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VISUALIZING AND PROFILING TISSUE LIPIDS BY ToF-SIMS IMAGING MASS SPECTROMETRY

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ABSTRACT

Time-of-flight secondary ion mass spectrometry (ToF-SIMS) is an emerging surface analytical method for chemical microanalysis of biological samples. While much work has been done with artificial materials and some with cultured cells and animal tissue, very little ToF-SIMS work has been done so far with clinically relevant tissue samples. The ability of ToF-SIMS to detect and visualize lipids in tissue was therefore evaluated in this project.

Study I: Patients with chronic kidney disease (CKD) display a variety of metabolic and nutritional disturbances, which contribute to various clinical complications and high mortality. The evaluation of plasma constituents in these patients reveals extensive lipoprotein and fatty acid composition abnormalities, but these changes may not necessarily reflect abnormalities in tissue lipid composition which conceivably could contribute to various alterations in the function of cells and tissues. In this work ToF-SIMS was used for detecting lipids in human adipose tissue from CKD patients. The results showed systematic variations in the lipid distribution between samples from different individuals, i.e. signal intensities from the saturated lipids relative to unsaturated lipids. These observations suggest that there exists a correlation between CKD and degree of saturation of the lipids.

Study II: Osmium tetroxide has long been used as a stain for unsaturated lipids but there has been disagreement about its site of binding in tissue. The spatial distributions of different lipids and osmium oxides in mouse adipose tissue were therefore analyzed by ToF-SIMS. Comparison of the lipid distributions before and after staining suggests that one type of osmium oxide binds specifically to glyceride lipids containing unsaturated C18 fatty acids. Another osmium oxide was co-localized with protein fragments. Chemical analysis, imaging and compatibility with tissue sections similar or identical to those in histology make ToF-SIMS highly useful for elucidating the chemical background of histological stains.

Study III: ToF-SIMS was used to analyze health human skin. The composition of the different layers of the skin was studied and compared by monitoring the localization of different inorganic substances and lipids. Although the signal at masses corresponding to intact lipids was less intense than from inorganic substances, ion images could be generated for several lipids, which showed a preferential localization close to the skin surface. Some of these lipids such as sapienic acid and lauric acid are considered to be antimicrobial and first line of defense against microbial colonization and infection.

These studies show that good quality ToF-SIMS spectra and ion images can be obtained from tissue sections similar or identical to those used in clinical histopathology. Many of the detected signals correspond to lipids and include a range of unsaturated lipids. ToF-SIMS will therefore be a useful method in the study of chronic diseases associated with local alterations in unsaturated lipids.

LIST OF PUBLICATIONS

I. Peter Sjövall, Björn Johansson, **Dalila Belazi**, Peter Stenvinkel, Bengt Lindholm, Jukka Lausmaa, Martin Schalling. TOF-SIMS analysis of adipose tissue from patients with chronic kidney disease. Applied Surface Science, 2008 May; 255: 1177-1180.

II. **Dalila Belazi**, Santiago Solé-Domènech, Björn Johansson, Martin Schalling, Peter Sjövall. Chemical analysis of osmium tetroxide staining in adipose tissue using imaging ToF-SIMS. Histochem Cell Biol, 2009 March; 132: 105-115.

III. **Dalila Belazi**, Annika Sääf, Birgit Hagenhoff, Felix Kollmer, Elke Tallarek, Björn Johansson, Magnus Nordenskjöld, Martin Schalling. ToF-SIMS mass spectrometric imaging of normal human skin. (Manuscript)

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LIST OF ABBREVIATIONS

AD	Atopic dermatitis
CAPD	Continuous ambulatory peritoneal dialysis
CKD	Chronic kidney disease
CVD	Cardio-vascular disease
DAG	Diacylglycerol
DM	Diabetes mellitus
DSIMS	Dynamic secondary ion mass spectrometry
FA	Fatty acid
FFA	Free fatty acid
GRF	Glomerular filtration rate
HDL	High-density lipoprotein
HL	Hepatic lipase
LB	Lamellar bodies
H&E	Hematoxylin and eosin
LDL	Low-density lipoprotein
LMIG	Liquid metal ion gun
LPL	Lipoprotein lipase
LRP	LDL-related protein
MAG	Monoacylglycerol
MALDI	Matrix-assisted laser desorption/ionization
MUFA	Monounsaturated fatty acid
PBS	Phosphate-buffered saline
PC	Principal Component
PCA	Principal Component Analysis
PI	Primary ion
PIDD	Primary ion dose density
PKC	Protein kinase C
PUFA	Polyunsaturated fatty acid
SB	Stratum basale
SC	Stratum corneum
SFA	Saturated fatty acid
SG	Stratum granulosum

SL	Stratum lucidum
SI	Secondary ion
SS	Stratum spinosum
SSIMS	Static secondary ion mass spectrometry
TAG	Triacylglycerol
ToF-SIMS	Time of flight secondary ion mass spectrometry
UFA	Unsaturated fatty acid
VLDL	Very low-density lipoprotein
WAT	White adipose tissue
Å	Ångström (10^{-10} m)

1 INTRODUCTION

In the last decade, mass spectrometry has become an important tool in the analysis of complex biological systems. Mass spectrometry gives simultaneous, non-presumptive detection of the major classes of bio-substances. The mass spectrometry used in biology was traditionally limited by its lack of imaging ability and unsuitable for work with tissue samples (Johansson, 2006). Each biological tissue is comprised of several cell types that work together to carry out the function of an organ. The ability to study distributions as well the concentrations of bio-compounds in a cell or tissue is important. Research and diagnosis is therefore much helped by methods that resolve individual cells or groups of related cells.

Time-of-flight secondary ion mass spectrometry (ToF-SIMS) has emerged as a surface analytical method for chemical microanalysis of biological samples. It allows the determination of specific bio-substances, allowing parallel detection of many analytes with microscopic imaging abilities (McDonnell, 2007). In ToF-SIMS, mass spectra are recorded from the outermost molecular layers of solid samples and ion images captured. Recent studies, including this thesis, have shown that the method is capable of providing chemical images of cells and tissues at resolutions down to 0.5 µm for specific molecules up to a few thousand daltons including a large set of lipids, without the need for chemical labeling or the application of a matrix to the sample. ToF-SIMS seems ideal for analysis of the chemistry of clinical tissue samples because it combines a comprehensive chemical analysis, particularly of lipids (mass spectrometry), with the ability to image tissues and cells at high resolution. Thus, ToF-SIMS makes it possible to visualize and quantify lipids in the context of tissue structure. While much work has been done with artificial materials (see above) and some with cultured cells and animal tissue, very little work has been done so far with clinically relevant tissue samples.

Based on our preliminary results and other research results involving diseases related to adipose tissue, lipids and inflammation (atopic dermatitis, kidney failure, atherosclerosis), the unsaturated fatty acids were omnipresent as a part of the final observations and as a cause/result of metabolism alterations (Sjögren et al., 2007), (Nikolakakis et al., 1999). There is excellent evidence that dietary fats can either counteract or worsen diseases such as inflammatory bowel disease, atherosclerosis, lung fibrosis, rheumatoid arthritis, and others. Despite strong evidence for such

modulation, its mechanisms are poorly unknown (García et al., 2006). While dietary effects on blood lipids have been studied with reasonable ease, effects on tissue and particularly the target cells have hitherto been more difficult to study. The ability of ToF-SIMS to detect and visualize unsaturated fatty acids in tissue was therefore evaluated in this project.

1.1 TOF-SIMS

The procedure of ToF-SIMS analysis is by introducing the solid sample into an analysis chamber, where it will be bombarded by a short-pulsed beam with high kinetic energy (1-25keV). The primary ions (PI) used for bombardment can be mono-atomic or polyatomic clusters. The impact of these PI with the sample surface results in a collision cascade at the outermost monolayer of the sample, passing some of the primary ion kinetic energy onto the surface, leading to emission of neutral particles and positively and negatively charged secondary ions (SI). These SIs are formed by attachment of protons, loss of hydrogen atom, fragmentation and sometimes rearrangement of the molecules (Grams, 2007). The SI within 10Å are emitted from the surface when their kinetic energy surpasses the binding energy of the surface (McDonnell, 2007). The arising SIs are then accelerated into a field-free drift region with a nominal kinetic energy (Schueler, 1992). The ToF-SIMS principle allows high transmission of SIs, high sensitivity, high mass range and high lateral resolution. The detection of the different ions is done in parallel; the high resolution allows the distinction between molecules with the same nominal mass (Schueler, 1992). The SIs are then accelerated by potential V_s and will travel a distance d in the field free region to reach the detector. The ions of same charge will have equal kinetic energies. The time it takes for these ions to reach the detector is used to calculate mass-to-charge ratio.

$(m\ v^2)/2 = q\ V_s = z\ e\ V_s = E_k$

$t = d/v \rightarrow t^2 = (m/z)(d^2/2V_s\ e).$

m/z is calculated from measuring t^2, since $(d^2/2V_s\ e)$ is constant. The particles with lowest mass will travel faster into the detector and vice versa (De Hoffmann, 2007). A mass spectrum is recorded that gives information about the molecular composition and

distribution of the analyzed surface. It has been observed the SI yield decreases with increasing m/z (McDonnell, 2007, Touboul et al., 2004).

Two different modes can be used with SIMS, depending of the ion dose. At low primary ion dose density, also called static SIMS, the analysis is made primarily on non-damaged surface area, while in dynamic SIMS (DSIMS), a very high primary ion dose density is used, and analysis is made on a molecularly damaged surface. DSIMS as opposed to static SIMS is surface erosive, enabling the depth profiling of a sample from the outermost atoms to a micron or more into the sample (Wise, 1995). The damaging effect of dynamic SIMS allows only detection of atoms or small fragments. In static SIMS less than 1% of the outermost monolayer is bombarded. A static limit has been calculated and is defined as the primary ion dose density (PIDD) beyond which artifacts emerge in the data. The PIDD for organic samples is considered to be 10^{12} primary ions/cm^2 (Kuo, 2007). ToF-SIMS in the static mode is specific for the outermost monolayer of a solid sample, leaving the surface almost intact. It requires much less than 1% of the surface to be removed by never bombarding the same spot more than once. Many studies have reported that the mass spectra reflect the virgin surface chemistry accurately if the analysis is below the static-limit (Clark, 1988). Static SIMS is mainly used to study solid samples and gives a high resolution chemical map of a solid sample by scanning a focused ion beam across its surface, as opposed to dynamic SIMS that causes surface erosion (De Hoffmann, 2007).

1.1.1 SIMS ionization mechanism

Imaging mass spectrometry is mostly based on either SIMS or MALDI principles (Todd et al., 2001), and each technique offers different capabilities. MALDI is mainly used for studying the spatial distribution of high-mass molecules and the typical spatial resolution is about 25 μm. SIMS on the other hand can provide high spatial resolution images but the signal often drops at m/z >1000. With the recent introduction in ToF-SIMS of liquid metal ion guns with a spot diameter > 100 nm, its mass range has been extended while retaining high spatial resolution.

1.1.2 Mass analysis

ToF is the mass analyzer used for this technique, which is either circular with electrostatic analyzers to direct the particle beams, or linear (the model used in this project) using a reflecting mirror. ToF analyzer is capable to detect simultaneously all masses of the same charge (Rivière. 1998).

1.1.3 ToF-SIMS for biological analysis

Thanks to the latest polyatomic cluster ion sources, the analysis of biological samples became possible, allowing efficient detection of organic species and their distribution laterally and in depth. However since biological cells and tissues usually have a very complex composition and since some molecules are fragmented within the mass spectrometer, the gathered information is sometimes difficult to interpret. Interpretation of biological data obtained with ToF-SIMS is still at its starting point, where tools and softwares are necessary in order to simplify the dataset but also to help interpretation, such as principal component analysis (PCA) and other multivariate analysis methods.

1.1.4 Artifacts (potential confounding factors)

Topography: When a sample contains a topography, its spatial distribution is distorted since it is presented in a 2D array. Other problems are also possible such as ionizations biases. That is why in SIMS, the angle of impact of PI is very important for the ionization yield. Another potential cofounding factor is the matrix effect. The matrix effect is defined as the dependency of an ion yield of an analyte to its chemical environment (McDonnell, 2007).

1.1.5 Primary ion sources

For SIMS mass spectrometry, the SI yield is directly dependent to the PI source. Different types of ion guns are available and could be classified in 3 different categories; surface ionization guns, liquid metal ion guns (LMIG) and gas ion guns.

1.1.6 Modes

Different modes of operation can be used during ToF-SIMS measurements, surface spectroscopy, imaging or depth profiling. These 3 modes allow the obtention of informations about the chemical composition and localization of different substances.

Quantitative information on the other hand is more difficult to get because of the matrix effect. But it is still possible to obtain semi-quantitative informations by normalization of the spectra. This is done by using an internal standard molecule that shows a signal intensity that is stable in different measurements. The emission intensity of the signals is presented then in comparison with the reference signal used for normalization. The imaging capability in ToF-SIMS allows the determination and the localization of different molecules at the surface of a sample. The combination of the operational mode the type of primary ions, angle of incidence, primary ion dose density and the topography of the analyzed material decides the quality of images. The depth profiling give an estimation of concentration changes at different depths. ToF-SIMS results improved with the increase in the emission intensity of SI due to the use polyatomic PI beams. However, apart from better sensitivity and lower fragmentation, large spot size of cluster ion beam was observed. Only liquid metal ion cluster sources combined the criteria of high lateral resolution and high cluster current (Grams, 2007). Gold cluster ion gun was very popular until ION-TOF GmbH demonstrated in 2006 a new generation of instruments with bismuth cluster ion source.

1.1.7 Improvement of ToF-SIMS for biological application

ToF-SIMS use for biological application necessitated improvement of the instrument for better mass and lateral/spatial resolution. The use of the right PI is primordial for SI emission and quality of the result.
Gallium ion source was advantageous compared to other ion sources with indium, argon and other PI. Later on gold cluster sources such as Au_2^+ and Au_3^+ showed an yield enhancement of ten-fold compared to the gallium. The main disadvantage with gold cluster ion source was the short lifetime of the source (Hill et al., 2004). The use of gold cluster PI gave a higher mass range allowing the imaging of different lipid classes of e.g. phospholipids, cholesterol and sulfatide.

1.1.8 Principal Component Analysis

Principal component analysis (PCA) is a statistical method used for analyzing data with a large number of variables. PCA reduces the number of variables when redundancy is suspected. When correlation between some variables is found, PCA reduces the number of variables by creating artificial ones that show the most of the variance. The first PCs are the one with most variation (Jolliffe, 2002).

1.2 LIPIDS

Lipids are made of different classes of molecules and share the same ability to dissolve in organic and nonpolar solvents. Lipids have a high energy value and are important for the fat-soluble vitamins and essential fats; therefore they are important dietary constituents (Botham, 2006, Koolman, 2005). Lipids can be hydrophobic or amphiphilic. Lipids have important biological functions that include fuel and energy storage in the adipocytes in form of neutral fats named triacylglycerols (TAG), as nutrients when amphipathic lipids are used to build cell membranes, as structural components of cell membranes, and as signaling molecules for signal transduction as secondary messengers or in the production of prostaglandins, mechanical and thermal insulator in the subcutaneous tissues and around various organs but also as mechanical insulators or electrical insulator for the cells (Holum 1998, Koolman 2005) necessary for the rapid propagation of depolarization along the nerves. Lipids can also act as hormones, mediators and vitamins (Koolman 2005). The lipid-protein combination is an important constituent in both membrane and mitochondria, and also functions as transporter for lipids in the blood (Botham 2006). Understanding lipids and their chemistry is obviously important in the study of diseases involving obesity, diabetes mellitus etc. Complex lipids such as cholesterol, phospholipids and glycolipids are major constituents of cell membranes. The hydrophilic groups of the phospholipids and glycolipids attract water to optimize contact of the molecules with the extra- and intracellular fluids. The hydrophobic groups force membrane molecules to be positioned in the interior of the membrane away from the water (Holum, 1998).

1.2.1 Fatty acids

Vegetable and animal fatty acids share the same features, they are monocarboxylic acids: RCO_2H, unbranched chain as R group. Usually FAs contain an even number of C atoms and can be saturated or unsaturated. FAs are constituted of 2 functional groups, the carboxyl group and the alkene double bond. Palmitic acid (C16:0) and stearic acid (C18:0) are the most abundant saturated fatty acids (SFA). FA with carbon atoms <16 are relatively rare in nature. Unsaturated fatty acids (UFA) like palmitoleic acid, oleic acid (most abundant), linoleic acid and linolenic acid have cis-double bonds. This cis-geometry of the alkene groups affects their melting point and the more kinks there are in the carbon chain, the lower is the melting point. Essential FA and fat-soluble vitamins are not formed in the human body (Holum, 1998).
Fatty acids can be free in the body or part of more complex molecules such as triacylglycerols. Free FA are oxidized by many tissues (liver, muscle) for energy.
If the FA has 2 or more double bonds, they are usually spaced at 3 carbons interval.
Essential fatty acids, necessary dietary constituents for humans are linoleic acid and alpha-linolenic acid. If linoleic acid is deficient, arachidonic acid becomes essential (Champe, 2008).

1.2.2 Triacylglycerols

The adipose tissue triacylglycerols (TAG) are mainly used as a source of energy and are completely hydrolyzed into FAs and glycerol by a lipase enzyme. Later on, the FA will be released and oxidized in the mitochondria, forming water and carbon dioxide. The digested triacylglycerols are hydrolyzed into monoacylglycerols (MAG), FA and diacylglycerols (DAG) by pancreatic lipase (Champe, 2008).

1.2.3 Phospholipids

Phospholipids are esters of glycerol or sphingosine. They are small and very polar and play an important role in the formation of cell membranes. Glycerophospholipids have phosphate unit and two acyl units and could be divided in two groups: phosphatides and plasmalogens. Sphinglolipids in the other hand are based on sphingosine (Holum, 1998). Phospholipids are the predominant lipids cell membrane and function as

reservoir for intracellular messengers and some proteins and act as anchors to cell membrane (Champe, 2008).

1.3 ADIPOSE TISSUE, AN ENDOCRINE ORGAN

During the last decade white adipose tissue has been recognized not only as an inert storage depot (Axelsson et al., 2006) but also as the largest endocrine organ and source for different hormones, proinflammatory cytokines, chemokines and adipokines (also called adipocytokines).

1.3.1 Role of white adipose tissue (WAT)

WAT can be found in all vertebrates, and it is only in the last decades that it has been recognized as an anatomically organized tissue with properties specific to the site. White adipocytes have a simple structure and are full of TAGs when there is an energy excess and deliver fatty acids by enzymatic hydrolysis of TAGs to tissues when it is required. WAT is also responsible for secreting leptin, which plays an important role in the regulation of appetite, body weight and the total amount of adipose tissue, by circulating in the blood stream and signaling to the brain about the amount of stored adipose tissue. Leptin decreases during fasting periods or weight loss and increases in case of overfeeding or weight gain. WAT also secrete pro-inflammatory cytokines, angiotensinogen, adipsin, acylation-simulating protein, adiponectin, retinol-binding protein, tumor necrosis factor alpha, interleukin 6, plasminogen activator inhibitor-1 and tissue factor (Trayhurn et al., 2001). The traditional view of WAT is that it comprises a long-term fuel reserve that can be mobilized during fasting by the release of fatty acids that are in turn oxidized in other organs. WAT also provides thermal insulation, and a mechanical role (Trayhurn et al., 2001). Furthermore it has an inflammatory role through preadipocytes functioning as macrophage-like cells and role in glucose regulation (Cousin et al., 1999). Leptin secreted principally from adipocytes and acts in the brain, in particular the hypothalamus and in peripheral organs. WAT therefore play an important role in metabolic regulation and physiological homeostasis. Fatty acids are the major secretory product of WAT. Cholesterol is also stored and used

in the synthesis of steroid hormones (glucocorticoids and sex hormones) (Trayhurn et al., 2001).

1.3.2 Adipose tissue structure

Different forms of adipose tissue can be found at different site of the body e.g. perirenal, mesenteric, omental, perineal and pericardial WAT. WAT adipocytes are monolocular and can be 100 μm or more in diameter. Inside each adipocyte a droplet of lipids is found determining the size of the cell. Adipose tissues are richly supplied with blood vessels, and the blood capillaries are situated where three adjacent adipocytes meet. The fat cells are surrounded by a basal lamina and the cytoplasm contains a small Golgi complex, filaments, free ribosomes and mitochondria (Ross, 1989).
Heredity and environmental factors determine the individual amount of adipose tissue (Ross, 1989). The main lipid of adipose tissues is triacylglycerols (>89%).
Visceral fat mass and adipocyte size has been associated with insulin resistance (Cases et al., 2000), (Vázquez-Vela et al., 2008). The removal of visceral fat mass, but not subcutaneous fat mass improved insulin sensitivity, but this observation does not mean that subcutaneous adipose tissue are not related to metabolic abnormalities. Increase in abdominal fat mass, either visceral or subcutaneous has shown to be related to dyslipidemia, glucose intolerance, hypertension, hypercoagulable state, and cardiovascular risk (Vázquez-Vela et al., 2008).

1.4 CHRONIC KIDNEY DISEASE (CKD)

Patients suffering from chronic kidney disease (CKD) have a permanent kidney damage that can be related to a previous disease or injury. CKD is reaching epidemic proportions (El Nahas et al., 2005). Statistics from the National Institute of Diabetes & Digestive & Kidney Diseases shows that more then 20 million Americans (the equivalent of 1 of nine adults) have CKD and most of these individuals do not know about it, and further 20 millions are at risk (http://kidney.niddk.nih.gov, visited 2009-09-12). In Sweden 2008, there were approximately 35 000 patients suffering from chronic kidney disease and about 7000 with kidney failure; the number of individuals with CKD is suspected to rise by 6-8% per year (http://www.internetmedicin.se/,

visited 2009-10-19). Patients with CKD have a high risk to develop cardiovascular disease (CVD) (Chmielewski et al., 2009) and show major proatherogenic lipid abnormalities (Harper et al., 2008) that could lead to a premature death (Chmielewski et al., 2009) even before dialysis becomes necessary (Harper et al., 2008), and at the same time possible treatments or therapies for CVD are usually contraindicated because of safety concerns and lack of evidence of benefit in this patient population suffering from renal insufficiency (Chmielewski et al., 2009). There are 5 different stages in CKD depending on the albuminuria and the glomerular filtration rate (GFR), where stage 1 means kidney damage with normal or increased GRF and stage 5 means kidney failure with GFR<15 or dialysis (Chmielewski et al., 2009).

1.4.1 CKD and lipid metabolism

Hypertriglyceridemia and low concentration of high-density lipoproteins (HDL) cholesterol are disorders often observed in CKD patients (Harper et al., 2008, Kaysen 2009). The expression of key enzymes responsible for lipolysis such as lipoprotein lipase (LPL) and hepatic lipase (HL) are diminished (Chan et al., 2007). A decreased expression of LDL-related protein (LRP) and VLDL receptor are also observed. Low HDL-cholesterol is also observed in CKD patients. LDL-cholesterol on the other hand seems to be usually within the normal range but LDL particles are often small and more dense then the normal and are believed to be highly atherogenic (Kwan et al., 2007). Disturbances of carbohydrates metabolism is also observed in CKD patients, such as insulin resistance or due to a primary disease such as diabetes mellitus (DM), one of the major causes of CKD (Uzma et al., 2009). CKD patients have a high risk of premature death, mainly due to cardiovascular diseases (CVD). Clear links have been established between kidney dysfunction and cardiovascular risk starting at early stage of CKD when GRF has reached 75ml/min. The risk of CVD increases progressively with decreased kidney function (Go et al., 2004, Hallan et al., 2006, Kottgen et al., 2007). Many CKD patients develop CVD very quickly. For this and other reasons, the conventional risk factors are unlikely to be the only explanation for the accelerated CVD (McCullough et al., 2008). The metabolic alterations in CKD and uremic toxins are also considered to be factors for developing CVD. Proatherogenic lipid abnormalities in CKD (Harper et al., 2008) can be fatal (Chmielewski et al., 2009) even before dialysis is needed (Harper et al., 2008). The usual therapies for CVD are often

contraindicated for safety reasons and lack of evidence that suggests benefit in this patient population suffering from renal insufficiency (Chmielewski et al., 2009). Hyper-lipidemia and dyslipidemia in form of increased triglycerides (TAG) and reduced HDL cholesterol, common abnormalities in CVD (Kaysen, 2009). Both respond to drug, diet and surgical treatment. However treatment efficiency is related to renal function. CKD Patients in stage 1 to 3 may respond to lipid-lowering therapies by a reduced CVD risk and preserved renal function (Kaysen, 2009). Besides high TAGs and low HDL cholesterol, blood analysis of CKD patients (cytokines, albumin…) show signs of inflammation associated with CVD. Reduced expression of lipolytic enzymes such as lipoprotein lipase (LPL) and hepatic lipase (HL) is found in CKD patients. The same observation is also made for the expression of LDL related protein (LRP) and VLDL receptor are also observed. While LDL-cholesterol is usually in the normal range, LDL particles often small, dense and believed to be highly atherogenic. Disturbed carbohydrate metabolism is also found in CKD, such as insulin resistance, this may be due to a primary disease such as diabetes mellitus (DM), a major cause of CKD (Uzma et al., 2009).

1.4.2 CKD and fat tissue

A relationship between obesity and insulin resistance is well known. In obesity increases circulating free FA and inflammatory signaling (Axelsson, 2008). Adipose tissue regulates metabolism by adipokine secretion and regulation of feeding behavior. Hypertrophy of adipocytes in obesity may lead to recruitment of tissue macrophages promoting inflammation in adipose tissue. Such a pro-inflammatory state stimulates lipolysis in adipose tissue, limits production of TAGs and promotes insulin resistance (Axelsson, 2008).

1.5 OSMIUM

Osmium is a rare metal that is considered to be in close association with iridium. Osmium tetroxide has a molecular weight 254,2 Da with a melting point at 41^{o}C and boiling point at 131^{o}C. It dissolves slowly in water and is very volatile at room temperature (Hayat, 1970).

Osmium (8) tetroxide (OsO_4) is a metallic ion used in cytology for fixation and staining of tissues. OsO_4 is a non-coagulant fixative and is also used as stain in microscopy. Reduction of osmium in osmiophilic tissue structures results in strong, electron-dense staining. OsO_4 also stabilizes some proteins without destroying tissue structural features, preventing coagulation during dehydration in alcohols.
OsO_4 has been widely used since the early age of electron microscopy because it preserves cytoplasmic details of biological materials, giving better results than fixation with acid fixation. It has been claimed that mixture of OsO_4 and glutaraldehyde cannot be surpassed by any other combination (Hayat, 1970).
Before the work of this thesis, very little was known regarding the degree of destruction tissue components due to OsO_4 fixation. In order to properly interpret histology results, it appears important during fixation to know the reaction product, the compound to which the fixative reacts and also the site of reaction. In some previous literature (Litman et al., 1972, Nielson et al., 1979), OsO_4 did not appear to alter or bind to saturated fatty acids so FA are more likely to be involved in the fixation process.
It is also known that oleic acid and its triglycerides olein reduce OsO_4 in contrast to saturated FA such as palmitic acid and stearic acid and triglycerides containing them.

1.6 SKIN

The skin is considered to be the largest organ of the human body. It makes up about 16% of the body weight and about 1,8 m^2 of surface area (Gawkrodger, 2002). The skin is the interface between body and external world (Freinkel, 2001), thereby protecting the body from the environment. The differentiated structure of the skin also acts as a body temperature holder, and is important for sexual attraction in some species (Gawkrodger, 2002). Loss of as little as 20% of the skin can lead to death (Gawkrodger, 2002).
The human skin is surprisingly complex but can be divided into two main layers; epidermis, dermis. The composition of the skin is heterogeneous, containing different types of cells. Characteristic for the epidermis is its rapid replication and reparation capacity. However, all skin layers offer some protection by presenting a barrier to small molecules (Freinkel, 2001). From evolution and change of environment from aquatic to terrestrial, the skin has adapted by producing lipids and forming an outer shell of dead

cells. Furthermore, the lipid phase evolved to include polar lipids for a better permeable barrier. These polar lipids are organized in the SC as bilayers parallel to the skin surface, preventing hydrophilic and hydrophobic substances from entering or leaving (Engström et al., 2000). Within the skin, several structures called appendages, i.e., sweat glands, hair follicles, sebaceous glands, aporine glands can be found (Freinkel, 2001). Healthy and abnormal skin and body areas differ in the term of skin thickness, density and composition (Freinkel, 2001, Gawkrodger, 2002).

The epidermal epithelium is a continuously renewed stratified squamous epithelium whose thickness varies from 0.1 and 1,4 mm, depending on location (Gawkrodger, 2002).
Thick epidermis is comprised by 5 different layers, from deep to superficial, the layers are; stratum germinativum also called stratum basale (SB), stratum spinosum (SS), stratum granulosum (SG), stratum lucidum (SL), and the stratum corneum (SC). However, SL is missing if the skin is thin.

1.6.1 Skin physical barrier

As mentioned, the major barrier of the skin is the exterior layer of the epidermis.
The SC consists of corneocytes and lipid-enriched intercellular domains that are critical to the physical barrier. The barrier formation starts at the basal layer as a result of the cell division differentiation. Terminal cell differentiation starts when the basal cells exit the cell cycle by becoming unable to adhere to the basement membrane (Segre, 2006). In the SS, the cells are strengthened by keratin filaments (Segre, 2006). During migration from SB to SC, biochemical reactions take place: keratins, cornified envelope-associated proteins and keratinocytes are produced. Lipids necessary for barrier function are produced then stored in the epidermal lamellar bodies of GL. The lamellar bodies contain different types of lipids: phospholipids, glucosylceramides, cholesterol, as well as hydrolytic enzymes needed for the conversion of the aforementioned lipids (except cholesterol) to free fatty acids and ceramides (Proksch et al., 2006).

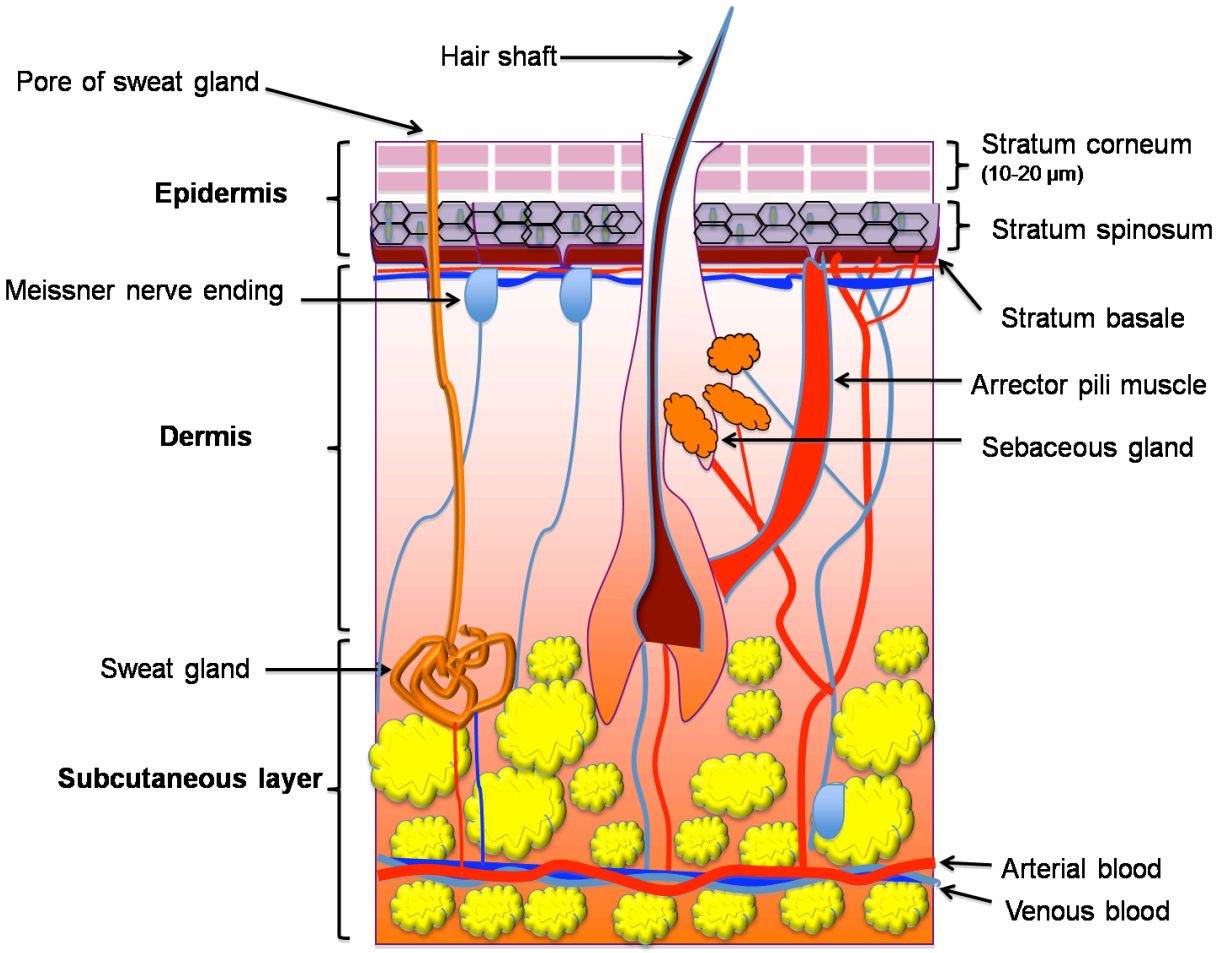

Figure 1: Schematic drawing of the different layers of the skin

1.6.2 Lipids and barrier function

Lipids are synthesized in all nucleated cell layers of the epidermis. These lipids are then stored in lamellar bodies (LB). When free FA and ceramides are synthesized from cholesterol, phospholipids and glucosylceramides by hydrolytic enzymes, lamellar membrane unit membranes form due to a sequence of changes in membrane structure. Proteases are secreted by LB and delivered to the SC (Proksch et al., 2006). The cholesterol, ceramides and free FA the SC are in equimolar quantities. SC also has smaller amounts of nonpolar lipids and cholesterol sulfate. The epidermis is a site of high lipid synthesis, completely autonomous from the circulating lipids (Proksch et al., 2006). This lipid synthesis is subject to regulation if the barrier is altered. There appears to be a relationship between skin barrier disturbances and altered lipid synthesis when skin barrier has been altered, a recovery response is provoked that delivers newly produced lipids to the extracellular fluid of the SC, driven by a local metabolic response at the injured site.

2 STUDY AIMS

The overall aim is to establish ToF-SIMS (Time of Flight-Secondary Ion Mass Spectrometry) imaging mass spectrometry as a mainstream research method in medicine. This licentiate thesis project will focus on adipose tissue and skin as tissues studied by ToF-SIMS.

-To investigate if ToF-SIMS can be used as a histological imaging method.

-To investigate if ToF-SIMS can be used to analyze the chemical composition of medical tissue samples.

-To investigate if ToF-SIMS can be used to confirm the chemical mechanisms postulated to underlie existing histological stainings.

3 MATERIALS AND METHODS

3.1.1 Sample preparation

3.1.1.1 Human adipose tissue (Study I)

Biopsies of subcutaneous (fat) tissue from six patients with chronic kidney disease (CKD) (at a time point close to start of dialysis treatment) and six control subjects were retrieved and immediately frozen to −80 °C. One tissue section (thickness 15 μm) from each individual was obtained using a cryosectioning device at −18 °C. All tissue sections were placed on the same glass slide and kept frozen at −20 °C until analysis. Immediately before analysis, the tissue sections were freeze-dried in a vacuum chamber by slowly (ca. 2 h) allowing the glass slide to thaw under vacuum conditions (10^{-2} to 10^{-4} bar).

3.1.1.2 Mouse adipose tissue (Study II)

Mice (C57BL) were briefly anesthetized with CO_2 and decapitated. The gluteal fat pads were collected, immediately frozen on dry ice and stored at −80°C. The tissues were further sectioned using a cryostat device (Leica Jung CM 3000) at approximately −30°C. The 15 μm thin sections were placed on two different pre-cooled glass slides. The sectioned tissue was fixed to the glass slides by gently warming the opposite side of the slide with the finger. The samples, properly stuck to the glass, were immediately refrozen at −80°C.

3.1.1.3 Osmium tetroxide preparation (study II)

All glass material, including an OsO_4 ampoule, were cleaned with concentrated HNO_3 and rinsed in distilled water prior to preparation of the OsO_4 solution. Waste was eliminated in a controlled way.
A 4% osmium tetroxide solution was prepared in distilled water and diluted in phosphate-buffered saline at the desired final concentration.

3.1.1.4 Staining (study II)

Three samples were prepared for ToF-SIMS analysis and taken out of the freezer before proceeding with the ToF-SIMS analysis. Sample 1 (unstained) was used as a reference sample for comparison and was not stained. Sample 2 was thawed, and covered with 1 ml of 0.33% OsO_4 solution. The sample was incubated 1 min at room temperature before washing six times in distilled water. The washed sample was then dried for 30 min before proceeding with the analysis. Sample 3 was thawed and covered with 1 ml of 1.0% OsO_4 solution, incubated for 1 min, then washed in distilled water six times and finally sequentially dehydrated in 50, 70, 80 and 95% alcohol solutions, respectively.

3.1.1.5 Human skin (study III)

Biopsies of skin tissue from 2 controls were retrieved from the "back "and immediately frozen to -80^{o}C. The skin was washed with salt-containing water just before extracting the biopsies. The tissue thickness about 15um from each individual was obtained using a cryosectioning device at -18 ^{o}C and placed on glass microscopy slides. The slide-mounted sections were stored at -80^{o}C and transported on dry ice. The analytical interests are to study the lateral distribution of lipids and other analytes in the human skin.

3.1.1.6 Staining (study III)

The two skin samples were stained with H&E. The samples were incubated in hematoxylin for 10 min and then washed 4 times with distilled water. The samples were later incubated during 5 min in eosin solution (1g eosin diluted in 100ml distilled water). They were then washed in distilled water 4 times and finally sequentially dehydrated in 50, 70, 80 and 95% alcohol solutions, respectively.

3.1.2 ToF-SIMS ANALYSIS

All ToF-SIMS analysis was carried out in a TOF-SIMS IV instrument (ION-TOF GmbH) using 25 keV Bi_3^+ primary ions, and low energy electron flooding for charge compensation.

3.1.2.1 Study I

The data were acquired with the instrument optimized for high mass resolution (bunched mode mass resolution m/Δm ~5,000, lateral resolution 3–5 μm) or for high image resolution (burst alignment mode mass resolution m/Δm ~300, lateral resolution 200 nm). The pulsed primary ion current was 0.1 pA in the bunched mode and 0.04 pA in the burst alignment mode. The accumulated ion dose was always kept below 1×10^{12} ions/cm^2. During analysis the primary ion beam was scanned over the analysis area, collecting separate mass spectra of the emitted secondary ions from 128 × 128 raster points (pixels). The recorded data were used to produce total area mass spectra and/or ion images showing the signal intensity distribution over the analysis area of selected secondary ion peaks representing specific compounds.
PCA was done using PLS-Toolbox v4.0 (Eigenvector Research Inc., Manson, WA) for MATLAB /The Mathworks, Inc., Natick, MA).

3.1.2.2 Study II

The instrument was optimized for high mass resolution (bunched mode), resulting in a mass resolution $m/\Delta m$ of ~5000 and a lateral resolution of 3–5 μm. The pulsed Bi_3^+ current was 0.1 pA, the acquisition time was 150 s and the analysis area was 500 μm × 500 μm, resulting in an accumulated primary ion dose of approximately 4×10^{10} ions/cm^2, well below the so-called static limit.

3.1.2.3 Study III

The analyzed area was 100x100 μm^2 and 500x500 um^2. The acquisition time was typically 100s. The pulsed Bi_3^+ current was 0.25 pA. The measurements performed were for surface mass spectrometry and mass spectrometric imaging.
Bunched mode was used for a high mass resolution and also for a high lateral resolution with focus of 3-5 μm. The chosen mode of operation (bunched) was chosen for its high resolution for identification of surface species.

4 RESULTS AND DISCUSSION

Our results indicate that ToF-SIMS is well suited for chemical imaging of several body tissues of relevance for the study of metabolic and vascular disturbances in diabetes and CKD. Several types of molecules such as lipids, minerals and proteins could be localized, semi-quantified and visualized. The method is really suitable for detecting signals from different phospholipids, sphingolipids, triglycerides, diacylglycerols, cholesterol and others lipids.

In the last decade there has been a number of studies that show the capacity of ToF-SIMS for analyzing biological materials, including different tissues such as mouse brain, human and mouse adipose tissue, cardiac tissue, muscle, kidney, single cultured cells (Aranyosiova et al., 2006, Johansson, 2006, Magnusson et al., 2008, Nygren et al., 2005, Sjövall et al., 2006, Sjövall et al., 2008). However, ToF-SIMS has seldom used for clinical studies. In the pre-ToF-SIMS period, it was impossible to obtain "fingerprintable" molecular mass information as a function of spatial distribution of polyatomic molecules consisting of more than two atoms (Francis et al., 2008). Most other mass spectrometry techniques such as electrospray with MS require sample preparation by homogenization and extraction, completely eliminating the possibility to obtain any kind of spatial information. The imaging version of MALDI-ToF on the other hand has a limited spatial resolution limiting its imaging capacity for biological cells. Other imaging surface analytical techniques such as FTIR, Raman and XPS can cause severe sample damage and have limited analysis capabilities and sensitivity. Another form of imaging mass spectrometry based on MALDI has also been used to study skin, making comparisons with H&E histology (Bunch et al., 2004). These authors successfully monitored the absorption of the antifungal ketoconazole through pig skin, although the spatial resolution (approx. 100 μm) is clearly inferior to that for other analytes in our present study.

The sub-studies of this project were chosen as examples of medical ToF-SIMS applications. We tried to show three different possible medical applications of ToF-SIMS technology: chemical analysis, imaging and study of a patient group. The investigations of human and mouse adipose tissue, and human skin were all possible by

means of ToF-SIMS. All our ToF-SIMS experiments were carried out on thin tissue sections of approximately 15 μm. Bunched mode was used for its high mass resolution in all three studies. Negative and positive spectra from adipose tissues and skin were captured and analyzed. Lateral distribution of a number of lipids, such as different fatty acids, glycerol lipids, phospholipids and other key constituents of cells, were imaged.

In paper I, II and III a large number of different lipids were identified in human and mouse adipose tissue and human skin, respectively. High signals representing different types of lipids were detected in negative and positive mode. The lipid profiling of the different analyzed biological samples served different purposes. Our studies add to evidence in the past few years of literature that has suggested that ToF-SIMS sensitivity and resolution permits cellular imaging of the several lipids and minerals of likely pathological importance in many diseases related to lipid changes.

4.1 PAPER I

In paper I, ToF-SIMS data was used to study the postulated causal role of abnormalities in adipose tissue in CKD. This lipid profiling was used for correlative study of the clinical data, which was expected to provide clues to underlying pathophysiological mechanisms. The subcutaneous adipose tissues from 6 CKD patients and 6 controls without known renal disease were analyzed. There was an obvious need to correlate the ToF-SIMS data with the clinical data of the studied individuals. For that purpose, PCA was used in order to compare the gathered data and investigate possible systematic variations in different lipids, and identify the mass peaks that showed the greatest variability. Intense signals representing different types of lipids from different areas of the samples (fatty acids, diaclglycerols and triacylglycerols) were detected. TAGs and FAs were mainly detected in the negative spectra while DAGs were detected in the positive spectra. Our results with PCA indicate systematic and rather major individual differences in the ratio between unsaturated and saturated lipids. The comparison between the data from CKD patients and controls showed that 3 of 6 patients have the highest values in PC1 that corresponds to high signal intensity from unsaturated lipids while 3 of 6 controls showed low PC1 values that corresponds to high signal intensity from saturated lipids. These results suggest a correlation between CKD and the degree of saturation in FAs, DAGs and TAGs lipids.

There is excellent evidence that dietary fats can either ameliorate or worsen diseases such as inflammatory bowel disease, atherosclerosis, lung fibrosis and rheumatoid arthritis. In a pilot study on the Island of Crete (Nikolakakis, et al., 1999), diet-related atherogenic patterns in continuous ambulatory peritoneal dialysis (CAPD) patients were explored. They recorded dietary data from a 3-day recall with common Greek food as diet. The results showed that CAPD patients had higher level of monounsaturated fatty acids (MUFA) (higher accumulation of oleic acid), lower levels of omega-6, omega-6/omega-3 and linoleic acid but no difference in total percentage of polyunsaturated fatty acids in adipose tissues, while the percentage of saturated FA was lower in patients (maybe due to reduced palmitic acid). This of course suggests a different metabolism of fat intake in CAPD patients. The study concluded that the observed differences in our study between the 2 groups with respect to the fatty acid composition of adipose tissue do not seem to be related to dietary factors.

Adipose tissue has an important role in the regulation of whole-body glucose homeostasis in both normality and disease (Guilherme et al., 2008). There is a lot of support for the hypothesis that mobilization of FFAs from adipose tissue into the circulation and high uptake of them into skeletal muscle promote insulin resistance, rather than excess body fat per se. The importance of adipose tissue in controlling whole-body metabolism by storing fat is strengthened by the finding that a lack of adipose tissue leads to increased blood concentrations of triglycerides and fatty acids and to insulin resistance in mice and humans (Frayn, 2001). The existence of adipose tissue is also needed for normal secretion of adipokines such as leptin and adiponectin, which enhance insulin sensitivity. Human and mouse lipodystrophies reduce adipokine secretion (Guilherme et al., 2008). These observations are consistent with the notion that normal insulin sensitivity and glucose homeostasis require functional adipose tissue in proper proportion to body size. Two roles of adipose tissue seem to contribute to this: the secretion of adipokines, that influence both metabolism and neuroendocrine control of behaviors and the storage of lipids as triglycerides, which attenuates the harmful effects of both circulating FFAs and ectopic triglyceride stores (Guilherme et al., 2008). Other lipids that modulate insulin secretion and/or insulin resistance at least under some conditions are the sphingolipids ceramide and sulfatides, which can also be studied with ToF-SIMS. There is now limited information about the actual lipid profile of adipose tissue in different stages of insulin resistance. Profiling of adipose tissue lipids will therefore likely be done in diabetes models and different stages of human

diabetes. In a study done by Sjögren et al., (2008) that investigates relationships between gene expression, desaturation index and insulin resistance in adipose tissue, showed that there is a relation between desaturase gene expression and desaturation index of adipose tissue fatty acids in case of insulin resistance. Elevated stearoyl-CoA desaturase (SCD) activity within adipose tissue was observed and related to insulin resistance. In our study, two of the three CKD patients that showed high signal intensity from unsaturated lipids had diabetes. Another working hypothesis is that the differences in saturation of lipid carbon chains make themselves heard through differential activation of protein kinase C; diacylglycerols with unsaturated fatty acid substituents have been described to be much better activators of protein kinase C than those with saturated carbon chains (Shinomura et al.,1991). Similar results has been reported (Magnusson et al., 2009) in skeletal muscle where they observed an increased in MUFA signals (mainly palmitoleic acid), and to a less extent an increased PUFA signals in ob/ob mice, but not differences in saturated FA signals between the ob/ob mice and controls. Increased palmitoleic acid has also been in children with abdominal obesity (Okada et al., 2005). We think that these individual differences in the ratio between unsaturated and saturated lipids in the study I are likely to influence disease risk, by differential activation of protein kinase C. Isoforms of PKC have been viewed as candidates for mediating the effects of fat oversupply on beta-cell function and insulin resistance (Schmitz-Pfeiffer et al., 2008, Reyland 2009)

Beside the saturation degree of dietary fats, a new attention is starting to emerge regarding nutritional interesterified fats and the position of FA in the TAG molecules "The sn-2 theory". Discussions regarding the relation between plasma lipids, CVD and the positional composition of dietary fats are starting to take place. It has been found that saturated fatty acids (SFA) are at the outer positions (sn-1 and sn-3) of TAG and unsaturated FA in the middle (sn-2) in vegetable TAG, whereas in animal fat, SFA are positioned in the middle (sn-2). The positional composition of TAG is thought to affect digestibility, atherogenicity and fasting lipids. TAG containing palmitic or stearic acid in the sn-2 position is thought to be better digested and considered to be more atherogenic (Berry et al., 2009). However, this has been only proven on animal and human infant studies.

A simple division of lipids into saturated and unsaturated ones may therefore too simplistic. A broad based search for peak patterns using PCA, common in ToF-SIMS work and used in our paper I, might therefore ignore differences in abundance of

particular lipids with a role in pathophysiology, such a broad approach might therefore be beneficially combined with a target study of relevant individual lipid species. However, some lipid species are indistinguishable using current ToF-SIMS methodology, usually because they have the same mass.

4.2 PAPER II

In our quest for unsaturated fatty acids in medical tissue samples, osmium tetroxide (OsO_4) was used for selective staining of unsaturated fatty acids in tissue samples (paper II), which were then studied using ToF-SIMS. In paper II, ToF-SIMS spectra were used to study osmium tetroxide staining by detecting and independently mapping the spatial distributions of different specific lipid species and osmium oxide species in mouse adipose tissue. Osmium tetroxide is thought to stain unsaturated lipids and not saturated lipids and multiple descriptions about its specific binding sites can be found in different works (Collin et al., 1974, Korn et al., 1967). The samples were analyzed by ToF-SIMS before and after staining just in order to investigate how OsO_4 reacts with unsaturated fatty acids. The results helped us to confirm the specificity of the stain and to understand the binding mechanism behind the staining which until now was uncertain and hypothetical. With ToF-SIMS, visualization of all unsaturated fatty acids was possible just by selecting the molecular masses of the molecules of interest. The result with osmium is evidence that osmium indeed stains unsaturated lipids, as proposed in the literature. Because of the strong reactivity of OsO_4, a comparison of the lipid distribution before and after osmium staining was necessary. The results suggest that two different types of osmium species form during this process; one type of osmium oxide that binds specifically to unsaturated C18 fatty acids and glyceride lipids (mainly triglycerides (TAG)) containing unsaturated C18 fatty acids, while another type of osmium oxide, with a lower Os/O ratio, not colocalised with the FA and glyceride lipids but with protein fragments. Another observation was that fragment of proteins and phospholipids showed a strong decrease in signal intensity after OsO_4 staining, indicating a possible removal from the tissue surface during incubation with OsO_4. This kind of loss during fixation/staining procedure has been described earlier by (Horobin 1982) and (Takahashi et al., 1989). However, the remaining proteins and phospholipids were strongly colocalised with type II osmium oxide. We also observed a redistribution of lipids in the adipose tissue after osmium staining suggesting, that

both physical and chemical modification is possible with this staining. However, this observation is for adipose tissue only, additional studies are needed to determine whether such modification is possible on other types of tissue. The co-localization observed between type I osmium oxide and unsaturated C18 fatty acids and glyceride lipids containing these fatty acids confirmed to some degree to the established view that OsO_4 binds to unsaturated lipids. However, some of the unsaturated fatty acids (C16 or C14) were not co-localized with the type I osmium, indicating that the OsO_4 binds mainly to unsaturated *C18* fatty acids chains. Such a discrimination between unsaturated C18 and C16 fatty acids was highly unexpected but, quite evident from our data. To our knowledge, no published results can either confirm or contradict our observation. The co-localization of type II osmium oxide and proteins/phospholipids agrees with the reported reaction of osmium oxide and proteins and aliphatic phospholipid side chains. The conclusion of this study is that fixation alter tissue and this can be checked by ToF-SIMS. Staining and fixation are usually needed to study the micro-anatomical structure and the chemical contents of tissues and cells by light or electron microscopy. This is largely done in order to protect the shape and content of the tissue from different kinds of degradation during processing. Different fixatives produce different chemical modifications, and leave the tissue in different physical states (Horobin 1982). Our study of osmium tetroxide as a staining dye suggests that chemical and physical modifications are indeed possible and provides insight into these modifications

4.3 PAPER III

Lipids were also detected in human skin samples and strong signal intensity of peaks representing different FAs were observed mainly superficially. These lipids were detected in different layers of the skin, but in the ion images the lipids were mainly visible in SC and at its surface. Some of these FA such as sapienic acid and lauric acid are considered to be antimicrobial and first line of defense against microbial colonization and infection (Drake et al., 2008). These antibacterial FA derive from sebaceous triglycerides. Cholesterol, DAGs and TAGs were also detected in skin but mainly localized at SC and its surface. These results confirm the reported composition of the SC composition, which is considered to be rich in lipids. High intensity signals for different minerals such as Ca, K, and Na were also observed. Elements such as Ca_2^+

and Zn are now known to function as second messengers or regulators. Ca^{2+} plays a role in cell differentiation and apoptosis (programmed cell death), which occurs in the final differentiation step between SG and SC. A balance between calcium and zinc may be needed for correctly timed apoptosis. ToF-SIMS gives strong signals for different minerals and allows to study their distribution in the different layers of the human skin. The strong peak at 430 Da in the positive spectra, presumably representing vitamin E, indicates that ToF-SIMS is a suitable method to study the presence and distribution in the skin of α-tocopherol, as has been previously described for other tissues. The only form of vitamin E that gives rise to a clearly visible peak is α-tocopherol. There is a major interest in using vitamin E (α-tocopherol and related compounds) as skin nutraceuticals. It has been proposed that better knowledge of the physiology of vitamin E, including its percutaneous penetration and skin barrier interactions could help develop more efficacious skin care products (Huang et al., 2009) and here it now appears that ToF-SIMS can be of help. This is also of interest for studying other types of tissues in order to detect possible mobilization vitamin E and its relation to different inflammatory states.
It was possible to visualize the human skin's anatomy and the chemical profile of the three main layers of the human skin. This suggest a possible use of ToF-SIMS in dermatology to help describe and understand the chemical composition of the skin in normal individuals as well as in skin diseases in which changes to lipid or inorganic content is suspected.

A very high mass resolution could be obtained with the bunching mode but at the cost of the spatial resolution, giving images considered to be fuzzy (Magnusson, 2008). The spatial resolution of ion images recorded in bunched mode is clearly worse that than that of light microscopy. The dermis and epidermis can be distinguished, but not the different layers within the epidermis. However the mass resolution of bunched mode is significantly better than that of burst alignment mode, and is necessary for the identification of many substances of medical interest. The high mass resolution of bunched mode allows an unambiguous identification of surface species. It was found that bunched mode is best suited to get an overview over the sample composition. Burst alignment mode in the other hand uses longer pulse times allowing a better spatial resolution. Burst alignment mode enabled the visualization of structures of the size of individual cells and their nuclei that merged into each other when bunched mode was used. But this affects the mass resolution by decreasing it. These two modes seems

to complement each other and therefore it is maybe more reasonable to analyze with both modes for an optimal mass and spatial resolution. For example, for a complete analysis of a skin sample, both bunched and burst alignment mode appear necessary, as each mode provides information about the sample that the other method omits.
The improved lateral resolution of a new generation of ToF-SIMS instruments (Kollmer, 2004) will be welcome, as it will help delineate and understand the different barrier layers of the epidermis and improve the analysis of many other cell and tissue structures elsewhere in the body.

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The present thesis has demonstrated possible different applications of ToF-SIMS technique and opened doors for different hypotheses regarding possible future studies. ToF-SIMS combines chemical analysis with imaging, and has been shown to function with tissue sections similar or identical to those in medical histology. The method is therefore highly useful to elucidate the chemical background of histological staining techniques. ToF-SIMS also enables the study of medical tissues without the need for fixation or staining of any kind, keeping the tissue intact from degradation resulting from chemical or physical alteration due to the preparation procedure, as we have shown in the case of osmium staining. With more widespread use and increased experience with medical ToF-SIMS, ToF-SIMS may become an important complement to microscopy when detailed chemical data about a tissue sample is needed. For the future work may be additional measurements, using ToF-SIMS or complementary techniques, to verify this observation of OsO_4 specificity and to determine how general is the material loss and possible chemical and physical modifications. ToF-SIMS mainly produces information about lipids and certain inorganic substances. It was found that our method (ToF-SIMS) is good at studying a variety of unsaturated and saturated lipids in place in clinical tissue samples. Since it was possible to observe differences between the CKD patients group and controls with respect to the fatty acid composition of adipose tissue which seem not to be related to dietary factors, further investigation is needed. A simple division of lipids into saturated and unsaturated ones may therefore be too simplistic. Since differences in saturation degree of lipids were previously described in CAPD (Crete study above) patients and in diabetes type 2 patients (Sjögren et al., 2008), it is of importance to study these groups separately in order to prevent mixing up diabetes phenotypes to CKD ones. Two of the three CKD patients that showed high signal intensity from unsaturated lipids had diabetes. Therefore a further investigation of these lipids is necessary, by studying them in relation to their number of double bonds and position and sub-grouping them into mono- or polysaturated fatty acids. A working hypothesis is that the differences in saturation of lipid carbon chains make themselves heard through differential activation of protein kinase C. Other lipids that modulate insulin secretion and/or insulin

resistance at least under some conditions are the sphingolipids, ceramide and sulfatides, which can also be studied with ToF-SIMS. There is now limited information about the actual lipid profile of adipose tissue in different stages of insulin resistance. Since a relationship has been found between desaturase genes expression and desaturation index of adipose tissue fatty acids in case of insulin resistance (Sjögren et al., 2008) accompanied with and elevated stearoyl-CoA desaturase (SCD) activity within adipose tissue, we think that the individual differences in the ratio between unsaturated and saturated lipids in the study I are likely to influence disease risk, by differential activation of protein kinase C. Isoforms of PKC have been viewed as candidates for mediating the effects of fat oversupply on beta-cell function and insulin resistance (Reyland 2009), (Schmitz-Pfeiffer et al., 2008).
It was also possible with ToF-SIMS to visualize the human skin's anatomy and the chemical profile of three main layers of the human skin. This suggest a possible use of ToF-SIMS in dermatology to help describe and understand the chemical composition of the skin in normal individuals as well as in skin diseases such as atopic dermatitis in which changes to lipid or inorganic content is suspected. ToF-SIMS is likely to be helpful in the characterization of effects of other treatments used in medicine.

Improvements in ToF-SIMS instrumentation during the current decade have produced an instrument that is clearly of interest to medical research. The simple sample preparation indicates that the method should also be under consideration for clinical diagnosis in case it produces information independent of information obtained for existing diagnostics. For future studies of tissue lipids, especially in the deeper layers of the skin, a higher sensitivity of the ToF-SIMS procedure would be advantageous. This might be achieved through optimization of procedures such as sample preparation and analysis time (recording times can probably be extended without problems) and/or improvements in equipment.

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