the y-axis the peptide count. The bar below the x-axis indicates the range covered in each subsequent plot.

2.3.6.1 Separation efficiency of IEF

To evaluate the efficiency of the IEF separation, the extent of overlap between consecutive fractions was investigated. First, extracted ion chromatograms of three randomly chosen precursors from the MALDI-MS run of all 72 IEF were generated (Fig 6 in **paper I**). As can be seen, precursors were observed in two or maximum three neighboring fractions. To evaluate the separation efficiency further, all peptides in three consecutive fractions were compared and peptides occurring in more than one fraction were identified. 8 % was found in both two fractions and less than 1 % was found in all three fractions (data not shown). This indicates a highly efficient separation for a complex peptide sample.

2.3.7 p*I* and retention time predictions

The IEF separation technique provides information about the p*I* of the peptides and this can be used to validate the peptide sequences matched to MS/MS spectra. The theoretical p*I* values of the peptides assigned with a confidence score ≥90 % were calculated using an algorithm kindly provided by Stephenson et al[17,139]. Figure 5 in **paper I** shows the distribution of calculated p*I* values of the peptides identified in one fraction analyzed by LC-MS/MS. Given adequate focusing and a well-performing prediction algorithm, all peptides in the fraction should have similar p*I* values. Indeed, the bulk peptide population of a representative fraction showed a standard deviation of 0.05 p*I* units. However, a few peptides showed deviating p*I* values. When examining these peptides in more detail, it was observed that most of them were modified. As the peptide p*I* calculation is done for non-modified sequences, peptides which have a shifted p*I* due to a post-translational modification will show up as outliers.

The work from **paper I** encouraged us to further explore the properties of narrow range peptide isoelectric focusing (IEF) as a separation strategy. In **paper II** we wanted to explore the possibilities of using a combination of narrow range peptide IEF and reversed phase chromatography to increase the information content of a proteomic analysis. Using the data generated in **paper I** for the peptide p*I* analysis and data from dog plasma and *in vitro* human liver microsomes for retention time analysis, experimental and predicted values for peptide p*I* and retention time were compared and general trends of peptide p*I* shifts, as well as shifts in retention time, induced by common modifications were analyzed. We observed that deamidations of glutamine and asparagines shift peptide p*I* by approximately 1.5 pI units, making the peptides more acidic. Additionally, a novel pI shift (+~0.4 pI units) was found associated with dethiomethyl Met modifications. Further, oxidation of methionine shortened the retention time of the modified peptides with 0.7 – 3.0 min (15 peptides; mean value 1.3 min), formylation at the N-terminus increased the retention time with 0.6 – 5.9 min (10 peptide pairs; mean value 2.8 min) and conversion of glutamate to pyro-glutamate prolonged the retention time with 2.5 – 7.1 min (4 peptide pairs; mean value 3.9 min).

2.3.7.1 Possible applications

To be able to use the predicted p*I* and retention time values, one relies heavily on the prediction algorithms at hand. While the behavior of non-modified fully tryptic peptides can be predicted with high accuracy in terms of both p*I* [17-18] and retention time [140-141], post-translationally modified peptides have been less explored. Recent work has been presented on extending the functionality of p*I*-prediction algorithms to include PTMs[142]. Future efforts to improve such algorithms and open modification search engines can only benefit from the degree of accuracy provided using narrow range IPG-IEF. An attractive application could be to use mass - p*I* - retention time filtering as a strategy for database reduction prior to open modification searches that is independent of MS/MS data acquisition. In such a strategy, a candidate list of protein accessions would be obtained by calculating which candidate peptide sequences fit in the recorded and calculated mass, p*I* and retention time windows. Such an approach could also take into consideration the most common modifications and amino acid alterations (Table 1 in **paper II**).

2.3.8 Lung cancer resistance mechanisms

In **paper I**, a small cell lung cancer cell line resistant to the cytotoxic drug Doxorubicin (H69AR) was analyzed and compared with its sensitive parental cell line (H69). Drug resistance is often associated with up-regulation of membrane-associated drug efflux systems and resistance to Doxorubicin has been shown to in part be due to an up-regulation of multidrug resistance-associated protein 1 (MRP1), which is one of the most important members of the ATP-binding cassette (ABC) protein family[108]. ABC transporters are transmembrane proteins that utilize the energy of adenosine triphosphate (ATP) hydrolysis to carry out certain biological processes including translocation of various substrates across membranes and non-transport-related processes such as translation of RNA and DNA repair[143].

In our study, 48 proteins were found to be more than a 2-fold differentially expressed between the two cell lines. One of them, Serca 2, was found to be down-regulated in the resistant cell line and this was validated by western blot analyses, fig 8 in **paper I**. Serca 2 is a Ca^{2+} pump with 10 transmembrane segments located in the ER membrane, responsible for pumping Ca^{2+} into the ER lumen. Sensitivity to apoptosis correlates with the total ER Ca^{2+} load and depends on the ability of cells to transfer Ca^{2+} from the ER to the mitochondria[144]. Ca^{2+} levels in the ER play an important role in the cell's ability to undergo apoptosis and down-regulation of the enzyme responsible for the influx of Ca^{2+} into the ER may contribute to the resistance phenotype of H69AR cells. Several cytoskeletal proteins were found to be up-regulated in the resistant cell line. These include plectin, vimentin and moesin. This could suggest a more rigid structure of these cells, making them less sensitive to environmental stress. Desmoplakin was down-regulated in H69AR. This protein is a component of desmosomes, intercellular junctions that tightly link adjacent cells. When culturing the cells, the resistant cells were less prone to grow in aggregates than the sensitive cells and this could be due to the down-regulation of desmoplakin.

2.3.9 Adrenocortical tumors

In **paper III**, the microsomal proteins of eight ACCs and six ACAs were analyzed. In an initial supervised prediction analysis, one of the ACCs clustered with the ACAs. Since this had been observed when performing microarray studies on the same material[145], this sample was removed from the subsequent analyses. Pathway analysis revealed that many proteins belonging to complex I in the mitochondrial respiratory chain (MRC) were down-regulated in ACCs compared to ACAs. A t-test was performed to identify significantly deregulated proteins and 69 were found to be up- or down-regulated. Aldolase A was up-regulated in ACCs compared to ACAs. This is a glycolytic enzyme responsible for the cleavage of the six-carbon sugar fructose 1,6-biphosphate into the two three-carbon fragments glyceraldehyde 3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP). This, together with the down-regulation of complex I proteins, indicates a metabolic shift in the ACCs where the glycolysis rate is higher and the MRC activity lower. This is known as the Warburg effect and is readily used in the clinic with the application of the imaging technique positron-emission tomography (PET) using the glucose analogue tracer ^{18}fluorodeoxyglucose (FdG).

IGF2 was up-regulated in ACCs and this is in agreement with several other studies on adrenocortical tumors[145-148]. The IGF2 gene is imprinted and is only expressed from the paternal allele. In the same region as IGF2, there are two other imprinted genes, H19 and p57Kip2 which are expressed only from the maternal allele. The reason for IGF2 overexpression in ACCs is often that there is a loss of heterozygosity and/or loss of imprinting, i.e. the paternal allele is expressed in two copies or the imprinting of the maternal allele is lost. This means that not only is IGF2 expression increased but the expression of the two other genes, H19 and p57Kip2 is lost or reduced. Since H19 is thought to be a tumor suppressor gene and p57Kip2 acts as a suppressor of cell cycle progression, this will further drive the tumorigenesis [149-150]. IGF2 has affinity for both receptors IGF1R and IGF2R[151]. Upon binding to IGF1R, the receptor tyrosine kinase activity is triggered, which leads to phosphorylation of itself and its major substrate, the insulin receptor substrate 1 (IRS-1). Phosphorylated IRS-1 can activate the Ras/Raf/MAPK and PI3-kinase/Akt pathways, and depending on the cell type, stimulate proliferation, differentiation, or both[152]. PI3-kinase activation can lead to anti-apoptotic signals, and components of this pathway are frequently amplified or mutated in cancers [153]. Elevated expression levels of IGF2 have been found in several cancer forms such as colorectal carcinoma [154].

One of the complex I proteins that was identified as down-regulated in ACCs was NDUFA13, also named GRIM-19 (**G**ene associated with **R**etinoic- and **I**nterferon-induced **M**ortality-19). GRIM-19 is a mitochondrial protein involved in complex I assembly and activity and is required for electron transfer activity [155]. It has been shown that GRIM-19 deficient mouse blastocysts had alterations in the mitochondrial structure, morphology and cellular distribution[156]. Dysfunctional mitochondria could lead to inability of the cells to undergo mitochondria-mediated apoptosis. In our mass spectrometry data we did not see a complete loss of GRIM-19 expression, but a 2-fold difference in expression when comparing ACAs and ACCs. The identification and quantification of GRIM-19 was based on 2 peptides, one at position 61-68 and the other

at position 70-81. Interestingly, western blot analyses could not detect any GRIM-19 at all in the malignant tissue (fig 6B in **paper III**). This could of course be due to sensitivity issues of the anti-GRIM-19 antibody or that the protein is completely lost but it could also suggest a defective protein, as suggested in[157]. He et al report of an alternatively spliced GRIM-19 in kidney cancer tissue, resulting in a longer transcript with a premature stop codon after exon 3. This could lead to a truncated protein. The antibody used in our study recognizes the full-length protein and the reason for the absent signal in the western blot analyses could be inability of the antibody to recognize any modified form of the protein.

Four benign tissues and four malignant tissues were evaluated for GRIM-19 expression with immunohistochemical methods. In the benign samples the expression patterns were grain-like (figure 7A). On the contrary, in the malignant samples there was a more general cytoplasmic staining pattern (figure 7B). This suggests a mitochondrial localization in ACAs and that this localization may have been lost in the ACCs, either due to loss of the protein or loss of functional protein. To check a possible role of STAT3 regulation by GRIM-19, STAT3 was analyzed by immunohistochemistry in the same tissues (figures 7C-D). There were no obvious differences between the benign and malignant tissues concerning STAT3 expression levels, but an indication of stronger staining in nuclei was observed for the malignant tissue, suggesting STAT3 relocalization (figure 7D). In some cells in the benign tissue, a grain-like expression pattern was observed; the same could not be seen in the malignant tissue (figure 7E). This could indicate a STAT3 mitochondrial localization in ACAs.

There could be a link between elevated expression levels of IGF2 and aldolase A and decreased expression of/non-functional GRIM-19. Activation of growth factor receptors leads to PI3K activation, which via AKT leads to increased glucose uptake and flux through the early part of glycolysis[158-159]. Additionally, tyrosine kinases, a common feature of oncogenes, can inhibit the later part of glycolysis through regulation of the M2 isoform of pyruvate kinase[160] and thereby intermediates of glycolysis can be used for amino acid and nucleotide synthesis. The reason for increased glycolysis in the ACCs examined in this study could be due to a combination of impaired mitochondrial function, due to down-regulation or loss of functional GRIM-19, and activation of growth factor receptors through IGF2 signaling.

In summary, by analyzing the microsomal protein composition of ACAs and ACCs, we observe a reduced expression of GRIM-19 in ACCs. It is not clear at this point if it is a down-regulation *per se* or a loss of functional GRIM-19, however indications point towards a re-localization of the protein, from mitochondria to the cytoplasm, and this re-localization could be due to a modified form of the protein in ACCs. Studies are on-going to confirm this hypothesis. Loss of functional or re-localized GRIM-19 could lead to dysfunctional mitochondria and STAT3 hyperactivation, which in turn could drive the tumorigenesis in ACCs. Further, we observe an increased glycolysis rate in ACCs, which also, in part, could be caused by dysfunctional mitochondria. However, the reason for the shift in metabolism is most probably multi-factorial, where e.g. hypoxia also could play a role. IGF2, identified as up-regulated in ACCs by us and others, may contribute to this metabolic shift, which increases proliferation of tumor cells.

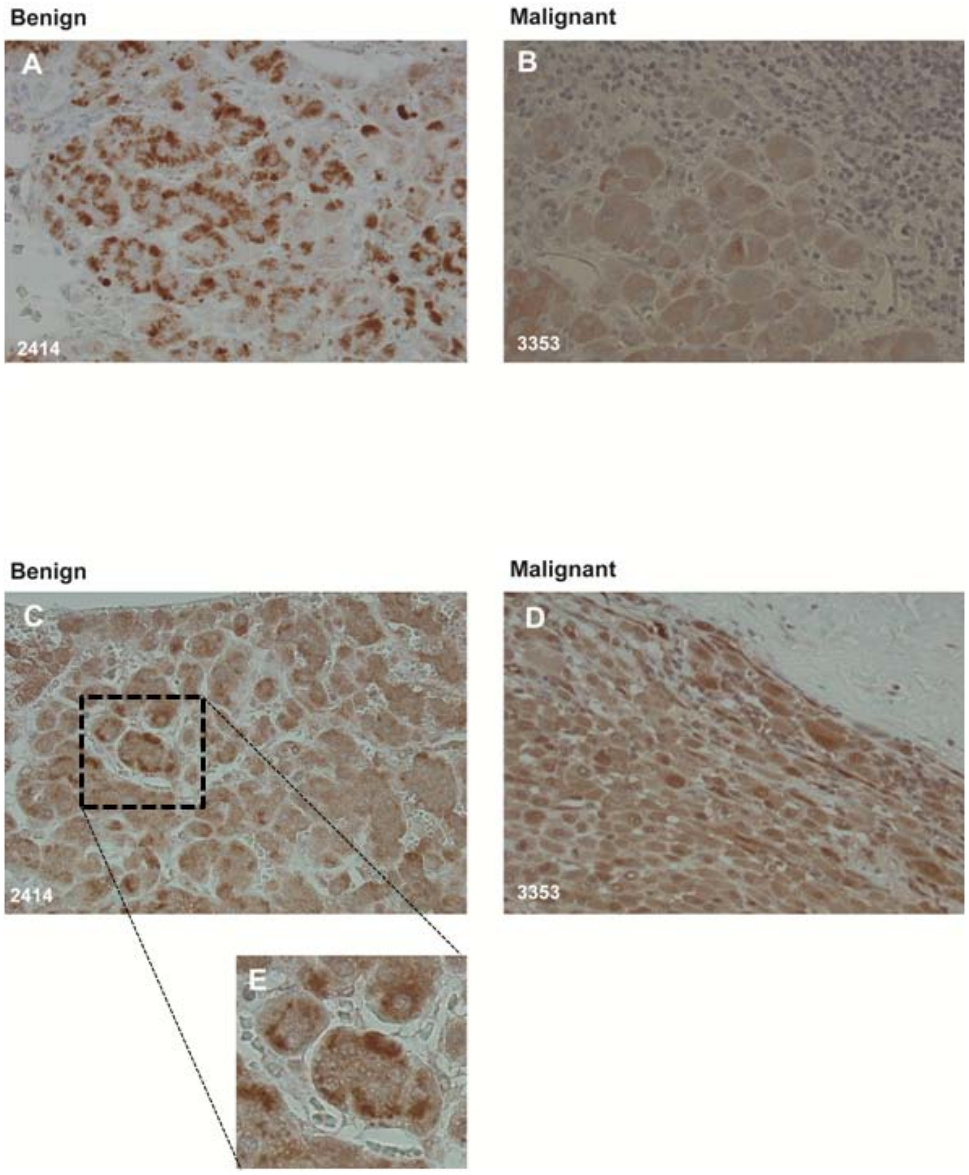

Figure7. Immunohistochemical analyses with anti-GRIM-19 (panels A and B) and anti-STAT3 (panels C-E). GRIM-19 staining in ACAs had a grain-like pattern, suggesting mitochondrial localization (panel A). In ACCs there was a more cytoplasmic staining (panel B). There was an indication of nuclear staining of STAT3 in ACCs (panel D), which was not observed in ACAs (panel C). Some cells in ACAs showed a grain-like staining pattern for STAT3 (panel E).

2.3.10 Sarcoidosis

In **paper IV**, we enriched for membrane-associated proteins from alveolar macrophages from eight sarcoidosis patients and six healthy controls. Alveolar macrophages were isolated from bronchoalveolar lavage (BAL) cells. By applying a shotgun proteomics approach on the MAP fraction, we obtained the protein profile on lung macrophages and a total of 1650 proteins were identified and relative quantitation was obtained on 423 proteins by using iTRAQ labeling. A PCA plot showed group clustering for sarcoidosis and controls, figure 3 in **paper IV**. The two clusters among the sarcoidosis patients can be explained by their diagnosis; non-Löfgren or Löfgren disease. An orthogonal projections to latent structures (OPLS) analysis was performed with healthy versus diseased. Proteins that had a small influence were removed iteratively to create a new predictive model. Accordingly, we reduced the number of proteins from 423 to 13 in a model with a predictability value $Q^2 = 0.718$ ($Q^2 > 0.45$ cut off to be considered as a good model) and a p-value of 0.0009 (figure 7 and table 2 in **paper IV**). Three of the proteins are involved in endocytic transport; MP1 (mitogen-activated protein kinase scaffold protein 1), C11orf59 and RAB7A. MP1 and C11orf59 are part of a protein complex called the Ragulator complex. This complex is anchored to lipid rafts in late endosome membranes via C11orf59, recruits mTORC1 to lysosomal membranes in amino acid signalling and is also involved in MAPK signalling[161]. Additionally, RAB7A has a role in the maturation of phagosomes[162]. One of the proteins is involved in host-virus interactions. i.e. complement component 1 Q subcomponent-binding protein[163]. The rest of the proteins in the OPLS model are involved in DNA repair, alcohol metabolism, protein folding and ribosomal activity.

Pathway analysis showed that energy-related (ATP synthesis), as well as phagocytosis pathways were altered. The oxidative phosphorylation pathway (p-value 1.02E-10) showed a down-regulation in sarcoidosis at the level of complex I, III, IV and partially V from the mitochondrial respiration system. The lungs are exposed to oxidants either endogenously, e.g. from the mitochondrial electron transport or during phagocytosis, or by exogenous pollutants. Reactive oxygen species (ROS) include a variety of radicals; e.g. superoxide anion ($O_2^{\cdot -}$), hydroxyl radical ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2). Redox imbalance, which can be caused by increased ROS formation, is associated with chronic inflammation in several lung disorders; COPD[164], asthma [165], acute lung injury, lung fibrosis and cancer [166]. High concentration of ROS can trigger tissue damage and changes in signal transduction. Studies have shown that imbalance in redox status leads to activation of NFκβ where ROS are directly implicated as second messengers by regulating ubiquitination and degradation of Ikβ [167]. In addition, it has been reported that the high exposure to oxidants induce mitochondrial dysfunction in the lungs [168] by oxidative damaged macromolecules (oxidatively dysfunctional proteins) which leads to increased protein degradation and dysfunction in mitochondria.

The pyruvate metabolism pathway (p-value 7.45E-09) was up-regulated in the sarcoidosis group. Pyruvate has been shown to be an endogenous anti-inflammatory molecule by protecting cells from ROS and suppressing both TNF-α secretion and NF-κB expression [169]. The up-regulation of pyruvate metabolism that we observe could be

an effect of mitochondrial dysfunction and ROS formation which leads to an up-regulation of endogenous pyruvate in order to protect the cells.

Additionally, in the results from the pathway analyses we could observe an up-regulation in phagocytosis related pathways in sarcoidosis, e.g. the Fcγ receptor-mediated phagocytosis in macrophages (p-value 1.47E-05) and the Clathrin-mediated endocytosis signaling (p-value 9.64E-09) (figure 7), both in Löfgren and non-Löfgren sarcoidosis patients. Macrophages have several phagocytotic mechanisms; for instance they can be initiated by the Fcγ receptor (FcγR) which was found up-regulated in sarcoidosis. The Fc region from IgG binds to FcR on AMs and initiates the phagocytosis. This mechanism mediates internalization of pathogens (bacteria, virus and parasites) into membrane-derived vacuoles and phagosome system which finally leads to degradation of pathogens and the presentation of specific antigens to memory T-cells in the lungs[170]. Activation of FcR by IgG opsonised particles induce phosphorylation of tyrosines in ITAM (immunoreceptor tyrosine-based activation motif) on the FcR which leads to recruitment of other proteins such as phosphatase Src and 5'PI downstream in signaling[171]. The FcR mediates signal transductions that regulate production of ROS, modulate actin cytoskeleton[172], and membrane fusion[173].

In summary, we report down-regulation of pathways related to the mitochondrial respiration system, which could lead to an imbalance in the oxidative homeostasis, in turn triggering ROS formation in sarcoidosis patients. This down-regulation was true only for non-Löfgren patients and this could in part explain their unresolved inflammation and prolonged disease course. The up-regulation of pathways related to pyruvate metabolism may be due to the cells trying to protect themselves from the increased ROS formation. We also observed up-regulation in phagocytosis-related pathways in sarcoidosis, which could be pathogen-mediated mechanisms of the alveolar macrophages.

2.4 GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

The overall aim of this thesis was to develop a proteomic method suitable for studies of integral membrane proteins in clinical material. Membrane proteins are under-represented in proteomic investigations due to their general low-abundance and hydrophobicity. They are an important class of proteins, involved in cell-cell interactions, signaling and transport. Two of the papers in this thesis are more methodological, while the last two have a more biological/clinical focus. The general conclusions are:

- ✓ *Membrane protein identification*

  15-22 % of the identified proteins were predicted to be membrane proteins. A predominant part of the membrane proteins originated from intracellular organelles, e.g. ER and mitochondria. The microsomal preparation method was shown to be reproducible and applicable to clinical material. The number of identified membrane proteins lies in line with top notations published so far. However the proportion soluble proteins in the fraction could be decreased and warrants further method development.

- ✓ *Isoelectric focusing*

  Narrow range IEF reduces the sample complexity by 2/3 while maintaining high proteome coverage; ~96 % of all human proteins are represented by at least one peptide in the pH range of 3.5 – 5.0. This increases the possibility of identifying low-abundant proteins, e.g. membrane proteins, and thereby enables to dig deeper into the proteome. The theoretical peptide p*I*s can also be used to validate the sequences and could lead to more proteins being identified. In **paper II** we explored the possibility of using the peptide p*I* together with retention time and mass as a filter for database reduction prior to open modification searches.

- ✓ *Adrenocortical tumors*

  Comparing the membrane proteome of ACCs with ACAs indicated a shift in metabolism towards increased glycolysis in favor of normal respiration via the electron transport chain in ACCs. GRIM-19 was found to be down-regulated in ACCs. This is a mitochondrial membrane protein required for correct assembly of complex I in the electron transport chain. It has further been shown to be a negative regulator of STAT3. Our analysis indicated a loss of mitochondrial localization of GRIM-19 in ACCs. This could have implications for the tumorigenesis of the ACCs, together with other factors such as elevated IGF2 levels.

- ✓ *Sarcoidosis*

  The membrane-associated protein population of alveolar macrophages isolated from Sarcoidosis patients was compared with healthy controls. We report down-regulation of pathways related to the mitochondrial respiration system,

which could lead to an imbalance in the oxidative homeostasis, in turn triggering ROS formation in sarcoidosis patients. This down-regulation was true only for non-Löfgren patients and this could in part explain their unresolved inflammation and prolonged disease course. The up-regulation of pathways related to pyruvate metabolism may be due to the cells trying to protect themselves from the increased ROS formation. We also observed up-regulation in phagocytosis-related pathways in sarcoidosis, which could be pathogen-mediated mechanisms of the alveolar macrophages.

2.4.1 Future perspectives

Even though gel-based methods have been introduced that are able to resolve membrane proteins, they lag behind shotgun methods in the number of identified proteins by a factor of at least 10. The challenge concerning successful identification of integral membrane protein using a shotgun approach is clearly the solubilization issue. Membrane proteins are predominately made up of hydrophobic domains and these domains need to be solubilized but at the same time available for enzymatic cleavage. Most peptides identified from membrane proteins come from their soluble domains, meaning that large parts of the proteins remain undetected, and by that they become even more low-abundant in a tryptic peptide sample. An attractive approach could be to first remove the soluble proteins and then target the hydrophobic domains of the membrane proteins by using e.g. shaving to get rid of the soluble parts. One could then use narrow range IEF in a pH range that is favorable for hydrophobic peptides.

One of the aims in **paper III** was to search for plasma membrane bound proteins that could differentiate between ACCs and ACAs. A plasma membrane protein could possibly become a target for new diagnostic strategies using e.g. PET scanning. Since most membrane proteins found with our method belonged to intracellular organelles, further enrichment/purification of the membrane fraction is needed in order to reach the more low-abundant plasma membrane proteins. Plasma membrane proteins are maybe the most interesting protein population among membrane proteins, especially regarding cancer which partly is regarded as a disease of aberrant signal processing, and is therefore a very interesting future research area.

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