Theses

Of the Doctoral

Dissertation

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Department of Analytical Chemistry

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Analysis of Fatty Acids in Diabetic Complications and their Diagnostic Application

Theses of the Doctoral Dissertation

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References

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Abstract

Diabetes mellitus is becoming a modern problem in the developed world, whether it be socially, medically or economically. We make significant contributions to its massive development through a hectic and unhealthy lifestyle. In the countries of the Western world, including ours, this increasingly restricts physical activity and also brings poor eating habits in the form of high-calorie and often difficult to digest meals. The aim of this dissertation is to analyze the composition of fatty acids in lipoprotein fractions of metabolic syndrome, hepatic steatosis, *diabetes mellitus* and cardiovascular diseases and compare them with a healthy control group. Another goal is to determine the activities of lipogenic enzymes that are affected by the insulin resistance of adipocyte, liver and muscle cells.

Abstrakt

Diabetes mellitus se stává novodobým problémem vyspělého světa, ať již po stránce sociální, lékařské nebo ekonomické. K jeho mohutnému rozvoji nezanedbatelnou měrou přispívá hektický a nezdravý životní styl, který v zemích západního světa, tedy i u nás, stále více omezuje pohybovou aktivitu populace a přináší také nesprávné stravovací návyky ve formě vysokokalorických a mnohdy těžko stravitelných pokrmů. Cílem disertační práce bylo provedení analýzy složení mastných kyselin v plazmatických lipoproteinových frakcích metabolického syndromu, jaterní steatózy, *diabetes mellitus*, kardiovaskulárních onemocnění a jejich vzájemné porovnání a porovnání se zdravou kontrolní skupinou. Dalším cílem bylo stanovení aktivit lipogenních enzymů, které jsou ovlivňovány inzulinovou rezistencí adipocytárních, jaterních a svalových buněk.

Keywords

lipid metabolism, fatty acids, diabetes, hepatic steatosis, gas chromatography, thin layer chromatography

Klíčová slova

lipidový metabolismus, mastné kyseliny, diabetes, jaterní steatóza, plynová chromatografie, tenkovrstvá chromatografie

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Introduction

Diabetes mellitus (DM) is a problem of the modern world that is growing significantly with the rise of civilization and the „western“ way of life. This disease interferes with many spheres of our well being, whether that be medical, economical or social. Type 2 DM (DM2), also known as non-insulin dependent DM, is a metabolic disease that usually develops in adulthood. It is related to obesity, decreased physical activity, unhealthy diets and the Western lifestyle in general. It is caused by a reduced sensitivity of the body's own tissues to insulin, a pancreatic hormone, which is the main factor that affects carbohydrate metabolism. The insulin is produced in beta islet cells. If there is a higher glucose level in a blood, the pancreas will secrete more and more insulin, and eventually, the overburdened beta cells will atrophy. Although the DM is identified as a disease of civilization, the first mention of it appeared more than three and a half thousand years ago in Ancient Egypt. The term DM was first used by Aretaeus of Cappadocia around the begining of our era. The main problem with DM is its unsatisfactorily compensated form. DM is the most commonn cause of kidney failure and blindness, and it has a very strong risk factor for the developement of atherosclerosis (if it has not already been developed). What is more, with the exception of atherosclerosis, DM is the most common cause of lower limbs amputation in the Czech Republic. An early diagnosis and a patient‘s disciplinecan contribute to the elimination, or at least significant mitigation, of all these adverse effects.

Not only can increased glucose levels or increased glycosidic haemoglobin be found in DM patients, but also changes in the concentration of other substances like lipids, amino acids and enzymes, . The main interest of this work is the concentration of fatty acids (FA) and activity of lipidic enzymes (desaturases and elongases). Their activity plays a key role in the biosynthesis of unsaturated higher fatty acids. The relationship among FA, glycosidic haemoglobin and enzyme activities is investigated herein. Although a large-scale significant improvement in the population‘s lifestyle is not expected, the results of this research could be used in the prevention, diagnosis and treatment of diseases of civilization including DM.

1 Definition of thesis objectives

The aim of this dissertation is to analyze the composition of FA in the plasma lipoprotein fractions of these diseases and compare them with a healthy control group (ZK). Another goal was to determine the activities of lipogenic enzymes that are affected by insulin resistence (IR) of adipocyte, liver and muscle cells. These diseases complicate the course of treatment for DM2. The changes in FA concentrations and lipogenic enzyme activities are useful in making an accurate diagnosis of the associated disease. At the same time, it ispossible to confirm the effect of IR on the individual tissues because plasma lipid fractions are formed in the corresponding organs.

These measurements were performed using a verified analytical method. The results of the analysis are mutually comparable, and provide the ability to determine the physiological intervals of FA concentrations in individual fractions for ZK and individual disease groups. These results also have an application as the diagnostic variables in the characterization of individual diseases. The activity indices of lipogenic enzymes for individual plasma fractions can have a similar use.

2 Theoretical part

2.1 Type 2 *diabetes mellitus*

A non-insulin dependent *diabetes mellitus* (NIDDM) is a metabolic disease which is increasing in occurance in the Central European population. This disease is characterized by not only an increased glucose and glycosidic haemoglobin (HbA1c) level but also the disruption of lipid metabolism, with all its inevitable consequences.[1]
Dietary FA are partly reflected by the FA profile of various biological media including adipose tissue (which reflects the intake over months to years), erythrocyte membranes, and plasma or serum (which reflectsthe intake over several weeks). However, the FA profile in biological tissues is also strongly dependent on the endogenous metabolism of FAs. It has been shown that the suppression of activity in the regulatory enzymes by experimental DM provoked an alteration in the FA composition of phospholipids in different tissues.[2]
Dyslipidemia of NIDDM patients may be characterized by the decreased content of plasma ω-3 and ω-6 polyunsaturated fatty acids (PUFA) and the increased content of cytotoxic saturated fatty acids (SFA) that worsen IR, which causes the disappearance of pancreatic β-cells and has proinflammatory effects. [3]-[6]
ω-3 PUFA helps to prevent glucose intolerance and has anti-inflammatory properties.[7],[8]

2.2 Diabetic complications and their diagnostic significance

2.2.1 Ischemic heart disease

Ischemic heart disease (ICHD) can take an acute or chronic form. This cardiac dysfunction is caused by an insufficient blood supply to the myocardium in the current coronary artery disease, which is usually coronary atherosclerosis.[9]
According to the World Health Organization (WHO), it is defined as a variable combination of changes in the intima of the arteries associated with the storage of lipids (cholesterol), polysaccharides and blood elements. In further developments, fibrous tissue is formed and accompanied by the deposition of calcium compounds. The disease begins at a young age. This chronic inflammatory disease is manifested by the formation of an atheroma plaque (atheroma) in the large or medium arteries. The atheroma consists of a soft lipid thrombogenic nucleus covered with a fibrous sheath, which contains muscle cells and inflammatory cells - macrophages, containing oxidized LDL. The most common manifestation of myocardial ischemia is *angina pectoris*. When the coronary artery suddenly narrows, astringent pain occurs in the upper or middle part of the patient's sternum. *Angina pectoris* can be categorised as stable or unstable. Stable *angina pectoris* occurs during increased exertion: walking up stairs, stress, when moving to a cold environment and the like. Patients with stable *angina pectoris* must stop these exertions to relieve the pain. The pain should disappear within about two minutes after the interruption of exertion or administration of nitroglycerin. Unstable *angina pectoris* manifests at rest and may resemble myocardial infarction with its long-lasting pain. These seizures are a manifestation of the inability to supply the myocardium to the coronary arteries and can only be detected on an ECG during their duration.[10]

In myocardial infarction, the pain is more severe and lasts longer than twenty minutes. It may be accompanied by vomiting, heart rhythm problems or severe back pain. After administration of nitroglycerin, the seizure does not subside. Myocardial infarction is caused by coronary artery occlusion and results in myocardial cell death in the affected area. A painless form of myocardial infarction is more common in DM.[10]

2.2.2 Non-alcoholic hepatic steatosis

Non-alcoholic hepatic steatosis (NAFLD) is a disease that mainly affects obese individuals, DM2 patients and cardiovascular diseases (CVD). The development of NAFLD and histological findings of individual stages can be seen in **Figure 1**. The prevalence of this disease is still growing. Currently, the disease affects about 25 % of all adults worldwide and 3-10 % of children. In developing countries, up to 35 % of obese children are affected. In patients with DM2, the prevalence of NAFLD is significantly higher: 70-90 % in adults and 48 % in children. The next stage of this liver disease is non-alcoholic liver steatohepatitis (NASH), which can progress to liver cirrhosis and also causes hepatocellular carcinoma (HCC). The prevalence of those subsequent stages in NAFL patients is 10-20 % for NASH and up to 25 % for fibrosis, cirrhosis and hepatocellular carcinoma (HCC). The emergence of NAFLD is clearly associated with the effects of civilization, and incidences of this disease are most prevalent in DM2 but difficult to diagnose. Risk factors for NAFLD are: gender, age over 45 years, DM2, obesity with BMI> 30 kg / m2 with visceral fat accumulation, dyslipidemia, serum TG level> 2.28 mmol / l (200 mg / dl), decreased HDL <0.9, AST / ALT ratio> 1 and hypertension> 140/90 mmHg in cardiac patients.[11]

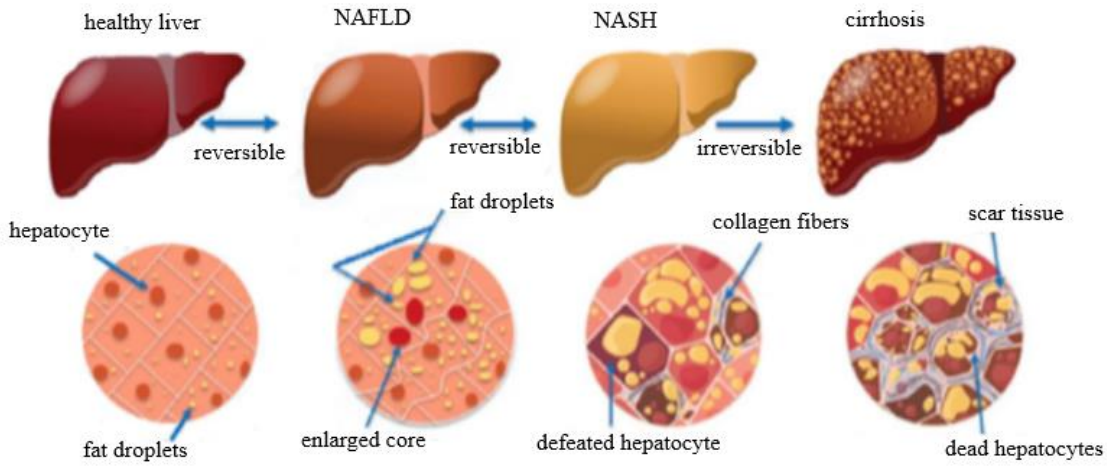


Figure 1: Development of non-alcoholic liver disease and histological findings of individual stages[12]

The amount of liver fat correlates with the overall obesity expressed by BMI and at the same time with the amount of visceral fat expressed as waist circumference. This finding is the basis for the so-called "portal hypothesis", which explains the onset of NAFLD by increased serum FFA concentrations derived from visceral fat. This ectopic fat is more metabolically active than other adipose tissue and accumulates a defense mechanism

called non-infectious inflammation. Visceral fat is infiltrated by macrophages and proinflammatory cytokines, and visceral fat becomes IR. When insulin can no longer inhibit lipolysis, the uninhibited hormone sensitive lipase produces increased amounts of FFA, which enter directly into the portal liver vein, become absorbed by hepatocytes and transform into acyl-coenzymes A (acyl-CoA), from which TG is further formed and stored in hepatocytes. In addition, elevated acyl-CoA levels cause hepatic IR diacylglycerol and induction of intracellular inflammation by activating nuclear factor κ-B (NF-κB).[13]

3 Processing methodology

3.1 Chromatographic separation

Before lipid separation through thin layer chromatography (TLC), proteins had to be removed from the samples. Denaturation of the sample‘s proteins was performed in a centrifuge tube using 500 μL of human plasma or serum (5 tubes for samples and one for standard) and 2.5 mL of reagent (isopropyl alcohol, n-heptane and phosphoric acid in the ratio 40:20:1). The resulting mixture was stirred using a vortex mixer and conditioned at room temperature for 10 minutes. 1 mL of methanol-toluene mixture (1:4) and 1.5 mL of distilled water was added to the contents fo the tube. The resulting mixture was centrifuged for 10 minutes at 1 700 x g. The top organic layer was moved into a clean tube and evaporated under nitrogen atmosphere to a volume of about 30 μL. Thus prepared, samples were applied to a glass chromatography plate covered with silica gel (**Figure 2)** and divided into five fractions: phospholipids (PL), diglycerides (DG), free fatty acids (FFA), triglycerides (TG) and cholesterol esters (EC). For preparation of the plate, a starting line was drawn 2.5 cm from the bottom of the plate. The other margins were 1 cm. The internal field of the board was split into 6 lines, each 3 cm wide. Mobile phase was prepared by mixing 160 mL of hexane, 40 mL of diethyl ether and 6 mL of acetic acid, 98 %. Chromatography took about one hour, untill the head reached the finish line at the top of the plate. On the dried plate, individual fractions in a „standard“ line were visualized using 2,7-dichlorofluorescein (0,2 g of 2,7-dichlorofluorescein in 100 ml of isopropyl alcohol). After drying, individual fractions were visible under UV light (240 nm). Then all prepared fractions were moved to tubes with teflon sealed caps.

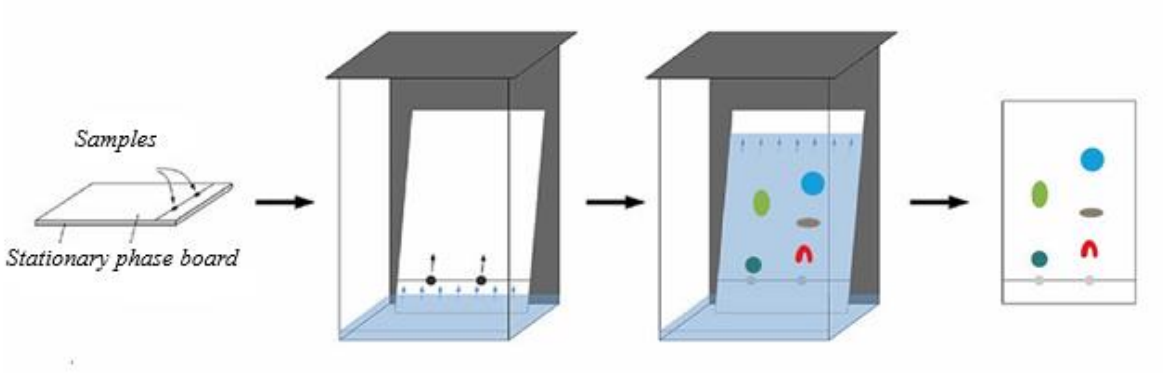


Figure 2: Thin layer chromatography – representation of retardation factor

3.2 Derivatization of fatty acids

All fractions were isolated. After the addition of internal standard (cis-13,16,19-docosatrienoic acid, 10 μg/mL) and methanol-toluene mixture (1:4), the samples were hydrolyzed and converted to the corresponding methyl esters of FA. This was carried out by adding 200 μL of acetyl chloride and heating to 100 °C for one hour. After cooling to laboratory temperature, the excess of acetyl chloride was neutralized by adding 5 mL of potassium carbonate solution, 6 %. After the addition of the carbonate solution, the tube was intensively stirred for two minutes and subsequently centrifuged at 1 700 x g for ten minutes. The top organic layer was moved into a clean tube and evaporated under nitrogen atmosphere in a volume of about 100 μL. The solution containing the preconcentrated lipid fractions was transferred to vials with chromatographic separation attachment, which were carefully sealed with a rubber septum cap.

3.3 Chromatographic analysis

Methyl esters of FA were separated using HP-88 column. The volume of the sample injected was 1 μL (split 1:10). The injector temperature was 250 °C, and the flame ionization detector temperature was 280 °C. The column temperature program was as follows: initial temperature of 130 °C for 1 min → gradient 2 °C/min to 176 °C hold for 2 min → gradient 1 °C/min to 186 °C hold for 1 min → gradient 0,1 °C/min to 190 °C hold for 1 min → gradient 1 °C/min to 220 °C hold for 4 min. Total analysis time took 92 minutes. The internal standard calibration curve was used for the quantification of individual FFA. We were able to determine 40 FA.

3.4 Calculation of enzyme activity indices

The 16:1n7/16:0 ratio was calculated as indice of stearoyl-CoA desaturase 1, i.e. 9-desaturase (9D) activity. Furthermore, the 20:4n6/20:3n6 and 20:3n6/18:2n6 ratios were calculated as indices of 5-desaturase (5D) and 6-desaturase (6D) activities, respectively. The ratio of 18:0 to 16:0 was calculated as an elongase activity indice.

3.5 Statistical analysis

The obtained chromatograms were subsequently integrated using GC ChemStation B04.03 software and the resulting data was statistically processed using MS Excel 2007, SigmaStat 3.5 and Statistica 12.0. Differences in variables between the groups were evaluated using Mann-Whitney test. A p-value less than 0.05 was considered statistically significant.

4 Results

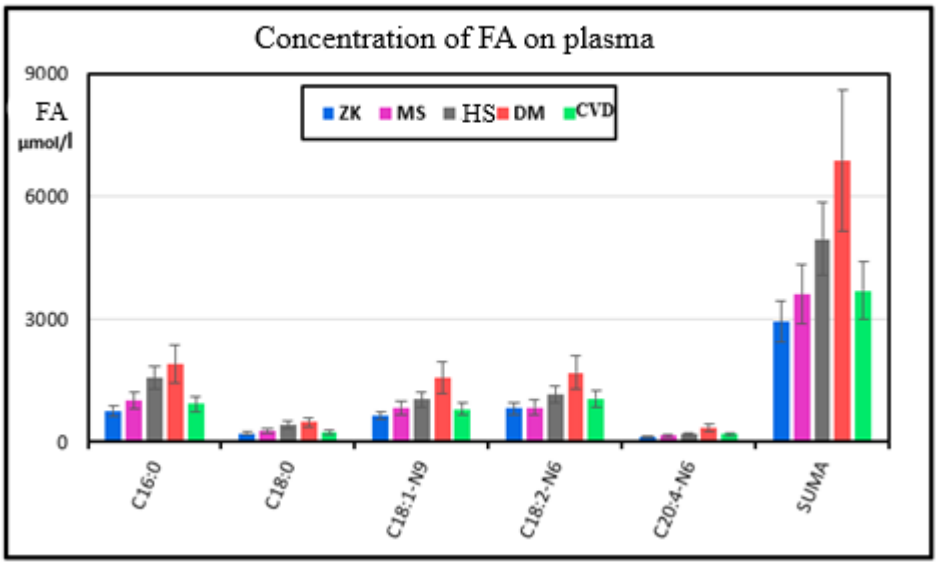
The most common health complications of DM2 are: MS, NAFL and CVD. These diseases are caused by excessive nutrient intake and unhealthy lifestyles, which are characterized by overeating, lack of physical activity, smoking, alcohol and drug use, stressful environment and other factors. Affected individuals are very often obese, have

high blood pressure, suffer from dyslipidemia, hypercholesterolemia, hyperglycemia and organ or generalized IR. These metabolic disorders are manifested by changes in the metabolism of lipoproteins, lipids, FA and their metabolites in blood, organs and organ cells, and subsequently affect FA concentrations in plasma lipoprotein fractions, namely PL, DG, FFA, TG and EC.[14]

The literature suggests that the triggering mechanism of the above diseases is an excessive supply of nutrients to the human body, which are stored in the form of TG, first in adipose tissue and later in other organs (liver, abdomen, muscles, etc.) [15]. Excess lipoproteins in the bloodstream cause dyslipidemia, characterized by increased concentrations of TG, cholesterol, EC and FFA. This significantly increases the availability of lipids as an energy source. FA, unlike glucose, penetrate organ cells by diffusion or facilitated diffusion and substantially increase the concentration of all lipid derivatives in the cytoplasm. They activate the production of TG, which are stored in cells, and phosphoacylglycerols, which are secreted into the circulation, and they increase the production of DG, which inhibits intracellular insulin signal transmission and induces IR (adipocyte, hepatocyte or myocyte) [16]. Excess lipoproteins in the circulation also cause IR lipoprotein and hormone sensitive lipases, which accelerate the hydrolysis of adipose TG to FFA and glycerol. Excess FFA is captured by the liver and transformed to TG secreted by the liver as VLDL into the bloodstream. The high concentration of VLDL is not comprehensively utilized by the muscles, and the TG contained in VLDL and IDL are again hydrolyzed by lipases to FFA and glycerol. This pathological cycle causes a permanent increase in circulating TG and leads to primary dyslipidemia. The long-term imbalance between the supply of FFA and glycerol to the liver and the excretion of VLDL leads to an increased deposition of TG in hepatocytes and the development of hepatic steatosis (HS). This pathological process is also accelerated by hepatic IR, which reduces the utilization of glucose for the production of ATP in mitochondria. It is stored in the form of hepatic glycogen, and at the same time it activates gluconeogenesis by activating key gluconeogenesis enzymes inhibited by insulin. In this way, the excess FA taken up by the hepatocytes are transformed into glucose, which is then excreted into the bloodstream. The long-term consequences of these metabolic changes in adipose, muscle and liver tissue are manifested as IR, dyslipidemia and hyperglycemia, which are major causes of the onset and progression of DM2.[17]

4.1 Fatty acid concatration in plasma

It is clear from the results that the levels of "total" FA increase in patients, and in addition to *cis*-palmitoleic acid and cardiac groups (CVD), *de novo* lipogenesis indices also increase. The following **Graph 1,** of the dependence of *cis*-palmitoleic acid on plasma palmitic acid, shows that the characteristics of these dependences are different in the studied patients compared to the control group (ZK), and documents a more than two-fold decrease of insulin activation in adipose tissue by *cis*-palmitoleic acid. In HS, *cis*-palmitoleic acid activity is independent of palmitic acid concentration and very low.



Graph 1: Concentration of FA on plasma

4.1.1 Fatty acid concentrations in the phospholipid fraction

From these results it is clear that in this fraction there are no significant changes in the overall composition of fatty acids in patients, with the exception of the concentration of *cis*-palmitoleic acid which is reduced in MS, HS, DM and CVD by 45 %, 36 %, 35 % and 16 %, respectively, when compared to the control group. As these decreases represent 12 % - 4.4 % of the total plasma concentration of *cis*-palmitoleic acid and 0.64 - 0.31 per thousand of the total fatty acid concentration in the phospholipid fraction, this change in *cis*-palmitoleic acid concentration is insignificant for the function of phospholipids as membrane lipids. Hepatic insulin resistance does not appear to be manifested in the synthesis of phospholipids in the liver. The structure of fatty acids in the phospholipid fraction is similar in patients with MS, HS and CVD and the control group, and total FA concentrations differ only in the standard deviation interval (ZK 808.8 ± 254, 7 µmol / l, MS 968.9 ± 267.9 µmol / l; HS 1141.5 ± 155.8 µmol / l; CVD 885.0 ± 229.2 µmol / l). In the group of DM, the total concentration of FA in this fraction is more than double (1824.9 ± 247.5 µmol / l), but this value is consistent with the total double concentration of FA in plasma compared to the control group. This correlates with diabetic dyslipidemia, which is characterized by almost double the levels of TG and cholesterol in the blood. Also, the activity coefficients of all monitored lipid enzymes are similar, and their differences are again in the range of standard deviations. It is clear that the synthesis of membrane phospholipids is independent of insulin resistance, elevated glucose concentrations and glycated hemoglobin in DM. The amount and structure of fatty acids in this fraction depends only on the need for the phospholipids that are necessary for the regeneration of the body's organ cells. In DM, this need is significantly higher due to ongoing internal inflammation caused by dysfunctional glycated proteins, non-infectious inflammation of hypertrophied adipose tissue by macrophages, ketogenesis of excess acetyl-CoA and reactions of formed oxoacids with both blood and organ proteins. This fraction is unsuitable for the diagnosis of these diseases. The concentration and composition of FA in this fraction are similar for all studied diseases, and usually differs in the interval of standard deviations. The

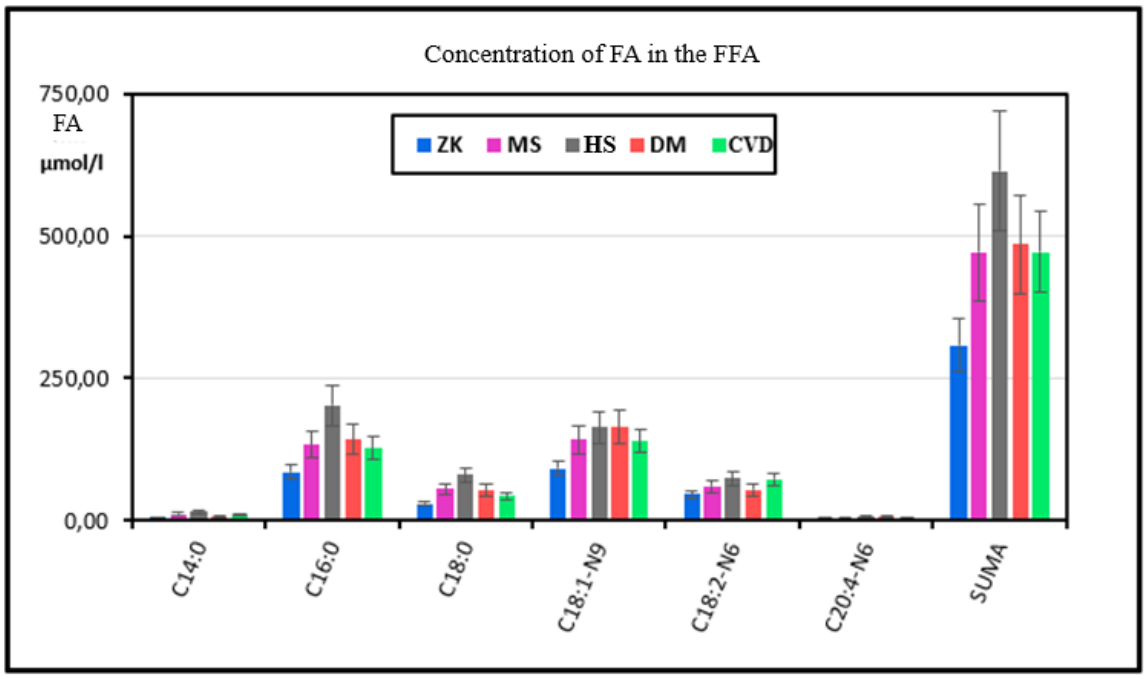
phospholipid fraction is a relatively conserved fraction, and the observed fatty acid composition does not provide new insights that are useful for the analysis of metabolic changes induced by IR.[18]

4.1.2 Fatty acid concentrations in the diacylglycerol fraction

From these results it is clear that in this fraction there is an increase in the concentrations of palmitic and stearic acids only in patients with HS and DM. These changes are not significant either, as they are probably due to almost twice the concentration of TG and EC in the plasma compared to the control group. Given that DG are predominantly derived from enterocytes, this finding is consistent with the literature [19]. 9D activity coefficients in JS and DM are lower than in the ZK by about half, but even this finding reflects unhealthy diet and obesity in patients who consume significantly more fats containing SFA acids than the healthy population [20]. This fraction is also not used for the diagnosis of these diseases because it is produced in enterocytes that are not affected by IR and has a minimal share in total plasma levels of FA (ZK of total FA = 2942.6 ± 406.3 μmol / l; FA in DG = 117.7 ± 24.5 μmol / l; resp. HS in FA = 4955.8 ± 886.0 μmol / l; FA in DG = 171.3 ± 24.8 μmol / l; DM in FA = 6886.5 ± 1496.5 μmol / l; FA in DG = 189.2 ± 32.7 μmol / l).

4.1.3 Fatty acid concentrations in the free fatty acid fraction

The results show that free fatty acid (FFA) concentrations are different from plasma FA concentrations, as shown in **Graph 2**. The highest total FFA concentration is in HS (614.4 ± 115.2 μmol / l), and is twice as high as the total concentration FA in the ZK (307.4 ± 60.0 μmol / l). Also in MS, DM and CVD, the total concentrations of FA are up to 50 % higher compared to the ZK (471.2 ± 103.3 μmol / l; 485.6 ± 71.3 μmol / l; 471.8 ± 126.8 μmol / l). This observation is consistent with current published results [21], and confirms obesity-induced IR of adipose tissue in MS and HS and decreased *cis*-palmitooleate concentration, which is reduced in the FFA fraction in MS and HS compared to the ZK (4.7 ± 2 .7 μmol / l; MS 2.9 ± 1.3 μmol / l; JS 3.3 ± 0.5 μmol / l). The concentration of *cis*-palmitooleate in DM and CVD is increased compared to the ZK (DM 5.6 ± 0.8 μmol / l, CVD 7.8 ± 2.9 μmol / l), this finding is related to the overall increased concentration of FA in plasma DM and the absence of IR in cardiac patients. As in the PL and DG fractions, the diagnostic significance of the detected concentrations of *cis*-palmitooleate in the FFA fraction is small. These experimental differences in concentrations confirm the effect of *cis*-palmitooleate on the existence of adipose tissue IR in MS and HS and on overall lipid metabolism. Further confirmation of adipose tissue IR in the FFA fraction can be found by comparing the activity indices of *de novo* lipogenesis and 9D-C16, and their changes in MS and HS are also consistent with the experimental results found. Higher *de novo* lipogenesis activity releases higher amounts of cytotoxic palmitic acid into the circulation, which is insufficiently detoxified in adipose tissue by conversion to *cis*-palmitooleate because 9D-C16 activity is inhibited by adipose tissue IR.[22]



Graph 2: Concentration of FA in the FFA

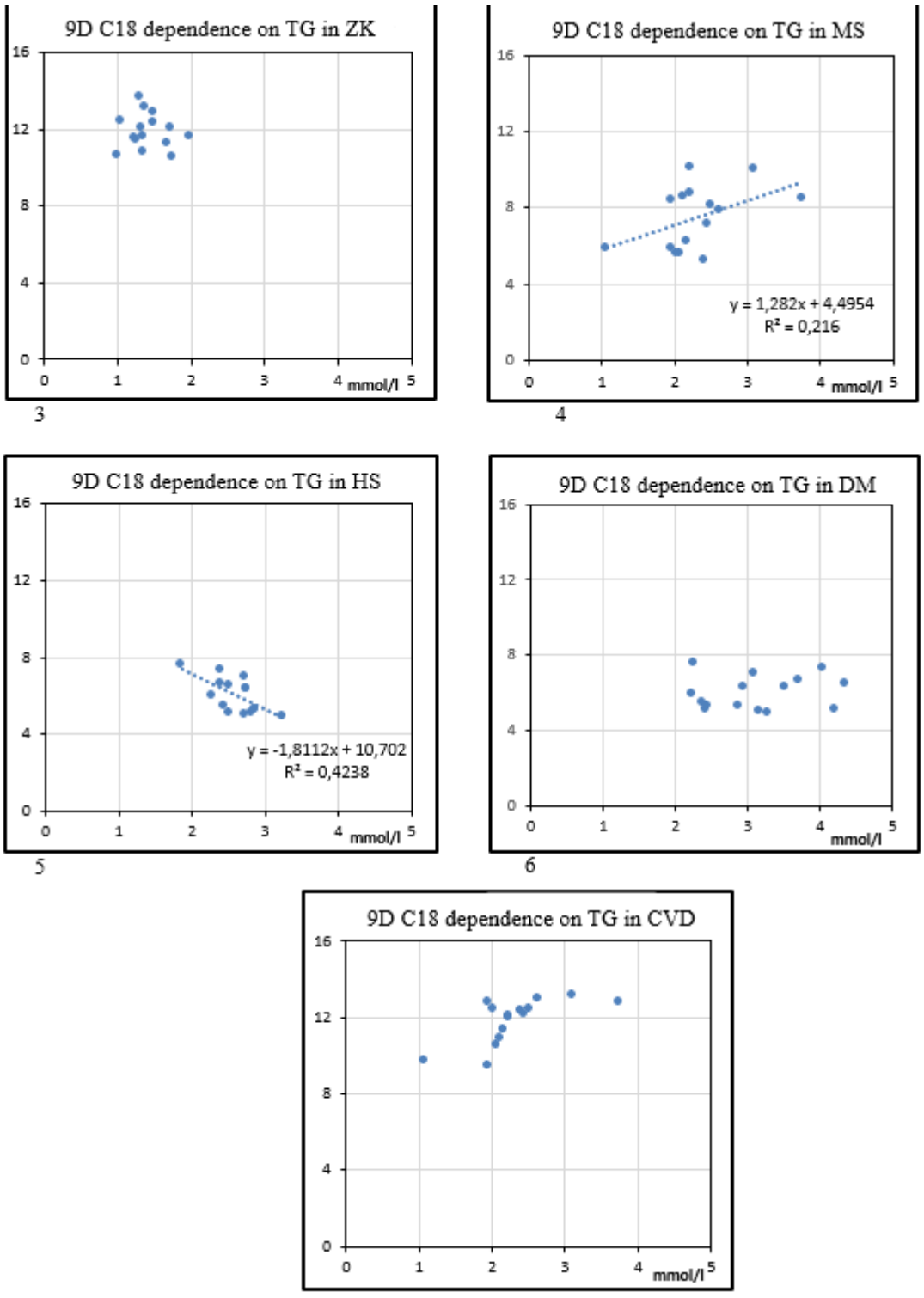
4.1.4 Fatty acid concentrations in the triacylglycerol fraction

The results show that the FA concentrations in the triacylglycerol (TG) fraction are similar to the total plasma FA concentration. There is an increase in SFA levels, which is higher than the increase in unsaturated FA levels. This is due to a decrease in the activities of all lipid enzymes except *de novo* lipogenesis, which, like in the FFA fraction, signals the production of a higher amount of cytotoxic palmitic acid. The decrease in the activity of all liver desaturases in MS, HS and DM compared to the ZK proves that another affected organ in these diseases is the liver, which has reduced enzyme activity to the point necessary for complete detoxification of SFA. We have previously published this finding in a study on the composition of FA in DM [23]. Enzyme activities in the CVD are similar to the ZK due to normal insulin sensitivity. The diagnostic use of reduced values of desaturase enzyme activity indices in DM has already been described in the literature [24] In MS and HS, this finding has not been sufficiently analyzed so far, due to the low number of published FA analyzes in these diseases. This decrease is sufficiently conclusive, and it is clear from **Graphs 3 - 7** that the 9D-C18 activity index has a different trend in MS and HS depending on blood TG concentrations. It is obvious that MS shows IR only in adipose tissue. In HS, it is a superposition of IR in adipose and liver tissue. In DM, the 9D activity index is independent of TG values, similar to the ZK, but the average value is significantly reduced. It is therefore possible to identify healthy individuals and patients with CVD from the detected values of 9D-C18 in the TG fraction (9D activity index in the ZK 11.858 ± 0.89, CVD 11.793 ± 1.12) and MS, HS and DM patients (9D activity index MS 7.458 ± 1.75; HS 5.987 ± 0.87; DM 5.978 ± 0.87). To divide MS, HS and DM patients into individual disease groups, it is possible to use the total values of FA concentrations in the TG fraction (FA in TG, ZK 746.8 ± 153.3 µmol / l; MS 1156.2 ± 354.9 µmol HS 1594.3 ± 223.4 µmol / l; DM 2576.8 ± 511.8 µmol / l). In DM, these

concentrations are 3.5 times higher than in healthy individuals, 2.2 times higher than in patients with MS and 1.6 times higher than in patients with HS. Based on these values, DM can be clearly separated from patients with MS and HS. The concentration of *cis*-palmitoleic acid can also be used to differentiate MS and HS patients because the differences found are the highest and sufficiently conclusive in this fraction (*cis*-C16: 1 in the ZK 9.4 ± 3.2 μmol / l; MS 11.3, 2 ± 5.2 μmol / l, HS 6.8 ± 3.0 μmol / l) and also the *de novo* lipogenesis activity index values (ZK 1.48 ± 0.22 μmol / l; MS 1, 72 ± 0.17 μmol / l, HS 2.3 ± 0.20 μmol / l).[25],[26]

The group of patients with CVD has FA concentrations similar to the ZK. These patients do not suffer from IR as patients with MS, HS and DM, and liver function is therefore not affected. The only significant difference is in the concentrations ω-6 and ω-3 fatty acids, where an increase in linoleic acid concentration (ZK 136.7 ± 28.7 μmol / l; CVD 175.2 ± 68.6 μmol / l), arachidonic acid (ZK 9.5 ± 3.7 μmol / l; CVD 16.2 ± 9.4 μmol / l) and the total concentration of all ω-6 fatty acids (ZK 163.2 ± 31.6 μmol / l; CVD 211.7 ± 70.9 μmol / l) was found. The concentrations of ω-3 fatty acids in the CVD group of TG remained almost identical (ZK 15.8 ± 3.1 μmol / l; CVD 16.4 ± 2.9 μmol / l). Linoleic acid is known to be oxidized in LDL particles to 9-hydroxy-10,12-octadienoic acid and 13-hydroxy-9,11-octadienoic acid, which are a risk indicator for atherosclerosis. Arachidonic acid also provides pro-inflammatory prostaglandins, thromboxanes and leukotrienes, which are involved in the inflammatory processes associated with atherosclerosis and myocardial infarction[27]. In a previously published study of cardiac patients, we studied the concentrations of selected FA in plasma erythrocytes and phospholipids and correlated them with indicators of inflammation, such as IL-6, serum amyloid A and malondialdehyde. We have found several FA (linoleic acid, dihomo-γ-linolenic, docosatetraenoic-n6, docosapentaenoic-n6) that have negative effects on the healing process after stent implantation. We did not confirm the anti-inflammatory effects of ω-3 fatty acids, with the exception of stearidonic acid (C18: 4-n3), but its concentration is significant only in the erythrocyte membrane. We also found other FA that positively or negatively correlate with the indicators of inflammation, and the results found in another publication were confirmed. [28] - [30]

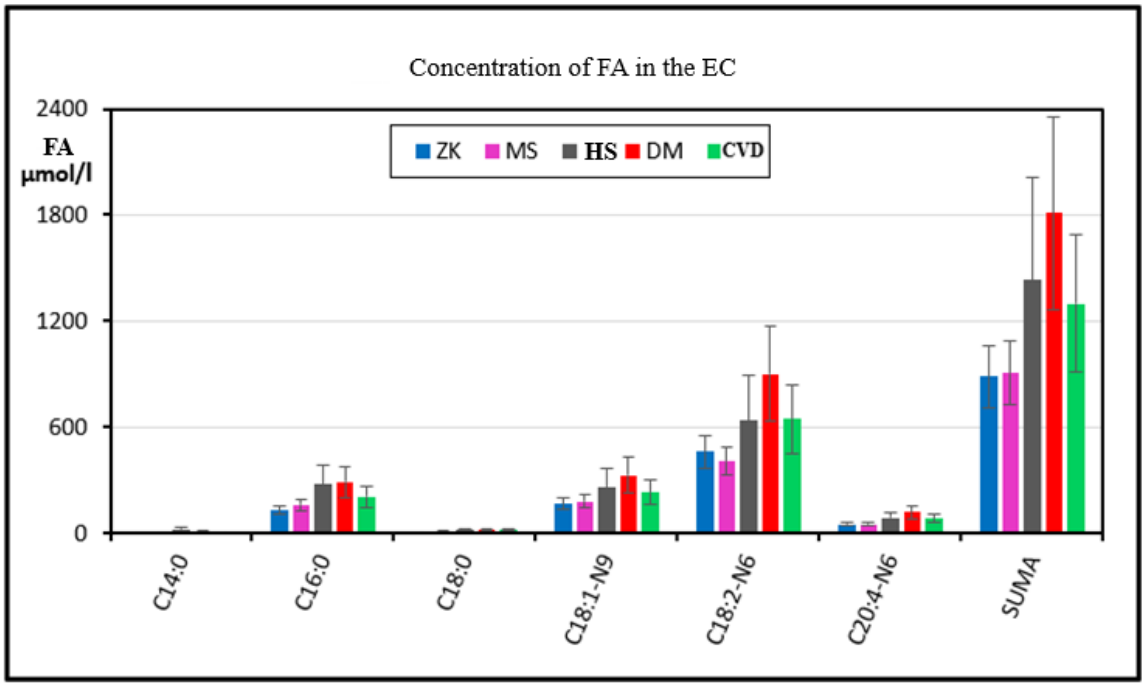
The TG fraction is the most frequently studied lipoprotein fraction, the FA concentrations found and the activity indices of lipid enzymes are directly related to the metabolic function of the liver and, like the VLDL fraction formed in the liver, provide diagnostically used information. In this fraction, we found the most changes in the levels of FA that are usable to characterize these diseases.



Graph 3-7: Dependences of 9D-C18 activity index on plasma TG concentrations

4.1.5 Fatty acid concentrations in the cholesterol esters fraction

The results show that the concentrations of FA in the EC fraction (**Graph 8**) are higher than in all groups of patients in the ZK and similar to the concentration of FA in the PL fraction. SFA levels (myristic, palmitic and stearic) also increase in this fraction, which is compensated by unsaturated FA levels (oil, linoleic and arachidonic) due to different EC synthesis compared to PL, DG and TG, but it is also affected by IR. Circulating EC transport is different from glycerol derivative transport, and therefore the composition of FA in this fraction also depends on the IR of all organs that are able to interact with lipoproteins, from chylomicrons to HDL. Another interesting finding in this fraction is the significant changes in the concentration of *cis*-palmitoleic acid, which has low concentration in MS and HS patients compared to the ZK and the DM and CVD (ZK 9.3 ± 3.3 µmol / l; MS 1.0 ± 0.8 µmol / l, HS 0.9 ± 0.4 µmol / l, DM 23.2 ± 8.0 µmol / l, CVD 13.6 ± 1.9 µmol / l). A similar finding has been reported in the literature as a consequence of a high-sugar diet in patients with MS [31]. Comparing the content of *cis*-palmitoleic acid in the TG fraction with the EC fraction in MS, it is clear that its concentration in TG is significantly higher. It is therefore possible to assume that most palmitoleic acid comes from hepatic desaturation of palmitic acid, because IR of adipose tissue production is significantly reduced (see *cis*-palmitoleic acid concentrations in the FFA fraction). This finding of low concentrations of *cis*-palmitoleic acid in the EC fraction is again usable as a diagnostic indicator of MS and HS, including reduced values of 9D -C18 in all groups of diseases.



Graph 8: Concentration of FA in the EC

Conclusion

The total content of FA in plasma corresponds to the already published results, and by their analysis, I determined the concentration intervals, for which the concentrations are 40 physiological FA from myristic acid (C14: 0) to tetracosaheptaenoic acid (C24: 6-N3), including most saturated, monounsaturated, essential and polyunsaturated fatty acids in individual patient groups. An interesting finding was a decrease in the concentration of *cis*-palmitoleic acid by 23 % in patients with MS and by 51 % in patients with HS. This decrease in the concentration of *cis*-palmitoleic acid was similarly reflected in the FFA fraction, and I found its concentration is significantly lower in the EC fraction. In accordance with the published information, I attributed this decrease to the IR of adipose tissue in MS patients and the combined IR of liver and adipose tissue in HS patients. These concentration values in the individual fractions are a quantitative, analytical evaluation of the importance of *cis*-palmitoleic acid as adipose tissue lipokine, which mediates the inter-organ transmission of information on their IR. The analysis of FA concentrations in the fraction of PL, DG and FFA showed only insignificant differences, and I see the benefit of the results in the quantification of the concentration intervals of individual acids. Furthermore, it can be stated that all the studied diseases have only a small effect on the concentration composition of FA in these fractions and do not provide significant information usable for diagnostic purposes. The fraction of TG is the most interesting from a diagnostic point of view, and the analysis of FA concentrations and activity coefficients of lipid enzymes allows the specification and diagnostic differentiation of individual groups of patients. The diagnostic use of reduced values of desaturase enzyme activity indices in MS and HS has not been described yet. However, the found decrease is sufficiently convincing, and has a different trend depending on TG concentrations in the blood. According to the values of 9D-C18 activity indices in the TG fraction, patients can be divided into individual disease groups. To divide MS, HS and DM patients into individual groups of diseases, it is possible to use the total values of FA concentrations in the fraction. In DM, these concentrations are 3.5 times higher than in the ZK, 2.2 times higher than in MS patients and 1.6 times higher than in HS patients. The concentration of *cis*-palmitoleic acid can also be used to differentiate MS and HS patients, where the differences found are the highest in this fraction and sufficiently convincing. It is also possible to use the values of *de novo* lipogenesis activity indices. The group of CVD patients has FA concentrations similar to the ZK. These patients do not suffer from IR like patients with MS, HS and DM, and liver function is therefore not affected. The only significant difference is in the concentrations of ω-6 and ω-3 fatty acids, where I found an increase in the concentration of linoleic and arachidonic acid and a noticeable increase in the total concentration of all ω-6 FA, at the same concentration of ω-3 FA compared to the ZK. This finding has also been discussed in the literature, and the benefit of these analyzes is the quantification of the concentration range of these acids and enzymes. In the PL fraction, I found significant changes in the concentration of *cis*-palmitoleic acid, which has low concentration in patients with MS and HS in comparison with the ZK and the DM and CVD. This finding of low concentrations of *cis*-palmitoleic acid in the EC fraction is again usable as a diagnostic indicator of MS and HS, including reduced values of 9D-C18 in all disease groups. For further diagnostic conclusions, it will be necessary to analyze the concentrations of other FA derivatives caused by peroxidation, oxidation,

cyclooxygenation and other enzymatic reactions, which are being studied to an increasing extent. For these purposes, the methodology of gas chromatography (GC) with FID detector will not be enough. However, it can become one of the routine methods in clinical biochemistry, similar to the already commonly used liquid chromatography, which for example is used in the analysis of catecholamines. Then, they will certainly find the use and concentration intervals of FA in plasma fractions and patients suffering from diseases associated with lipid metabolism disorders.

List of abbreviations

5D	5 desaturase
6D	6 desaturase
9D	9 desaturase
acyl-CoA	acyl-coenzymes A
ALT	alanine aminotransferase
AST	aspartate aminotransferase
BMI	body mass index
CV	coefficient of variation
CVD	cardiovascular disease
DG	diacylglycerols
DM	*diabetes mellitus*
DM2	*diabetes mellitus type 2*
ECG	electrocardiogram
FA	fatty acid
FFA	free fatty acid
FID	flame ionization detector
GC	gas chromatography
HbA1c	glycated hemoglobin
HCC	hepatocellular carcinoma
HDL	high density lipoproteins
HS	hepatic steatosis
IR	insulin resistance
ICHD	ischemic heart disease
LDL	low density lipoproteins
MS	metabolic syndrome
NAFL	non-alcoholic fatty liver
NAFLD	non - alcoholic hepatic steatosis disease
NASH	hepatic steatohepatitis
NF	nuclear factor
NIDDM	non-insulin dependent *diabetes mellitus*
PL	phospholipids
PUFA	polyunsaturated fatty acid
SFA	saturated fatty acid
TG	triacylglycerols
TLC	thin layer chromatography
VLDL	low density lipoproteins
WHO	World Health Organization)
ZK	control group

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