Study of the diacylglycerol composition in the liver and serum of mice with prediabetes and diabetes using MeV TOF-SIMS

Marijana Popović Hadžija[1]*, Zdravko Siketić[2], Mirko Hadžija[1], Marko Barac[2], Iva Bogdanović Radović[2]

[1]Division of Molecular Medicine, Laboratory for mitochondrial bioenergetics and diabetes

[2]Division of Experimental Physics, Laboratory for ion beam interactions

Ruđer Bošković Institute, Bijenička cesta 54, 10000 Zagreb, Croatia

***Corresponding author at:** Laboratory for mitochondrial bioenergetics and diabetes, Division of Molecular Medicine, Ruđer Bošković Institute, Bijenička cesta 54, 10000 Zagreb, Croatia, E-mail address: mhadzija@irb.hr, fax: 385 1 456 10 10

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Abbreviations: MeV TOF-SIMS, Time-of-Flight Secondary Ion Mass Spectrometry using MeV ions; HFD, high-fat diet; SFD, standard-fat diet; STIM, Scanning Transmission Ion Microscopy; DAGs, diacylglycerols; FAs, fatty acids; PC, phosphocholine

Abstract

Aims: Hepatic insulin resistance, induced by fat, occurs before peripheral resistance and leads to prediabetes and diabetes. If insulin resistance is detected earlier, lifestyle changes could prevent or delay disease development. Therefore, we analysed lipids in the liver and serum of prediabetic and diabetic mice by MeV TOF-SIMS with a focus on diacylglycerols (DAGs) as the best predictor of (liver) resistance.

Methods: Glucose impairment was spontaneously developed or induced by HFD in NOD/LtJ mice, and prediabetic and diabetic mice were selected according to their glucose levels. MeV TOF-SIMS was applied to image the lipid–distribution in the liver and to relatively quantify lipids related to insulin resistance in both the liver and serum.

Results: The same lipids were detected in the liver and serum but with different intensities between mice. The intensity of DAGs and fatty acids was higher in the diabetic than that in the prediabetic liver.

Imaging of liver tissue showed a more compact density of prediabetic (non-fatty) than diabetic liver with DAG remodelling in diabetes. DAGs, which are greatly increased in diabetic serum, were successfully detected and quantified already in prediabetes.

Conclusion: MeV TOF-SIMS applied to the serum presents an excellent tool for *in vivo* monitoring of disease development over time.

Keywords: lipids, liver, serum, diabetes, MeV TOF-SIMS

1. Introduction

Our modern lifestyle and mechanized society often include physical inactivity accompanied by the consumption of a high-calorie diet [1]. The consequence is energy imbalance, where caloric intake exceeds expenditure, thus contributing to the current epidemic of obesity.

The liver is the major site for converting excess dietary carbohydrates and fats into fatty acids (FAs) and triglycerides, which are then deposited first in liver cells and later in adipose tissue. It is normal to have a small amount of fat in the liver, but the build-up of fat in the liver is connected with specific hepatic insulin resistance, metabolic syndrome, and prediabetes, which increases the risk for developing type 2 diabetes [2].

Experiments with high-fat diet (HFD)-fed rodent models have shown that consuming a HFD contributes to the chronic increase in circulating free FAs. A high rate of FAs is delivered into the liver through the liver-specific fatty acid transport protein 5 (FATP5), resulting in increased levels of long-chain fatty acids (LCoAs) [3,4], which can be converted to diacylglycerols (DAGs). DAG is a glyceride consisting of two fatty acid chains covalently bonded to a glycerol molecule through ester linkages, thus forming 1,2-diacylglycerols and 1,3-diacylglycerols. Therefore, saturated fatty acids are usually bonded at the C1 position and unsaturated fatty acids at the C2 or C3 position. The accumulation of DAGs containing saturated FAs in various intracellular compartments such as the membrane or lipid droplets in hepatocytes may interfere with β-cell function and insulin signalling, causing liver-specific insulin resistance [5-7].

The link between intrahepatic DAG accumulation and hepatic insulin resistance could be attributed to the activation of protein kinase Cε (PKCε), which is the predominant PKC isoform activated in the liver with a much greater affinity for DAGs [8, 9]. Lipid-induced enzyme activation inhibits insulin receptor substrate-2 (IRS-2) tyrosine phosphorylation by the insulin receptor kinase, leading to the inability of insulin to activate hepatic glycogen synthesis and the inability to suppress hepatic glucose production.

Intrahepatic fat vacuoles and hepatic insulin resistance in mice and rats can be induced with only 3 days of high-fat feeding before the development of obesity, muscle lipid accumulation or peripheral

insulin resistance [8]. It has been demonstrated that ectopic lipids in the liver are specifically associated with hepatic insulin resistance. Among all correlated lipid components, Magkos et al. [10] demonstrated that the hepatic DAG content, but not hepatic ceramide content, was the best predictor of hepatic insulin resistance in obese humans. Additionally, shifts in lipids and lipoproteins at the periphery were detected early in the progression of metabolic syndrome in overweight rhesus monkeys, certainly by the time insulin resistance has developed and possibly even in advance of that [11]. Among them, the plasma diacylglycerol composition has been suggested as a biomarker of metabolic syndrome onset in rhesus monkeys [11].

After months or years of (hepatic) insulin resistance, blood sugar begins to rise, leading to prediabetes, when the glucose level is between 6.1-6.9 mmol/L (according to the WHO criteria for the diagnosis of prediabetes based on the fasting glucose level). In clinical settings, to check if someone has prediabetes, doctors commonly measure the fasting plasma glucose level and/or fasting blood glycosylated haemoglobin. At the same time, doctors do not test for insulin resistance since the hyperinsulinaemic-euglycemic clamp and intravenous glucose tolerance test, as the most reliable methods available for estimating insulin resistance, are time consuming and expensive. If we can determine a marker of insulin resistance in the target tissues and/or in the serum earlier, it would be possible to make some immediate and lasting lifestyle changes that could help prevent not only the onset of type 2 diabetes but also all related complications, including heart disease, vision loss, nerve damage, and kidney failure. Taking into account all of the aforementioned factors, it is clear that determining DAGs in liver tissue and/or serum could play an important role in the prediction of early events in the development of glucoregulatory impairment.

To our knowledge, there are several different ways to identify DAGs in biological samples. Most available kits use the competitive binding enzyme immunoassay system on microtitre plates. In that case, DAGs from the sample are captured on a pre-coated DAG-specific antibody and further visualized by applying enzyme-linked secondary antibody and substrate for producing visible signal, which is measured spectrophotometrically. The concentration of DAGs in the sample is determined by comparing the optical density of the samples to the standard curve.

Most of those kits are suitable for the analysis of cell lysate, plasma or serum, but some of them are able to measure the DAG content in tissue homogenate and collected supernatant, which can be applied on the microtitre plate.

DAGs in tissue can also be measured by thin layer chromatography (TLC) or liquid chromatography-mass spectrometry (LC-MS). For that purpose, tissue should be frozen and DAGs isolated through several steps that involve chloroform/methanol extraction, evaporation to dryness and re-dissolving in hexane-methylene chloride-ethyl ether and separation from triglycerides. The eluted DAG fraction is then evaporated to dryness and re-dissolved in solvent suitable for LC-MS analysis. Since the procedure for tissue preparation is complex and requires tissue homogenization, information about the 2D distribution of DAGs is lost.

It is obvious that all mentioned methods for measuring DAGs have some limitations, such as using species-specific primary antibodies, creating standard curves requirements for protocol optimization. To better understand the composition of biological samples as well as to evaluate chemical and structural pathological changes in the tissue, methods are required that offer detailed chemical information with high spatial distribution and sensitivity. The method that satisfies those conditions is Time-of-Flight-Secondary Ion Mass Spectrometry (TOF-SIMS).

TOF-SIMS was originally developed as a surface analysis technique for the identification and 2D molecular imaging of organic and inorganic materials by detecting secondary molecular ions released from the sample surface by a primary ion beam [12-14]. In recent years, the potential of the technique for implementation in life sciences has been widely explored. Fundamental advances, such as lateral resolution below 1 μm and high sensitivity for the examination and characterization of lipids, allow the utilization of TOF-SIMS for studying lipid-related diseases [15,16]. Therefore it is possible to detect multiple lipid species directly from the sample surface, which is ideal for the analysis of complex biological systems under physiological or pathological conditions.

In recent years, the capability of TOF-SIMS for surface analysis and structural investigations in biological samples has been greatly improved by using either cluster ion sources [17] or MeV ions for excitation, making identification of molecular species in the sample at the micron level easier.

We have already successfully applied MeV TOF-SIMS for imaging human adenocarcinoma cells at the subcellular level [18]. The goal of the present study was to determine early events in the development of glucoregulatory impairment in the prediabetic (spontaneously developed) and HFD-induced diabetic mouse model, with a focus on lipids in the liver tissue and also in the serum. In the liver, changes not only in the content of DAGs (as an insulin resistance predictor) but also in the content of FAs and cholesterol (whose increased levels are found in steatosis) have been determined. To our knowledge, this is the first application of MeV TOF-SIMS to determine the lipid content in mouse serum. The level of DAGs in the serum of prediabetic and diabetic mice correlates well with their blood glucose levels. Therefore, MeV TOF-SIMS is an excellent tool for tracing a lipid-based biomarker (DAGs) in the assessment of metabolic disruption and insulin resistance at an early phase, when it is possible to prevent or delay type 2 diabetes.

2. Materials and Methods

2.1. Treatment of mice and preparation of mouse liver tissue and serum

To detect and measure compounds linked to hyperglycaemia/insulin resistance in complex biological samples (liver tissue and serum), the MeV TOF-SIMS method was applied. For that purpose, normoglycaemic NOD/LtJ mice (both sexes at 8 weeks of age) were fed with either SFD (11.4% fat, 4 mice) or HFD (58% fat, 4 mice) for 4 weeks. Both groups of mice were age-matched and housed in identical environments. For detection of hyperglycaemia, blood glucose levels were measured every week by glucometer in a blood drop taken from the tail vain. Before the measurement, mice were fasted for 6 hours. All procedures were approved by the Ministry of Agriculture of Croatia, (No: UP/I-322-01/17-01/43 525-10/0255/17/3 from 19 May 2017) and carried out in accordance with associated guidelines of the EU Directive 2010/63/EU.

Two representative mice on different diets were selected for further MeV TOF-SIMS analysis according to their blood glucose levels in the last two measurements. The fasting glucose level in the blood of mouse on SFD was 6.3 mmol/L (fasting blood glucose level between 6.1 and 6.9 mmol/L is considered as prediabetes), but mouse on HFD was hyperglycaemic with a glucose concentration of

8.6 mmol/L (fasting blood glucose level equal to or higher than 7.0 mmol/L characterizes diabetes). Whole blood (20-30 μl) taken from the tail vain was collected into the appropriate tube and allowed to form a clot. The clot was removed by centrifugation, and the resulting serum was pipetted in a clear vial and stored at -80°C until MeV TOF-SIMS measurements. The animals were sacrificed by cervical dislocation, and the liver was excised. In order to preserve liver morphology and the primary distribution of molecular species, a portion of the tissue was immediately embedded in Tissue-Tek OCT Compound for Cryostat Sectioning (Sakura, The Netherlands), cryo-preserved in isopentane cooled with liquid nitrogen and stored at -80°C until cryo-sectioning and further analysis.

OCT-embedded blocks of frozen liver tissue were removed from the freezer and sectioned inside a cryostat-microtome held at -25°C. The liver tissue sections were selected to be 5 μm thick to allow transmission of 9 MeV O^{4+} ions through the sample to use high lateral resolution mode for MeV TOF-SIMS measurements [18]. One of the sections was immediately placed onto a 100-nm-thick Si_3N_4 window and freeze-dried overnight. The time for transferring the sample from the freeze-drying equipment to the vacuum chamber where MeV TOF-SIMS measurements were performed was kept below 30 minutes. Additional cryo-sections of mouse liver were stained by fat-soluble Sudan III dye for the visualization of intra-hepatic fat vacuoles.

In pilot experiments, the MeV TOF-SIMS measurement of the embedding OCT medium was performed in order to obtain the OCT molecular spectrum and to see if the mass peaks coming from the OCT interfere with the mass peaks relevant for the present measurements of liver tissue.

A portion of the mouse liver was fixed in 10% neutral buffered formalin, dehydrated in alcohol and cleared in xylene embedded in paraffin and cut into 5-μm-thick sections. Then, thin sections were stained with haematoxylin and eosin (H&E) according to the standard procedure [19]. Just before MeV TOF-SIMS analysis, serum samples were removed from -80°C. For each sample, a volume of 5 μl was dispersed as a thin film on a clean silicon wafer, dried for 2 minutes and immediately placed in the vacuum chamber for MeV TOF-SIMS measurements.

2.2. MeV TOF-SIMS analysis

Measurements were performed using a setup for MeV SIMS with linear TOF installed at the Ruđer Bošković Institute heavy ion microprobe. For homogeneous samples such as blood serum, only mass spectra were collected using the MeV TOF-SIMS setup with the pulsed ion beam. Since the used MeV TOF-SIMS spectrometer is linear type, without any timing focusing elements, mass resolution is $M/\Delta M \approx 400$ for M=262 [20]. More details about the experimental setup can be found in Tadić et al. [20]. Focused 8 MeV Si^{4+} ions were scanned over an area of approximately 500x500 μm^2. The typical lateral beam resolution was approximately 8x8 μm^2.

In order to improve lateral resolution for imaging, a continuous beam was applied instead of the pulsed primary beam, allowing the start signal for TOF from the charge particle detector placed behind the thin transmission target. This detector is usually used for Scanning Transmission Ion Microscopy (STIM), a technique that is based on the energy loss of ions in the sample that can provide information about the sample density distribution. Therefore, together with molecular images, a STIM image of the sample density distribution was also recorded [26]. For the liver tissue measurements, 9 MeV O^{4+} ions were scanned over an area of approximately 360x360 μm^2, and the typical lateral beam resolution was approximately 1x1 μm^2. As shown in Stoytschev et al. [21], secondary molecular ion yields are, among other parameters, directly proportional to the electronic stopping power of the primary ions used in the organic material, and therefore, for the sensitivity, it would be better to use silicon ions instead of oxygen as we have used for serum measurements. However, as ions have to be sufficiently energetic to reach the STIM detector after passing through the 5-μm-thick liver tissue sample, 9 MeV O^{4+} ions were selected.

Mass spectra and 2D images from liver tissue were collected using the in-house developed data acquisition system SPECTOR [22]. Positive and negative secondary molecular ions were extracted from the sample surface through the ±4 kV potential difference toward the extractor and linear TOF. Data acquisition was controlled using SPECTOR software, while data processing and normalization were performed using the MATLAB software MSiReader [23] and mMass software [24]. All SIMS maps were normalised to the total number of counts, as is usually the case in TOF-SIMS imaging. Colour scale bars, with the amplitude representing the number of counts, are indicated to the right of each image.

3. Results and Discussion

3.1. HFD-induced mouse model of fatty liver

Although the NOD strain of mice spontaneously develops an autoimmune type of diabetes, consuming a HFD leads to the faster development of impaired glucose tolerance and its complications (such as obesity and insulin resistance). The effect of high calorie intake could go directly through the promotion of beta-cell apoptosis [25,26] and indirectly through favoured lipid accumulation in hepatocytes, which has a dangerous effect on liver function, causing a high risk of developing liver insulin resistance [27, 28].

In addition to the liver, muscles and fat can also become resistant to insulin with disease progression. The pancreas must produce more insulin to help glucose enter the cells due to insulin resistance in the target tissue. In people who already have some insulin resistance, prediabetes, the condition when blood glucose level is higher than normal (fasting blood glucose level between 6.1 and 6.9 mmol/L), usually occurs. If the pancreas could not make enough insulin to overcome a weak response to insulin, the blood glucose level will reach the diabetes diagnosis level (≥7.0 mmol/L). It is estimated that 25% of persons with prediabetes will fully develop diabetes over three to five years.

In order to prevent or delay the disease progression, recommendations to the people with prediabetes are to eat healthier and to lose some body weight.

It was already shown that specific lipids accumulated in the liver are associated with hepatic insulin resistance, which can be induced by a HFD [8]. Because resistance to insulin action in the liver is developed earlier than that in the periphery, it is very important to detect hepatic insulin resistance at the very beginning, when changes in the lifestyle or the appropriate therapy can be applied. Among hepatic lipids, the DAG content is the best predictor of liver insulin resistance [10].

As already mentioned, available methods for DAG detection involve complex protocols for sample preparation, and none of the methods provide information about the spatial distribution of DAGs in tissues or cells. Here, we demonstrate that MeV TOF-SIMS can be used for the identification of not only DAGs (as a predictor of hepatic insulin resistance) but also cholesterol, phosphocholine (PC) and FAs, directly on the liver cryo-sections obtained from mice fed with a SFD or HFD. Since all these

types of lipids are normally present in the liver, we were interested in observing changes in their levels (particularly the level of DAGs) after treatment of mice with HFD in which they already developed impaired glucose tolerance and hyperglycaemia.

This is important since the measurement of the 2D distribution across diseased tissue can reveal areas of abnormal chemistry, such as scarcity or over-abundance of a particular compound, thus connecting cellular dysfunction with anatomical specificity.

One of the characteristics associated with high calorie intake is body weight gain. NOD mice on a HFD were approximately 3 g heavier than mice on a SFD at the end of the experiment. According to the expectation, the average glucose value was higher (7.7 mmol/L) after consuming a HFD compared to the glucose level of mice fed with a SFD (6.0 mmol/L). Mice fed with a SFD or HFD that were selected for MeV TOF-SIMS analysis had fasting glucose levels of 6.3 mmol/L (prediabetes) and 8.6 mmol/L (diabetes), respectively, confirming that the HFD induced metabolic disturbance. First, we explored the grade of liver deterioration caused by treatment with HFD for 4 weeks. The staining of SFD and HFD liver paraffine-embedded or cryo-sections revealed that the tissue morphology was preserved (Fig.1. upper and lower panels). In order to confirm the storage of fat in the vacuoles, standard Sudan III dye for the visualization of lipid droplets within the cytoplasm of hepatocytes was used for both mice. Fat vacuoles were predominately present in the liver of mouse fed with a HFD compared to the mouse fed a SFD (Fig. 1, lower panel). This confirms that our mouse model established by HFD is characterized by hyperglycaemia and also by fatty liver.

3.2. Imaging of liver tissue and measurement of biochemical composition

Since the sample preparation techniques, which should preserve the chemical and spatial integrity, are crucial for TOF-SIMS imaging, we have used the recommended technique for mouse liver preparation involving cryo-fixation in OCT medium, cryo-sectioning and freeze-drying [29].

The Scanning Transmission Ion Microscopy (STIM) imaging technique was first applied to assess the cryo-sections of 5-μm-hick liver tissue obtained from mice fed a SFD (Fig. 2a) or HFD (Fig. 2b). The area shown in the image is approximately 360x360 μm^2. On the left side of Fig. 2a, part of the Si_3N_4 frame can be seen where ions were completely stopped. The colour bar represents the density

distribution from the highest (dark blue colour) to the lowest density (red colour). Black arrows indicate lipid vacuoles. From the figures, it can be concluded that the energy loss of 9 MeV O^{4+} ions is larger for the SFD (Fig. 2a) than that for the HFD (Fig. 2b), indicating that the tissue of the SFD liver is more dense than that of the HFD liver. Moreover, the STIM image of the HFD liver displays lipid vacuoles in the analysed area (Fig. 2b, arrows), which are mostly absent in the image of the liver from the mouse fed with SFD (Fig. 2a), which correlates well with the numerous fat vacuoles detected by Sudan III dye (Fig.1, lower panel). Red colour presents regions with lower energy loss, which morphologically might correspond to the boundary between adjacent liver segments (Fig. 2).

The observed difference in the density of the liver tissue is due to the changes in biochemical composition as a consequence of consuming a high-fat diet, which is confirmed by measuring mass spectra. As shown in Fig. 3a, the most pronounced peaks in the spectra (measured in the positive ion mode) belong to cholesterol (m/z 369.4), DAGs (DAG 32, m/z 552 and DAG 34, m/z 578) and PC (m/z 760). It should be emphasized that the mass peaks of cholesterol and DAGs from the mouse fed with the HFD (Fig. 3a, red) are much higher than the same mass peaks belonging to the mouse fed with the SFD (Fig. 3a, black). This correlates with the fatty liver that developed in the mouse on a HFD, which was also confirmed by Sudan III staining. Additionally, the FA mass peaks (measured in the negative ion mode) are significantly higher in the liver of HFD-fed mouse than in the liver of SFD-fed mouse (Fig. 3b). Therefore, two groups of FAs were identified: the first group FA16 (C16:1 (m/z 253.2) and C16:0 (m/z 255.2)) and the second group FA18 (C18:2 (m/z 279.2), C18:1 (m/z 281.2) and C18:0 (283.2)). It is assumed that FA ions are primarily part of TAGs and phospholipids [2].

As the specific role of DAGs in hepatic insulin resistance [28, 30] has already been explained, we further assessed the composition and spatial distribution of DAG32 and DAG34 from mice fed with a SFD and HFD on the same liver sections displayed in Figure 2. The images recorded in the positive ion mode show a homogenous distribution of DAGs throughout the entire SFD liver section (Fig. 3c, upper panel), while in the HFD liver, not only a higher number of accounts but also the spatial remodelling of DAGs (Fig. 3c, lower panel) was observed. DAGs are dispersed over the analysed area, but the largest amount is localized at the boundary of segments.

As can be concluded from Fig. 3a, some small amount of DAGs exists already in SFD-fed mouse but peaks for DAG32, DAG34 and DAG36 are significantly more pronounced in the liver of HFD-fed mouse most likely due to the high calorie intake or fragmentation of TAGs [31]. Cholesterol, as a membrane lipid and mass spectrometric-friendly lipid molecule, displayed a higher signal in fatty liver than in non-fatty liver as a consequence of the disproportionate of caloric intake relative to expenditure. Some lipids are not detected *in situ,* such as fatty acids with 20 carbon atoms, which might be either due to the poor desorption/ionization yields or to intense fragmentation of these compounds [30].

We can summarize that the same lipid classes are present in the liver samples of both mice but with variations in their relative intensities; DAGs and FAs showed a higher intensity in the diabetic mouse with a fatty liver compared to the mouse with non-fatty liver. Our results are in agreement with the TOF-SIMS results obtained on the tissue samples of people suffering from non-alcoholic fatty liver disease [15, 32, 33]. Additionally, the method is successfully applied to image the location and to quantify a single compound or even a set of compounds in diseases such as colon dysplasia, osteoporosis, breast cancer or chronic obstructive pulmonary disease [34, 35, 36, 37]. To our knowledge, the only work in which TOF-SIMS was used to identify the changes in the lipid composition within mouse tissue (skeletal muscle, liver and adipose tissue) in response to the dietary intake of omega-3 fatty acids was published by Sjovall et al. [2]. However, there is lack of data about the dietary-induced events in insulin-targeted tissues that precede or cause the loss of glucose homeostasis. Here, we have shown that disrupted glucose homeostasis is due to the increase in DAGs and FAs in the liver.

3.3. MeV TOF-SIMS spectra of serum

It is obvious that HFD-induced disease in mouse liver stems from dysfunctional metabolic processes of lipids. The changes in lipid metabolism, transport and storage occur early in the progression of impaired glucose tolerance, certainly by the time of insulin resistance or possibly even before it. As demonstrated above, it can be related to the increased accumulation of DAGs and FAs in the mouse liver of HFD-fed animals compared to the SFD-fed animals (Fig. 3a, b). Studies by other authors

[10,11] have shown that DAGs are specifically responsible for liver insulin resistance and consequently impaired glucose homeostasis. The increase in glucose concentration could be detected in plasma at the very beginning, in the state of prediabetes that presents an intermediate state of hyperglycaemia. Therefore, it is very likely that metabolic changes caused by the accumulation of lipids in the liver in this state also have an effect on the periphery through the different intensities of DAGs and/or FAs. In order to test our assumption, we applied the MeV TOF-SIMS method to analyse serum from the same SFD- and HFD-fed mice from which liver samples were taken.

Only 5 µl of serum was dispersed on a clean silicon wafer as a fine, thin film and dried only a few minutes before the MeV TOF-SIMS measurement. As shown in Figures 4a, b, serum samples produce very clear mass spectra in positive and negative mode, with the most pronounced peaks from cholesterol, DAGs, PC and FAs. Among all those peaks, only peaks belonging to DAGs are greatly increased in the serum of HFD-fed mouse compared to the serum of mouse fed with SFD. This is consistent with results from the liver tissue, where higher levels of DAGs in HFD- than in SFD-fed mouse were measured. This is in agreement with the observations of other researchers found in the literature [10, 11] that the DAG composition could be taken as an early plasma marker for assessment of a metabolic syndrome risk or a marker of liver resistance.

It should be noted that the levels of FAs and cholesterol in the serum of HFD- and SFD-fed mice are almost equal, although simultaneously the same compounds in the liver are present at slightly higher levels in the HFD- than in the SFD-fed mouse (comparison of Figs. 3 and 4). From that, it can be concluded that the level of cholesterol and FAs in the mouse liver is below a critical capacity of accumulation to affect the periphery. The increase in both components with diabetes development (increasing hyperglycaemia), could be expected, but this should be investigated in the future. As far as the authors know, the MeV TOF-SIMS method was applied for the first time to analyse serum, and even a small volume (5 µl) was more than sufficient to perform the analysis. It should be emphasized that in the serum, mass spectrum peaks belonging to several lipid species could be clearly seen as well as the differences in the relative peak intensities.

4. Conclusions

The goal of this preliminary study was to determine events in the spontaneously developed or HFD-induced impairment of glucose homeostasis (state of prediabetes and diabetes), with a focus on lipids in the target tissue (liver) and body fluid (serum). The MeV TOF-SIMS method was applied to image the distribution of lipids in liver cryo-sections and to quantify the relative amount of lipid species related to insulin resistance/hyperglycaemia (particularly DAGs) in both liver and serum. The most pronounced peaks were peaks belonging to DAGs, FAs, cholesterol and PC. However, the peak intensities varied between the prediabetic and diabetic mouse and between the type of sample (liver *vs* serum). DAGs and FAs showed a higher intensity in the liver of diabetic mouse with a fatty liver compared to the prediabetic mouse with a non-fatty liver. Moreover, we observed the spatial remodelling of DAGs in the diabetic liver. The image of the density distribution showed a more compact density of prediabetic (non-fatty) liver than diabetic liver (fatty liver) due to accumulated fat in hepatocytes.

Among all peaks detected in serum, only peaks of DAGs were significantly increased in the serum of diabetic mouse. However, it should be emphasized that the DAG level was successfully detected already in the periphery of prediabetic mouse, and it could be compared to the level found in the serum of diabetic mouse.

Increased amounts of DAGs in the serum might be a distinctive marker providing information about increased accumulation of lipids in the liver (confirmed by Sudan III dye in our animal model) and the development of liver insulin resistance. These results clearly demonstrate that serum (not only specific tissues) can be useful for studying metabolic changes, and the combination of the MeV TOF-SIMS method and serum presents an excellent tool for *in vivo* investigation because serum is much easier to prepare than tissue samples, and a very small amount of serum is sufficient for the analysis. An animal does not have to be sacrificed, and samples can be taken from the same animal, allowing monitoring of disease development over time, which will be the subject of our future work.

Although the MeV TOF-SIMS method cannot acquire absolute quantitative data, the relative quantification comparing mass spectra of healthy and pathological samples is possible. The method is

highly informative because the detected compounds have a high biological significance, providing new insights into the understanding of hepatic lipid metabolism in normal and diabetic conditions.

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Author contributions

M.P.H., established animal model, performed biomedical research, wrote the paper

Z.S., designed research by MeV TOF-SIMS, performed MeV TOF-SIMS analysis, wrote the paper

M.B., collected and analysed the MeV TOF-SIMS data

M. H., interpreted the data relevant to the animal model

I. B. R., the principal investigator of the project, wrote the paper

The authors declare no conflicts of interest.

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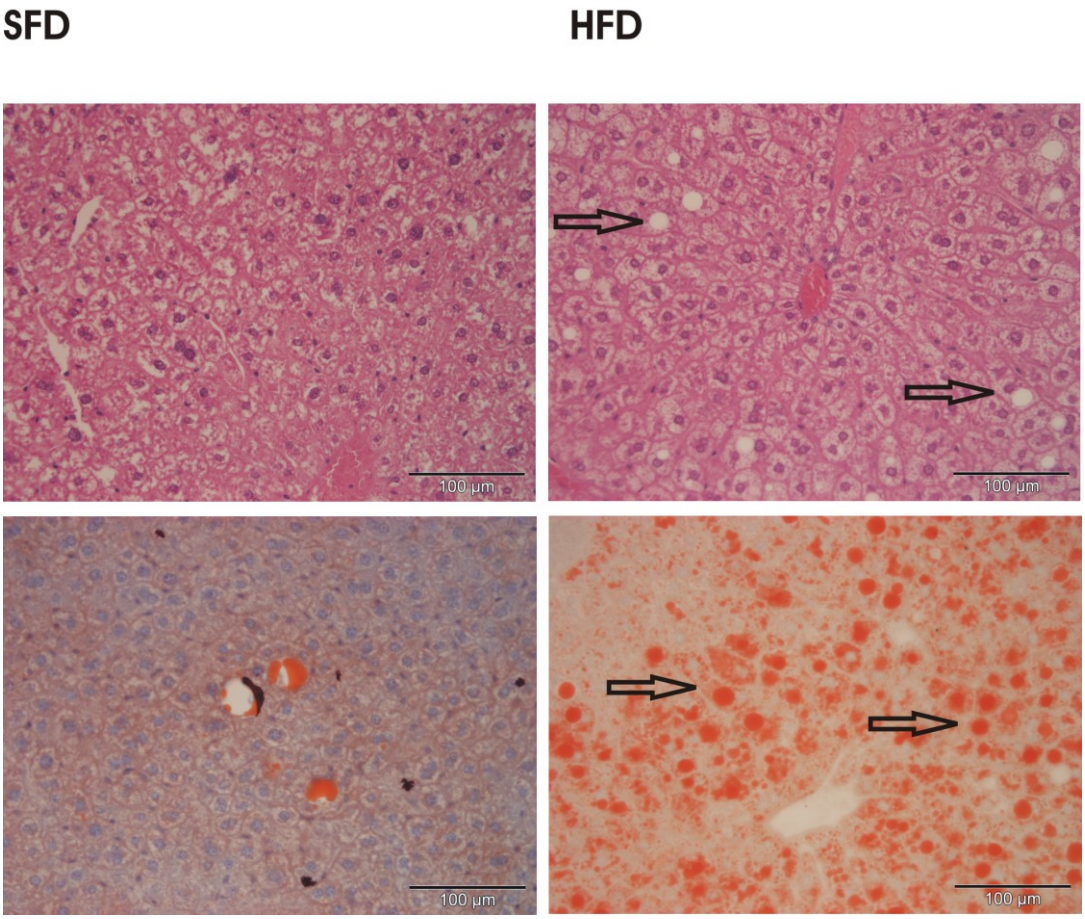

Fig. 1. Liver sections of mice fed a standard-fat diet (SFD) and a high-fat diet (HFD) stained with H&E (upper panel) and with Sudan III (lower panel). The vacuoles and lipid droplets are marked with arrows.

a) b)

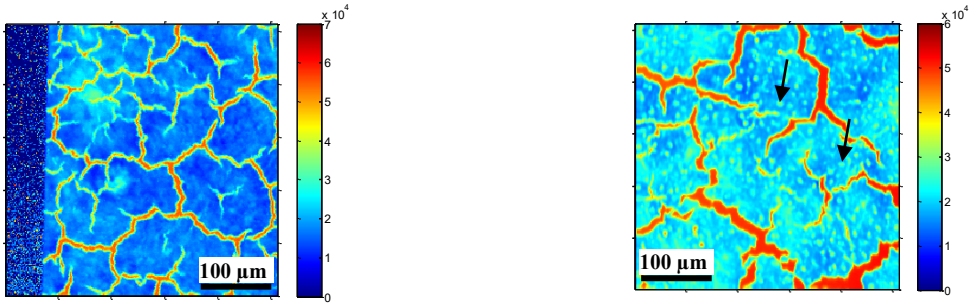

Fig. 2. Scanning Transmission Ion Microscopy (STIM) image of 5-µm-thick liver tissue samples of mice fed with SFD (a) or HFD (b). The area of the image size is approximately 360x360 µm^2. The colour bar represents the density distribution from the highest (dark blue colour) to the lowest density (red colour). Black arrows indicate lipid vacuoles. From the figures, it can be concluded the energy loss of 9 MeV O^{4+} ions is larger for SFD (a) than for HFD (b) liver tissue, indicating that normal liver tissue is more dense than tissue with fat vacuoles. On the left side of (a), part of the Si_3N_4 frame can be seen where ions were completely stopped.

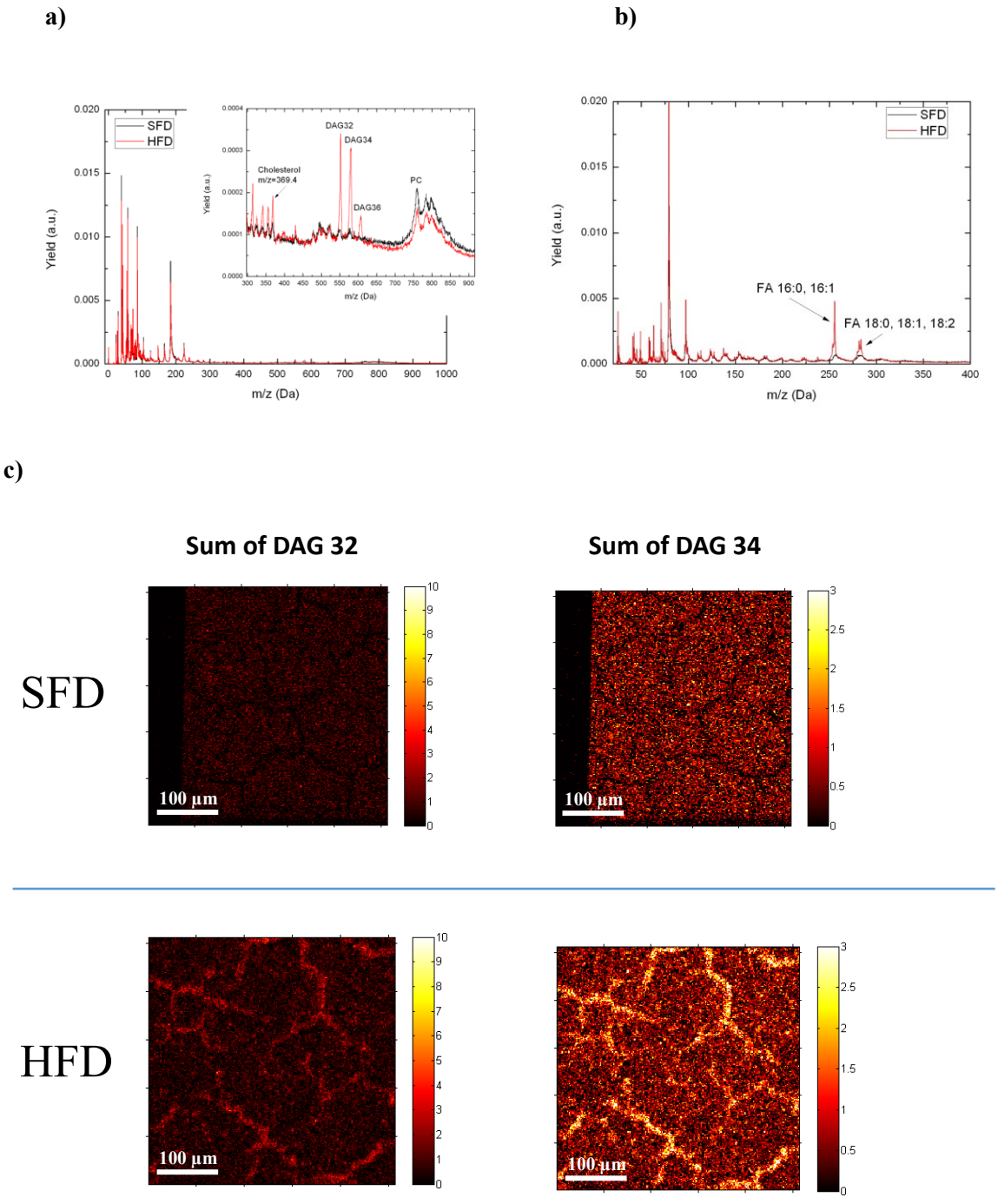

Fig. 3. Mass spectra in positive (a) and negative (b) ion mode collected from liver cryo-sections of mice fed with SFD (black) or HFD (red). The peaks belonging to cholesterol, DAGs, PC and FAs are

marked in the mass spectra. The spatial distribution (c) of DAG 32, m/z=552 and DAG 34, m/z=578 in the liver obtained from mouse on SFD (upper panel) or HFD (lower panel). Data are collected from the same tissue area shown in Figure 2.

a) b)

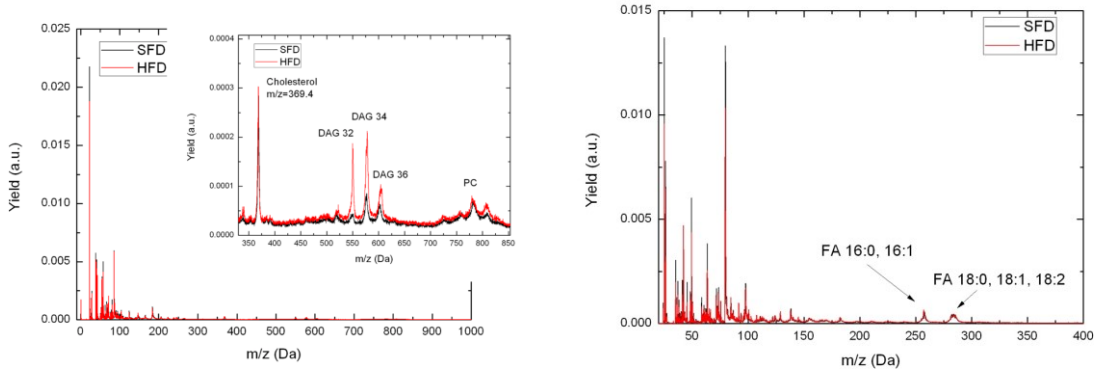

Fig. 4. Mass spectra collected in the positive (a) and negative (b) ion mode of serum obtained from mice fed with SFD (black) and HFD (red). The peaks belonging to cholesterol, DAGs, PC and FAs are marked.