CHALMERS

Mass spectrometry of the subcutaneous interstitial fluid obtained by microdialysis in situ in lean man: A novel in vivo application of proteomics

Master of Science Thesis

EMANUEL FRYK

Department of Chemical and Biological Engineering
Division of Life Sciences

CHALMERS UNIVERSITY OF TECHNOLOGY
Göteborg, Sweden, 2011

Mass spectrometry of the subcutaneous interstitial fluid obtained by microdialysis in situ in lean man: A novel in vivo application of proteomics

EMANUEL FRYK

This work was performed at the Wallenberg laboratory,
at the Sahlgrenska Academy.

Supervisor: Per-Anders Jansson

Examiner: Carl Johan Franzén

Department of Chemical and Biological Engineering

CHALMERS UNIVERSITY OF TECHNOLOGY

Göteborg, Sweden 2011

Mass spectrometry of the subcutaneous interstitial fluid obtained by microdialysis in situ in lean man: A novel in vivo application of proteomics

EMANUEL FRYK

Department of Chemical and Biological Engineering
Chalmers University of Technology
SE-412 96 Göteborg
Sweden
Telephone + 46 (0)31-772 1000

ABSTRACT

Background: Obesity and the metabolic syndrome is a global problem, and it is known that the adipose tissue plays a crucial role in its development. Many methods exist to study this problem , but there is a need of a minimally invasive in vivo technique. The combination of microdialysis with Mass spectrometry could present a further insight into what mechanisms in the adipose tissue that promote development of the metabolic syndrome.

Aim: To develop and evaluate a method for studies of the adipose tissue secretome in vivo.

Method: Basic protocols for microdialysis and Mass spectrometry were adapted to one another before subcutaneous dialysates of 8 healthy subjects were run through the protocol. The proteins detected in all subjects were checked through a database for information regarding origin, function, size and if they were secreted or not. A general overview of the results was also made investigating the relation to protein concentration in the dialysate and the number of different proteins detected for each additional subject.

Results: 531 proteins were detected, of which 63 were found in all subjects. Out of these 63 proteins, 87% were identified as secreted proteins, about half of them having an inflammatory or cell damage response function whereas the other proteins were transporters, metabolic proteins or had other functions. About 50% of the proteins were described as plasma proteins, a third had an unknown origin and all the remaining but one protein had an origin from a cell type within the adipose tissue. The correlation between the protein concentration in the collected dialysate and the amount of detected proteins was low. For each subject added to the study additional proteins unique for that sample were detected. There was a low variation between subjects regarding proteinconcentration of individual proteins.

Conclusion: The method provides a unique insight to the adipose tissue secretome which previously did not exist. However, there is a large amount of cell damage and inflammation which to some extent may be caused by the inserted catheters. Therefore, when applying this method to a comparative study between two groups, inflammatory components will have to be interpreted with caution. Nevertheless, there are indications that the protocol could be further refined by simple means.

SAMMANFATTNING

Bakgrund: Övervikt och det metabola syndromet är globala problem och det är känt att fettväven spelar en betydande roll i denna utveckling. Det finns idag många metoder för att studera detta problem, men det saknas en minimalt invasiv in vivo-teknik. Kombinationen av mikrodialys med mass spektrometri skulle kunna ge nya insikter i vilka mekanismer i fettväven som kan driva fram det metabola syndromet.
Syfte: Att utveckla och utvärdera en metod för att studera fettvävens sekretom in vivo.
Metod: Grundläggande protokoll för mikrodialys och mass spektrometri anpassades mot varandra innan dialysatet från 8 friska forskningspersoner analyserades. De proteiner som återfanns hos samtliga deltagare jämfördes mot en databas avseende ursprung, funktion, storlek samt om det är ett känt sekreerat protein. En generell överblick av resultaten genomfördes också där korrelation mellan proteinkoncentration i dialysatet och antal detekterade proteiner kontrollerades.
Resultat: 531 proteiner detekterades, varav 63 återfanns i samtliga prover. Av dessa var 87 % sekreerade och hälften hade en inflammatorisk eller cellskade-signalerande funktion. Övriga proteiner fungerade som transportörer, metabola proteiner eller hade en annan funktion. Hälften av proteinerna var beskrivna som plasmaproteiner, en tredjedel hade ett okänt ursprung och övriga hade ett tidigare känt ursprung från fettvävsceller i alla fall utom ett. Korrelationen mellan proteinkoncentration i dialysatet och antalet detekterade proteiner var låg. För varje deltagare i studien som analyserades detekterades ytterligare proteiner som var unika för provet. Det var en låg spridning i proteinkoncentration mellan individer för enskillda proteiner.
Slutsats: Metoden ger en insikt i sekretomet hos fettväven in vivo. Det finns ett stort inslag av proteiner som är länkade till inflammation och cellskada vilket till viss del säkerligen orsakas av vävnadsskada i samband med provtagninen. Det finns indikatorer på att protokollet med små medel kan förbättras ytterligare. Metoden ger en tidigare ej tillgänglig insikt i fettvävens sekretom, men när man går vidare med en komparativ studie bör man vara medveten om att inflammatoriska inslag kan vara svåra att tolka då dessa kan orsakas av en vävnadskada vid kateterinsättning.

ACKNOWLEDGEMENTS

First of all, I would like to thank my supervisor Per-Anders Jansson for giving me the opportunity to work on this project, for all support, trust and responsibility I have been given throughout this journey. This is truly what makes a person develop. I would also like to thank Thomas Larsson for guidance and help in the field of Mass spectrometry, this area would still be a black hole without your help. Nor would this project have been possible without the work done by Per-Anders Jansson´s group, thank you all.

I am also very grateful to Carl Johan Fransén for taking on the responsibility to examinate this work. Last of all I would like to thank my family for giving me the time to write and completing what I once started.

CONTENTS

1 INTRODUCTION

1.1 Background

Obesity and the metabolic syndrome represent an example of a global health problem[1]. It is known that the adipose tissue has an important role in the development of the metabolic syndrome[2]. Several proteins contributing to this have been identified and different approaches have been tested to give a description of possible patophysiological mechanisms in the adipose tissue[3-5]. Proteomics enables the possibility to get a large overview of what goes on in the adipose tissue with the high precision qualitative data it provides. Much proteomic research today is performed on different cell types cultured in an artificial environment and only few methods exist to acquire in vivo data from the adipose tissue where many different cells in addition to adipocytes exist. Through microdialysis, the tissue can be studied in vivo and in situ, giving the possibility to overview the interactions between different cell types without the invasiveness of taking a biopsy. Microdialysis is however mainly used to analyse smaller molecules and the combination of microdialysis and proteomics is almost non-existing. It is hypothesized that microdialysis may be used through a proteomic approach to study the adipose tissue secretome, discover new mechanisms as to the development of the metabolic syndrome in man.

1.2 Aim

The aim for this thesis was to develop a functional method for an in vivo proteomic analysis of the adipose tissue, by linking the two independent techniques microdialysis and Mass spectrometry to one another.

1.3 Strategies

A standard protocol for protein-analysis using LC MS/MS-techique is adapted to the material acquired when performing microdialysis in the subcutaneous adipose tissue. The functional protocol for Mass spectrometry-analysis is applied to a group of eight healthy volunteers and the results are evaluated.

2.BACKGROUND THEORY

2.1 Understanding the problem

This study is performed on a healthy population. The purpose however is to continue this work with a comparison between healthy subjects and patients, either at risk, or with a developed metabolic syndrome. To understand why this work is important, it is crucial to know basic facts about the insulin resistance syndrome often called the metabolic syndrome.

2.1.1 Obesity

Obesity can be described as having such an increase in adipose tissue mass that it affects the health. It is however impossible to measure the amount of adipose mass on a living person in a precise way and therefore one is forced to use indirect methods. A minor increase in body weight can depend on numerous things, an increase of muscular mass or growth of bone. Massive weight gain is however directly related to an increase in the mass of adipose tissue. A widely used way to determine obesity is therefore the relation of body height to weight, adjusted through the formula presented below.

$$BMI = \text{weight (kg)}/\text{height}^2 \text{ (m)}$$

The value obtained is referred to as the body mass index, BMI, where a value >30 is considered as obesity. In Sweden a approximately 10% of the population is obese, an increase of 50% since the 1980´s. Military recruits with obesity have increased more than four times in the last 20 year period, showing that this problem also is affecting young people [1]. More than 40% of the adult population in Sweden have a BMI >25, classing them as overweight. It is known that 30% of all obese people will develop type 2 diabetes, one of many reasons why obesity is a problem to reckon with. [1]

2.1.2 Etiology and pathogenesis.

Obesity has the background in a lasting positive energy balance. Since the energy intake is higher than the consumption, weight gain is inevitable. One hypothesis has been tested that obesity is caused by a lower thermogenesis, a mechanism where the body generates heat of extra calories to maintain a steady weight, another hypothesis is that obese people have a more efficient energy absorption from the gut and thereby a lower energy loss through faeces and urine. There exists no

support for either of these hypotheses today.

The regulation of the appetite by circulating hormones, has been shown to be expressed differently in obese than in normal weight individuals. Many of these findings are however still only in animal models and trials to translate findings from animals to humans are yet to be successful. Looking at twin-studies one can estimate that about 50% of all obesity has a genetic component. Although this means that the genes have a causal connection to obesity, it does not mean that obesity in these individuals is inevitable. A fact which is obvious considering that it takes milleniums to change genetic conditions, while there today is an epidemic increase in obesity world wide, which has happened over decades. Epidemiological studies point to the fact that our lifestyle has changed considerably the last decades and that this has decreased our energy expenditure, while our energy intake has gone the other way and increased. [1]

2.1.3 Type 2 diabetes mellitus

As mentioned, 30 % of all obese people will develop type 2 diabetes, a disease characterized by a defect metabolic regulation. It is often described as a resistance to insulin, a hormone responsible for many metabolic effects in the body where regulation of blood glucose is the most prominent one. Known mechanisms with an effect on insulin action are physical exercise, high levels of adrenaline, glucagon, growth hormone and cortisol which are hormones secreted in stressful situations [1]. Exercise increases the sensitivity to insulin, whereas the insulin antagonistic hormones mentioned increase the resistance of insulin. What insulin resistance means is that the insulin levels required in a healthy individual are not sufficient to maintain a stable blood glucose in a diabetic patient. The resistance is most obvious in tissues such as skeletal muscles, the liver and adipose tissue, otherwise highly sensitive tissues to insulin[1]. In addition to type 2 diabetes, insulin resistance is highly relevant for the metabolic syndrome, which is a criteria based diagnose. The WHO definition requires a diagnosed type 2 diabetes, impaired glucose tolerance (IGT), impaired fasting glucose, or an insulin resistance in combination with at least two of the following criterias [6];

1. An elevated blood pressure >140/90 mmHg,
2. A dysregulation in blood lipids with serum triglycerides $\geq$ 1.7 mmol/L and HDL-Cholesterol $\leq$ 0.9 mmol/L (male) and $\leq$ 1.0 mmol/L (female),
3. Central obesity with a waist to hip ratio > 0.90 for males or > 0.85 for females, or a body mass index (BMI) above 30 kg/m^2.

4. Microalbuminuria: urinary albumin excretion ratio ≥ 20 µg/min or albumin:creatinine ratio ≥ 30 mg/g

This diagnose describes people with a wider metabolic dysregulation besides being insulin resistant, also taking other aspects into consideration besides the glucose metabolism.

2.1.4 Inflammation in the adipose tissue

When studying the adipose tissue of obese and type 2 diabetic patients, biomarkers indicating inflammation will be detected. These signs can be found although no macroscopic findings of an inflammation may be seen. This condition is therefore referred to as a ”low grade” inflammation, and is believed to be caused by a surplus of metabolites and nutrients in the tissue. A theory presented as to why this inflammation starts is that the adipose tissue is evolutionary related to other parts of the immune system such as the liver and the haematopoietic cell-lineage (which contains the white blood cells). All these tissues have the same origin. In the fruit fly (Drosophila), these tissues are all still incorporated as one organ, the fat pad. Comparing the liver and adipose tissue in humans, there are still similarities to be seen. Both the adipose tissue and the hepatic tissue are well perfused and are in close proximity to immune cells. The adipocyte also has similarities with the white blood cells regarding complement activation and production of inflammatory mediators [7]. Going deeper into the pathogenensis, it is shown that there is an enlargement of the adipocytes in obese individuals. These enlarged cells have a decreased secretion of the hormone adiponectin which has an anti-inflammatory effect and increases the insulin sensitivity. There is also an increased secretion of pro inflammatory cytokines and chemokines and an increased recruitment of macrophages to an expanded adipose tissue layer. Accordingly an adipose tissue with enlarged cells exibits insulin resistance. As insulin besides having metabolic effects also may be anti-inflammatory, a resistance to insulin combined with the other effects in the tissue lead to a vicious negative spiral. [8]

2.1.5 Inflammation in general

When cells in the body come to harm, the tissue responds to this in a highly complex way to enable healing and elimination of harmful agents in the area affected. This reaction is called inflammation. From a macroscopic perspective, it can be divided into four reactions. The tissue becomes reddish, warmer, swells up and starts to ache. The causes of this reaction are, an increased blood perfusion and also an increase in the vascular permeability in the tissue, making it possible for plasma

proteins and blood cells to leave the bloodstream and enter the tissue to take care of the damage in the area [9]. This is regulated through a long list of different signalling molecules. The mechanism for triggering inflammation can be one of several. Damaged cells from the body will start to leak if damaged, causing DNA and mitochondria to enter the extracellular space which should contain none of this. Another stimulus can be a specific surface marker from for example bacteria such as lipopolysaccaride (LPS) , a molecule highly abundant in bacteria but non-existing in humans. When such a stimulus has started an inflammatory process, local cells in the tissue such as macrophages and endothelial cells will initiate expression of specific signalling proteins secreted or presented on the cell surface which will recruit other circulating cells from the immune system circulating in the bloodstream. Other proteins from these cells called cytokines and chemokines will spread in the circulation. These molecules may affect the metabolism and body function if the stimulus is strong enough. Systemic effects are exemplified by the activation of the complement system and triggering of acute-phase proteins. They represent two groups of proteins mainly synthesized in the liver aimed for a better handling of the cell damage. The complement system is a large group of proteins which can form complexes and eliminate bacteria and infected cells. Moreover, acute phase proteins have a major role in resetting the body to an inflammatory state causing fever [9].

2.2 Understanding the cell biology in subcutaneous adipose tissue

Obesity with its complications in type 2 diabetes and the metabolic syndrome is a growing problem. The adipose tissue in obese individuals has been known to promote the development of type 2 diabetes [10]. Furthermore it is shown that there is a chronic inflammation taking place in the adipose tissue of obese and type 2 diabetes patients [7]. It is therefore crucial to find novel means to investigate the adipose tissue organ. The primary objective of this study is to detect and later characterize proteins secreted from cells in the adipose tissue.

2.2.1 Adipose tissue architecture

The adipose tissue is defined as a connective tissue with a high content of adipocytes. It is divided into brown and white adipose tissue, where the total adipose tissue mass in adult humans is constituted almost entirely of the white type. This thesis will only discuss the white adipose tissue. The adipose tissue exists either directly under the skin, there called subcutaneous fat, or as a coating around internal organs, the visceral fat. Since this project is a study of subcutaneous fat, that will also be the focus in the following sections. Current knowledge demonstrates that adipose tissue has

several functions, not only a protection of vital organs, heat insulation and energy storage, but also for hormone secretion. When studying a histological sample of fatty tissue, one will to a large extent only see tightly packed big round cells, adipocytes. It is still relevant to keep in mind that this is a connective tissue containing several other cell types such as preadipocytes, fibroblasts, macrophages and also capillaries with microvascular endothelial cells [11].

2.2.2 Endocrine function

Thus, adipose tissue has an endocrine function, and this is believed to contribute to the development of type 2 diabetes in obese individuals. Several hormones have been described as being produced in adipocytes, such as leptin, a hormone regulating hunger and satiety, angiotensinogen, regulating blood pressure and adiponectin which affects plasma levels of both glucose and lipids. Inflammatory cytokines such as TNF-α and IL-6 are also produced by the adipose tissue [6].

2.2.4 The secreted protein

Proteins destined for secretion from the cell are produced in the rough endoplasmic reticulum (rER). There are ribosomes attached to the surface that produces the proteins before they are forwarded from the rER to the Golgi apparatus. The Golgi apparatus is a sorting station where proteins are modified and directed through vesicular transport to their destinations. Besides delivering proteins out from the cell, other proteins are designated for other organelles of the cell where they are needed. The destination of a protein is decided based on a signalling peptide on the N-terminus of the protein. [12]

Cells use molecules to communicate with other cells and thereby enabling them to act together. Many molecules involved in cell communication are proteins. There are several different ways how cells can communicate between one another. There are two methods through which cells communicate which are interesting in this study. These are called endocrine and paracrine signalling. In both endocrine and paracrine signalling, molecules are secreted and cells in contact with a communicating fluid will all receive the signal regardless of the distance to the signalling cell. The difference between the two types is that paracrine communication uses the extracellular space for protein secretion, whereas endocrine signalling cross-talks through the blood. This means that paracrine signalling only will have effect in the tissue where the cell is present, while endocrine signals reach most cells of the body. [13]

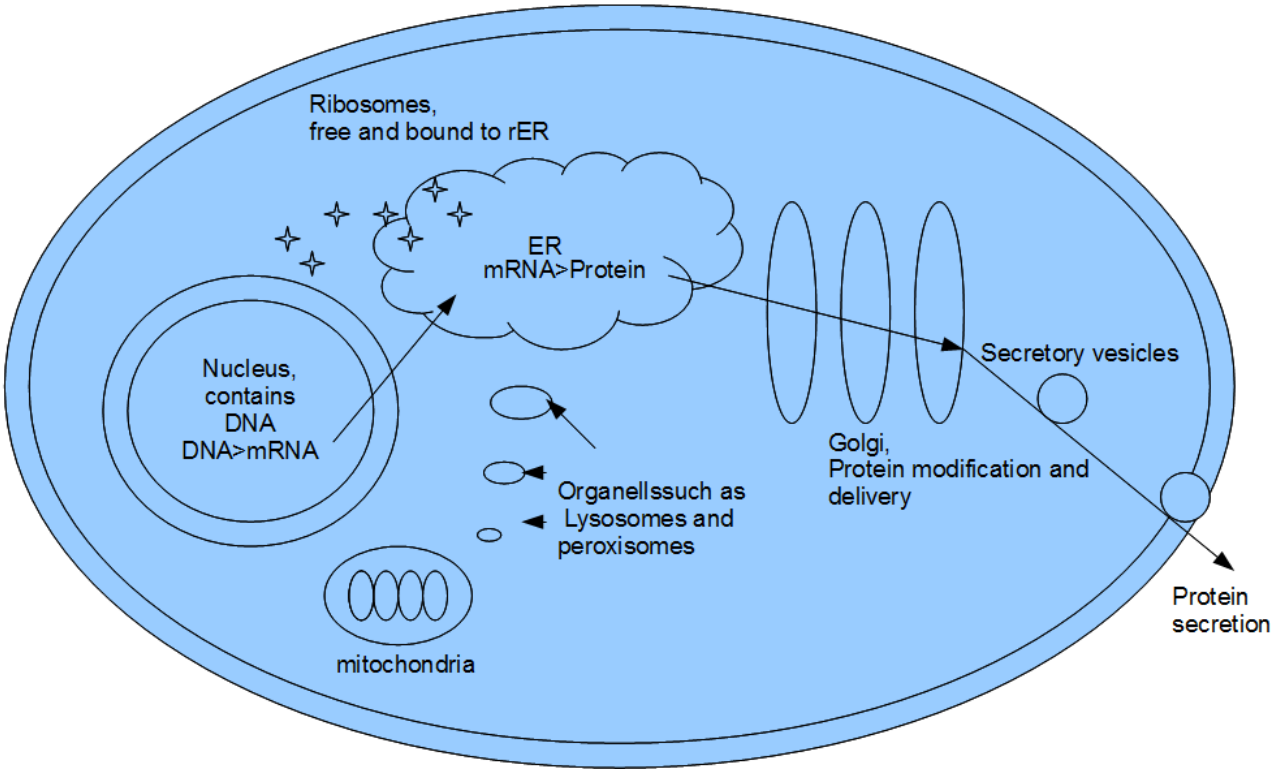


Fig 1: The anatomy of the cell and the pathway for secreted proteins, from DNA to secreted protein.

2.2.6 Protein structure

Proteins are large molecules, polymers constructed from a unique combination of 20 different amino acids in an unlimited amount. These amino acids are covalently bound through a peptide bond. Accordingly, another word for protein is polypeptide. The different amino acids have different functional sidegroups, giving each amino acid a unique chemical function. These polymers also fold in 3 dimensions, giving each protein its own functional conformation. This fold takes place during synthesis in the ribosome and is based on a wide variety of variables, the order of the amino acids, their hydrophobicity, help from other proteins called assembly factors and the combination of α-helixes and β-sheets. Proteins can also be complemented by non-amino acid components, such as metal ions and smaller organic molecules, contributing to an even greater versatility in protein function and structure. [13]

2.2.7 Protein function

Proteins have many different functions inside and outside the cell. A protein can, when described, be separated into a body, with not so important functional features, and one or many functional

sites. These sites are special epitopes on the protein surface where the side groups of the amino acids are focused out from the protein, forming a surface with a highly specialized chemical environment, combined with a complex three dimensional structure. It is such a microclimate that gives the protein its proper function. These epitopes are in textbooks often compared to keyholes, where only an equally unique molecule can bind, the key in this analogy. These molecules are called ligands. The active sites do not necessarily have to interact with a ligand. They can also bind to other proteins forming bigger structures or bind to surfaces as a cover. These functional sites make proteins capable of providing a wide variety of properties to a cell. The proteins can be structural components in the cytoskeleton, they can form enzymes, catalysing chemical reactions or act as transporters of other molecules which otherwise would not be possible to distribute. They can also act as messengers or signalling molecules allowing cells to communicate and cooperate as a system in the tissue. [13]

2.2.8 Protein regulation

Protein activity must be regulated for proper cell function. This can be accomplished by different mechanisms. The amount of protein available will of course matter for the final effect. This can be adjusted in different ways. First, the cell is made up from different compartments, removing a protein from its native compartment will deactivate it in this area. Second down regulating the protein production is another way of decreasing the activity. Yet another way of deactivating a protein is to change the phosphorylation pattern of a protein. This is done by proteins referred to as kinases and phosphatases. Usually proteins are activated by the binding of a phosphate group through kinase activity, a mechanism called phosphorylation or deactivated by de phosphorylation through phosphatase activity. The phosphorylation produces a conformational change leading to the activation of the protein. In the same way, a protein can be activated by cleaving of the protein or by binding a ligand. When a protein and a ligand bind together, a change in the conformation can occur. This change of conformation can then open up or trigger another active site, giving the protein another function after interacting with the ligand. Missfolded proteins are also effectively removed through ubiquitation. This is a complex regulatory system where ubiquitone proteins are bound to proteins which are missfolded, directing them to proteasomes in the cell where the proteins are immediately destroyed. [13]

2.3 Finding a solution

Understanding the consequences of an increased prevalence of obesity and also knowing that this partly may be caused by the adipose tissue, it is natural that the next step should be to try to characterize proteins from the adipose tissue. As cells use proteins to a large extent for communication and this cross-talk takes place either by paracrine or endocrine signalling, how can this knowledge be used to get a deeper insight to the role of the adipose tissue in the development of type 2 diabetes and the metabolic syndrome?

2.3.1 Analysing proteins in the tissue

There is strong evidence that the adipose tissue has a significant role in the development of type 2 diabetes in obese individuals, due to the secretion of certain proteins. However, the mechanisms are still not known. A proteomic approach, describing which proteins that appear in the subcutaneous interstitial fluid at certain conditions, might be able to provide new insights to this issue [10]. Hitherto, many reports approach the area from different directions. The complete proteome of the adipocyte has been described, listing a total of 3287 proteins, based on the commercial cell line 3T3-adipocytes [14]. There are also a few studies describing the secretome in the adipose tissue [15]. Human adipose tissue biopsies have been cultured and 100 secreted proteins were described [16]. Moreover animal studies revealed that 37 secreted proteins were detected from adipocytes in mice [4] and yet another 12 proteins were found in cultured 3T3-adipocytes [3]. In plasma, a comparison has been made between the protein expression in type 2 diabetes patients compared to a group of healthy subjects [5]. Importantly, there is still no comparison made between subject groups as to the adipose tissue secretome, and the knowledge of in vivo experiments is limited to that of biopsies. This is not optimal as the tissue is taken from its location in the body closing down all cross-talk to other organs and at the same time massively damaging the tissue through the use of surgical instruments.

2.3.2 Principles of microdialysis

Dialysis is ultrafiltration over a semipermeable membrane [17]. Microdialysis is the same as dialysis only scaled down. The catheters are thin tubes with a diameter of about a millimeter, which have a wall constructed of a dialysis membrane. The membranes have a specified cut off at a certain molecular size, and are commercially available for many different applications and in different sizes and models. The catheters are constructed to mimic small blood vessels. When perfusing the

catheter at a low flow rate with a saline solution the perfusate will equilibrate to the outside of the catheter, making it possible to study the chemical environment of a subject in vivo [18]. Probes used for sampling of small organic components such as glycerol have a high accuracy and can be used for direct measurements of the extracellular levels, when calibrated correctly [19]. The probes used for protein collection have a lower precision and also have some other problems associated to them. The pore sizes of the probes are not all of the same size, providing fewer pores for the larger proteins than for the small. It has also been noted that the mass transport wears off over time decreasing the yield when running for long times and reaches a low yield steady-state. Another difference using the probes for large molecular sampling is that they will not provide the real tissue concentration, they can only be used to measure differences between two groups [20].

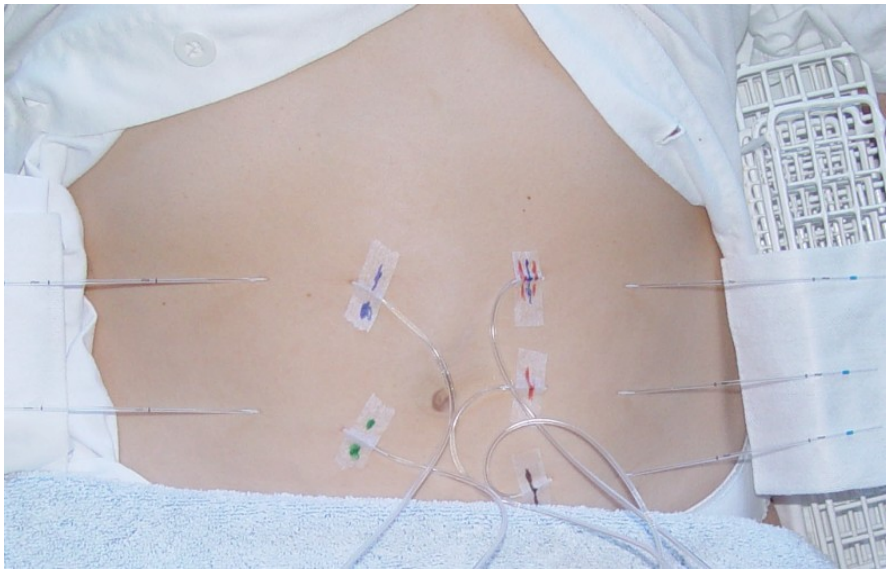


Fig 2: Microdialysis catheters inserted through the subcutaneous fat around the umbilicus of a subject.

2.3.3 Applications of microdialysis

Microdialysis is a well used technique for investigating the adipose tissue in the metabolic syndrome. The technique has been used both for studying chemokines and specific proteins such as adiponectin, often in response to insulin stimulation [21-23], but also for monitoring metabolic processes involving glucose and lactate in the adipose tissue [24]. In common for previously published studies approaching the adipose tissue with microdialysis is that they have focused on predetermined molecules. No proteomic approach has been attempted to clarify what takes place in the adipose tissue in the metabolic syndrome.

2.3.4 Pros and cons with microdialysis

The strengths of microdialysis: First, it is a quite easy technique to perform. Second, it also gives data directly from the tissue of interest, without a need to take a biopsy from the patient. To sum up, microdialysis enables an in vivo measurement of a tissue [18]. It is also a great benefit that this technique can be performed in humans, giving the data a higher significance than if taken from a mouse model or cultured cells. There are, however, also some drawbacks to this method. Because of the observation that flow rate of the microdialysis pumps for an optimal yield is low, and the fact that a patient has a limited area for insertion of subcutaneous catheters on their abdomen, it is a time consuming method to collect large quantities of extracellular fluid. One µl/minute flow per catheter compared to the seconds it takes to fill a tube with venous blood from the forearm is a real difference. The cost per sample is therefore also comparatively high considering the price for the catheters, staff and reimbursing the donor. Since the dialysate is collected over long times and the sample is kept close to the donor in a glass capillary, it is hard to prevent protein degeneration and contamination of the sample. There will also be some damage to the tissue, as the catheter is inserted through the incision of a needle. The procedure induces some leakage from cells and also triggers a cell response to the trauma.

2.3.5 Principles of Mass spectrometry of peptides

Mass spectrometry is a method used for determination of the molecular masses of a chemical compound. The principal steps of Mass spectrometry can be divided in to three parts. First, the sample is ionized, second the ions are passed through an analyzer which separates the ions according to mass to charge ratio before they reach a detector, which is the final step. [25]

2.3.6 Step 1- ionization

It was for long only possible to perform Mass spectrometry on smaller molecules with a size no larger than 500 m/z. There was no technique for ionizing larger molecules available. Today, there are several methods based on different principles, all from electrospray ionization to laser bombardment on a special surface or matrix. The method used in this paper is called electrospray ionization [25]. In electrospray ionization, the sample is passed through a high voltage needle producing charged droplets. The charged droplets are nebulized in nitrogen, which evaporates the droplets, producing ionized peptides. The benefits of this technique are that the ionization can be created at atmospheric pressure and in room temperature. It is also a highly precise method,

producing very accurate data. [25, 26]

2.3.7 Step 2 and 3- analysis and detection

After ionization the ions are separated in an analyser and detected in a detector. There are several different methods to be used for the separation. Two different analysers were used in this work, a Linear Trap Quadrupole mass analyser (LTQ) and a Fourier transform (FT) analyser. The LTQ analyser is an instrument of lower resolution, but is useful when analysing complex samples as it is swift in switching between scanning for masses and generating fragmentation spectra of detected ions. Downstream of the LTQ another analyser was used, the FT analyser. The FT analyser is of a high resolution and traps ions using a high magnetic field, detecting them through cyclotron resonance.[26]

2.3.8 LC-MS/MS

To detect proteins with high confidence, liquid chromatography-tandem Mass spectrometry (LC-MS/MS) is used. This means that a sample of proteins digested with an enzyme such as trypsin, now turned into peptides, is first separated through a liquid cromatography using a gradient of acetonitrile and water. The sample is separated by the peptides hydrophobicity and increases the number of peptides possible to detect. This is followed by analysis through tandem Mass spectrometry where the sample is first screened in a first analyser, and where highly charged ions are broken down further using a 'collision-induced dissociation' (CID), breaking the peptide bonds of a peptide. Information is generated regarding the mass of a peptide, but through CID also mass-information of all fragments of this peptide. By combining the information of the full length peptide and its fragments, an amino acid sequence can be determined. The sequences acquired are then compared to a data base for human proteins where a number of different criterias can be set. Among these are; which digestive enzyme was used, the allowable number of missed cleavage-sites and how many unique peptides that should be demanded. The harder the criteria are set the more confident will the list of proteins be but it will certainly also be shorter. [26]

2.3.9 Quantification of proteins using Mass spectrometry-emPAI

Mass spectrometry was for a very long time simply a descriptive technique. To quantify Mass spectrometry-data special techniques using labelled isotopes were a must in the experimental procedure. There was no way of taking a protein sample already available, such as human plasma

from a patient for quantification of the content using Mass spectrometry. But in 2005, two groups independently presented techniques based on similar ideas about how quantification using already acquired Mass spectrometry data could be performed. One of these methods was named PAI, protein abundance index, and was a calculation of the relative amount of a protein in a sample based on the amount of detected peptides from the protein, related to the total amount of detected peptides in the sample. This method was later adjusted using a logarithmic function giving emPAI wich has been verified as giving accurate data comparable to other protein quantification methods such as BCA protein assay (BCA stands for bicinchoninic acid) and Bradford analysis. [27]

2.3.10 Proteomics and subcutaneous microdialysis in combination

This thesis is a work investigating the compability of two methods: Microdialysis and proteomics, using Mass spectrometry. To give a perspective of the novelty of this combination, it is of interest to know that a search on Pub Med, a database for all published medical research, gave the following results when conducting a search. ”*Microdialysis”*: 13 509 hits [28], *”Microdialysis and Proteomics”*: 19 hits [15], *”Proteomics”*: 28 211 hits [29]! The 19 articles produced within this field almost exclusively deal with the central nervous system and all are either strictly reviews in the area or have a direct clinical approach. No articles have been published with an aim to evaluate the methodology as such. Therefore, it is easy to say that this is not a well investigated field, and accordingly the results when breaking this new ground will be hard to predict.

3. METHOD

3.1 optimisation of the protocol

Starting these experiments, the protocol was based on a standard protocol used for Mass spectrometry-analysis of arbitrary protein solutions in water at the Proteomics Core Facility, University of Gothenburg. This is based on a protein separation using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS_PAGE), followed by the preparation protocols described in appendix 2 and 3.

3.1.1 Acquisition of material

Subcutaneous dialysates were acquired from healthy males between 18 and 65 old, not using any medications and, in addition, all of them were non-smokers. The probe used in these experiments was a custom-made hollow fibre, the Plasmaflo OP-02 (Asahi, Japan) with a molecular cut-off at 3 MDa. The probe is made from polyethylene and has a pore size of approximately 0.3μm. After the microdialysis procedure the yield, ie the dialysate volume, of the catheters was assesed and the most functional catheters were selected. Clotted catheters have a blocked lumen which prevents a full flow through the catheter. This could have a negative effect on the membrane function by sterically blocking the pores. The osmotic balance between the inside and outside of the catheter could also be shifted secondary to changes in intraluminal pressure if the tubing was bent in situ, possibly affecting the mass transport over the membrane. For this reason only the catheters generating dialysate volumes expected from the set flow and time were selected. The collected dialysate was cleared from debris such as broken cell walls and contaminating particles by micro-filtration before it was separated into three tubes. 50 μl was taken for protein quantification, 300 μl for Mass spectrometry-analysis and the rest was frozen in -80 C for re-runs or further analyses.

3.1.2 Choice of perfusate solution

In microdialysis, a physiological saline is often used as the perfusate solution. This is complemented with the addition of a macromolecule to mimic the extracellular fluid. This macromolecule could be albumin or a carbohydrate polymer of similar size. The addition of macromolecules is used to prevent an outflow of perfusate to the extracellular room. This outflow could otherwise reduce the yield of protein in the microdialysate. Initially in this project, there had

been problems with albumin dominating the sample content, a problem commented on further in later sections. For this reason, no additional albumin was added to the solution. Test runs were performed using a carbohydrate polymer added to the solution, but this polymer could not be removed in the downstream processing making it impossible to run the samples on a gel. The polymer bound to much water and the sample volume could not be reduced to a level appropriate for SDS-PAGE analysis. Therefore, the microdialysis was performed using physiological saline as the perfusate, using no additives.

3.1.3 Removal of higly abundant plasma proteins

When running the first pilot, a large clot clouded the middle of the gel. As it is only possible to load a limited amount of protein on a gel, such a high content of a single protein would lead to very low amounts of all other proteins. The clot was assumed to be caused by albumin, judging from its location on the gel and the fact that albumin is highly abundant in human plasma. A purification cartridge for plasma was therefore purchased from Agilent biosystems (Human 14 Multiple Affinity Removal System Spin Cartridge), removing the 14 most abundant plasma proteins from the sample and thereby enabling a larger amount of all other proteins to be loaded on a gel and later analysed in Mass spectrometry.

3.1.4 Deciding amount of dialysate to be collected

When running a gel, each lane is preferably loaded with an amount of protein between 15-50 µg. Determination of protein concentration showed that dialysates contain about 1-2 µg protein/µl microdialysate of protein. Of this about 90% of the protein was removed using the cartridge from Agilent biosystems. Counting low, we therefore decided that 300 µl of dialysate volume was needed, providing a minimum of 30 µg of protein to be loaded to the gel and analysed in Mass spectrometry. An additional 50 µl was needed for BCA analysis and also extra dialysate should be collected if further analyses were needed. A catheter generates optimally 60 µl/hr, but often a lower volume than this. It was also decided that no more than 6 catheters should be placed in any one subject, 3 catheters placed on each side of the abdomen. The fact that catheters sometimes clotted and perfusion stopped, as well as the risk of perforating a small blood vessel leading to blood contamination of the dialysate also meant that there had to be room in the design for exclusion of a catheter with technical problems. Adding all these pieces of information together it was decided that 6 catheters should be run for 3 hours, and that dialysates had to be fractionated, so that the glass

capillary collecting dialysates from each catheter was changed once every hour. This gave a total dialysate volume of approximately 800 µl from each subject, making it possible to re-run an entire sample if necessary.

3.1.5 Adapting the cartridge protocol for the subcutaneous dialysate

Once solving the problem with plasma proteins and collection of dialysates, another problem appeared. The protocol for the Agilent cartridge was adapted for plasma, a solution with a considerably higher protein concentration. Also, it only allowed for 12 µl of sample to be passed through the cartridge in one run. This protocol would require almost 30 runs of the cartridge for each sample. The protocol was therefore modified, taking the low protein concentration of the dialysate into consideration, and tests were performed with 60 µl of sample in one run. This implicates that the cartridge only had to be run for 5 times for each patient. The gels that were run in these tests showed no large clots of plasma proteins, and the Mass spectrometry data were not clouded with an over representation of albumin, as demonstrated in the results section. This modification was therefore seen as successful and applied to the protocol.

3.1.6 Adapted Agilent antigen cartridge protocol

The cartridge was initially perfused with 4 ml of Buffer A at a very low flow rate. 300 µl of sample were then diluted in 1700 µl of buffer A. Out of these 2 ml, 400 µl at a time were loaded to the cartridge and spun in a table top centrifuge at 40 · g for 1-2 min until the whole volume had passed through the cartridge. This was repeated until the total volume of 2 ml had passed through the instrument. The cartridge was then reset perfusing it first with 2 ml of Buffer B, followed by 4 ml of Buffer A. The instrument was stored in 8 °C when not in use. Both Buffer A and Buffer B are solutions provided when purchasing the cartridge and have a classified content. The cartridge and the buffers are, however, developed for the use of protein analysis on a human material and should not contain any substances which could interfere with the results.

3.1.7 Desalting and concentration of the dialysates

The use of the cartridge required dilution of the sample in Buffer A, a saline solution delivered along with the cartridge. This resulted in an increase in volume from 300 µl to 2 ml. The volume possible to load on a commercial pre-cast gel is no more than 40-50 µl. To concentrate the dialysates all samples were precipitated in acetone. A benefit of using acetone precipitation to concentrate the sample is that this procedure also removes salts, of which Buffer A contained

plenty.

3.1.8 Precipitating and redissolving the sample

Acetone precipitation was performed, mixing the sample with -20 °C acetone in a relation of 1:4. The mix was placed in a -20 °C freezer for 1 h and then centrifuged for 30 min in 5100 g. The sample was then decantated and the pellet was redissolved in 10 µl loading buffer and 10 µl milliQ water on ice, and further suspended in an ultrasonic bath for 20 min.

3.1.9 Adaptations to the SDS-PAGE protocol due to remains of salt in the dialysate

In spite of the acetone precipitation, much salt yet remained in the sample loaded to the gel, resulting in strange shapes of the lanes. Therefore, the gel was always started with a slow run at 50 V for 30 min, before increasing the voltage to 200 V for 50 min. Buffer A was also precipitated alone and loaded as blanks on each side of the lane containing the dialysate, in order for the dialysate to move in a straight line.

3.1.10 Protein quantification using the BCA-kit

In order to assess the dependence of the protein-concentration for the final Mass spectrometry-results, the protein concentration was measured for the first 5 samples using a BCA-kit (Thermo Fisher Scientific, USA). The procedure followed the manual without any modifications.

3.1.11 Preparation for Mass spectrometry step 1: SDS PAGE

All the Mass spectrometry -preparation steps were performed in the clean-room at the Proteomics core facility laboratory at the University of Gothenburg.

The sample was fractionated according to size using 1-d gel-electrophoresis on a pre-cast 10% polyacrylamide gel of 1 mm thickness. For the complete protocol see Appendix 1.

3.1.12 Preparation for Mass spectrometry step 2: digestion of protein into peptides

The lane was cut out using a scalpel and divided into 15 equally sized pieces. Each piece was then cut up in to cubes measuring 1^3 mm and placed in a maximum recovery Eppendorf tube. This generated 15 tubes per sample which all were washed in 4 cycles of 30 min each, first three times in AA50 and the last time in MA50 (AA50 and MA50 are explained in Appendix 1). The samples were then dried using a speed-vac® vacuum-centrifuge. After having dried the samples 15 µl of

trypsin solution was added and the samples were incubated in 37 °C overnight. The complete protocol is shown in appendix 2.

3.1.13 Preparation for Mass spectrometry step 3: eluation of peptides from gel

Having digested the proteins into peptides, the peptides were eluted in three cycles. The buffer was then dried out using a speed-vac®, and the samples were redissolved in 100% acetonitrile. The full protocol is presented in Appendix 3.
These ready-to-analyze samples were then analysed in the mass spectrometer by the personnel at the Proteomics Core Facility and the acquired raw peptide data were transformed into protein data by Thomas Larsson at the Wallenberg laboratory through the UniProt database.

3.2 Data analysis

The data acquired consisted of 8 lists with detected proteins, one from each subject. These data were pooled for one total list of detected proteins (Appendix 5). The proteins that were found to be in common for all subjects, a total of 63 proteins, are presented as a list in Appendix 4. As one purpose of this report is to investigate the feasibility to apply this method for comparisons between groups of subjects, it is the latter list of the 63 proteins found in all subjects which is of the greatest interest. For this reason a database search was conducted only for these proteins. The database used was UniProt and for all 63 proteins four properties were investigated. These were: the protein size, the tissue specificity of the protein, whether the protein was known to be secreted and the functional classification of the protein. The method was also evaluated in the following parameters: total amount of detected proteins, amount of proteins detected in each sample and the observed variation, the dependence of protein concentration in the dialysate for the number of detected proteins, the increase in number of detected proteins for each additional subject analysed and the amount of protein in each sample that constituted the 63 proteins in common for all samples.

4. RESULTS & DISCUSSION

4.1 Optimisation of the technique - results

The modifications made in the protocol are described in detail in Methods and therefore only summarized here. The changes made to the initial protocol to be able to perform Mass spectrometry-analysis of subcutaneous microdialysate were as follows:

1. Physiological saline was used as perfusing solution, without any additives.
2. High abundant plasma proteins were removed using a cartridge from Agilent.
3. The cartridge protocol was optimised for microdialysate instead of plasma.
4. Desalting and concentration of the microdialysate samples was accomplished through the use of acetone-precipitation.
5. Each sample was sonicated for 20 min whilst redissolving, prior to being run on a SDS-PAGE.
6. All gels were started at a low voltage of 50V for 30 min before increasing the voltage to 200 V for 50 min, and blanks containing only precipitated buffer A was loaded on each side of the sample lane.

4.2 optimisation of the technique - discussion

4.2.1 Deciding on an appropriate solution as perfusate.

The selection of an appropriate perfusion solution during microdialysis is a difficult question to handle. Earlier studies have used saline with the addition of bovine serum albumin [22-24]. The addition of albumin is however not appropriate when the solution is later to be analysed in the proportion between different proteins, among them albumin. As the protein concentration is determined for all samples the addition of albumin would also interfere with this step. Moreover, because the abundance of albumin from the donor itself caused problems in the analysis, it was believed to be counter-productive to add even more albumin to the perfusion liquid. On the other hand, as albumine is effectively removed in the new protocol, a small addition of albumine might be possible to use in order to improve the final results. Ideally the perfusate would be a fluid with the same properties as the extracellular fluid, which is being studied, but without containing any proteins that could interfere with the Mass spectrometry-analysis. The initial trials were therefore performed with Voluven®, an aqueous solution constituted of large carbohydrate polymers also used as a substitute for human plasma in hospitals. This solution, however, caused great problems,

binding large amounts of water, which made it impossible to reduce the volume to a size possible to load on a gel. It was therefore decided to settle for a basic saline solution without additives, and this performed well after desalting the sample. A concern with this solution is that, since there are no macromolecules in the sample, a mass transport of perfusate over the membrane may therefore take place, causing less proteins to be extracted from the subcutaneous extracellular fluid to the perfusion solution. This should however be a systematic problem equal for all dialysates analysed.

4.2.2 Removal of abundant plasma proteins and adaptation of cartridge protocol.
The cartridge functioned well after adjusting the protocol for subcutaneous dialysates and as can be demonstrated in the data the removed proteins were no longer in majority in the Mass spectrometry-data. Although the cartridge is specific in the removal of the 14 plasma proteins there are most likely losses of other proteins to the cartridge. This could happen both through the unspecific binding of proteins to the inside of the cartridge wall and to the antibodies of the cartridge, but also as smaller proteins can use larger proteins as transporters. If the larger transport-proteins, such as albumin, are removed the smaller proteins will also be lost. Knowing that the cartridge is used in the same manner in all samples, this should mainly render a systematic error and only affect how many different proteins are detected, not the proportion between the proteins when comparing the dialysates. Of course, some of these smaller proteins could be present in a level close to what is possible to detect in this analysis. These proteins could then be detected in certain samples and not in others, affecting the proportion between proteins in the samples. But if they are close to the detection limit, these proteins will be of such a low content that their effect on the relative masses of the other proteins also should be insignificant.

4.2.3 Desalting and concentrating the samples using acetone-precipitation.
Acetone precipitation was the method selected for concentrating and desalting the samples, being a well used method with good experiences in the lab. Other methods considered were to dry the sample in a vacuum centrifuge [3], or to use a C-18 cartridge, which binds proteins reversibly allowing for a change of solute [30]. The use of a vacuum centrifuge was not an option as it only concentrates the sample and all salts remain but in greater concentrations. The reason for proceeding with acetone precipitation instead of a C-18 cartridge was because although the protein binding is reversible in a C-18 cartridge, there may be losses of protein to the cartridge. The findings made by Bernay et al [30], where such a cartridge had been used were also somewhat

limited compared to the total list of proteins described in this work. Although this work looks very similar in methodology but applied to a completely different tissue, the results may differ for many other reasons. Still, it was the fear of further protein losses led to the decision to work with acetone precipitation. In hindsight, redissolving the precipitated pellet probably was one of the most uncertain steps in this work, which might have affected the results. Sonicating the sample for 20 min when redissolving the sample for SDS-PAGE is not believed to have any negative effects on the final resluts. However, the addition of the sonication did not completely eliminate all pellets so there are probably still significant losses of proteins caused by adopting acetone precipitation. The pellet produced from precipitation was sometimes hard to redissolve and at times a small unresdissolvable pellet had to be left when transferring the samples to the SDS-PAGE. A possible way forward could be to use a C-8 cartridge, which is works in the same way as a C-18 cartridge, only it is shorter and thereby the losses to the cartridge wall could be somewhat reduced.

4.2.4 Starting the gel at a low voltage to remove salt.

Starting the gel on a low voltage is a method used for running out salt from a sample. This along with loading precipitated Buffer A in the adjacent lanes resulted in very neat lanes. No problems are believed to come from these techniques.

4.3 Quantitative data from the experiments

4.3.1 Total number of different proteins detected - results

The total list of detected proteins was interesting to know, because this could constitute a reference database in upcoming experiments. These proteins could be described as probable to have either an origin, or at the least a function in the adipose tissue. Data were pooled from all dialysates demonstrating a total of 531 detected proteins. Of these, 63 proteins were identified in all samples. The complete list of all detected proteins is presented in appendix 4, and the 63 proteins in common for all samples are presented in Appendix 3.

4.3.2 Proteins detected in any one sample -results

A key object of this study was to evaluate the method, since this approach to the adipose tissue was novel. It was important to study the reproducibility of the method and the number of proteins found in any one sample was therefore of interest. When studying this, a span between 123 and 267 proteins was found, presented in table 1. The average number of proteins detected in any one

sample was 193 proteins, with a standard deviation of 59.

Table 1: The number of detected proteins for all samples, also the average and standard deviation in the group.

1	2	3	4	5	6	7	8	Average	Standarddeviation
219	217	144	161	139	267	274	123	193	59,2

4.3.3 The number of different proteins detected - discussion

In total, 531 different proteins were detected. Previously published work around adipose tissue secretome have presented less than or around 100 proteins [3, 4, 16]. These 531 proteins are therefore an unlikely good result. When comparing the 8 subjects, 63 proteins appear in all samples. This number is more in line with previously published work and, moreover, also has a strong support as it is based on several independent observations in the present study. When inserting a microdialysis catheter through the skin and further through the subcutaneous tissue a substantial number of cells will be damaged. For this reason a 30 min washout period was introduced where the dialysate is discarded prior to the sharp dialysate collection. Contaminations from broken cells and from the skin might still appear in any one sample. These unrepresentative proteins may vary, why the list of proteins observed when all detected proteins are added is much longer. Thus, many of the 531 proteins are likely to be contaminations.

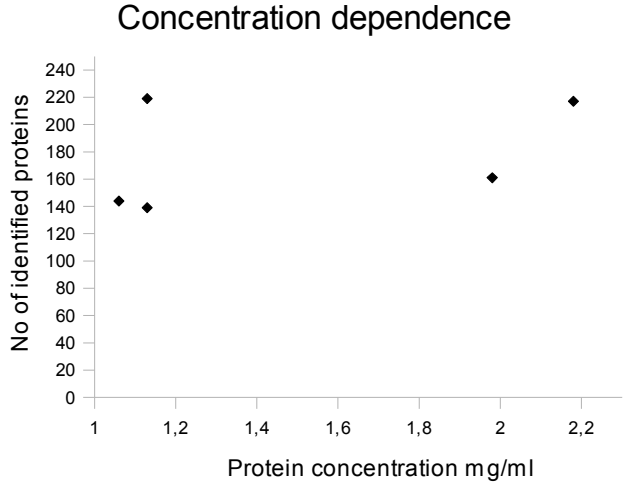
There was also a big difference in the number of proteins that were detected in each sample. The range between 123 and 274 proteins in the dialysates could probably to a large extent be caused by the processing of the samples, from dialysate collection to Mass spectrometry-analysis. Further improvements to the protocol, such as the use of a C-8 cartridge, might even out this number, bringing the mean yield above 200 proteins and reducing the standard deviation below 59 which is considered high. New proteins that could be added to the present list of the 63 in common proteins, will most likely be found in the complete list of the 531 detected proteins.

4.3.4 Protein dependence of the protocol - results

Throughout this project there were many obstacles in adapting protocols to the subcutaneous dialysates. There were quite a few processing steps between the microdialysate being collected and looking at the final Mass spectrometry data. In any and all steps, one must account for certain losses of protein. A measurement which could be performed early in the processing was protein quantification using a BCA-kit. It was interesting to know if the initial protein concentrations had a

major effect on the acquired Mass spectrometry-data. This would have been an indication as to how robust the protocol was. A high correlation between protein concentration and the amount of peptides found should support an idea that the protocol has a minimal impact on the final result of the Mass spectrometry-data. This theory being based on the fact that a low protien content would mean that many low abundant proteins would be present in the samples in such a low amount that they would fall below the detection limit.
There was also another reason why protein quantification could be of interest. The dialysate collected was initially fractionated both depending on which catheter it was taken from and also on time. For this reason if BCA-quantification could give a hint on the quality of the final Mass spectrometry-data, BCA-analysis could be of help in selecting which catheters the dialysates should be collected from to give as strong data as possible.

However, no obvious correlation could be identified between the protein concentration and the number of detected proteins in these experiments. Both samples of higher and lower protein concentrations varied between approximately 150 proteins and above 200 proteins detected, which is also illustrated in Figure 3



.

Fig 3. The number of identified proteins is plotted against the protein concentration for the first five subjects.

4.3.5 Protein dependence of the protocol & proteins detected in any one sample - discussion

As previously mentioned there is a problem in the fact that the number of detected proteins in the sample with most detected proteins is more than twice the size of the sample with the shortest detection list. What is most disturbing is that there is no good explanation as to why this difference exists. The sample with 123 detected proteins, sample 8, was completely re-run once, yet with an equally poor outcome. The colour intensity and the number of bands in the lanes of a gel are indicators of how much and how many different proteins should be expected from the Mass spectrometry-analysis. Sample 8 had a very colourful lane with many bands, which indicated that this should be a promising dialysate. For this reason, one can suspect that there is a step downstream from the SDS-PAGE that causes problems. A putative step which may exemplify this, is when treating the samples with trypsin. If the amount of liquid is to small for the trypsin to reach all of the dried gel pieces, or if the gel pieces do not get soaked through, so that they are too dry, the trypsin will not function properly. The trypsin can be diluted in 50 mM ammonium bicarbonate if the total volume of gel pieces is large, in order to guarantee a well soaked sample. If this indeed is a problem the volume of 15 µl should be increased. Since sample 8 had to be re-run, there was not enough sample left to perform a protein quantification with a BCA-kit. Therefore the precise protein concentration for this sample can not be known nor can its importance be discussed.
Another contributor to the low number of detected proteins in sample 8 is the fact that the performance of one of the Mass spectrometry-analysers has been reduced. It has been determined that this is the case, but it is not known in what magnitude this error lies.
Whether the protein concentration was the main contributor to the spread of the results or not can still be discussed on a general level, as the first 5 samples were analysed using the BCA-kit. An increase in number of detected proteins can be seen correlating to the protein concentrations, but there is a major spread in the data and the increase is small both compared to differences in protein concentrations and the difference in number of proteins detected. Collectively, this supports the idea that there is one or several steps in the dialysate processing that contribute to the variation in the number of detected proteins of the samples.

4.3.6 Effect of the number of subjects on the total number of detected proteins - results

In order to acquire as large a database as possible it was interesting to see if the 8 subjects were sufficient to present the full picture of the secretome or if an increase in the number of participating subjects would give a more complete picture of the result. As can be seen in the Figure 4, depicting

the cumulative result of proteins found for each extra subject added to the study, there was an increase in proteins identified for each subject added to the experiment. Although only a small increase was seen when adding the results from the last subject.

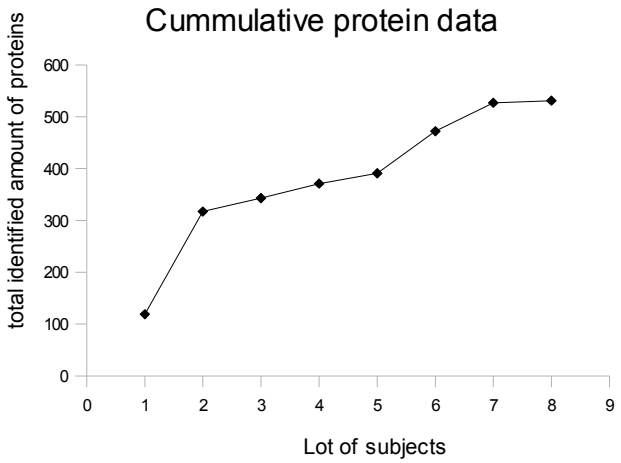


Fig 4. The graph shows how the list of total number of detected proteins increases for each subject added.

4.3.7 Effect of the number of subjects on the total number of detected proteins - discussion

Figure 4 suggests that more secreted proteins may indeed be found if the amount of subjects were to be increased. One could be led to believe that the number of proteins detected are stabilising around a value of 550 proteins when studying the increases in how many new proteins are found for each additional subject analysed. But one should keep in mind that the curve in Figure 4 only describes part of the data it is based on. The last sample analysed in this study, sample 8, had the least number of detected proteins of all subjects. Should the position of sample 8 been shifted with any other sample, the change in number of detected proteins probably would show a greater increase, and one would not be led to believe that a maximum value for how many different proteins could be detected was reached. Most likely, additional proteins would be detected if further subjects were to be included in this study.

4.3.8 Percentage of total sample protein mass constituting the 63 common proteins - results

Figure 5 demonstrates how much of the total protein mass in all samples that was constituted by the 63 proteins in common for all subjects. There was quite a small difference between the samples, the results ranging from 61-85% of the total number of proteins. An interesting observation was that the dialysates with the highest percentage, sample 3, 4, 5 and 8 were obtained from subjects with the

lowest count of detected proteins. On the other hand, samples with the highest count, sample 6 and 7 were among the samples with the lowest mass-percentage of the common proteins.

4.3.9 Percentage of total sample protein amount constituting the 63 common proteins - discussion

The observation that the samples with the largest number of detected proteins had a lower fraction of the 63 in common proteins, compared to the samples with fewer detected proteins could be expected. It could be explained through an idea that the mass fraction of the in common proteins is actually about the same in all samples, approximately 60 %, assuming that all subjects have a similar protein expression in the extracellular space and that all proteins act in the same way regarding mass transport over the membrane. Why the fraction of the 63 in common proteins later increase to over 80 % during processing in the samples with the lowest number of detected proteins, could be due to losses of low-abundant proteins during the way. These losses may or may not be avoidable, but simply by being aware that this is the case could contribute to a better handling of the quantitative data. These data also show that even if the method very well detects 63 common proteins, there is a small mass in the samples which is constituted by a very large number of proteins which are lost in some samples. As the mass fraction of the 63 common proteins increases from one sample to another, from around 60 percent to above 80 percent, over one hundred proteins also fall below the detection level.

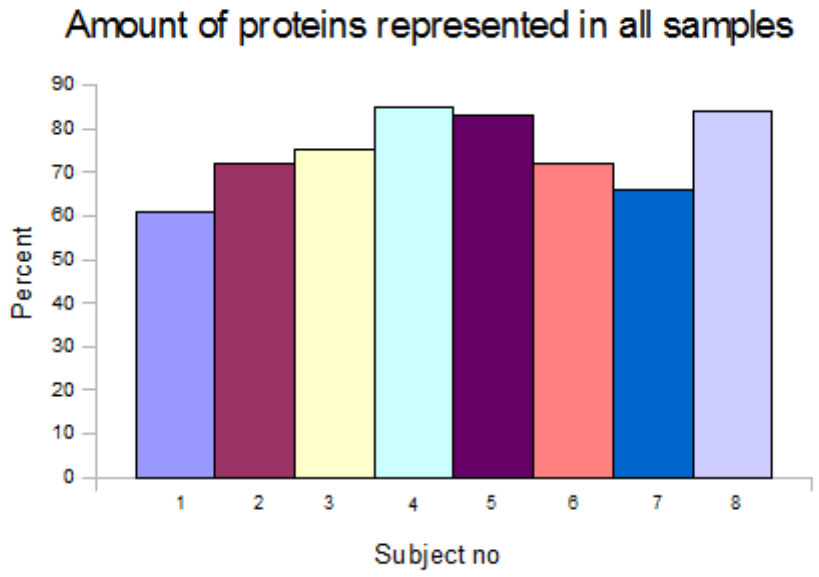


Fig 5. The percentage of the total sample protein mass which was constituted by the 63 proteins found in all samples, presented for each subject.

4.4 Qualitative data of protein function

4.4.1 Amount of secreted proteins - results

To study the adipose tissue in vivo it is of essence to be minimally invasive, this meaning that no cells should be harmed. For this reason all proteins detected should be secreted. However, there are reasons why such a result can not be expected. In this study 87% of the proteins in common for all samples were secreted.

4.4.2 Amount of secreted proteins - discussion

To find that 87 % of the 63 proteins were found to be secreted is a very good result, as many different reasons may contribute to a much lower number. First of all, proteins can be secreted in two different ways. The main pathway is through the Golgi network which is regulated through a signalling sequence. It is almost exclusively proteins secreted in this fashion that will be presented as secreted when searching in a database. However, there are non-classical ways for proteins to be secreted. The mechanism is not known, but what is known is that certain proteins are definitely secreted, but when analysed lack a signalling sequence. It could be that some of the proteins detected in this way actually are secreted, but are done so in a non-classical way which is not known at present. Another reason why the percentage could have been much lower is due to the fact that needles are inserted into the tissue and some cells must be damaged as a consequence of this. High abundant cytosolic proteins should therefore appear in the sample. To avoid contamination of punctured cells, all catheters were perfused for 30 min at a flow rate of 1 µl/min prior to the sampling period and the dialysate is discarded. Judging from the data, this was a sufficient time as almost all 63 proteins are classified as secreted.

4.4.3 Protein function - results

There is a theoretical risk adopting subcutaneous microdialysis that this procedure may induce a significant cell damage following the insertion of the catheters and that this in turn may trigger an inflammatory process. Because a comparative study is planned with an aim to investigate the metabolic syndrome, based on this method, it is essential to know that relevant proteins of potential interest are found. The data presented in figure 6 is also available in Appendix 4, but for a clearer presentation some functional groups have been put together. What can be noted is that cell-damage, inflammation, acute phase proteins, complement system and coagulation proteins constituted more than half of the proteins in common for all samples. Besides from these, proteins with a metabolic

effect constituted 1/10 and transport proteins and the highly versatile group 'other' constituted 1/6 each of the detected proteins. It is in these three last groups that interesting proteins for the metabolic syndrome may be found, without the concern that it is an artificially induced protein which is detected.

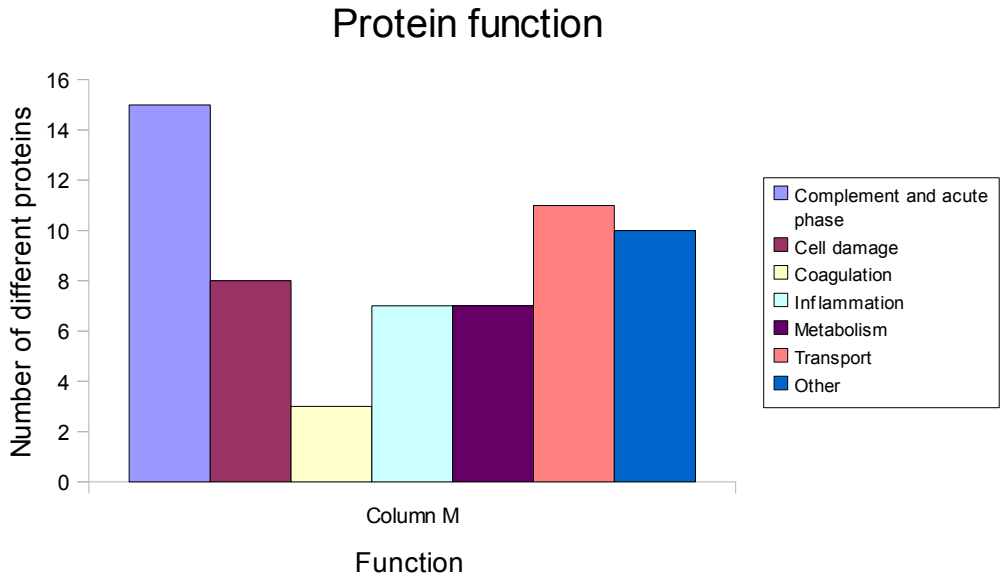


Fig 6. The 63 proteins detected in all samples are here categorized after the functionality of each protein.

4.4.4 Protein function - discussion

It is rather disappointing to find that more than half of the 63 proteins are indeed signs of cell damage, inflammation, coagulation, acute phase proteins or complement system proteins. As previous data show close to all of these proteins are secreted. So it is not a question of leakage from the cells, but these proteins are commonly secreted when something is damaged or out of order in the tissue. It is unavoidable not to cause any cell-damage when inserting foreign material into the body, and the fact that the catheters are inserted for over 3 hours also contributes to the high representation of these proteins. However, some of these proteins are most likely present in the tissue in a normal state as well, but their presence will be more dominant during tissue stress or cell damage. The difficult part is to know which of them and to what extent represent a normal state and which proteins represent cell-damage and stress. Inflammation is a central part when studying the metabolic syndrome. How then should one view these results in relation to the applicability of the method for studying the metabolic syndrome? First of all, it is in a way a good thing that so many inflammatory proteins can be detected. There is a risk that proteins not found in the dialysate can

not be detected using this method. From that point of view, microdialysis combined with proteomics could be an excellent method for analysing the inflammatory response in the adipose tissue. The proteins detected in this healthy population would then constitute a reference for the comparison to another group. On the flip side of the coin is the fact that it is not the inflammatory response of a needle insicion that is of interest when studying inflammation in the adipose tissue. Any result taken from such a study would have to find a way to meet this critique.
The other half of the proteins are more uncomplicated to analyse. These have a wide variety of functions including metabolic proteins and different transporters. Any of these could prove to have a different expression in a group with an affected metabolism, which may open the door for completely new insights into the metabolic syndrome.

4.4.5 Tissue origin - results

The origin of the proteins is of importance as it is the aim of this study to explore proteins in the adipose tissue. As mentioned in the background, the adipose tissue is constituted of adipocytes, preadipocytes, fibroblasts, endothelial cells/blood vessels and macrophages. The absolute majority of the proteins described originated from the plasma, followed by many proteins of unknown tissue origin. Besides from these findings there were seven proteins where the origin was from any of the cells previously mentioned as belonging to the adipose tissue, or from the extracellular space. There was also one outlier which was a protein with an origin previously described only from white blood cells, B-cells to be more specific. B-cells are not typically involved in the adipose tissue of healthy subjects.

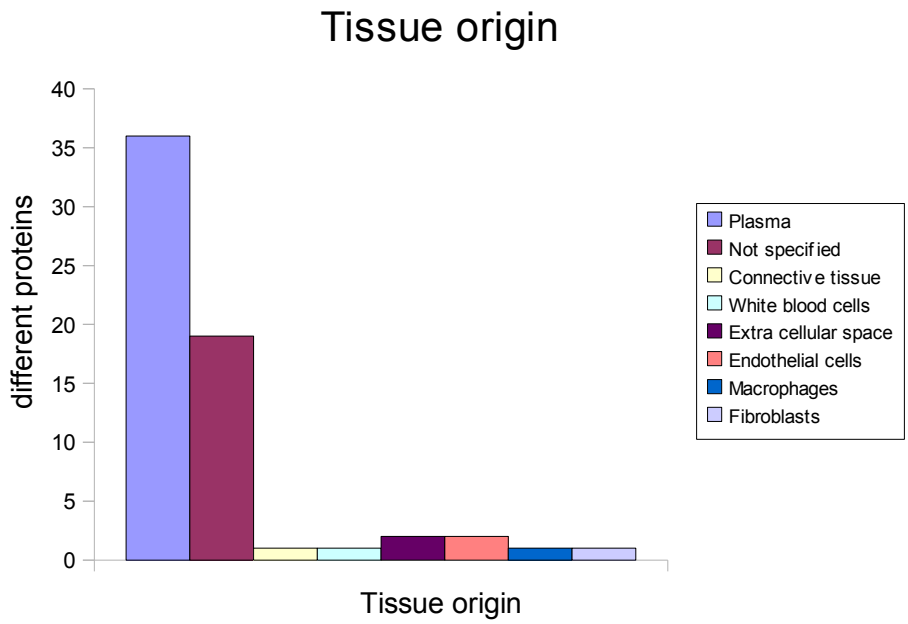


Fig 7. This figure illustrates the originating tissue for the 63 proteins detected in all subjects.

4.4.6 Tissue origin - discussion

Of 63 proteins, 62 were previously described as having an origin either from the adipose tissue, plasma or having an unspecified origin when searching the database Uniprot. The last one, Lymphoid-restricted membrane protein, is a protein that is involved in vesicle fusion in B- and T-cells. Why this intracellular protein appears in the samples of all 8 healthy subjects is a mystery that is hard to explain. B- and T-cells should not be present in the adipose tissue, and even if this was the case, other intracellular proteins from these cells should also be detected. It can be that this is an artefact of the data processing from the Mass spectrometry-spectrum. It could also be that this protein also is present in other cells in the adipose tissue that have leaked but that this is previously unknown.

It is very comforting to see that all other proteins are either previously known to belong to the adipose tissue, the plasma or have an undescribed origin. The high number of plasma proteins is no problem as most secreted proteins will end up in the plasma after being secreted. It would be interesting to proceed with these proteins, comparing the protein levels detected here with the levels detectable in plasma from another location such as the forearm using microdialysis. Should the protein levels detected in the adipose tissue be higher compared with plasma, that would strengthen the hypothesis that these proteins have a significant role in this tissue.

What also can be seen from these results is a strength of these novel techniques. All the major cell

types of the adipose tissue have made, if only one or two, contributions to the secretome. This shows that it is not sufficient to only study individual cell lines in vitro, as it is the interaction between the cells which completes the picture.

4.4.7 Protein size - results

Proteins were detected over a wide size range, the smallest with a size of below 100 amino acids and the largest being overe 2000 amino acids long. However, the absolute majority of the detected proteins were between 200 and 600 amino acids long. The size distribution of the 63 proteins is presented in Figure 8.

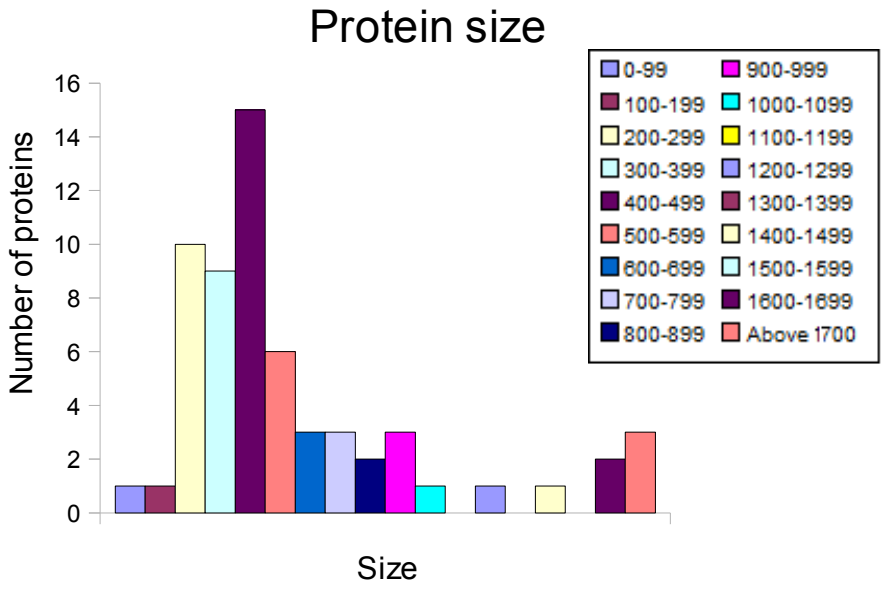


Fig 8. The size distribution for the 63 proteins found in all subjects shows a peak close to proteins of 400-499 amino acids length.

4.4.8 Protein size - discussion

The results show that proteins of all sizes can be detected using the combination of microdialysis and Mass spectrometry, from less than 100 amino acids to over 1700 amino acids. However it is for the protein sizes ranging from 200-600 amino acids that most proteins are detected. Fourteen highly abundant proteins have been removed from the sample using the Agilent cartridge. These proteins had an even size distribution ranging from around 200-1600 amino acids [31]. It is therefore assumed that this step in the processing does not affect the results significantly. However, the use of

the cartridge could still affect the results in another way. Small proteins sometimes use larger proteins such as albumin as a transporter in the blood. This means that the removal of albumin may also remove small proteins from the sample. For this reason, it could be that some small proteins are lost during processing due to the use of the cartridge.

4.4.9 Variance between samples - results

Using emPAI, quantitative information can be acquired using Mass spectrometry, giving a mass percentage estimate for each protein in a sample based on the Mass spectrum information. For a comparative study to be conducted, these data need to be stable within the group allowing a significant change between populations to be detected. Calculating an approximate 95% confidence interval for a normally distributed sample of sufficient size is done by taking the mean for the sample group and subtracting two times the standard deviation for the lower boundary, and adding two times the standard deviation for the higher boundary. Presenting this data for all 63 proteins is not relevant but for a future comparative study it is of relevance to get an estimate how large change in protein concentration that would be needed to detect a significant difference. The lower limit for such a confidence interval would for almost all proteins be estimated close to 0, knowing that mean for all 63 proteins being 0.05 mass%, where many proteins also have a negative value. The upper limit of the confidence interval is, presented as a mean for all 63 proteins, 1.97 times the mean for any protein. This means that a change around a 2-fold increase is needed comparing data with another group, to end up beyond the 95 percentile.

4.4.10 Variance between samples - discussion

This calculation is rough, if not only by assuming that the sample is normally distributed. With such a low n-value, a t-test should be used and this would require larger differences between the groups. But it is for an upcoming study good to have an idea in what magnitude the change will have to lie in order to get a significant result. A change in protein expression of 2-3-fold is not much, as many proteins are regulated by signalling cascades increasing the expression many times over. In agreement, if there were to exist any real difference between two groups, there should not have to be any fear that this would be missed due to shortcomings of this method.

4.4.11 Positive controls - Results

The complete protein list was searched for a number of previously known proteins [2, 32]

originating from the adipose tissue and that have an already described effect in the metabolic syndrome. Plasminogen activator inhibitor-1 (PAI-1), visfatin, omentin, leptin, resistin, TNF-α, interleukin 6 (IL 6), interleukin 8 (IL 8), fibrinogen, C-Reactive Protein (CRP), Complement C3 were not detected in any of the samples. However, adiponectin, complement factor B and D as well as angiotensinogen were detected. The number of samples in which they were found is presented in table 2.

Table 2: The following controls were detected in the accounted number of samples.

Protein	No of samples
Adiponectin	1
Complement factor B	8
Complement factor D	6
Angiotensinogen	8

4.4.12 Positive controls - Discussion

There are known explanations for some of the proteins which were not found. Two of the proteins which were not detected, fibrinogen and complement C3 are among the high abundant plasma proteins which are cleared away using the Agilent cartridge. These two proteins were therefore not expected to be identified, and the fact that they are not there should only be interpreted as a control for the functioning of the Agilent cartridge.

Visfatin is a quite new discovery and it is not certain if it is actually a secreted protein, or if it is only detected in plasma due to cell damage. Omentin is known to be secreted from visceral fat, ie. the intra abdominal fat, and not from subcutaneous fat which is the tissue of focus in this project. Resistin is still controversial as to its role in humans and TNF is mainly found in obese and type 2 diabetes patients [32].

Adiponectin has previously been studied using the same method for acquiring the dialysate but with a different detection method [22]. The fact that adiponectin is found in only one sample should therefore point to the fact that certain proteins will be missed using this technique. That could also be the case for the remaining proteins which were not found. They might still be in the sample, but are somehow lost in the processing. What should be noted is that all missing proteins are shorter than 200 amino acids long, a fact which supports the idea that there is a methodological problem which causes these losses [31]. A possible explanation to what this might be is an unspecific binding of these proteins to the membrane of the dialysis catheters, as polyethylene is a material well known to be coated with proteins shortly after insertion into the body [33]. The catheters used are as mentioned custom-made and there is a potential for further improvement in this area. Today

no alternative materials or surface treatments are commercially available, although it is probably only a matter of time before such alternatives will be in common use. Another explanation to the losses of smaller proteins could be that these proteins are lost when removing other plasma-proteins through the Agilent cartridge. What is encouraging is that the other three proteins detected were done so in all or almost all samples. This gives a good comfort for reproducibility and they could be counted in as proteins which an upcoming comparison between groups could study.

5. CONCLUSION

5.1 THE METHOD

Considerable variations between samples in how many proteins were detected, and the low correlation between the number of detected proteins and the initial protein concentration indicate that there are improvements still to be made in the processing of the samples. Two things should be further tested before a comparative study is initiated. First, an evaluation of the benefits in using a commercially available C-8 cartridge for concentrating and desalting the sample after the removal of high abundant plasma proteins. Second, the volume of trypsin should be increased to 30 µl by diluting the trypsin-mother solution with 50 mM ammonium bicarbonate. Both these changes are believed to have a potential of increasing the reproducibility of the samples.

5.2 EVALUATION OF THE RESULTS

The results from this study show that the method is indeed functional to investigate the secretome of the adipose tissue in vivo. Almost all proteins were secreted, which shows that the leakage from damaged cells was minor, although the method detects many proteins signalling for cell-damage and inflammation, these were also secreted proteins to most part. Many celltypes in the adipose tissue are represented and proteins are detected over a large size span. However, there are indications that smaller proteins are lost in processing. The data also indicate that the variation between subjects, studying the proteins found in all samples, is moderate. Initiating a comparative study, cell-damage and inflammatory proteins would have to act as a baseline in any comparison and as the inflammatory component is so high in the study of healthy subjects.

6. FUTURE WORK

6.1 Further experiments

There are two further experiments which should be made to improve the protocol prior to proceeding with a comparative study. The purchase of a C-8 cartridge and comparison of this method of desalting and concentrating proteins with acetone precipitation should be done. This may be arranged on a relative small group of 2-3 subjects where dialysates enough for two complete runs through the protocol should be secured. All collected material should be pooled introducing a minimum of confounders. In addition, an increase in trypsin may also be tested by performing re-runs of subjects who have delivered a poor yield of proteins.

6.2 Estimation of in-subject variation

It would also be interesting to see the in-subject variation for the present data. This should preferably also be done before the comparative study, so that this variation could be taken in to consideration prior to a decision of the sizes of the groups studied.

6.3 Comparative study

The application of the protocol provided in this report on a comparative study between healthy subjects and a group of type 2 diabetics should be initiated as soon as the minor adaptations of the protocol mentioned above have been evalutated. Furthermore, to optimize the design of such a study, modified microdialysis catheters might also be placed in a forearm vein on each subject to provide more accurate information about the tissue specificity of the protein data obtained from subcutaneous adipose tissue. Moreover, considerations will have to be made regarding the size of the groups compared. In order to get significant results they should at least be of the same size or larger than the group analysed in this study.

REFERENCES

1. Berglund G, Engström-Laurent A, Lindgren S, Lindholm N (Edit.), Internmedicin, 4th ed, Liber AB, Stockholm, 2006
2. Summers LK., Adipose tissue metabolism, diabetes and vascular disease-lessons from in vivo studies., Diab Vasc Dis Res. 2006 May;3(1):12-21.
3. Kratchmarova I, Kalume DE, Blagoev B, Scherer PE, Podtelejnikov AV, Molina H, Bickel PE, Andersen JS, Fernandez MM, Bunkenborg J, Roepstorff P, Kristiansen K, Lodish HF, Mann M, Pandey A., A proteomic approach for identification of secreted proteins during the differentiation of 3T3-L1 preadipocytes to adipocytes., Mol Cell Proteomics. 2002 Mar;1(3):213-22
4. Chen X, Cushman SW, Pannell LK, Hess S., Quantitative proteomic analysis of the secretory proteins from rat adipose cells using a 2D liquid chromatography-MS/MS approach., J Proteome Res. 2005 Mar-Apr;4 (2): 570-7
5. Li RX, Chen HB, Tu K, Zhao SL, Zhou H, Li SJ, Dai J, Li QR, Nie S, Li YX, Jia WP, Zeng R, Wu JR., Localized-statistical quantification of human serum proteome associated with type 2 diabetes., PLoS One. 2008 Sep 16;3(9):e3224
6. Alberti KG, Zimmet PZ, Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation., Diabet Med. 1998 Jul;15(7):539-53
7. Hotamisligil G. S., Inflammation and metabolic disorders., Nature. 2006 Dec; 444, 860-867
8. Gustafson B, Hammarstedt A, Andersson CX, Smith U, Inflamed Adipose Tissue: A Culprit Underlying the Metabolic Syndrome and Atherosclerosis, Arterioscler Thromb Vasc Biol 2007;27;2276-2283; originally published online Sep 6
9. Mölne J, Wold A., Inflammation, 1st ed, Liber AB, Stockholm, 2007
10. Scott EM, Carter AM, Findlay JB. M. Et al., The application of proteomics to diabetes., Diab Vasc Dis Res. 2005 May; 2(2): 54-60
11. Ross H. R., Pawlina W., Histology A Text and Atlas With correlated cell and molecular biology, 5th ed., Lippincott Williams & Wilkins, Baltimore, 2006
12. Mathews CK, van Holde KE, Ahern KG, Biochemistry, 3rded, Addison Wesley Longman, Inc., San Francisco, 2000.

13. Alberts B, Jhonson A, Lewis J, Raff M, Roberts K, Walter P, Molecular Biology of the Cell, 4th ed, Garland Science, New York, 2002.
14. Adachi J, Kumar C, Zhang Y, Mann M., In-depth Analysis och the Adipocyte Proteome by Mass Spectormetry and Bioinformatics., Mol Cell Proteomics, 2007 Jul; 6: 1257-73
15. http://www.ncbi.nlm.nih.gov/pubmed?term=microdialysis%20proteomics, 2011 03 04
16. Alvarez-Llamas G, Szalowska E, de Vries MP, Weening D, Landman K, Hoek A, Wolffenbuttel BH, Roelofsen H, Vonk RJ., Characterization of the Human Visceral Adipose Tissue Secretome., Mol Cell Proteomics, 2007 April; 6(4): 589-600.
17. Ramström H (Edit.), Läkemedelsboken 2009-2010, 17th ed, Apoteket AB, Sundbyberg, 2009
18. http://www.microdialysis.se/the-company/the-technique, 2011 03 21
19. Jansson PA, Larsson A, Smith U, Lönnroth P., Glycerol Production in Subcutaneous Adipose Tissue in Lean and Obese Humans., Departments of Medicine IIand Radiation Physics, University ofGoteborg, Sahigren's Hospital, S-413 45 Goteborg, Sweden
20. Clough GF., Microdialysis of Large Molecules., Submitted: March 25 , 2005 ; Accepted: May 15 , 2005; Published: October 26, 2005
21. Murdolo G, Hammarstedt A, Sandqvist M, Schmelz M, Herder C, Smith U, Jansson PA., Monocyte chemoattractant protein-1 in subcutaneous abdominal adipose tissue: characterization of interstitial concentration and regulation of gene expression by insulin., J Clin Endocrinol Metab. 2007 Jul;92(7):2688-95.
22. Murdolo G, Hammarstedt A, Schmelz M, Jansson PA, Smith U.,Acute hyperinsulinemia differentially regulates interstitial and circulating adiponectin oligomeric pattern in lean and insulin-resistant, obese individuals., J Clin Endocrinol Metab. 2009 Nov;94(11):4508-16. Epub 2009 Oct 9.
23. Murdolo G, Herder C, Wang Z, Rose B, Schmelz M, Jansson PA., In situ profiling of adipokines in subcutaneous microdialysates from lean and obese individuals., Am J Physiol Endocrinol Metab. 2008 Nov;295(5):E1095-105.
24. Højbjerre L, Sonne MP, Alibegovic AC, Dela F, Vaag A, Meldgaard JB, Christensen KB, Stallknecht B., Impact of physical inactivity on subcutaneous adipose tissue metabolism in healthy young male offspring of patients with type 2 diabetes., Diabetes. 2010 Nov;59 (11): 2790-8. Epub 2010 Sep 7.
25. Wilson K, Walker J, Principles and Techniques of Practical Biochemistry, 5th ed, Cambridge University Press, Cambridge, 2000

26. Wysocki VH, Resing KA, Zhang Q, Cheng G., Mass spectrometry of peptides and proteins, Mass spectrometry of peptides and proteins., Methods. 2005 Mar;35(3):211-22. Epub 2005 Jan 20. Review
27. Ishihama Y, Oda Y, Tabata T, Sato T, Nagasu T, Rappsilber J, Mann M.Ishihama Y, Oda Y, Tabata T, Sato T, Nagasu T, Rappsilber J, Mann M., Exponentially modified protein abundance index (emPAI) for estimation of absolute protein amount in proteomics by the number of sequenced peptides per protein., Mol Cell Proteomics. 2005 Sep;4(9):1265-72.
28. http://www.ncbi.nlm.nih.gov/pubmed?term=microdialysis, 2011 03 04
29. http://www.ncbi.nlm.nih.gov/pubmed?term=proteomics. 2011 03 04
30. Bernay B, Gaillard MC, Guryca V, Emadali A, Kuhn L, Bertrand A, Detraz I, Carcenac C, Savasta M, Brouillet E, Garin J, Elalouf JM., Discovering new bioactive neuropeptides in the striatum secretome using in vivo microdialysis and versatile proteomics., Mol Cell Proteomics. 2009 May;8(5):946-58. *Epub 2009 Jan 21.*
31. www.uniprot.org, 2011 03 11 - 2011 03 26
32. Rosen ED, Spiegelman BM., Adipocytes as regulators of energy balance and glucose homeostasis., Nature. 2006 Dec 14;444(7121):847-53.
33. Hu WJ, Eaton JW, Ugarova TP, Tang L., Molecular basis of biomaterial-mediated foreign body reactions.,Blood. 2001 Aug 15;98(4):1231-8.

APPENDIX 1

PLASMA PURIFICATION CARTRIDGE PROTOCOL (AGILENT)

1. The cartridge is prepared by flushing it with 4 ml of buffer A with a syringe.
2. 350 µl sample, cleaned from clutter with ultrafiltration cartridge.
3. 50 µl are kept for protein quantification using a BCA-kit.
4. The other 300 µl are mixed with 1700 µl buffer A.
5. Cartridge is loaded with 400 µl of the mix at a time and the solution is passed through by centrifugation at 30·g in 1 minute. This is repeated until all 2 ml has passed through the cartridge.
6. Cartridge is reset by flushing it with first 2 ml of buffer B, then followed by flushing it with 4 ml of buffer A.

Buffer A and B are unspecified solutions delivered by the cartrige manufacturer.

SDS-PAGE-PROTOCOL

1. Prepare 800 ml MOPS (morpholino-propanesulfonic acid) solution.
2. Redissolve the acetone precipitated protein in 10 µl running buffer and 10 µl milliQ H20.
3. Sonicate for 20 min.
4. Heat samples for 10 min in 90 ºC.
3. Spin down clutter in the samples for 5 min using a tabletop centrifuge.
4. Load samples onto gel, load blank samples on each side of each sample.
5. Start gel on 50 V for 20 min.
6. Increase voltage to 200 V and run for ~50 min.
7. Place gel in 40/10 gel fixation solution.
8. Wash gel 3·5 min in milliQ H2O.
9. Stain with Coomassie blue for 1 h.
10. Wash gel 2·5 min and then place in mQ H2O overnight.
11. Store gel in a laminated bag in 2% HAc in cooler for further analysis.

APPENDIX 2

DAY 1 OF MASS SPECTROMETRY PREPARATION

1. Cut out lane and divide into 15 pieces.
2. Divide each piece into 1^3 mm small grains and place in 1.5 ml maximum recovery Eppendorf tubes.
3. Add 100 µl AA50 (50 % acetonitrile + 50 % 25 mM NH_4HCO_3) to each tube.
4. Incubate while shaking for 20 min.
5. Centrifuge briefly and remove buffer.
6. Repeat step 3-5 another 2 times.
7. Wash a fourth time, now with 100 µl MA50 (50 % MetOH + 50 % 25 mM NH_4HCO_3).
8. Centrifuge and remove the MA50.
9. Dry samples in a Speed Vac® for 1-2 h.
10. Add 15 µl of Trypsin (10 ng/µl).
11. Incubate overnight.

APPENDIX 3

DAY 2 OF MASS SPECTROMETRY PREPARATION

1. Inhibit the trypsin with 10 µl TFA-1A60 (2 % TFA, 75 % Acetonitrile in mQ H2O)
2. Incubate while shaking for 20 min.
3. Prepare extraction solutions as follows:

    I. Extraction solution: 500µl Acetonitrile, 480 µl mQ H2O, 20 µl TFA 10%
    II. Formic acid 0.1 % in mQ H2O.

4. Spin the samples briefly.
5. Transfer the supernatant to small maximum recovery Eppendorf tubes.
6. Add 20 µl of extraction solution to the gel pieces.
7. Shake for 30 min.
8. Spin samples short.
9. Transfer the supernatant to the small Eppendorf tubes.
10. Discard the gel pieces and dry the samples in a Speed vacc for 1-2 h.
11. Redissolve in 18 µl 0.1 formic acid.
12. Shake for 30 min.
13. Spin down contaminating particles for 20 min at 13000 rpm.
14. Transfer 15 µl of each sample to glass vials.

APPENDIX 4

Protein	Size (aa)	Secreted	Origin	Function	Average (mass%)	St. dev
1. Extracellular superoxide dismutase [Cu-Zn]	240	Yes	Endothelial cells	Antioxidant	0,3	0,26
2. Transaldolase	337	No	Not specified	Metabolism	0,24	0,12
3. Histone H1.3	221	No	Not specified	Not specified	0,17	0,08
4. Inter-alpha-trypsin inhibitor heavy chain H3		Yes	Not specified	Protease inhibitor	0,21	0,14
5. Complement factor H	1231	Yes	Plasma	Complement system	0,51	0,11
6. Inter-alpha-trypsin inhibitor heavy chain H1	991	Yes	Not specified	Protease inhibitor	0,98	0,29
7. Peptidase inhibitor 16	463	No	Not specified	Protease inhibitor	0,09	0,05
8. Corticosteroid-binding globulin	405	Yes	Not specified	Transporter	0,45	0,2
9. Kallistatin	427	Yes	Plasma	Other	0,49	0,35
10. Lamin-A/C	664	No	Not specified	Other	0,19	0,15
11. Lysozyme C	148	Yes	Not specified	Inflammation	1,39	1,08
12. Complement C2	752	Yes	Not specified	Inflammation	0,4	0,14
13.Serum amyloid P-component	223	Yes	Plasma	Cell damage	1,24	0,81
14. Lumican	338	Yes	Connective tissue	Transporter	1,36	0,62
15. Apolipoprotein B-100	4563	Yes	Plasma	Transporter	0,83	0,57
16. Alpha-1-antichymotrypsin	423	Yes	Plasma	Inflammation	5,02	1,91
17. Alpha-2-antiplasmin	491	Yes	Plasma	Acute phase	0,37	0,14
18. Vitamin D-binding	474	Yes	Plasma	Transporter	2,13	0,96

protein						
19. Carbonic anhydrase 1	261	No	Not specified	Metabolism	1,41	1,08
20. Complement C5	1676	Yes	Plasma	Inflammation	0,52	0,17
21. Pigment epithelium-derived factor	418	Yes	Plasma	Other	2,29	0,82
22. Lymphoid-restricted membrane protein	555	No	White blood cells	Transporter	0,06	0,03
23. Leucine-rich alpha-2-glycoprotein	337	Yes	Plasma	Not specified	0,74	0,45
24. Alpha-2-HS-glycoprotein	367	Yes	Plasma	Acute phase	0,84	0,19
25. Alpha-1B-glycoprotein	491	Yes	Plasma	Not specified	1,32	0,52
26. Kininogen-1	644	Yes	Plasma	Metabolism	0,52	0,23
27. Zinc-alpha-2-glycoprotein	298	Yes	Plasma	Metabolism	0,95	0,38
28. Plasma protease C1 inhibitor	500	Yes	Not specified	Complement system	1,75	0,42
29. Complement factor B	764	Yes	Not specified	Complement system	1,4	0,51
30. Antithrombin-III	464	Yes	Plasma	Coagulation	7,37	3,66
31. Protein S100-A9	114	Yes	Plasma	Inflammation	5,73	3,41
32. Prothrombin	622	Yes	Plasma	Acute phase	0,18	0,07
33. Zinc finger CCCH domain-containing protein 13	1668	No	Not specified	Not specified	0,03	0,02
34. Attractin	1429	Yes	Plasma	Inflammation	0,08	0,03
35. Annexin A2	339	Yes	Extra cellular space	Transporter	0,15	0,07
36. Gelsolin	782	Yes	Fibroblasts	Other	1,79	0,53
37. Angiotensinogen	485	Yes	Plasma	Other	1,28	0,49
38. Carboxypeptidase N	545	Yes	Not specified	Cell damage	0,1	0,02

subunit 2						
39. Beta-2-glycoprotein 1	345	Yes	Plasma	Metabolism	0,23	0,1
40. Apolipoprotein A-IV	396	Yes	Plasma	Transporter	7,49	6
41. Ceruloplasmin	1065	Yes	Plasma	Transporter	1,57	0,27
42. Protein S100-A8	93	Yes	Endothelial cells, Macrophages		2,14	1,69
43. Thyroxine-binding globulin	415	Yes	Plasma	Metabolism	0,67	0,24
44. Glutathione peroxidase 3	226	Yes	Plasma	Antioxidant	0,32	0,11
45. Heparin cofactor 2	449	Yes	Plasma	Inflammation	0,38	0,17
46. Complement component C7	843	Yes	Not specified	Complement system	0,19	0,1
47. Complement C1q subcomponent subunit C	245	Yes	Not specified	Complement system	0,43	0,19
48. Actin, cytoplasmic 1	375	No	Not specified	Other	4,83	3,12
49. Afamin	599	Yes	Not specified	Transporter	0,74	0,26
50. Inter-alpha-trypsin inhibitor heavy chain H2	946	Yes	Plasma	Metabolism	1,59	0,48
51. Hemopexin	462	Yes	Plasma	Metabolism	2,33	0,87
52. Fibronectin	2386	Yes	Extra cellular space	Acute phase	0,33	0,2
53. Complement factor I	583	Yes	Plasma	Complement system	0,15	0,11
54. Inter-alpha-trypsin inhibitor heavy chain H4	930	Yes	Plasma	Acute phase	1,49	0,48
55. Plasma serine protease inhibitor	406	Yes	Plasma	Coagulation	0,47	0,25
56. Complement C4-A	1744	Yes	Plasma	Complement system	0,73	0,19
57. Protein AMBP	352	Yes	Plasma	Cell damage	0,2	0,11

58. Plasminogen	810	Yes	Plasma	Coagulation	0,49	0,18
59. Retinol-binding protein 4	201	Yes	Plasma	Transporter	0,86	0,39
60. Complement component C8 gamma chain	202	Yes	Not specified	Complement system	1,11	0,57
61. Complement component C8 alpha chain	584	Yes	Not specified	Complement system	0,25	0,08
62. Complement component C9	559	Yes	Plasma	Complement system	0,51	0,33
63. Tetranectin	202	Yes	Plasma	Transporter	0,32	0,17

APPENDIX 5

1. 14-3-3 protein beta/alpha
2. 14-3-3 protein epsilon
3. 14-3-3 protein gamma
4. 14-3-3 protein sigma
5. 14-3-3 protein zeta/delta
6. 5-azacytidine-induced protein 1
7. 5~-3~ exoribonuclease 1
8. 6-phosphofructokinase, liver type
9. 6-phosphogluconate dehydrogenase, decarboxylating OS=Homo sapiens GN=PGD PE=1 SV=3
10. 78 kDa glucose-regulated protein OS=Homo sapiens GN=HSPA5 PE=1 SV=2
11. A kinase anchor protein 1, mitochondrial
12. A-kinase anchor protein 11
13. Actin-related protein 2 OS=Homo sapiens GN=ACTR2 PE=1 SV=1
14. Actin-related protein 2/3 complex subunit 2 OS=Homo sapiens GN=ARPC2 PE=1 SV=1
15. Actin-related protein 2/3 complex subunit 4 OS=Homo sapiens GN=ARPC4 PE=1 SV=3
16. Actin-related protein 3 OS=Homo sapiens GN=ACTR3 PE=1 SV=3
17. Actin, alpha cardiac muscle 1
18. Actin, cytoplasmic 1
19. Adenylate kinase isoenzyme 1
20. Adenylosuccinate synthetase isozyme 2 OS=Homo sapiens GN=ADSS PE=1 SV=3
21. Adenylyl cyclase-associated protein 1 OS=Homo sapiens GN=CAP1 PE=1 SV=4
22. Adiponectin
23. Afamin
24. Alcohol dehydrogenase 1A OS=Homo sapiens GN=ADH1A PE=1 SV=2
25. Aldo-keto reductase family 1 member C1
26. Alpha-1-antichymotrypsin
27. Alpha-1B-glycoprotein
28. Alpha-2-antiplasmin
29. Alpha-2-HS-glycoprotein
30. Alpha-2-macroglobulin-like protein 1
31. Alpha-actinin-1 OS=Homo sapiens GN=ACTN1 PE=1 SV=2
32. Alpha-actinin-2
33. Alpha-actinin-3
34. Alpha-actinin-4 OS=Homo sapiens GN=ACTN4 PE=1 SV=2
35. Alpha-adducin
36. Alpha-amylase 1 OS=Homo sapiens GN=AMY1A PE=1 SV=2
37. Alpha-enolase
38. Angiopoietin-related protein 7 OS=Homo sapiens GN=ANGPTL7 PE=1 SV=1
39. Angiotensinogen
40. Ankyrin repeat and SOCS box protein 15
41. Ankyrin repeat domain-containing protein 5 OS=Homo sapiens GN=ANKRD5 PE=2 SV=2
42. Annexin A1
43. Annexin A2
44. Annexin A3
45. Annexin A5
46. Antithrombin-III
47. AP-1 complex subunit beta-1
48. AP-1 complex subunit gamma-like 2
49. Apolipoprotein A-IV
50. Apolipoprotein A-V OS=Homo sapiens GN=APOA5 PE=1 SV=1
51. Apolipoprotein B-100
52. Apolipoprotein C-II
53. Apolipoprotein C-III
54. Apolipoprotein D
55. Apolipoprotein E
56. Apolipoprotein M
57. Apolipoprotein(a)
58. ARF GTPase-activating protein GIT2 OS=Homo sapiens GN=GIT2 PE=1 SV=2
59. Arginase-1 OS=Homo sapiens GN=ARG1 PE=1 SV=2
60. Armadillo repeat-containing protein 3
61. ATP-dependent DNA helicase 2 subunit 1
62. ATP-dependent DNA helicase Q5
63. Attractin
64. Autocrine motility factor receptor, isoform 2 OS=Homo sapiens GN=AMFR PE=1 SV=2
65. Azurocidin
66. Baculoviral IAP repeat-containing protein 6
67. BAI1-associated protein 3
68. Basement membrane-specific heparan sulfate proteoglycan core protein
69. BAT2 domain-containing protein 1 OS=Homo sapiens GN=BAT2D1 PE=1 SV=2
70. Beta-2-glycoprotein 1
71. Beta-2-microglobulin
72. Beta-actin-like protein 2
73. Beta-Ala-His dipeptidase
74. Beta-centractin
75. Beta-enolase
76. Beta-galactoside alpha-2,6-sialyltransferase 1
77. Beta-nerve growth factor
78. Bifunctional aminoacyl-tRNA synthetase
79. Biotinidase
80. Bleomycin hydrolase OS=Homo sapiens GN=BLMH PE=1 SV=1
81. Brain-specific angiogenesis inhibitor 1-associated protein 2-like protein 1
82. Bromo adjacent homology domain-containing 1 protein
83. BTB/POZ domain-containing protein 16 OS=Homo sapiens GN=BTBD16 PE=2 SV=2
84. C2 tensin-type domain-containing protein ENSP00000371290
85. C4b-binding protein alpha chain
86. Cadherin-5 OS=Homo sapiens GN=CDH5 PE=1 SV=4
87. Calmodulin
88. Calneuron-1
89. Carbonic anhydrase 1
90. Carbonic anhydrase 2
91. Carbonic anhydrase 3
92. Carboxypeptidase B2
93. Carboxypeptidase N catalytic chain
94. Carboxypeptidase N subunit 2
95. Cardiotrophin-like cytokine factor 1
96. Cartilage acidic protein 1
97. Cartilage oligomeric matrix protein
98. Caspase-14
99. Caspase-8
100. Catalase
101. Cathelicidin antimicrobial peptide
102. Cathepsin D
103. Cathepsin H OS=Homo sapiens GN=CTSH PE=1 SV=3
104. Cation-independent mannose-6-phosphate receptor
105. Cell division protein kinase 10
106. Cell surface glycoprotein MUC18
107. Centrosomal protein of 192 kDa
108. Ceruloplasmin
109. Chitinase-3-like protein 1 OS=Homo sapiens GN=CHI3L1 PE=1 SV=2
110. Chitotriosidase-1 OS=Homo sapiens GN=CHIT1 PE=1 SV=1
111. Chloride intracellular channel protein 1 OS=Homo sapiens GN=CLIC1 PE=1 SV=4
112. Cholinesterase
113. Clathrin heavy chain 1
114. Clusterin
115. Cofilin-1
116. Cofilin-2
117. Coiled-coil domain-containing protein 54
118. Coiled-coil domain-containing protein 87
119. Collagen alpha-1(I) chain
120. Collagen alpha-3(V) chain
121. Collagen alpha-3(VI) chain
122. Complement C1q subcomponent subunit A
123. Complement C1q subcomponent subunit B
124. Complement C1q subcomponent subunit C
125. Complement C1r subcomponent
126. Complement C1r subcomponent-like protein
127. Complement C1s subcomponent
128. Complement C2
129. Complement C4-A
130. Complement C5
131. Complement component C6
132. Complement component C7
133. Complement component C8 alpha chain
134. Complement component C8 beta chain
135. Complement component C8 gamma chain
136. Complement component C9
137. Complement factor B
138. Complement factor D
139. Complement factor H
140. Complement factor H-related protein 1
141. Complement factor H-related protein 3
142. Complement factor I
143. Conserved oligomeric Golgi complex subunit 5
144. Coronin-1A OS=Homo sapiens GN=CORO1A PE=1 SV=4
145. Corticosteroid-binding globulin
146. Creatine kinase M-type
147. CWF19-like protein 2
148. Cyclin-dependent kinase-like 1
149. Cystatin-A
150. Cystatin-C
151. Cystatin-M
152. Cystatin-SN
153. Cystic fibrosis transmembrane conductance regulator
154. Cytochrome b
155. Cytochrome P450 4X1
156. Cytoskeleton-associated protein 2
157. Delta-aminolevulinic acid dehydratase OS=Homo sapiens GN=ALAD PE=1 SV=1
158. Delta-like protein 4 OS=Homo sapiens GN=DLL4 PE=1 SV=1
159. Delta(14)-sterol reductase OS=Homo sapiens GN=TM7SF2 PE=2 SV=3
160. Dermatopontin

161. Dermcidin
162. Desmoplakin
163. Diacylglycerol kinase alpha OS=Homo sapiens GN=DGKA PE=1 SV=2
164. Disabled homolog 2-interacting protein
165. Disheveled-associated activator of morphogenesis 1
166. Disintegrin and metalloproteinase domain-containing protein 5
167. DNA-dependent protein kinase catalytic subunit
168. DnaJ homolog subfamily B member 13 OS=Homo sapiens GN=DNAJB13 PE=2 SV=1
169. Dopamine beta-hydroxylase
170. Doublesex- and mab-3-related transcription factor C2 OS=Homo sapiens GN=DMRTC2 PE=2 SV=2
171. Dynein heavy chain 10, axonemal
172. Dynein heavy chain 3, axonemal
173. Dystrobrevin alpha OS=Homo sapiens GN=DTNA PE=1 SV=1
174. E3 ubiquitin-protein ligase RNF123
175. Egl nine homolog 2 OS=Homo sapiens GN=EGLN2 PE=1 SV=1
176. ELAV-like protein 3
177. Elongation factor 2 kinase
178. Elongation of very long chain fatty acids protein 7 OS=Homo sapiens GN=ELOVL7 PE=2 SV=1
179. Endothelial protein C receptor OS=Homo sapiens GN=PROCR PE=1 SV=1
180. Endothelin-converting enzyme 1
181. Epidermal growth factor receptor kinase substrate 8-like protein 1
182. Epidermal growth factor receptor substrate 15
183. ERO1-like protein alpha
184. Eukaryotic translation initiation factor 2-alpha kinase 3 OS=Homo sapiens GN=EIF2AK3 PE=1 SV=2
185. Eukaryotic translation initiation factor 4 gamma 2 OS=Homo sapiens GN=EIF4G2 PE=1 SV=1
186. Extracellular glycoprotein lacritin
187. Extracellular matrix protein 1
188. Extracellular superoxide dismutase [Cu-Zn]
189. Eyes absent homolog 2
190. Ezrin
191. F-box only protein 24
192. Fanconi anemia group J protein
193. Fatty acid-binding protein, adipocyte
194. Fatty acid-binding protein, epidermal
195. Fetuin-B
196. Fibroblast growth factor 12
197. Fibronectin
198. Fibulin-1
199. Ficolin-3
200. Filaggrin-2
201. Filamin-A OS=Homo sapiens GN=FLNA PE=1 SV=4
202. Filamin-C
203. Flavin reductase OS=Homo sapiens GN=BLVRB PE=1 SV=3
204. Forkhead-associated domain-containing protein 1
205. Forty-two-three domain-containing protein 1
206. Four and a half LIM domains protein 1
207. Fructose-bisphosphate aldolase A
208. Fructose-bisphosphate aldolase C OS=Homo sapiens GN=ALDOC PE=1 SV=2
209. Galectin-7 OS=Homo sapiens GN=LGALS7 PE=1 SV=2
210. Galectin-related protein OS=Homo sapiens GN=GRP PE=1 SV=2
211. Gelsolin
212. Glia maturation factor beta OS=Homo sapiens GN=GMFB PE=1 SV=2
213. Glucose-6-phosphate isomerase
214. Glutathione peroxidase 3
215. Glutathione reductase, mitochondrial OS=Homo sapiens GN=GSR PE=1 SV=2
216. Glutathione S-transferase omega-1 OS=Homo sapiens GN=GSTO1 PE=1 SV=2
217. Glutathione S-transferase P
218. Glyceraldehyde-3-phosphate dehydrogenase
219. Glycerol-3-phosphate dehydrogenase [NAD+], cytoplasmic
220. Glycogenin-1 OS=Homo sapiens GN=GYG1 PE=1 SV=4
221. Golgi-associated plant pathogenesis-related protein 1 OS=Homo sapiens GN=GLIPR2 PE=1 SV=3
222. GTPase-activating Rap/Ran-GAP domain-like protein 3
223. Haptoglobin-related protein
224. Heat shock 70 kDa protein 1
225. Heat shock 70 kDa protein 1L
226. Heat shock cognate 71 kDa protein
227. Heat shock protein beta-1 OS=Homo sapiens GN=HSPB1 PE=1 SV=2
228. Heme-binding protein 2 OS=Homo sapiens GN=HEBP2 PE=1 SV=1
229. Hemopexin
230. Heparin cofactor 2
231. Hepatocyte growth factor activator
232. Histidine-rich glycoprotein
233. Histone H1.3
234. Histone H2A type 1-A
235. Histone H2B type 1-A
236. Histone H3-like
237. Histone H4
238. Histone-lysine N-methyltransferase MLL4
239. Histone-lysine N-methyltransferase SETD8
240. Homeodomain-interacting protein kinase 1
241. Hornerin
242. Hyaluronan-binding protein 2
243. Hyaluronidase-4
244. Immunoglobulin superfamily containing leucine-rich repeat protein
245. Immunoglobulin-like and fibronectin type III domain-containing protein 1
246. Inactive dual specificity phosphatase 27
247. Insulin receptor substrate 2 OS=Homo sapiens GN=IRS2 PE=1 SV=2
248. Insulin-like growth factor-binding protein 3 OS=Homo sapiens GN=IGFBP3 PE=1 SV=2
249. Insulin-like growth factor-binding protein 6
250. Insulin-like growth factor-binding protein complex acid labile chain
251. Inter-alpha-trypsin inhibitor heavy chain H1
252. Inter-alpha-trypsin inhibitor heavy chain H2
253. Inter-alpha-trypsin inhibitor heavy chain H3
254. Inter-alpha-trypsin inhibitor heavy chain H4
255. Interferon-induced GTP-binding protein Mx2 OS=Homo sapiens GN=MX2 PE=1 SV=1
256. Interferon-induced, double-stranded RNA-activated protein kinase OS=Homo sapiens GN=EIF2AK2 PE=1 SV=2
257. Interleukin-5
258. Intraflagellar transport protein 74 homolog
259. IQ domain-containing protein E OS=Homo sapiens GN=IQCE PE=1 SV=2
260. Isocitrate dehydrogenase [NADP] cytoplasmic
261. JmjC domain-containing protein 5
262. Kallistatin
263. KH domain-containing, RNA-binding, signal transduction-associated protein 1
264. Kinectin
265. Kinesin-like protein KIF27
266. Kinetochore-associated protein 1
267. Kininogen-1
268. L-lactate dehydrogenase A chain
269. L-lactate dehydrogenase B chain
270. L-selectin
271. Lactotransferrin
272. Lamin-A/C
273. Leucine-rich alpha-2-glycoprotein
274. Leucine-rich repeat LGI family member 2
275. Leucine-rich repeat-containing protein 15
276. Leucine-rich repeat-containing protein 40 OS=Homo sapiens GN=LRRC40 PE=1 SV=1
277. Leucine-rich repeat-containing protein 61 OS=Homo sapiens GN=LRRC61 PE=2 SV=1
278. Leucyl-cystinyl aminopeptidase
279. Leukocyte elastase
280. Leukocyte elastase inhibitor
281. Leukotriene A-4 hydrolase OS=Homo sapiens GN=LTA4H PE=1 SV=2
282. Lipocalin-1
283. Long-chain fatty acid transport protein 6
284. Lumican
285. Lymphoid-restricted membrane protein
286. Lysosome-associated membrane glycoprotein 2
287. Lysozyme C
288. Macrophage colony-stimulating factor 1
289. Macrophage-capping protein
290. Malate dehydrogenase, cytoplasmic
291. Mammaglobin-B
292. Mannan-binding lectin serine protease 1 OS=Homo sapiens GN=MASP1 PE=1 SV=3
293. Mannose-binding protein C OS=Homo sapiens GN=MBL2 PE=1 SV=2
294. Mannosyl-oligosaccharide 1,2-alpha-mannosidase IC
295. Matrix metalloproteinase-9
296. Mediator of RNA polymerase II transcription subunit 23
297. Mediator of RNA polymerase II transcription

subunit 25
298. Mediator of RNA polymerase II transcription subunit 30
299. Merlin
300. Metalloproteinase inhibitor 1
301. Microfibril-associated glycoprotein 4
302. Mimecan
303. Moesin OS=Homo sapiens GN=MSN PE=1 SV=3
304. Monocyte differentiation antigen CD14
305. Mucin-5AC (Fragments)
306. Multiple inositol polyphosphate phosphatase 1
307. Myeloperoxidase
308. Myocilin
309. Myoglobin
310. Myomegalin
311. Myosin regulatory light chain MRLC2 OS=Homo sapiens GN=MYLC2B PE=1 SV=2
312. Myosin-14 OS=Homo sapiens GN=MYH14 PE=1 SV=1
313. Myosin-2
314. Myosin-7
315. Myosin-9 OS=Homo sapiens GN=MYH9 PE=1 SV=4
316. Myosin-binding protein C, slow-type
317. Myotrophin OS=Homo sapiens GN=MTPN PE=1 SV=2
318. N-acetylmuramoyl-L-alanine amidase
319. N(G),N(G)-dimethylarginine dimethylaminohydrolase 2
320. NADH dehydrogenase [ubiquinone] iron-sulfur protein 7, mitochondrial
321. NADPH:adrenodoxin oxidoreductase, mitochondrial
322. Neural cell adhesion molecule 1
323. Neuronal acetylcholine receptor subunit alpha-4
324. Neuronal pentraxin-2
325. Neutral alpha-glucosidase C
326. Neutrophil collagenase
327. Neutrophil gelatinase-associated lipocalin
328. NF-kappa-B inhibitor zeta
329. Non-structural polyprotein 1AB
330. NTPase KAP family P-loop domain-containing protein 1
331. Nuclear cap-binding protein subunit 2-like
332. Nuclear pore complex protein Nup153 OS=Homo sapiens GN=NUP153 PE=1 SV=2
333. Nuclear pore complex protein Nup98-Nup96
334. Nuclear receptor ROR-beta
335. NudC domain-containing protein 2
336. Occludin OS=Homo sapiens GN=OCLN PE=1 SV=1
337. Osteoclast-stimulating factor 1 OS=Homo sapiens GN=OSTF1 PE=1 SV=2
338. Oxysterol-binding protein-related protein 7
339. PACRG-like protein
340. Pantetheinase OS=Homo sapiens GN=VNN1 PE=1 SV=2
341. Parathymosin
342. PDZ and LIM domain protein 5
343. Pentatricopeptide repeat-containing protein 1
344. Peptidase inhibitor 16
345. Peptidyl-prolyl cis-trans isomerase A
346. Peptidyl-prolyl cis-trans isomerase B OS=Homo sapiens GN=PPIB PE=1 SV=2
347. Peptidyl-prolyl cis-trans isomerase FKBP1A OS=Homo sapiens GN=FKBP1A PE=1 SV=2
348. Peroxiredoxin-1 OS=Homo sapiens GN=PRDX1 PE=1 SV=1
349. Peroxiredoxin-2
350. Peroxiredoxin-6 OS=Homo sapiens GN=PRDX6 PE=1 SV=3
351. Phosphatidylethanolamine-binding protein 1
352. Phosphatidylinositol-glycan-specific phospholipase D
353. Phosphoglucomutase-1 OS=Homo sapiens GN=PGM1 PE=1 SV=3
354. Phosphoglycerate kinase 1
355. Phosphoglycerate mutase 1
356. Pigment epithelium-derived factor
357. Piwi-like protein 4
358. Plasma kallikrein
359. Plasma protease C1 inhibitor
360. Plasma serine protease inhibitor
361. Plasminogen
362. Plastin-2
363. Platelet basic protein OS=Homo sapiens GN=PPBP PE=1 SV=3
364. Platelet glycoprotein Ib alpha chain
365. Pleckstrin homology domain-containing family G member 6
366. Polyadenylate-binding protein 1 OS=Homo sapiens GN=PABPC1 PE=1 SV=2
367. Polymerase I and transcript release factor
368. Probable asparaginyl-tRNA synthetase, mitochondrial
369. Probable E3 ubiquitin-protein ligase HERC3
370. Profilin-1
371. Prokineticin-2 OS=Homo sapiens GN=PROK2 PE=1 SV=2
372. Prolyl 4-hydroxylase subunit alpha-3 OS=Homo sapiens GN=P4HA3 PE=1 SV=1
373. Prostaglandin-H2 D-isomerase
374. Proteasome subunit alpha type-3 OS=Homo sapiens GN=PSMA3 PE=1 SV=2
375. Proteasome subunit alpha type-5 OS=Homo sapiens GN=PSMA5 PE=1 SV=3
376. Proteasome subunit alpha type-6 OS=Homo sapiens GN=PSMA6 PE=1 SV=1
377. Proteasome subunit beta type-4
378. Protein AHNAK2
379. Protein AMBP
380. Protein cappuccino homolog
381. Protein DJ-1
382. Protein FAM65A OS=Homo sapiens GN=FAM65A PE=1 SV=1
383. Protein FAM71B
384. Protein FAM83G
385. Protein kintoun
386. Protein KIAA0323
387. Protein LAP4
388. Protein LLP homolog
389. Protein S100-A12 OS=Homo sapiens GN=S100A12 PE=1 SV=2
390. Protein S100-A4
391. Protein S100-A6
392. Protein S100-A8
393. Protein S100-A9
394. Protein S100-P OS=Homo sapiens GN=S100P PE=1 SV=2
395. Protein very KIND OS=Homo sapiens GN=KNDC1 PE=2 SV=2
396. Protein Z-dependent protease inhibitor OS=Homo sapiens GN=SERPINA10 PE=1 SV=1
397. Proteoglycan 4 OS=Homo sapiens GN=PRG4 PE=1 SV=2
398. Prothrombin
399. Protocadherin beta-16 OS=Homo sapiens GN=PCDHB16 PE=1 SV=2
400. Protocadherin Fat 2
401. Protocadherin gamma-B2 OS=Homo sapiens GN=PCDHGB2 PE=2 SV=1
402. Purine nucleoside phosphorylase OS=Homo sapiens GN=NP PE=1 SV=2
403. Putative heat shock 70 kDa protein 7
404. Putative protein TMEM137 OS=Homo sapiens GN=TMEM137 PE=5 SV=1
405. Putative tropomyosin alpha-3 chain-like protein OS=Homo sapiens PE=5 SV=2
406. Pyruvate kinase isozymes M1/M2
407. Ral-GDS-related protein OS=Homo sapiens GN=RGL4 PE=2 SV=1
408. Ras-related protein Ral-A
409. Receptor-interacting serine/threonine-protein kinase 1
410. Regulator of G-protein signaling 3
411. Reticulocalbin-1
412. Retinol-binding protein 4
413. Rho GDP-dissociation inhibitor 1 OS=Homo sapiens GN=ARHGDIA PE=1 SV=3
414. Rho GDP-dissociation inhibitor 2
415. Rho guanine nucleotide exchange factor 15
416. Rhox homeobox family member 1
417. Ribosome production factor 1
418. RILP-like protein 1
419. RNA polymerase II transcription factor SIII subunit A2 OS=Homo sapiens GN=TCEB3B PE=1 SV=2
420. Rootletin
421. Ryanodine receptor 1
422. Ryanodine receptor 2
423. S1 RNA-binding domain-containing protein 1 OS=Homo sapiens GN=SRBD1 PE=1 SV=2
424. Sarcoplasmic/endoplasmic reticulum calcium ATPase 2 OS=Homo sapiens GN=ATP2A2 PE=1 SV=1
425. Secretoglobin family 1D member 1
426. Selenium-binding protein 1 OS=Homo sapiens GN=SELENBP1 PE=1 SV=2
427. Selenoprotein P
428. Serine/threonine-protein kinase 13
429. Serine/threonine-protein kinase 36 OS=Homo sapiens GN=STK36 PE=1 SV=2
430. Serine/threonine-protein kinase TAO2
431. Serine/threonine-protein phosphatase 4 regulatory subunit 4
432. Serotransferrin
433. Serpin B3 OS=Homo sapiens GN=SERPINB3 PE=1 SV=2
434. Serpin B6 OS=Homo sapiens GN=SERPINB6 PE=1 SV=3
435. Serpin I2
436. Serum amyloid A-4 protein
437. Serum amyloid P-component

438. Serum paraoxonase/arylesterase 1
439. Sex hormone-binding globulin
440. SH3 domain-binding glutamic acid-rich-like protein
441. SH3 domain-binding glutamic acid-rich-like protein 3
442. Short transient receptor potential channel 1 OS=Homo sapiens GN=TRPC1 PE=1 SV=1
443. Sialic acid-binding Ig-like lectin 16
444. Sickle tail protein homolog OS=Homo sapiens GN=SKT PE=1 SV=2
445. Signal peptide peptidase-like 2C OS=Homo sapiens GN=SPPL2C PE=1 SV=2
446. Small VCP/p97-interacting protein OS=Homo sapiens GN=SVIP PE=2 SV=1
447. Solute carrier family 45 member 4
448. SPARC OS=Homo sapiens GN=SPARC PE=1 SV=1
449. Spermatogenesis-associated protein 7 OS=Homo sapiens GN=SPATA7 PE=2 SV=3
450. Stromal interaction molecule 1
451. Sulfhydryl oxidase 1 OS=Homo sapiens GN=QSOX1 PE=1 SV=3
452. Superoxide dismutase [Cu-Zn]
453. Suppressor of IKK-epsilon OS=Homo sapiens GN=SIKE PE=1 SV=1
454. Synaptotagmin-1 OS=Homo sapiens GN=SYT1 PE=1 SV=1
455. Talin-1 OS=Homo sapiens GN=TLN1 PE=1 SV=3
456. TATA-binding protein-associated factor 172
457. TBC1 domain family member 2A
458. Telomeric repeat-binding factor 2 OS=Homo sapiens GN=TERF2 PE=1 SV=2
459. Tenascin-X
460. Tetranectin
461. Tetratricopeptide repeat protein 23-like OS=Homo sapiens GN=TTC23L PE=2 SV=2
462. Tetratricopeptide repeat protein 37
463. TFIIH basal transcription factor complex helicase XPB subunit
464. Thrombospondin-1 OS=Homo sapiens GN=THBS1 PE=1 SV=2
465. Thyroxine-binding globulin
466. Tigger transposable element-derived protein 4 OS=Homo sapiens GN=TIGD4 PE=1 SV=1
467. Transaldolase
468. Transcription regulator protein BACH2 OS=Homo sapiens GN=BACH2 PE=2 SV=1
469. Transcriptional adapter 2-beta
470. Transgelin OS=Homo sapiens GN=TAGLN PE=1 SV=4
471. Transgelin-2 OS=Homo sapiens GN=TAGLN2 PE=1 SV=3
472. Transient receptor potential cation channel subfamily M member 8
473. Transitional endoplasmic reticulum ATPase OS=Homo sapiens GN=VCP PE=1 SV=4
474. Transketolase
475. Translation initiation factor IF-3, mitochondrial
476. Transmembrane protease, serine 13 OS=Homo sapiens GN=TMPRSS13 PE=2 SV=2
477. Transmembrane protein 114 OS=Homo sapiens GN=TMEM114 PE=2 SV=2
478. Trem-like transcript 1 protein OS=Homo sapiens GN=TREML1 PE=1 SV=2
479. Triosephosphate isomerase
480. Tripartite motif-containing protein 6
481. tRNA guanosine-2~-O-methyltransferase TRM11 homolog OS=Homo sapiens GN=TRMT11 PE=1 SV=1
482. Troponin I, fast skeletal muscle
483. Troponin I, slow skeletal muscle
484. Troponin T, fast skeletal muscle
485. Troponin T, slow skeletal muscle OS=Homo sapiens GN=TNNT1 PE=1 SV=4
486. Trypsin-3 OS=Homo sapiens GN=PRSS3 PE=1 SV=2
487. Tryptophan 5-hydroxylase 2
488. Two pore calcium channel protein 2
489. Tyrosine-protein kinase BTK OS=Homo sapiens GN=BTK PE=1 SV=3
490. Ubiquitin OS=Homo sapiens GN=RPS27A PE=1 SV=1
491. Uncharacterized protein C15orf33
492. Uncharacterized protein C1orf106 OS=Homo sapiens GN=C1orf106 PE=2 SV=1
493. Uncharacterized protein KIAA2012
494. Uncharacterized protein LOC113230
495. UPF0360 protein C11orf60
496. UPF0526 protein
497. UPF0550 protein C7orf28
498. UPF0587 protein C1orf123 OS=Homo sapiens GN=C1orf123 PE=1 SV=1
499. UPF0634 protein KIAA1641
500. Vacuolar protein sorting-associated protein 13B
501. Vacuolar protein sorting-associated protein 13D
502. Vacuolar protein sorting-associated protein 37A
503. Vacuolar protein sorting-associated protein 45
504. Vasohibin-2
505. Vasorin
506. WD repeat-containing protein 19
507. WD repeat-containing protein 60
508. Vimentin
509. Vinculin
510. Vitamin D-binding protein
511. Vitamin K-dependent protein C OS=Homo sapiens GN=PROC PE=1 SV=1
512. Vitamin K-dependent protein S
513. Vitronectin
514. VPS10 domain-containing receptor SorCS3 OS=Homo sapiens GN=SORCS3 PE=2 SV=2
515. Xin actin-binding repeat-containing protein 2 OS=Homo sapiens GN=XIRP2 PE=2 SV=2
516. Xaa-Pro dipeptidase OS=Homo sapiens GN=PEPD PE=1 SV=3
517. Zinc finger CCCH domain-containing protein 13
518. Zinc finger CCHC domain-containing protein 11 OS=Homo sapiens GN=ZCCHC11 PE=1 SV=3
519. Zinc finger MYM-type protein 3
520. Zinc finger protein 180 OS=Homo sapiens GN=ZNF180 PE=1 SV=2
521. Zinc finger protein 239
522. Zinc finger protein 324B OS=Homo sapiens GN=ZNF324B PE=2 SV=1
523. Zinc finger protein 483
524. Zinc finger protein 551 OS=Homo sapiens GN=ZNF551 PE=1 SV=3
525. Zinc finger protein 597
526. Zinc finger protein 618
527. Zinc finger protein 677
528. Zinc finger protein 81 OS=Homo sapiens GN=ZNF81 PE=1 SV=3
529. Zinc finger protein DZIP1
530. Zinc finger SWIM domain-containing protein KIAA0913 OS=Homo sapiens GN=KIAA0913 PE=1 SV=1
531. Zinc-alpha-2-glycoprotein