Protein Profiling and Type 2 Diabetes

TEA SUNDSTEN
Dissertation presented at Uppsala University to be publicly examined in Auditorium minus, Gustavianum, Akademigatan 3, Uppsala, Friday, March 14, 2008 at 09:00 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English.

**Abstract**


Type 2 diabetes mellitus (T2DM) is a heterogeneous disease affecting millions of people worldwide. Both genetic and environmental factors contribute to the pathogenesis. The disease is characterized by alterations in many genes and their products. Historically, genomic alterations have mainly been studied at the transcriptional level in diabetes research. However, transcriptional changes do not always lead to altered translation, which makes it important to measure changes at the protein level. Proteomic techniques offer the possibility of measuring multiple protein alterations simultaneously.

In this thesis, the proteomic technique surface enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF MS) has been applied and evaluated in the context of T2DM research. Protocols for pancreatic islet and serum/plasma protein profiling and identification have been developed. In addition, the technique was used to analyze the influence of genetic background versus diabetic environment by determining serum protein profiles of individuals with normal glucose tolerance (NGT) and T2DM with or without family history of diabetes. In total thirteen serum proteins displayed different levels in serum from persons with NGT versus patients with T2DM. Among these proteins, apolipoprotein CIII, albumin and one yet unidentified protein could be classified as being changed because of different genetic backgrounds. On the other hand, ten proteins for instance transthyretin, differed as a result of the diabetic environment.

When plasma protein patterns of NGT and T2DM individuals characterized by differences in early insulin responses (EIR) were compared, nine proteins were found to be varying between the two groups. Of these proteins five were identified, namely two forms of transthyretin, hemoglobin α-chain, hemoglobin β-chain and apolipoprotein H. However no individual protein alone could explain the differences in EIR. In conclusion, SELDI-TOF MS has been successfully used in the context of T2DM research to identify proteins associated with family history of diabetes and β-cell function.

**Keywords:** Type 2 diabetes, Protein profiling, SELDI-TOF MS, Proteomics

_Tea Sundsten, Department of Medical Cell Biology, Box 571, Uppsala University, SE-75123 Uppsala, Sweden_

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ISSN 1651-6206
urn:nbn:se:uu:diva-8458 (http://urn.kb.se/resolve?urn=nbn:se:uu:diva-8458)
Till Fafa

If you had one shot,
or one opportunity,
to seize everything you ever wanted.
   In one moment.
Would you capture it,
or just let it slip?

Eminem, Lose Yourself

Till Mamma

Little girl
Never forget her eyes
Keep them alive inside
I promise to try – it’s not the same

Madonna, Promise to try
List of Papers

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<tr>
<td>2DGE</td>
<td>2-dimensional gel electrophoresis</td>
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<tr>
<td>ACN</td>
<td>Acetonitrile</td>
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<td>ANOVA</td>
<td>Analysis of variance</td>
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<td>Apo</td>
<td>Apolipoprotein</td>
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<td>CM10</td>
<td>Cationic exchanger (formerly called WCX2) protein array</td>
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<tr>
<td>CV</td>
<td>Coefficient of variance</td>
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<td>CyDye</td>
<td>Cyanine dye</td>
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<td>DIGE</td>
<td>Difference gel electrophoresis</td>
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<td>EIR</td>
<td>Early insulin response</td>
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<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>EAM</td>
<td>Energy absorbing molecule</td>
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<td>FHD</td>
<td>Family history of diabetes</td>
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<td>GRP</td>
<td>Glucose regulated protein</td>
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<td>H50</td>
<td>Reverse phase protein array</td>
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<tr>
<td>Hb</td>
<td>Hemoglobin</td>
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<td>HOMA</td>
<td>Homeostasis model assessment</td>
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<td>IGT</td>
<td>Impaired glucose tolerance</td>
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<td>IMAC30</td>
<td>Immobilized metal affinity capture protein array</td>
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<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography tandem mass spectrometry</td>
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<tr>
<td>MALDI-TOF</td>
<td>Matrix assisted laser desorption ionization time-of-flight</td>
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<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>NGT</td>
<td>Normal glucose tolerance</td>
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<td>NP20</td>
<td>Normal phase protein array</td>
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<td>OGTT</td>
<td>Oral glucose tolerance test</td>
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<tr>
<td>PCA</td>
<td>Principal component analysis</td>
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<tr>
<td>PPI-B</td>
<td>Peptidyl-prolyl isomerase B</td>
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<tr>
<td>PMF</td>
<td>Peptide mass fingerprinting</td>
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<td>SAX2</td>
<td>Anionic exchanger protein array</td>
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<tr>
<td>SDPP</td>
<td>Stockholm Diabetes Prevention Program</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<tr>
<td>SELDI-TOF</td>
<td>Surface enhanced laser desorption ionization time-of-flight</td>
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<tr>
<td>S/N</td>
<td>Signal to noise ratio</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>SPA</td>
<td>Sinapinic acid</td>
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<td>T1DM</td>
<td>Type 1 diabetes mellitus</td>
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<td>T2DM</td>
<td>Type 2 diabetes mellitus</td>
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<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TTR</td>
<td>Transthyretin</td>
</tr>
<tr>
<td>ULSAM</td>
<td>Uppsala Longitudinal Study of Adult Men</td>
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<tr>
<td>WCX2</td>
<td>Cationic exchanger (now called CM10) protein array</td>
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<td>WHO</td>
<td>World Health Organization</td>
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Introduction

Normal glucose homeostasis

Blood glucose homeostasis describes the balance of glucose ingestion and hepatic glucose production on one side, and peripheral glucose uptake and utilization on the other side. This equilibrium is maintained by complex interplay of several glucose-elevating hormones like glucagon, cortisol, growth hormone and catecholamines, and one glucose-lowering hormone, insulin [1].

After a meal, the blood glucose concentration increases, leading to insulin secretion from the pancreatic β-cells of healthy individuals. Within ten minutes after a glucose load the blood insulin rises to a maximum level, the so-called first or early phase of insulin release [2]. If the blood sugar concentration remains high, the β-cells continue to release insulin, resulting in the second or late phase of insulin release [3]. Insulin lowers the blood glucose by acting on three main target tissues, namely muscle, liver and adipose tissue (Figure 1). First, the glucose uptake and utilization in muscle and adipose tissue is enhanced. In liver and muscle cells glycogen synthesis is enhanced, while breakdown is suppressed, resulting in net storage of glycogen. Glucose release from liver is suppressed by inhibition of enzymes of the gluconeogenetic pathway.

The opposite reactions happen in the fasting state, when blood glucose and insulin levels are low. Glucose production is then promoted by enhanced hepatic gluconeogenesis and glycogenolysis. At the same time, glycogen production and glucose-uptake in insulin-sensitive tissues is decreased, leading to elevation of the blood glucose level [1]. Besides glucose, several other factors can also promote insulin secretion, including amino acids, fatty acids, gastrointestinal peptides and neuronal factors [1].
Figure 1. Schematic illustration of the glucose-lowering effects of insulin. Insulin lowers the blood glucose by targeting liver, skeletal muscle and adipose tissue. The glucose up-take and utilization in muscle and adipose tissue is increased. In liver and muscle glycogen synthesis is enhanced and glycogen break-down is suppressed, resulting in net storage of glycogen. Also, liver gluconeogenesis is inhibited. All these effects lead to lowering of blood glucose concentration.

Diabetes mellitus

Diabetes mellitus is a heterogeneous group of metabolic disorders, which are all characterized by hyperglycemia [4]. When pancreatic β-cells fail to secrete adequate amounts of insulin, periods of hyperglycemia occur. These periods of elevated blood glucose concentrations damage both small and large blood vessels, leading to micro- and macrovascular complications. Microvascular complications include neuropathy, nephropathy and retinopathy, while macrovascular complications include coronary artery disease, cerebrovascular disease and peripheral arterial disease [5].

The worldwide prevalence of diabetes has increased dramatically over the past two decades. The World Health Organization (WHO) estimates that more than 180 million people worldwide suffer from diabetes and the num-
ber is estimated to more than double by 2030 [6]. Diabetes is classified on the basis of the pathogenic mechanisms causing hyperglycemia. The two main types of diabetes are type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM) [7]. T1DM is usually diagnosed in children, teenagers and young adults, and was formerly known as insulin-dependent diabetes or juvenile diabetes. The disease is caused by an autoimmune destruction of pancreatic \( \beta \)-cells causing insulin deficiency and absolute dependence of insulin therapy [1].

T2DM constitutes about 90 \% of all diabetes cases [7]. The disease is more common among adults, and was formerly called non-insulin dependent diabetes or adult-onset diabetes. Abdominal obesity is a prominent risk factor for developing T2DM [8], and the majority of the T2DM patients are overweight [9, 10]. Thus, the increased incidence of T2DM may at least partly be explained by increased obesity [11, 12]. T2DM is caused by inadequate insulin secretion by the pancreatic \( \beta \)-cells [13]. In T2DM a prominent feature is the decreased ability of insulin to act effectively on target tissue, so called insulin resistance [14]. Insulin resistance is, however, compensated by \( \beta \)-cells hypersecreting insulin, thus normalizing blood glucose levels [13]. The increased demands may lead to \( \beta \)-cell exhaustion and development of overt T2DM. The exact mechanisms of insulin resistance are not yet elucidated, but defects at both receptor and postreceptor levels have been described [15]. Multiple genetic and environmental risk factors have been shown to contribute to insulin resistance, including ethnicity, obesity, sedentary lifestyle, family history of diabetes and low birth weight [16]. Also adipose tissue can contribute to insulin resistance by releasing free fatty acids and adipocytokines [17-19]. Many adipocyte-derived signaling molecules, like adiponectin, leptin and resistin have important regulating roles in muscle and hepatic lipid and glucose metabolism [8].

The pathogenesis of T2DM is far from fully understood, but it is clear that the disease has a strong genetic component as well as an environmental component [7, 20]. The disease is characterized by altered expression of many genes and their products in several tissue types [21, 22]. Historically, mainly genomic alterations have been studied in diabetes research. Wide scan genomic projects and mRNA microarray studies have been very informative [23, 24]. However, not all transcriptional changes lead to altered translation, which makes it important to also measure expression changes at the protein level. Proteomic analysis also offers an opportunity to study post-translational modifications like glycosylations or phosphorylations, which are often crucial for protein activity or function.
Proteomic techniques

In the field of proteomics many different techniques have evolved all capable of separating sample proteins in various ways. Most of the methods use properties of the proteins including hydrophobicity, isoelectric point and mass in the separation step. In diabetes research, 2-dimensional gel electrophoresis (2DGE) and ProteinChip arrays have mainly been used [25-34]. However, since neither of these techniques inherently identifies the separated proteins, they are usually combined with additional identification approaches. Typically, protein identification is performed by peptide mass fingerprinting (PMF), which involves separating the proteins by gel electrophoresis followed by in-gel tryptic digestion and matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). Subsequently, data base searches are performed, aimed at obtaining the correct identity of the protein.

2DGE

One method of visualizing the proteome is 2DGE [35]. The complex mixture of sample proteins is separated in two steps. The first separation is the isoelectric focusing, where proteins are separated based on their isoelectric point, using gel strips with an immobilized pH-gradient. In an electric field, the proteins migrate to their isoelectric points, the location where they have no net charge. The second separation is performed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), so the proteins are separated based on their individual masses (Figure 2). After separation, the proteins need to be visualized by staining. Some commonly used stains include Coomassie brilliant blue, silver and SYPRO Ruby, all with different properties [36, 37].

The resulting gels containing protein spots are then analyzed by image analysis software, to detect and quantify differences. One important factor in making 2DGE better is improving the image analysis. Often images are not perfectly super-imposable, making the gel editing and analysis time-consuming. Image analysis can be improved by utilizing difference gel electrophoresis (DIGE) and the cyanine dyes (CyDyes) [38]. Two samples are then labeled with different CyDyes, with different excitation and emission wavelengths. Afterwards, the samples are mixed and proteins separated by 2DGE. After excitation the resulting gel images are overlaid to show differences between the samples.
To identify proteins of interest found by 2DGE, the spots are excised from the gels and digested by trypsin. Trypsin cleaves the protein at certain cleavage sites, resulting in a peptide mixture, which can be subjected to MALDI-TOF MS. The resulting mass spectrum is a characteristic fingerprint of the original protein. By comparing the experimental peptide fingerprint with peptides in databases, the identity of the protein can be obtained.

Depending on the gel size and the pH-gradient used, the method can resolve up to 5,000 proteins in one run. As little as 1 ng of protein can be present in one spot [36] and the method is also quantitative. Generally, the
2DGE method preferentially detects high-abundant proteins and is not good at separating proteins either with very low or high masses, with poor solubility or extreme isoelectric points [39]. No standard methods for separating proteins over the entire mass range between 5-500 kDa are available. To overcome this limitation, several gels optimized for different mass ranges can be combined. One drawback with 2DGE in the context of diabetes research is that substantial amounts of sample material are needed, which limits the use of the technique using for instance human islets.

LC-MS/MS

Liquid chromatography tandem mass spectrometry (LC-MS/MS) has also been applied in the context of diabetes research [40-42]. In the case of LC-MS/MS the sample proteins are separated by chromatographic columns and subsequently analyzed by MS [43]. The method for separating the sample proteins is often by hydrophobic interactions between sample proteins and columns. The columns are eluted under acidic conditions using water and organic solvents like acetonitrile (ACN). Currently one experiment using LC-MS/MS is capable of identifying several hundred proteins. However, since losses during the fractionations are large, also here sufficient amounts of starting material are needed. The method is not inherently quantitative.

SELDI-TOF MS

Surface enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF MS) [44] is capable of detecting protein patterns of complex samples [45, 46]. The SELDI-TOF MS is a three-step-method, where crude biological samples are first applied on ProteinChip array surfaces (Figure 3). The array surfaces capture sample proteins by biological or chemical interactions. After sample binding, interfering components like salts and detergents are washed away. Only the substances interacting with the array surface are retained. Energy absorbing molecules (EAM) are applied on the array surfaces causing the bound proteins and EAM to co-crystallize. In the second step the ProteinChip reader is used to detect the bound proteins. The laser beam causes the proteins bound to the array to ionize and transform into gaseous phase, thus making the proteins able to fly in a vacuum tube in an electric field. The voltage difference gives all the proteins the same starting energy, which results in mass-dependent time-of-flights. From the time-of-flight recordings the ProteinChip reader derives the individual masses of the proteins. The ProteinChip software is the third step in the process, controlling all aspects of data collection and analysis such as sample group comparisons, cluster analysis and statistical calculations between data sets. One advantage with the SELDI technique is that it only re-
quires minute amounts of sample material and both resolution and detection below 10 kDa are good [47].

Figure 3. SELDI-TOF MS technique consists of three parts: the ProteinChip array, the ProteinChip reader and the ProteinChip software. The sample is applied on the surface of the array, which captures a subset of sample proteins due to surface-protein interactions. After washing away salts, detergents and unbound proteins, a matrix substance is added on the array. The proteins of the sample are transformed to gaseous form and made to fly inside the reader flight tube. Finally, the flight times are analyzed by the software, which also controls all other aspects of data collection and analysis.
Sample proteins can be visualized in spectral or gel views. In spectral view every peak represents an individual protein, while in gel view proteins are displayed as bands. Different tissue types generate characteristic SELDI spectra, as exemplified in Figure 4.

**Figure 4.** Protein spectra of different mouse tissues generated by SELDI-TOF MS. The anionic exchanger array and binding with 100 mM phosphate buffer (pH 6) was used to generate the spectra. Most of the detected proteins are present in the low mass weight range, which is characteristic for the SELDI-technique.

General considerations when using proteomics

Many proteomic techniques, including the SELDI technology, preferentially capture high-abundant proteins, like the acute phase reactants in blood [48]. Sample pre-fractionations can be performed to eliminate high-abundant proteins from the samples, but one must keep in mind that the remaining sample proteins might be influenced [49]. In all kind of proteomic work the samples need to be correctly collected and handled before and during the analysis [50, 51]. Many aspects like type of blood collection tube, clotting time and temperature of blood samples influence the results and have been evaluated [52]. Further, correct calibration and automation of sample preparation steps contribute to reproducible results [53].
The role of proteomics in diabetes research

In many diseases screening and diagnosis can be performed by analyzing specific proteins or combinations of proteins. Both blood and other tissue samples can be used for such screening purposes. During recent years proteomic approaches have been used to develop better diagnostic and prognostic markers [54, 55].

In the case of diabetes the blood glucose level is a reliable and easily obtained diagnostic marker. Therefore, the proteomic techniques will probably not be used so much for diagnostic purposes. On the other hand the proteomic approaches are relevant for investigating the pathophysiological mechanisms of the disease, since T2DM is a polygenic disease with expected alterations in many proteins [21, 22]. In this context, whole pancreata [26, 56], pancreatic islets [25, 41, 57, 58], different β-cell lines [59-61], muscle cells [58, 62], adipose tissue [58, 63-65], liver [58, 66, 67] and blood [28, 29, 33, 34, 59, 68-72] have been investigated using proteomics. The main aims of the studies have been to characterize the proteomes of different tissues connected to diabetes and to investigate different pathophysiological aspects of the disease. Also, effects of treatments on proteins have been evaluated [30, 31].

Pancreas, pancreatic islets and β-cell lines

In tissue protein reference maps a large number of proteins of the tissue are separated and identified. As the name implies, the maps are generally used as references, when comparing for example protein expression profiles of control tissue with tissue from individuals with a certain disease. Some years ago, the human pancreatic protein reference map was created [56]. This reference map contained in total 302 identified proteins. In the pancreatic reference map, the exocrine and endocrine proteins were not separated. For diabetes research, the islet proteome is, however, more interesting and both mouse [25, 58, 73] and human islets [41, 57] have been proteomically characterized. The mouse islet reference map contained 44 identified proteins [58]. A human islet reference map has also been created [57]. In this work several of the identified proteins for example glucose regulated protein (GRP)78, GRP94, calreticulin, annexin and cytokeratins had previously been implicated in T2DM pathophysiology. Recently, a human islet peptide database was created by pooling and lysing islets and analyzing them by 2D-LC/MS/MS [41]. Over 29,000 peptides were detected and identified corresponding to 3,365 identified proteins.

Many specific aims in diabetes research have been investigated using proteomics. With the aim of investigating how glucose affects mouse islet proteins, a 2DGE approach was applied [25]. The authors found actin, α-enolase, cytokeratin 8, endoplasmic, glucose regulated proteins, heat shock
proteins, peroxiredoxins, prohormone convertase 2, protein disulphide isomerase, superoxide dismutase, tubulin, and V-type H+-ATPase (V1 subunit A) to be up-regulated in islets exposed to 11 mM glucose. In contrast, exocrine proteins and secretagogin were down-regulated in these islets compared with freshly isolated islets.

In diabetes research different cell lines are frequently used to elucidate different cellular mechanisms. The INS-1E cell insulin secretory granule proteome has recently been analyzed [74]. The presence of 130 granular proteins was determined, among which Rab37 and VAMP8 were suggested to be associated with granules. Glucose responsive and non-responsive MIN6 β-cells have also been investigated [61]. Using DIGE, several proteins were shown to be altered and suggested to play a part in glucose responsiveness. Most of the identified proteins were involved in cell metabolism. Particularly, proteins associated with the endoplasmic reticulum and involved in oxidative stress were found to be decreased in glucose non-responsive cells.

When the pancreatic proteome of mice with diet-induced T2DM was investigated by 2DGE the two proteins REG1 and REG2 (regenerating islet-derived protein) were up-regulated in the type 2 diabetic group, probably due to β-cell proliferation [26]. GSHPX1 (cellular glutathioneperoxidase) protein levels were decreased in type 2 diabetes mice. This down-regulation was interpreted to contribute to the progressive deterioration of the β-cell function. Pancreatic regeneration was also investigated by Yang et al [27]. By pancreatectomizing rats and analyzing the remaining pancreatic proteome by 2DGE, early adaptive changes of the regenerating pancreas were investigated. Over 90 up-regulated pancreatic proteins were found in the pancreatectomized group. Many of the changed proteins were proteins involved in cell growth and proliferation, but other protein variations indicated that the protein synthesis machinery in the regenerating pancreas was inhibited.

The T2DM animal model ob/ob mouse was used in another study applying liquid chromatography and mass spectrometry [42]. Pancreatic protein alterations in the low molecular mass range (below 20 kDa) were correlated to hyperglycemia and deranged insulin secretion characteristic for the disease. The main islet hormones insulin, glucagon and islet amyloid polypeptide (IAPP) were increased in ob/ob islets. Other up- and down-regulated peptides, like syncollin, were also found in the study, but their roles in β-cell dysfunction needs to be evaluated. The ob/ob mouse has also been used in another study, where the effects of rosiglitazone on islet protein expression were investigated [30]. When comparing with lean littermates, four ob/ob islet proteins were found to be modulated by rosiglitazone treatment. These proteins were identified as tropomyosin isoform 1, adipocyte fatty acid-binding protein, profilin and profilin fragment.

Another area of research, where proteomic techniques have been applied, is when effects of treatment, on for instance islet proteins, are investigated. When treating rat islets with imidazoline, which increases insulin release at
high glucose concentrations, 22 proteins were differentially expressed [31]. Among these proteins HSP60, protein disulfide isomerase and calreticulin all were involved in the protein folding machinery and up-regulated by imidazoline. Calgizzarin, calcyclin and annexinI are part of Ca$^{2+}$ binding and were also differently expressed. Pyruvat kinase, α-enolase and protein kinase C inhibitor are involved in the metabolism and were differently expressed.

Blood, serum and plasma

T2DM is a disease where many organs are affected [1]. Since all tissues are in contact with blood, proteins secreted or leaking from the different tissues are reflected in the blood. Blood insulin concentration at different stages of diabetes is a good example of how a single protein in the blood directly reflects changes in β-cell physiology. The easy accessibility of blood makes it attractive to study changes also of other blood-borne proteins, which may be related to different aspects of the disease. Therefore, profiling of blood proteins has been performed in context of many diseases.

An important consideration when performing blood protein profiling is the overall composition of blood proteins, where a limited number of proteins, like albumin and immunoglobulins, account for a large part of the total blood protein amount [46, 75-77]. To aid detection of medium- and low-abundant proteins, depletion of high-abundant proteins by different methods has been utilized [76, 78]. On the other hand, when pre-fractionations are used, the risk of losing potentially interesting proteins increases [49]. With the development of different methodologies capable of simultaneously measuring large numbers of proteins present in biological samples, blood protein profiling has become an important area for studying the pathophysiology of diabetes [29, 34, 68]. One of the first studies using blood from patients with T2DM was done by Jiang et al., who searched for altered proteins in the red blood cell membranes in T2DM [28]. In total 42 protein spots were found to be differently expressed when comparing the red blood cell membranes of T2DM individuals to healthy controls. Out of the 42 differently expressed proteins, 27 were up-regulated and 15 down-regulated. Two of the up-regulated proteins were identified as flotillin-1 and arginase. The results led to another study where the interactions of flotillin-1 and arginase were investigated [79]. The results showed that soluble arginase located to the red blood cell membranes through interaction with membrane bound flotillin-1. This interaction in turn, was shown to increase the arginase activity, also present in T2DM patients.

Another early study using a proteomic approach on human serum in the context of T2DM and insulin resistance was performed to mine low-abundant proteins, using an approach where fractionated serum samples were analyzed with SELDI MS [33]. When comparing serum from patients with T2DM or insulin resistance to control serum, haptoglobin and hemo-
globin were found to be elevated. Also, several other proteins involved in the inflammatory response, like α-2 macroglobulin, fibrinogen, complement C3 and C1 inhibitor, were altered when comparing serum from persons with insulin resistance with control serum. Many of the found proteins had been connected to diabetes in various ways. For instance, haptoglobin has been associated with glucose and lipid metabolism in many ways [80], while fibrinogen has been shown to be related to the development of T2DM [81]. To get new insights into the molecular alterations implicated in diabetes, serum protein profiles of normal rats and rats with streptozotocin-induced diabetes were analyzed [34]. Eight proteins were found, all increased in diabetes. One of the increased proteins was indirectly identified as C-reactive protein. Even if the protein has previously been shown to be increased in T2DM, and also predicts the development of the disease in humans [82], it is however not a strong acute phase reactant in rats [83].

Serum proteomics have also been used to investigate the effect of anti-diabetic agents from plants and fungi. In one study, the anti-hyperglycemic effects of green tea on serum proteins were investigated [32]. In this study, serum samples from mice with or without diabetes were first protein profiled using SELDI. Seven proteins were found to be more than three-fold decreased while seven were more than three-fold increased, when comparing mice with T2DM to control mice. To find the specific proteins, which were affected by green tea, the mice with T2DM were re-investigated after green tea administration, and compared with saline-treated control mice. When the two sets of results were compared, one protein specific for diabetes and sensitive for green tea was found. This unidentified protein with the mass 4212 Da was significantly reduced both in type 2 diabetes and by the green tea administration. In another study, the anti-hyperglycemic effect of fungal polysaccharide treatment on protein patterns monitored by 2DGE was evaluated in normal rats and rats with streptozotocin-induced diabetes [71]. In total about 50 proteins were found to be differentially regulated and 20 spots were identified as diabetes-associated proteins. When the protein patterns were monitored over time, different patterns were found. Nine proteins (albumin, apolipoprotein A1, apolipoprotein E, haptoglobin β, immunoglobulin kappa-chain, kallikrein binding protein, transthyretin monomer, transthyretin tetramer and vitronectin) were significantly changed during diabetes induction and were restored to healthy levels after treatment. Seven proteins (α1-inhibitor III, apolipoprotein A-IV, ceruloplasmin, fetuin β, hemopexin, serine protease inhibitor and transferrin) changed upon diabetes induction, but were not restored by treatment.

Also, effects of the Japanese traditional medicine Kampo on the plasma proteome have been determined [69]. The plasma protein expression profiles of spontaneously diabetic rats with nephropathy were compared to normal rats and ten proteins were found to have different levels. Some of these peaks were altered by the kampo treatment, which was interpreted by the
authors to be proteins with special association with the development of nephropathy.

**Diabetic complications**

Even if the diagnosis of diabetes is easily obtained by blood glucose measurements, there is a need for prognostic markers for the associated complications. Recently, serum proteome analysis of humans with T2DM and nephropathy was investigated [70]. The aim of this study was to identify more specific and accurate markers for prediction of diabetic nephropathy. The authors focused on proteins that showed gradual alteration when comparing with the increasing severity of the nephropathy. Six proteins behaved in that manner namely c-type lectin domain family 3, ficolin 3 precursor, hemopexin precursor, complement factor I light chain, apolipoprotein E and extracellular glutathione peroxidase. In another investigation, SELDI-TOF MS was used to find urinary protein markers for prediction of the clinical course of nephropathy connected with T2DM [84]. Two proteins were identified and suggested to be markers of nephropathy.
Aims

Many individual proteins have been shown to vary between healthy individuals and persons with insulin resistance and T2DM. Examples of such proteins are interleukin-6, resistin, leptin, adiponectin and visfatin [85-89]. Many of the circulating proteins have also been connected, in various ways, to the pathogenesis of the disease. Rather than measuring single protein alterations, the approach chosen in the present thesis investigated many proteins, protein profiles, to answer specific questions. The idea was to first characterize the sample (pancreatic islets or human serum/plasma) and subsequently analyze changes of protein profiles connected to the differences in sample phenotypes. However, in the beginning of the 21st century not much proteomic work had been done in the field of diabetes research, especially not with the selected method, SELDI-TOF MS. Therefore, it was crucial to start by thoroughly investigating the methodological aspects of the chosen approach.

It can be assumed that that proteomic studies will generate results showing variations in many individual proteins. To be able to draw biological conclusions of the findings it is therefore not only important to identify the changed protein, but also to investigate the cause of the change. In case of T2DM, a protein change can either be a part of the pathogenesis or a consequence of the disease. Example of that kind of distinction is the genetic background and diabetic state. Since decreased β-cell function is an early sign of T2DM, it is also important to elucidate which proteins that can be linked to impaired β-cell function.

The specific aims of the thesis were:
1. to evaluate the SELDI-TOF MS technique for finding and identifying differently expressed pancreatic islets proteins.
2. to evaluate the SELDI-TOF MS technique for finding and identifying differently displayed serum proteins in individuals with T2DM.
3. to determine the influence of the genetic background versus diabetic environment on differentially displayed proteins by measuring and comparing serum protein profiles of individuals with normal glucose tolerance to individuals with T2DM.
4. to investigate if differentially displayed plasma proteins can explain the impaired β-cell function observed in humans with low early insulin response.
Material and methods

Islet isolation and culture (Paper I)
Islets were isolated from adult male C57BL/6J mice (B&K, Sollentuna, Sweden) by collagenase digestion. The pancreata were removed from the animals and cut into pieces. The tissue pieces were shaken vigorously in collagenase A (Roche Diagnostics GmbH, Mannheim, Germany) solution at 37°C to free the islets from exocrine tissue. Subsequently, the islets were cultured in RPMI 1640 culture medium supplemented with 3 or 11 mM glucose with or without 0.5 mM oleate. The fatty acid was complexed with 0.5 % fatty acid free BSA and the same amount of BSA was also present in culture media without any fatty acid. The procedures involving animals were in conformity with international and national laws and approved by the local Animal Research Ethics Committee at Uppsala University.

Measurements of islet insulin release and oxygen tension (Paper I)
Dynamic islet insulin release was measured from single islets, placed in 10 μl-chambers in a closed perifusion system and perifused with a flow rate of approximately 150 μl/min [90]. The perifusion media contained 1 mg/ml albumin, 125 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl₂, 1.28 mM CaCl₂, 25 mM HEPES and 3 mM glucose (pH 7.4). After an initial 60 min equilibration period, samples were collected every minute for 8 min. Subsequently, the glucose concentration in the medium was raised to 11 mM and the sampling continued for an additional 32 min. The insulin concentration in the samples was measured with a competitive enzyme-linked immunosorbent assay (ELISA) using microtiter plates coated with guinea-pig insulin antibodies. The rate of insulin release was normalized to islet dry weight. The early and late phases of insulin release were defined as the average insulin secretion rate 2-6 min and 20-30 min after glucose elevation, respectively.

Islet metabolism was also investigated by measuring intra-islet oxygen tension with Clark-type microelectrodes [91]. Individual islets were attached to poly-lysine coated cover slips, which were used as the bottoms in open Sykes-Moore perifusion system. The microelectrodes were inserted into the islets by a micromanipulator and the electrode position was monitored by
The tip of the electrode was positioned 25-50 μm into the islet to record intra-islet oxygen tension (pO₂). The perifusion rate was 150-200 μl/min and the perifusion media contained 1 mg/ml albumin, 125 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl₂, 1.28 mM CaCl₂, 25 mM HEPES and 3 mM glucose (pH 7.4). After recording the basal oxygen tension in the presence of 3 mM glucose, the glucose concentration was elevated to 11 mM.

Differences in insulin secretion and oxygen tension between experimental groups were evaluated using ANOVA with Fischer’s posthoc test. P-values <0.05 were considered significant. Values were expressed as means ± SEM.

Study participants

Study participants Paper II

The study participants in paper II consisted of two women and four men of whom three had T2DM and were treated with anti-diabetic drugs or diet. The drug treatment was discontinued two days prior to the study. The study subjects were between 54 and 65 years old, had normal blood pressure, did not have any other metabolic or cancerous disease and were non-smoking. All subjects had normal thyroid, liver, cardio-pulmonary and kidney function as determined by medical history, physical examination and blood chemistry screening. The study participants with T2DM were patients at the Diabetic unit at Enköping hospital, while the control persons were recruited by advertisement. The participants fasted over night and in the morning an intravenous catheter was placed in the antecubital vein. Blood was drawn from the catheter and plasma was prepared for glucose concentration measurements by collecting blood in EDTA containing tubes. After centrifugation (2400 g) of the tubes, plasma was separated and the glucose concentration was measured. Serum was prepared for protein profiling measurements. In such blood samples, PEFAbloc® was added before the samples were allowed to clot. The serum samples were aliquoted and stored in -70°C until analysis. Samples for blood chemistry were taken and analyzed according to standard procedures at the Enköping hospital laboratory. Subsequently, the study participants received 75 g of glucose per os (time point 0 min) and blood samples were collected after 120 min for glucose concentration measurements. The plasma glucose concentrations were determined by the glucose oxidase reaction using Granutest 250 glucose (Merck, Darmstadt, Germany). By applying the WHO criteria [4], three individuals had normal glucose tolerance (NGT) and three had T2DM. The study protocol was approved by the Ethics Committee at the Medical Faculty, Uppsala University, Uppsala, Sweden and written informed consent was obtained from all participants.
Study participants Paper III

This study was performed on previously healthy men enrolled in the Stockholm Diabetes Prevention Program (SDPP) [92, 93]. The SDPP cohort consists of men between 35-54 years old, who were characterized in 1992-1994 using questionnaires and oral glucose tolerance tests (OGTT). In paper III, 60 individuals were chosen from the cohort, out of which 20 individuals had NGT, 20 individuals had impaired glucose tolerance (IGT) and 20 individuals were diagnosed to have T2DM. The groups were age and weight matched. In all groups there were 10 persons with and 10 persons without family history of diabetes (FHD). FHD was defined as known diabetes in at least one first-degree relative or at least two second-degree relatives. Whereas individuals with FHD were selected to have low β-cell function, the individuals without FHD had high β-cell function. The estimation of β-cell function was made by homeostasis model assessment (HOMA-β) [94]. The study protocol was approved by the Ethics Committee at Karolinska Institutet, Stockholm, Sweden.

Study participants Paper IV

The study was performed on previously healthy men enrolled in the Uppsala Longitudinal Study of Adult Men (ULSAM) [95-97]. This population-based cohort consists of men living in Uppsala and born between 1920 and 1924, who have been investigated several times at different ages. Investigations at age 71 formed the baseline in this study. Three groups were selected from the ULSAM cohort. The first group consisted of 10 healthy individuals with normal blood pressure and BMI <25 kg/m². Further, they were characterized by OGTT to have NGT, and were within the highest tertile of the early insulin response (EIR) distribution in the NGT group. The second group consisted of 10 individuals with T2DM who were within the highest tertile of the EIR distribution in the T2DM group. Finally, the third group consisted of 10 individuals with T2DM who were within the lowest tertile of the EIR distribution in the T2DM group. Fasting plasma samples from these subjects were obtained and stored frozen (-70°C) until protein profiling was performed. EIR was defined as the ratio of the 30-min increment in insulin concentration to the 30-min increment in glucose concentration after a 75 g OGTT. Samples obtained at ages 71, 77 and 82 years were used in order to investigate protein stability over time. The study protocol was approved by the Ethics Committee at the Medical Faculty, Uppsala University, Uppsala, Sweden.
Islet protein measurements

Islet protein profiling (Paper I)

Mouse islets were protein profiled using ProteinChip arrays and SELDI-TOF MS. The islets were first washed with ice-cold PBS containing 1 mM PE-FAbloc®. Afterwards, approximately 50 islets/sample were lysed by vigorous shaking in 25 μl lysis buffer (50 mM Trizma® base buffer, 8 M urea, 3 % CHAPS, 1 % ABS14 and 3 mM PEFAbloc®). Samples were diluted to 75 μl with 50 mM Trizma® base buffer and centrifuged 10 min at 10,000 rpm to pellet any remaining cell debris. The total protein content of the samples was determined (Bio-Rad, Hercules, CA). Samples were aliquoted and stored frozen (-80°C) until analysis.

Islet protein profiling was performed with ProteinChip arrays (Ciphergen, Freemont, CA), with different surface chemistries. Anionic exchanger (SAX2), cationic exchanger (CM10, formerly WCX2) and reverse phase (H50) arrays were tested. Islet samples were thawed on ice, vortexed, centrifuged and diluted with different binding buffers. For SAX2 arrays, binding was performed in 100 mM Trizma® base buffer supplemented with 0.1 % Triton X100 (pH 9), for CM10 in 100 mM ammonium acetate buffer supplemented with 0.1 % Triton X100 (pH 4) and for H50 in 100 mM phosphate buffer supplemented with 30 % ACN (pH 6). The arrays were placed in a bioprocessor and pre-washed with 200 μl of the same binding buffer as used for diluting the sample. The bioprocessor can hold 12 arrays, and allows larger sample and buffer volumes to be used. The binding buffer was discarded and 90 μl of sample was added. The bioprocessor was sealed and protein binding was performed over-night at 4°C with gentle shaking. Next day, the excess samples were discarded and the arrays washed once with 200 μl of binding buffer and three times with 200 μl of binding buffer without Triton X100. After two final washes with 1 mM HEPES (pH 7.4) the arrays were air-dried. Saturated (100 %) sinapinic acid (SPA) solution was prepared by mixing 5 mg SPA with buffer consisting of acetonitrile (ACN), trifluoroacetic acid (TFA) and water (50:0.5:49.5 vol/vol). The 50 % SPA solution was prepared by diluting the solvent phase with an equal volume of the same buffer. To each spot 3 x 0.5 μl of a 50 % solution of SPA was applied. Arrays were read in a ProteinChip reader (PBSII, Ciphergen, Freemont, CA). Peak normalization, detection, clustering and alignment were performed with supplied Ciphergen ProteinChip Software 3.1. The mass accuracy was calibrated externally using the All-in-1-peptide molecular weight standard. Differences in islet protein expression levels were evaluated by ANOVA with Fischer’s posthoc test, and p-values <0.05 were considered significant.
Islet protein identification (Paper I)

Identities of the differentially expressed proteins, discovered by SELDI-TOF MS, were obtained by peptide mass fingerprinting (PMF). First, the islet proteins were separated by SDS-PAGE. Aliquots containing 10 μg protein were mixed with SDS-PAGE sample buffer. Proteins of the islet samples and pre-stained molecular marker proteins (Fermentas GmbH, St. Leon-Rot, Germany) were resolved on 12 % SDS-polyacrylamide gels. Gel pieces were blindly excised from the gel, by using the pre-stained marker proteins as position references. Proteins were eluted from the gel by mincing, sonicating and vortexing the gel piece in 50 μl elution buffer containing formic acid, ACN, isopropanol and water (50:25:15:10 vol/vol). The eluate was transferred to a new tube and the gel piece was again washed with 50 μl elution buffer. The two eluate volumes were pooled and speed-vacuum dried, then the pellet was solubilized in 10 μl of 0.5 % octyl-gluco-pyranoside and diluted to 100 μl in 100 mM phosphate buffer (pH 6) supplemented with 0.1 % Triton X100. The presence of the protein of interest in the eluate was then verified by re-applying the eluate on SAX2 array.

To obtain material for PMF, islet lysates were subjected to another SDS-PAGE and gels were stained with Bio-Safe Coomassie (Bio-Rad, Hercules, CA). Based on the information of the localization of the biomarkers in the gel, bands were excised from the gels and trypsin was added to the gel piece. When an unknown protein is digested by trypsin, the protein is cleaved at specific cleavage sites. This results in a characteristic mixture of peptides or a peptide fingerprint, which is often enough for identifying the original protein. To identify the protein the search program Mascot (Matrix Science, London, UK) was used. Swiss-Prot was used as the protein sequence database and peptide masses were compared to the theoretical peptide masses of all available proteins from mammalian taxonomy. No missed cleavages were allowed and the mass tolerance was ± 0.1 Da. The criteria used to accept identifications included the extent of sequence coverage, number of peptides matched and molecular weight search (MOWSE) score. Information about the identified protein and its putative function was found at the ExPASy Molecular Biology Server at Swiss-Prot (http://www.expasy.org/sprot/).

Serum and plasma protein measurements (Papers II, III and IV)

Serum and plasma protein profiling

Serum and plasma protein patterns were determined by SELDI-TOF MS using ProteinChip arrays. Serum/plasma samples were thawn on ice, quickly vortexed and centrifuged. One part serum/plasma was denaturized with two
parts denaturation buffer (50 mM Trizma® base buffer, pH 9, containing 9 M urea, 2 % CHAPS). The denaturized samples were vortexed during 30 min at 4°C and subsequently centrifuged (12,000 g) for 5 min. Finally, the samples were diluted 50 times with appropriate binding buffer (see below).

Two arrays with different surface chemistries were used to capture proteins from the samples. The arrays were placed in a bioprocessor and the immobilized metal affinity capture (IMAC30) arrays were first pre-activated during 5 min by washing with 50 μl 100 mM copper(II)sulphate twice. The copper(II)sulphate solution was discarded and the arrays briefly washed with water. Then the arrays were equilibrated during 2 x 5 min with binding buffer (100 mM sodium phosphate buffer, pH 6). Cationic exchanger (CM10) arrays were equilibrated during 2 x 5 min with binding buffer (100 mM acetate buffer, pH 4). After equilibration, the serum/plasma samples were applied on the spots of the arrays. Binding was performed for 1 h by gentle agitation at room temperature. Excess sample was discarded and the arrays were washed with binding buffer (3 x 125 μl) and twice quickly with water. The arrays were removed from the bioprocessor and allowed to air-dry. Saturated solution of SPA matrix was diluted to 50 % and applied on the spots (2 x 1 μl).

Time-of-flight spectra were generated in the SELDI reader by averaging laser shots at different laser intensities, depending on the mass range studied. The mass accuracy was calibrated externally using the All-in-1-protein molecular weight standard. In papers III and IV also an external calibration was performed using known peaks. Peak normalization, peak detection, clustering and alignment were performed with ProteinChip Software 3.1. Mass spectra of serum protein patterns were obtained from each study subject. The ProteinChip software settings for peak detection were set, and clusters were selected for further analysis if their heights were significantly different when comparison was performed between the groups using either the Mann-Whitney U-test (papers II and III) or ANOVA (paper IV). P-values <0.05 were considered significant.

Serum and plasma protein identification

The discovered differently displayed proteins of interest were identified by a procedure containing several steps including sample fractionation, SDS-PAGE, elution and verification of biomarkers, excision of relevant gel bands, in-gel trypsin digestion, mass determination of the tryptic fragments and peptide database comparison (Figure 5).
Figure 5. Identification procedure of a specific protein in a complex sample. The sample, where the protein originally is found, is first purified by fractionations. The purified fraction is separated by SDS-PAGE in two parallel lanes. One protein band is then eluted and the presence of the correct protein in the eluate is confirmed by SELDI-TOF MS. The other protein band is excised and in-gel trypsin digested. Finally, the identity is obtained by mass determination of the tryptic fragments and peptide database comparisons.
Sample fractionations

Since serum/plasma contains thousands of proteins, the first step in the identification process was to reduce sample complexity. This was achieved by several fractionation steps. First, anionic fractionation was done, using Q ceramic HyperD F spin columns. The columns were first equilibrated with a buffer (50 mM Trizma®base buffer with 0.9 M urea and 0.2 % CHAPS). Denaturized serum/plasma (250 μl) was diluted with another 250 μl of the same buffer and bound to the resin during 30 min on a mixer at room temperature. The column was then centrifuged (1 min, 500 g) and the flow through fraction was collected into an eppendorf tube. The column was then step-wise washed with 500 μl of buffers with decreasing pH 9, 7, 5, 4, 3 and finally an organic buffer (16.7% isopropanol, 33.3% ACN, and 0.1% TFA). The fractions were applied on both normal phase (NP20) arrays and the specific array used when finding the protein. To further purify the anionic fractions, size fractionation by microcon YM-50 columns (Millipore, Billerica, MA) or hydrophobic fractionation using reverse phase beads (RPC Poly-Bio beads, Ciphergen) were performed. For the size fractionation, the sample was added to the size fractionation column and concentrated by centrifugation. With repeated washing steps using a solution of 0.1 % TFA supplied with an increasing percentages of ACN (10 %, 20 %, 30 %, 40 %, 50 % and 60 %), the serum/plasma sample was further purified. These fractions were also analyzed with NP20 arrays, to locate the proteins of interest to the different fractions. When performing the hydrophobic fractionation, the hydrophobic beads were equilibrated for 1 h at room temperature in a 0.1 % TFA solution containing 10 % ACN. The beads were spun down by centrifugation and the supernatant was removed. The sample to be purified was adjusted to a final concentration of 10 % ACN/0.5 % TFA and mixed with the beads. To allow proteins to bind to the beads, the beads and the sample were shaken at room temperature for 30 min. Subsequently, the tube was centrifuged, and the supernatant was removed by aspiration. Bound proteins were then eluted by step-wise washes with increasing percentages of ACN (10 %, 20 %, 30 %, 40 %, 50 % and 60 %) in 0.1 % TFA. Fractions were removed between washing steps and analyzed on NP20 arrays. Fractions containing the proteins of interest were speed-vacuum dried in a concentrator (Eppendorf, Hamburg, Germany) to remove the buffer.

Protein separation by electrophoresis

The purified fractions containing the proteins of interest were separated by SDS-PAGE. The samples were first resuspended with SDS-PAGE sample buffer and then resolved in parallel lanes on gels. Also, pre-stained standard was run on the gel. After electrophoresis, the gels were stained with colloidal coomassie. From the first lane protein bands of the relevant masses were excised, cut into smaller pieces and passively eluted. The elution was
achieved by shaking the gel pieces for 30 min in 300 μl of solution containing water, methanol and ACN (50:40:10 vol/vol). Afterwards, the solution was removed and the gel pieces were washed with 200 μl of 50 % ACN in 50 mM ammonium bicarbonate buffer (pH 8). After 30 min shaking, the buffer was removed and the gel pieces dehydrated for 15 min in 200 μl 100 % ACN. Subsequently, the ACN was removed and the gel pieces were heated during 5 min in 60°C. Approximately 30 μl of an elution buffer (45 % formic acid, 30 % ACN, 10 % isopropanol and 15 % water) was added to the tubes, which were vortex-mixed briefly and left on a shaker at room temperature over-night. Subsequently, 1 μl of the eluent was applied on an NP20 array, to see if the desired protein had been eluted from the gel.

Peptide mass fingerprinting

When eluates contained a protein of interest, the corresponding band of the second lane was excised and the coomassie stain was removed by incubating with solutions consisting of water, methanol and acetic acid (50:40:10 vol/vol) and subsequently with 100 mM ammonium bicarbonate buffer containing 50 % ACN. The gel pieces were dehydrated in 100 % ACN and then rehydrated by adding 20 mM ammonium bicarbonate buffer containing 10-20 ng/μl trypsin. The gel pieces were incubated over night at 37ºC. The tryptic digestion of the unknown protein yielded a characteristic peptide mixture or a peptide fingerprint of the protein. This unique set of peptides was used to identify the protein. The peptide mixture was analyzed by MALDI-TOF MS yielding the peptide masses, which were compared with protein databases with masses of peptides derived from known proteins. The search program Mascot (Matrix Science, London, UK) and the protein sequence database Swiss-Prot were used and the peptide masses were compared to the theoretical peptide masses of all available proteins from mammalian taxonomy. The criteria used to accept identifications included the extent of sequence coverage, number of peptides matched and molecular weight search (MOWSE) score. Information about an identified protein and putative function was found at the Swiss-Prot database at the ExPASy Molecular Biology Server (http://www.expasy.org/sprot/).

Indirect protein identification

The indirect protein identification approach took into account that peaks at 15.1 and 15.8 kDa were previously identified as α- and β-chains of hemoglobin (Hb) [98]. Pure Hb (Sigma-Aldrich) was dissolved in buffer (50 mM Trizma®base, 9 M urea, 2 % CHAPS, pH 9), diluted 1:10 in binding buffer (100 mM acetate buffer, pH 4) and applied on a cationic exchanger array. The resulting spectrum was compared with the plasma spectrum peaks.
Confirming protein identities

Western blotting (Paper I)

The identity of a protein of interest was confirmed by western blotting. Samples, which included both purified protein and crude islet lysates, were mixed with SDS-PAGE sample buffer and reduced by boiling for 5 min. After electrophoresis, proteins were transferred onto PVDF membrane and immunoblot analyses were performed with anti-peptidyl-prolyl isomerase B (PPI-B) (Abcam, Cambridge, UK) and anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. Immunoreactive bands were imaged with Fluor-S MultiImager MAX (Bio-Rad) and quantified with Quantity One software (Bio-Rad). Student’s t-test was used to compare the groups and p-values <0.05 were considered significant.

Albumin analysis (Paper III)

The albumin peak identity was confirmed by depleting five serum samples of albumin using the ProteoExtract Albumin Removal Kit (Merck, Darmstadt, Germany). The samples were subsequently analyzed by CM10 arrays and SELDI. Amounts of albumin in serum from the SDPP individuals were also measured spectrophotometrically using brom cresol purple as reagent (albumin/BCP assay, Roche Diagnostics, Basel, Switzerland).

Hemoglobin analysis (Paper IV)

The concentration of Hb was measured in the original plasma samples, using an ELISA for human Hb (Bethyl Inc, Montgomery, TX, USA).
Results and discussion

Islet protein profiling by SELDI-TOF MS (Paper I)

Evaluation of methodological aspects

In the mass spectra detected with the SELDI-TOF MS system, the number of peaks can be calculated with the Biomarker wizard algorithm within the software. The Biomarker wizard uses the peak signal to noise (S/N)-ratio and the number of spectra, where a particular peak is present (percentage of spectra) to include or exclude the peaks. When lysates from 32 different islet preparations were analyzed with increasing stringency of the software settings (higher S/N and higher percentage of spectra), the number of detected peaks dropped rapidly with increasing stringency. Next, protein profiles from 24 different islet preparations were analyzed using three different types of ProteinChip arrays and binding buffers. The SAX2 arrays (pH 9), CM10 arrays (pH 4) and H50 arrays (pH 6) were tested. Most detected peaks were present in the low mass range. Peaks captured by the SAX2 surface were more evenly distributed over the entire mass range, while 32 and 43% of the peaks detected by CM10 and H50 arrays were between 3-6 kDa. Although the same proteins may be captured by different surfaces, by combining different arrays many unique protein species can be detected.

A ProteinChip array captures a subset of proteins present in a sample. By altering the array and/or binding conditions the binding of certain proteins can be favored. On the SAX2 array, the largest number of peaks was obtained when binding at pH 6. At both lower and higher pH, less peaks were detected. The decreased number of captured proteins at higher pH, could be caused by insulin binding to the array surface, and thereby hampering the binding of other less-abundant islet proteins.

To be able to study differences in protein levels, the analytical system needs to be reproducible and quantitative. To test the reproducibility, the same islet lysate was applied on eight surfaces on a SAX2 array. Peak intensities had a coefficient of variance of less than 10%. To test the quantitativeness an islet lysate was diluted (40, 80, 160 and 320 times) and applied on a SAX2 array. Peak intensities showed strong linear correlation ($R^2>0.95$) to the logarithm of the dilutions. For optimal reproducibility and quantitativeness, parallel processing of samples was crucial.
Effects of oleate and glucose on islet insulin release, oxygen tension and protein profiles

Glucose and oleate are known to both regulate β-cell gene expression [99, 100] and function [101, 102]. In particular, long-chain fatty acids such as oleate have an inhibitory effect on glucose-induced changes in metabolism and insulin secretion after prolonged exposure [100, 103, 104]. To evaluate the SELDI-TOF MS system in experimental islet research, islets were cultured at basal (3 mM) or stimulatory (11 mM) glucose concentration in the absence or presence of 0.5 mM oleate for 24 or 48 h.

Following culture, islets were first functionally characterized by measuring insulin release and intra-islet oxygen tension from individual islets perfused with 3 or 11 mM glucose. In islets cultured for 24 h, basal oxygen tension was decreased in islets cultured at 11 mM glucose compared to islets cultured at 3 mM glucose. When 0.5 mM oleate was present during culture, the basal oxygen tension was further lowered. No changes in basal insulin secretion were seen. When the glucose concentration in the perfusion buffer was increased to a stimulatory level the intra-islet oxygen tension decreased in all groups. The reduction was greater in islets exposed to 0.5 mM oleate in combination with 3 mM glucose than in islets exposed to 3 mM glucose alone and the reduction was even greater in islets cultured at 11 mM glucose. No additional effect of 0.5 mM oleate was observed in the latter islets. The decreased oxygen tension was paralleled by increased insulin secretion. When the culture period was extended to 48 h, the beneficial effect of 0.5 mM oleate on oxygen tension and glucose stimulated insulin secretion seen in islets cultured at 3 mM glucose was no longer evident. Furthermore, islets cultured in the presence of 11 mM glucose and 0.5 mM oleate showed reduced metabolic and secretory responses to a stimulatory glucose concentration compared to islets cultured at 11 mM glucose alone.

Secondly, islets were lysed and protein profiled using SAX2 surfaces (pH 6). Peak definition criteria were set to S/N of 5 and percentage of spectra containing the peak to ≥25 %. In total 25 peaks were detected. The expression levels were determined for every peak in the four culture conditions. Islets cultured in 11 mM glucose showed more oleate-regulated proteins than islets cultured in 3 mM glucose. When glucose is elevated, there is a rise in the cytosolic levels of malonyl-CoA, which inhibits carnitine palmitoyltransferase-1 (CPT-1) [105]. The nutrient source of the β-cell is thereby shifted from fatty acids towards glucose, leading to cytosolic free fatty acid accumulation [105], which in turns affects gene and protein expression [100]. On the other hand, in the presence of low glucose, fatty acids are the primary energy source for pancreatic β-cells [106]. Therefore, it can be speculated that inclusion of oleate in the presence of 3 mM glucose will not lead to similar increase in cytosolic free fatty acid levels as observed when oleate is intro-
duced together with 11 mM glucose and that the islet gene and protein expression might not be altered to the same extent.

Since it was found that the reproducibility of SELDI-TOF MS data was dependent on parallel processing of samples, an independent data set of islet lysates was analyzed by SELDI-TOF MS exactly as the original data set. Of the original 23 peaks discovered, 16 (70 %) peaks were also discovered in the new data set. All the significantly changed proteins except one (peak at 17063 Da) were also significantly changed in the new data set. In order to visualize the effects of treatment and differences in time of analysis a principal component analysis (PCA) was performed on the 16 peaks shared by the two data sets. The PCA component 1 and 2 explained 60 % of the observed variance in the data. The first conclusion of the PCA is that in both data sets the samples from oleate treated islets cluster differently from non-oleate treated islets, verifying that treatment effects are reproducibly detected by SELDI-TOF MS analysis. The second conclusion is that in addition to the treatment effects, there is a clustering effect that is due to differences in time-point of analysis. This might be caused by different SPA preparations or changes in reader laser efficiency. The results emphasize, that samples that are to be compared must also be processed in parallel.

Islet protein identification and verification

To be able to make biological implications of proteomic data, it is often essential to obtain the identity of the differently expressed proteins. In contrast to the 2DGE approach, where proteins are accessible as separate spots [25, 107], differentially expressed proteins discovered by SELDI-TOF MS are not inherently separated. The islet proteins were first separated and purified by SDS-PAGE. During the purification steps, the presence of the correct protein of interest was tracked by SELDI re-analysis using the same buffer and surface conditions as those used initially, thus taking advantage of the SELDI technique.

We chose to identify a protein appearing at 25 kDa, which was lowered in islets cultured at 11 mM glucose and 0.5 mM oleate. The protein was purified by separating the crude islet lysate proteins by SDS-PAGE. Gel pieces in the relevant mass range were blindly excised, proteins were eluted from the gel pieces and eluates were analyzed by the SELDI-system. One eluate was shown to contain the 25 kDa protein. In the next step the islet sample was again subjected to SDS-PAGE and the gel was stained. The relevant gel piece was excised and trypsin was added. Following digestion the tryptic fragments were eluted and their masses determined by MALDI-MS. Comparison of these fragments with Swiss-Prot database resulted in a match (MOWSE score 92.6) to the *Mus musculus* protein peptidyl-prolyl cis-trans isomerase B precursor (PPI-B) or cyclophilin B.
The protein identity was verified by western blotting. Both gel eluates and islet lysates were tested for PPI-B immunoreactivity. A band of appropriate molecular size was detected by an antibody against PPI-B only in lanes, where the eluates had been demonstrated to contain the 25 kDa peak. In addition, lysates obtained from islets cultured for 48 h at 11 mM glucose in the absence or presence of oleate were subjected to western blotting using the anti-PPI-B antibody. When the immunoreactivity in the 25 kDa region was quantified, protein levels in islets exposed to oleate were 54 % lower than in islets exposed to 11 mM glucose alone, thus confirming the 36 % reduction seen in the peak intensity obtained by SELDI-TOF MS.

PPI-B is an endoplasmic reticulum (ER) resident protein [108] belonging to the abundantly expressed and evolutionary conserved immunophilin protein family including the cyclophilins and the FK506 binding proteins [109]. The protein was detected on a human islet proteome map [57]. The protein is involved in protein folding where it accelerates the process by catalyzing the slow steps of initial folding and rearrangement of proline containing polypeptides [110].

Serum protein profiling by SELDI-TOF MS (Paper II)

Serum/plasma protein profiling using SELDI-platform has been applied on large patient cohorts with the aim of finding reliable diagnostic and prognostic markers of diseases like breast and prostate cancer [54, 55]. In large patient cohort studies, patterns of differentially displayed serum proteins have been discovered that with high accuracy distinguish afflicted from healthy individuals [54]. Since such patterns may prove useful in themselves for diagnosis without knowing the identities of the varying proteins in the pattern, often only mass/charge of these proteins have been published [111]. In diabetes research the situation is different, since the blood glucose level is a reliable and easy to measure marker of the disease state. In diabetes research the SELDI-technology will probably therefore not be used so much for diagnostic and prognostic purposes. In contrast, the proteomic platform is highly relevant as a tool for hypothesis generation or for investigation of the disease pathophysiology. T2DM is polygenic [21, 22] and levels of many blood-borne proteins can be expected to be altered. With this background, we wanted to investigate changes in serum protein patterns in individuals with T2DM by SELDI-TOF MS.

Evaluation of methodological aspects

The aim of paper II was to see whether differing serum proteins could not only be discovered, but also identified in T2DM individuals using SELDI-TOF MS. The CM10 and IMAC30 surfaces were found to be equally effi-
cient to retain proteins. These experiences are in line with those of other studies, where serum or plasma protein profiling has been conducted [32, 34, 54, 55, 69, 112, 113]. Whereas three differentially displayed proteins were captured by both arrays, the remaining proteins were either detected by the CM10 or IMAC30 array. These results illustrate the usefulness of employing different arrays and binding conditions, which will adsorb different subsets of proteins, and that some proteins are common to the different subsets especially when arrays have similar surface chemistries.

Reproducibility of the arrays was tested by applying five equal aliquots of a serum sample onto spots of the CM10 array. SELDI-TOF MS spectra were generated and the intensities of six peaks were determined for each spot. The coefficient of variance (CV) was around 10 % (4.1-12.6) for all six peaks as shown in Figure 6.

![Figure 6. Reproducibility of the Protein-Chip arrays and SELDI-TOF MS analysis. Aliquots of the same serum sample were applied on five different spots of a cationic exchanger array. The intensities of six peaks (denoted A-F) were analyzed.](image)

<table>
<thead>
<tr>
<th>Peak</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average</td>
<td>6.0</td>
<td>7.8</td>
<td>1.5</td>
<td>1.3</td>
<td>2</td>
<td>28.8</td>
</tr>
<tr>
<td>SD</td>
<td>0.71</td>
<td>0.67</td>
<td>0.13</td>
<td>0.05</td>
<td>0.25</td>
<td>1.67</td>
</tr>
<tr>
<td>% CV</td>
<td>11.9</td>
<td>8.58</td>
<td>8.71</td>
<td>4.09</td>
<td>12.58</td>
<td>5.80</td>
</tr>
</tbody>
</table>
Quantitativeness of the same arrays was tested by diluting a serum sample and determining peak intensities of three peaks for each dilution (10, 20, 40, 80, 160 and 320 times). The peak intensities decreased as the sample was diluted and showed strong linear correlations ($R^2$ 0.85-0.93) when plotted against the logarithm of the dilutions (Figure 7).

**Figure 7.** Quantitativeness of the cationic exchanger arrays was tested by diluting a serum sample (in six steps) and determining peak intensities of three peaks (denoted A-C) for each dilution. The peak intensities decreased as the sample was diluted and showed strong linear correlations ($R^2$ 0.85-0.93) when plotted against the logarithm of the dilutions.
Serum protein identification
An important aspect of the present work was to evaluate how readily the differently displayed proteins were identified. The strategy was to reduce sample complexity by fractionation, separate proteins of the fractions by SDS-PAGE, digest separated proteins with trypsin and identify the digested proteins by PMF.

When fractionating the crude serum sample, the existence of the protein of interest was verified by applying the fraction on NP20 array and the initially used CM10 array. Fractions which contained the protein of interest, reasonably well separated from other proteins, were subjected to SDS-PAGE. By passively eluting proteins from bands in relevant mass range and re-applying the eluates on NP20 arrays, the presence of a certain protein in a given band could be verified. When the presence of the protein in the gel piece was confirmed, a similarly obtained gel piece was subjected to trypsin digestion and the identity of the protein obtained by PMF. We were able to identify four out of six proteins initially discovered using the CM10 array. In conclusion, in paper II the SELDI-platform was evaluated for diabetes work using blood samples. The approach could be used to both detect and identify serum proteins, which may be implicated in the pathophysiology of the disease.

Serum protein profiles of NGT and T2DM individuals with or with no family history of diabetes (Paper III)
Failure of the β-cell is a characteristic of T2DM [114] and observed early during the development of the disease. Although environmental factors including a sedentary lifestyle contribute to this development [115], recent advances in identifying genes associated with the disease has underscored the importance of the genetic background for this development [21, 116-118]. Indeed, β-cell failure is observed especially in persons with a family history of the disease [119]. In an attempt to determine the relative importance of the diabetic environment versus genetic background for the development of β-cell failure, serum protein profiles of NGT and newly diagnosed T2DM individuals were compared. By including persons with differences both in β-cell function (estimated by HOMA-β) and FHD, we had the opportunity to determine whether a specific protein variation depended on genetic or environmental factors.

Serum proteins profiles of NGT (n=20) and newly diagnosed T2DM (n=20) individuals were protein profiled using CM10 arrays and the SELDI-system. Altogether 66 peaks were detected out of which 13 showed differing serum levels between the NGT and T2DM groups. Five proteins were lowered and eight were elevated in serum from T2DM. In a second comparison,
the individuals were categorized into persons with low β-cell function with FHD and persons with high β-cell function without FHD. Three of the initially found proteins were rediscovered and interpreted to be a consequence of genetic factors. Ten proteins were not rediscovered and interpreted to be unrelated to FHD, but rather varying because of the diabetic environment. Among these proteins potentially depending on the genetic background two were identified as apolipoprotein CIII (ApoCIII) and albumin. One of the proteins more related to the diabetic environment was identified as transthyretin (TTR).

Since IGT is a state in between NGT and overt T2DM, serum levels of the 13 differing proteins were investigated in persons with IGT (n=20). For all proteins except two, mean peak intensities were intermediate to those observed in NGT and T2DM. The fact that most proteins correlated with the degree of glucose tolerance strengthens their links, genetic or environmental, to the disease.

Plasma protein profiles of T2DM individuals with low or high early insulin response (Paper IV)

Failure of the β-cell [114] is a major contributor to the low early insulin response (EIR) to a glucose challenge, which is also a characteristic and predictor of T2DM [95, 120]. The aim of paper IV was to investigate variations in plasma protein levels in persons with T2DM and differences in EIR, and compare these protein levels with the levels in NGT individuals. Therefore plasma samples of individuals with NGT with high EIR (n=10), T2DM with high EIR (n=10) and T2DM with low EIR (n=10) were profiled using SELDI-TOF MS. Since the glycemic environment in the two T2DM groups was comparable, differences in EIR might reflect variations in the genetic background. Nine proteins with different plasma concentrations were found. Most of these proteins showed different levels when comparing NGT to T2DM, irrespective of differences in EIR.

Among the differentially displayed plasma proteins, TTR and both α- and β-chains of Hb were found to be lowered in individuals with T2DM. The latter observation was confirmed by measuring plasma Hb with an ELISA, which showed higher levels in NGT compared to T2DM individuals. Apolipoprotein H (ApoH) was decreased in plasma from T2DM individuals with high EIR compared to NGT individuals. The levels of ApoH in T2DM individuals with low EIR were not reduced compared to the levels found in NGT subjects.

A longitudinal protein analysis was performed to investigate if the differently displayed proteins showed variation over time. Of the initial 30 study participants only seven were re-investigated at both ages 77 and 82 years.
None of these persons had T2DM and low EIR, which is not surprising since both morbidity and mortality are higher in this group of patients. All nine proteins found in the initial investigation, remained unchanged over time, supporting the validity of the found variations. The results also demonstrate, that the plasma samples had most likely been handled and stored with minor protein degradation.

Changes in plasma proteins in T2DM can either be regarded as reflecting the cause of the disease, an effect of the disease or both. No single protein in the present study could by itself explain the differences seen in EIR, reinforcing the polygenic nature of T2DM. The differences in plasma proteins are interpreted as probably manifestations of the disease state, rather than being causative. The conclusion is consistent with the results from paper III, where the majority of serum protein alterations between NGT and T2DM were caused by the diabetic environment, rather than genetic factors.

Role of the differently displayed serum and plasma proteins in T2DM

Albumin
In paper III, the lowered serum albumin levels in T2DM individuals were measured by two independent methods. The observation has also been made in other studies examining T2DM etiology in humans [121] and streptozotocin-treated rats [71]. Lowering of albumin is a characteristic change in inflammation [122], which is suggested to be a part of the pathogenesis of T2DM [123]. Decreased circulating levels of the protein in T2DM have also been connected with impaired kidney function [124]. Since the individuals with T2DM were all newly diagnosed and did not report any kidney disorders, it seems unlikely that the observed decrease in serum albumin reflects enhanced urinary albumin excretion. In addition, albumin was not only lower when individuals with T2DM were compared with individuals with NGT, but also when NGT individuals with and without FHD were compared. Increased urinary excretion of albumin has, nevertheless, been observed in normoalbuminuric patients with T2DM [125], which may contribute to explain our findings. When analyzing the serum spectra, not only albumin but also the albumin dimer (132 kDa) and the double-charge peak of albumin (33 kDa) were lower in T2DM, which supplies further evidence to the validity of the findings.

Apolipoprotein CIII
The 9446 Da protein was identified in paper II as ApoCIII. The apolipoprotein, which was shown in paper III to be increased in T2DM serum, is a ma-
Apolipoprotein H

ApoH (β2-glycoprotein-I) is secreted from the liver [132] and exists in plasma both as free ApoH and bound to lipoprotein particles [133]. In study IV, ApoH was decreased in plasma from T2DM individuals with high EIR compared to NGT individuals. The protein has been implicated in many physiological pathways, including lipoprotein metabolism [134] and coagulation [135]. Previously, it has been shown that plasma ApoH concentrations are increased in patients with diabetes compared to healthy individuals and strongly correlated with total plasma cholesterol [136]. On the other hand, ApoH has been shown to be decreased during an inflammatory response [137]. Markers of inflammation have also been associated with the development of T2DM [123, 138], which may explain the decreased levels in plasma from T2DM individuals found in the present investigation. However, the levels of ApoH in T2DM individuals with low EIR were not reduced compared to the levels found in NGT subjects.

Hemoglobin

Hb α- and β-chains were shown in paper IV to be lowered in plasma from T2DM individuals. The proteins were identified by aligning the peaks with the peaks of pure human Hb, using an approach described previously [98]. An increase of plasma Hb could be explained by red blood cell lysis and subsequent release of Hb. This explanation is not likely, since the blood samples were collected and plasma was prepared at different time points during a period of several months. In addition, Hb levels were consistently higher in NGT individuals also at ages 77 and 82 years, making hemolysis not a plausible explanation.

Transthyretin

TTR is mainly produced in the liver and choroid plexus, but also within the pancreatic islets [139], where it has been shown to increase the cytoplasmic Ca^{2+} concentration and promote insulin release [140]. TTR levels display low variations across healthy cohorts [52] and can therefore be considered a robust marker of disease although it is an acute phase protein. The blood profile of TTR is well-characterized, showing several peaks around 13.8-
14.1 kDa [141-143]. The peaks all represent TTR, native and with different modifications.

In this thesis, the peak at 13.9 kDa was first identified as transthyretin in paper II. The peak has also previously been identified by others [98]. In paper III the levels of TTR were found to be decreased in T2DM individuals, and the same result was found in paper IV. The finding aligned well with results reporting lowering of TTR in streptozotocin-treated rats [29]. Reduced levels of TTR have also been associated with inflammatory conditions [144] and T1DM [145]. In patients with insulin resistance and diabetes several acute phase reactants like C-reactive protein, have been shown to be increased in serum [81]. This has been interpreted to be a part of the low-grade inflammation present in these patients [123, 146], which could also explain the lowered TTR found in both paper III and IV.
Conclusions

In the present thesis the proteomic technique SELDI-TOF MS was applied and several methodological aspects of the technique were evaluated. First, a protocol for profiling and identifying pancreatic islet proteins was developed. From this work it was concluded that the anionic exchanger array and binding at pH 6 were suitable for mouse islet protein profiling. To get reproducible results of SELDI-TOF MS data, parallel processing of samples was important. Also, one oleate-regulated protein was identified as PPI-B. Secondly, a protocol was optimized for detecting and identifying differently displayed serum/plasma proteins in humans with NGT or T2DM. This protocol was then used to address specific questions regarding serum/plasma profiles in T2DM. The influence of the genetic background versus diabetic environment was determined by analyzing differentially displayed serum proteins of NGT and T2DM individuals with or without family history of diabetes. In total 13 serum proteins displayed different levels in serum from persons with NGT versus patients with T2DM. Among these proteins, apolipoprotein CIII and albumin and one yet unidentified protein could be classified as being changed because of different genetic backgrounds. On the other hand, 10 proteins for instance transthyretin differed as a result of the diabetic environment. In addition, plasma protein variations in persons with differences in β-cell function characterized by EIR were studied. In total nine proteins were found to be varying between the groups. Of these proteins five were identified, namely two forms of transthyretin, hemoglobin α-chain, hemoglobin β-chain and apolipoprotein H. No individual protein alone could explain the differences in EIR.
Future perspectives

The results from paper II, III and IV indicate that specific proteins differ in serum and plasma from T2DM individuals when compared to healthy controls. This inspired us to further investigate if the proteins could be considered to be causing β-cell defects. In paper III, ApoCIII was found to be increased in T2DM serum and dependent of genetic factors. Previously, it has been shown that ApoCIII is also increased in T1DM serum [130]. Serum from T1DM patients also increased the activity of voltage-gated Ca\textsuperscript{2+}-channels in β-cells, resulting in increased intracellular Ca\textsuperscript{2+} and cell apoptosis. These effects were inhibited by Ca\textsuperscript{2+}-channel blockers. ApoCIII was also shown to result in apoptosis. When RIN-cells were cultured together with ApoCIII-antibodies the effects of ApoCIII were inhibited [130].

Our first specific aim was to investigate if culturing INS-1E in the presence of ApoCIII increased cell apoptosis. Therefore, INS-1E cells were seeded into plates and cultured in regular culture medium for 2 days. Thereafter the cells were cultured for additional 24 h in culture medium with or without 10 μg/ml ApoCIII. After culturing, apoptosis was determined. In cells cultured in the presence of ApoCIII, apoptosis was significantly increased.

The second specific aim was to elucidate the pathway by which ApoCIII induced apoptosis. Previously it has been shown that one pathway which leads to apoptosis in INS-1E cells is the endoplasmic reticulum (ER) stress response [147-149]. However, if apolipoproteins can activate the ER-stress response and thereby cause cell death, has not been investigated. Therefore, two proteins involved in the cellular ER-stress response, namely CHOP and phosphorylated-eIF2α were measured by western blotting in INS-1E cells cultured with or without ApoCIII. Cells were lysed, vortexed for 30 min in 4ºC and centrifuged. The sample protein concentrations were measured, sample proteins were separated by SDS-PAGE and subsequently transferred to PVDF-membranes. The membranes were blocked, washed and probed with primary and secondary antibodies towards the specific proteins. Levels of CHOP and peIF2α were standardized to total protein and total eIF2α, respectively. The protein levels of CHOP and peIF2α were not different between cells cultured in the absence or presence of ApoCIII, indicating that the apoptosis was not caused by the activation of the ER-stress response.
Swedish resumé

Hos friska personer stiger blodsockerkoncentrationen efter en måltid. Det leder till att de insulinproducerande öarna i bukspottkörteln genast frisätter insulin, kroppens enda blodsockersänkande hormon. Om insulinfrisättningen av någon anledning störs, resulterar det i att blodsockerkoncentrationen förblir hög.


de beroende på arv respektive miljö bestämmas. De flesta proteinvariatio-
ernas visade sig bero på den diabetiska miljön, och inte på ärftliga faktorer.
Två proteiner som visade sig bero på ärftliga faktorer identifierades som
albumin och apolipoprotein CIII. I delarbete IV undersöcktes också protein-
skillnader i blodet mellan tre försöksgrupper med varierade funktion i de
insulinproducerande cellerna. Trots att flera proteiner visade varierande ni-
våer i blodplasma, kunde inget enskilt protein förklara skillnaden i cellfunk-
tionen.
Acknowledgements

This work was performed at the Department of Medical Cell Biology, Uppsala University, Sweden. Financial support was received from the Swedish Medical Research Council (72X-14019), the European Foundation for the Study of Diabetes, the Swedish Foundation for Strategic Research, the Swedish Diabetes Association, the Swedish Medical Association, the Swedish Foundation for International Cooperation in Research and Higher Education, the Novo Nordisk Foundation, the Swedish Society for Medical Research, Göran Gustafsson Foundation, Marcus and Amalia Wallenberg Foundation, Magnus Bergvall Foundation, Filip Lundbergs Foundation, Family Ernfors Fund, Anna-Maria Lundins stiftelse and Syskonen Svenssons Fund.

Jag vill varmt tacka:

Min handledare Peter Bergsten, som för hundra år sedan entusiastiskt introducerade mig i forskningens fantastiska värld och inspirerade mig att fortsätta.


Tidigare och nuvarande institutionsprefekterna Godfried Roomans och Arne Andersson för vårutflykter och julfester.

Leif Jansson för kritisk genomläsning av min avhandling.

Erik Gylfe för alla ominstallationer av Word.

Bo Hellman och Eva Grapengiesser för trevliga samtal om både forskning och framtid.

Övriga medarbetare på institutionen, särskilt tack till Birgitta, Karin, Göran, Marianne och Agneta.
Gamla och nya doktorandkollegor för alla pizzamöten, roliga fester och delad ångest över doktorandlivet!

Stor kram till solstrålarna i grupp Gylfe-Bergsten, som jag under åren delat pipetter, inkubator, fikalista och mjölkpaket med. **Fredrik, Henrik, Russ, Johanna, Jian-Man, Staffan, Michael, Kristina, Linnéa, Anders, Oleg, Meftun, Elaine, Sophia, Hanna, E-ri, Kristofer, Ernest, Meri, Olof, Jenny, Anne, Tian och Heléne.** Ni gör definitivt BMC mindre grått!

Alla mina världsbästa vänner från lekparken när jag var liten till lekparken nu. Särskilt stora kramar till mina catch-me-if-I-fall-människor **Katarina, Emma och Anna.**

Övriga familjemedlemmar för support.

**Anne** och **Janne,** för att ni trott på mig i alla lägen och alltid uppmuntrat och stöttat mig.

Slutligen många pussar till min egen familj med kulupulu **Henrik,** gosseparvel **Axel** och spruttibangbang **William.** Ni är och förblir viktigast.
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A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)