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Analytical characterization of adipose tissue structure and composition: A novel approach towards diagnosis of metabolic disturbances in the human body

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In this work the combination of several analytical techniques, such as Fourier transform infrared spectroscopy (FTIR), nuclear magnetic resonance (NMR) spectroscopy, scanning electron microscopy (SEM) and metal content determination using atomic absorption spectroscopy (AAS), was used for the characterization of adipose tissue samples taken from volunteer obese patients. The obtained results provided information about the adipose tissue chemical and structural composition of adipose tissue layers in the human body, as well as the main microstructural features. It was demonstrated for the first time to the best of our knowledge that these methods are indispensable tools in order to investigate some special features of the human adipose tissue, identifying its chemical composition and structure. From the obtained results we concluded that such characterization of the adipose tissue is an essential step for the possible prediction of appearance of symptoms of different diseases.

Key words: adipose tissue, characterization, FTIR, NMR, chemical analysis, SEM

INTRODUCTION

Obesity is defined as an unhealthy excess of body fat which increases the risk of medical illness and premature mortality [1]. The obesity prevalence and severity is growing dramatically in many developed and developing countries. In the European Union (EU) the levels of obesity and overweight have been rising as well in the last decades [2]. Obesity has been clearly associated with numerous pathophysiologic processes and comorbidities, such as metabolic syndrome (MetS), type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD), which is the most common cause of death in the Western World. However, obesity related with metabolic disturbances varies widely among obese individuals, there is evidence that not all of them are at increased cardiometabolic risk, and that differences exist between individuals with upper body fat and those with lower body fat distribution [3]. As opposed to the extent of subcutaneous adipose tissue (SAT), the increase in visceral adipose tissue (VAT) is associated with increased metabolic disturbances and CVD. An individual with a normal body mass index (BMI) but with an increased VAT is at higher risk of developing metabolic disturbances than an obese person with less VAT [4]. Although the development of obesity is easily attributed to excess intake of calories, the underlying reasons for the metabolic disturbances and health risks associated with obesity are still unclear.

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Adipose tissue is generally considered as a storage depot for excess energy, which is stored as triglycerides. It was long considered as a passive organ, but the adipose tissue has been described recently as an endocrine organ with important physiological roles [5]. There is conflicting information about differences between the fatty acid composition of SAT and VAT. Varying proportions of fatty acids from the adipose tissue may be related to atherosclerosis and other diseases and might exert a direct influence on serum lipids that may differ depending on the adipose tissue region [6, 7].

The conventional methods for determining the composition of fats are gas and liquid chromatography [8–12]. However, it is clear that new alternative analytical procedures for the analysis of adipose are very much desired [13–15]. In this work the adipose samples were taken from volunteer obese patients and analysed using FTIR and ^{1}H NMR spectroscopies. Also, the aim of the present study was to investigate the distribution of different metals (Na, K, Mg, Ca, Cr, Mn, Fe, Cu, Zn and Ni) in different layers of adipose tissue from obese patients. SEM was also used for the determination of human fats surface morphological features.

EXPERIMENTAL

All subjects included in our study were recruited from 5 volunteer patients at the Department of General Surgery, Vilnius University Hospital, where they had been referred for obesity surgery. Men and women aged 18–65 years with a BMI more than 30 kg/m^2 were enrolled in the study. Exclusion criteria were contraindications for surgery and patient's refusal. During the laparoscopic gastric banding surgery three samples of adipose tissue were taken: subcutaneous (A), preperitoneal (B) and visceral (C). The study protocol was approved by the Lithuanian Ethics Committee, with the aim and design of the study explained to each subject, who in turn gave their informed consent.

On the onset of surgery, adipose tissue biopsies were taken from subcutaneous, preperitoneal and visceral (omental) adipose tissues (5 g from each region) which in turn were washed out in normal saline solution and frozen immediately. Adipose tissue samples were stored in –70 °C temperature before the chemical analysis was performed. The adipose tissue samples were homogenised and extracted using a modified Folch extraction procedure [16–18]. The lipids were extracted by 20 ml of chloroform/methanol (2:1, v/v).

The infrared (FTIR) spectra were recorded on a Perkin Elmer Frontier FT-IR spectrometer. ^{1}H NMR spectra were recorded on a Bruker Ascend 400 spectrometer operating at 9.4 Tesla, corresponding to the resonance frequency of 400 MHz for the ^{1}H nucleus, equipped with a direct detection four-nuclei probe head and field gradients on axis *z*. The samples were analyzed in 5 mm NMR tubes. The ^{1}H NMR samples were prepared by dissolving 0.5 mL fat in 2 mL $CDCl_3$. The chemical shifts are reported in ppm, using the TMS as an internal standard. The typical parameters for ^{1}H NMR spectra were 45° pulse, 20 ppm spectral window, and 16 scans. The average acquisition time was approximately 1.5 min. The main metals (Na, K, Mg, Ca, Cr, Mn, Fe, Cu, Zn and Ni) in different layers of adipose samples were determined by flame atomic absorption spectroscopy (FAAS) using a Hitachi 170-50 spectrometer. The instrumental parameters were adjusted according to the manufacturer's recommendations [19–21]. For morphological characterization of the adipose tissue specimens a scanning electron microscope (SEM) Hitachi SU-70 was used.

RESULTS AND DISCUSSION

FTIR spectra in the range of wavenumbers from 4 000 to 500 cm^{-1} for 15 adipose tissue samples were recorded. As was mentioned in the Experimental part, the adipose tissue was taken from 3 layers of the adipose tissue (subcutaneous (A), preperitoneal (B) and visceral (C)) of 5 patients (samples 1, 2, 3, 4 and 5). It is interesting to note that the FTIR spectra of the adipose tissue samples investigated qualitatively were nearly the same, irrespective of the sample origin. The representative spectra recorded for specimens 1, 2 and 5 are shown in Fig. 1. As seen in Fig. 1, the FTIR spectra of adipose samples obtained from different patients and different tissues contain broad and sharp bands located at 2 950–2 800 cm^{-1} as well as weaker bands centred at around 3 150, 1 350 and 900 cm^{-1}. These bands correspond to the ν(C-H) stretching vibration of methylene and alkyl groups [22]. The sharp and intensive absorption band located at 1 743–1 710 cm^{-1} is attributed to the stretching vibrations of C=O possibly in the triacylglycerol functional group [23]. FTIR spectra of all samples contain the same bands located at 1 680–1 600 cm^{-1} and 1 200–1 000 cm^{-1} which correspond to C=C and CO–O–C vibrations, respectively. These observations let us state that unsaturated bonds in the fatty acids and esters are present in the investigated samples. The broad bands centred at ~800–700 cm^{-1} correspond to -CH_2-CH_2- vibrations in long alkyl chains. Interestingly, the intensity of these bands are quite different in the spectra for different adipose samples. This only one slightly different observation is visible in all FTIR spectra confirming the presence of different fatty acids in the adipose tissues. For example, absorption peaked at approximately 757 cm^{-1} is very intensive from the preperitoneal (B) layer for samples 1 and 5, and very weak for sample 2. On the other hand, for sample 2 this absorption is intensive in the FTIR spectrum of adipose tissue taken from the visceral (C) layer. The absorption peaks at ~722 cm^{-1} are very similar for all samples, however, the intensities of the peaks located at ~668–669 cm^{-1} in FTIR spectra again slightly differ for different specimens. According to FTIR analysis results we could conclude that the main functional groupings in the adipose tissue of different obese patients are the same, however, the real chemical composition of the samples obtained from subcutaneous, preperitoneal and visceral layers of different patients could be also different. From these measurements, it is clear that FTIR spectroscopy could be used for the analysis and direct qualitative characterization of adipose tissue composition.

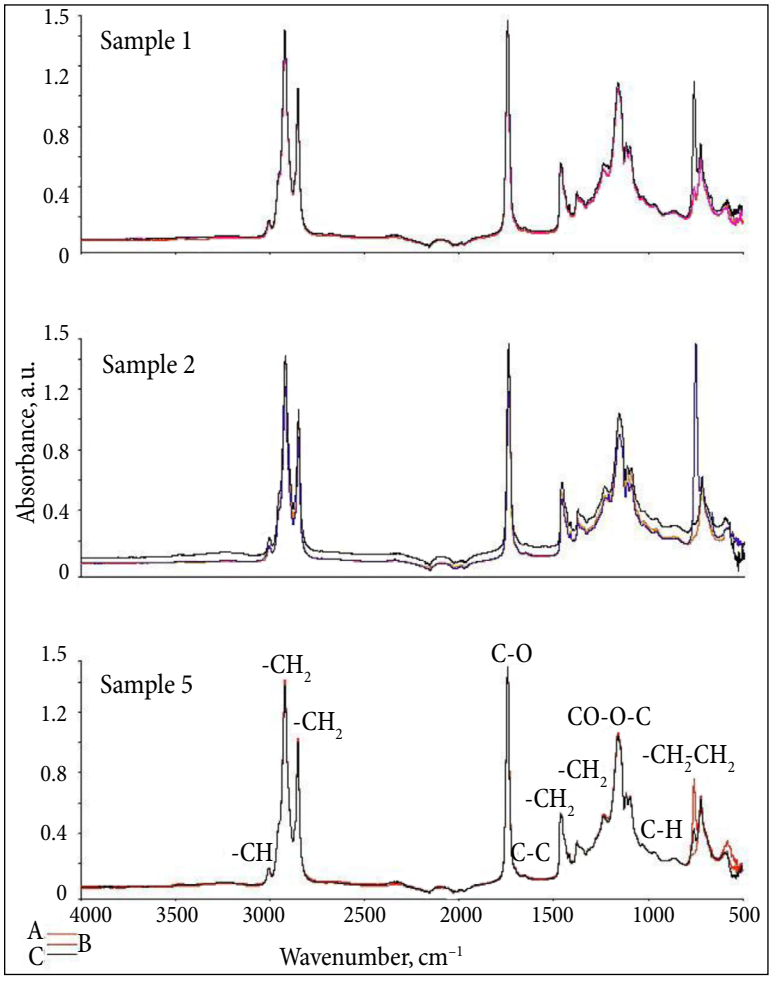

Fig. 1. FTIR spectra of adipose tissue samples taken from different layers of the adipose tissue of three patients

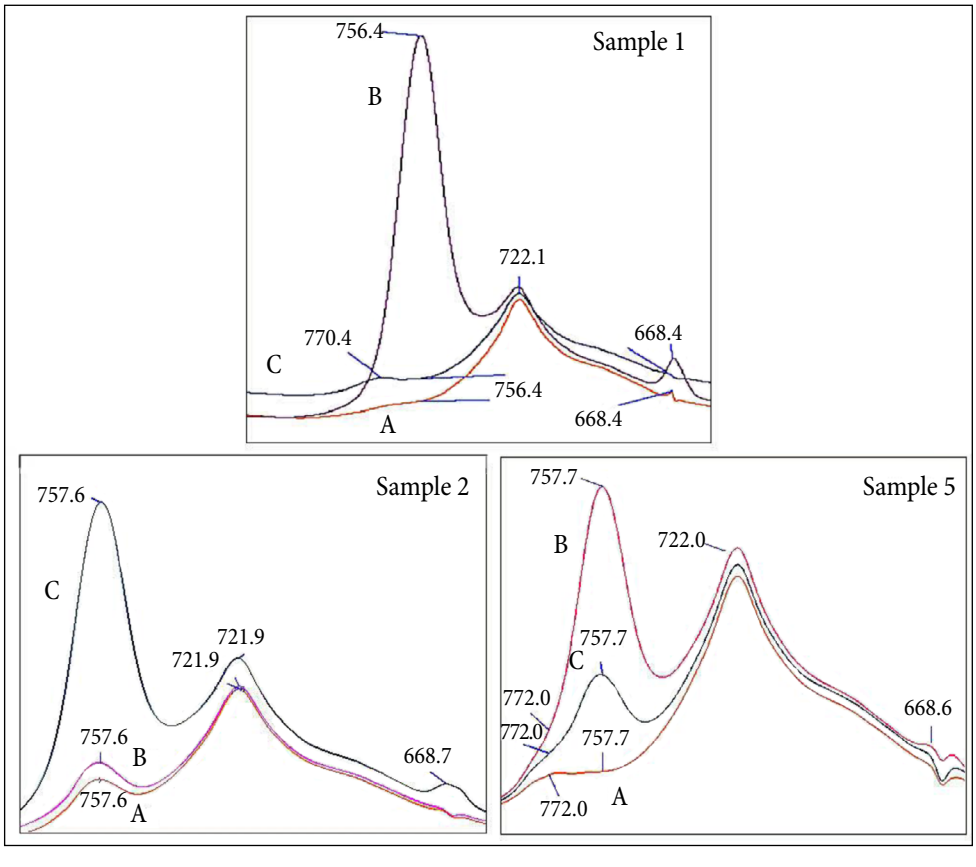

Fig. 2. Fragments ~800–700 cm^{-1} of FTIR spectra which correspond to $-CH_2-CH_2-$ vibrations in long alkyl chains of the adipose tissue samples taken from different layers (subcutaneous A, preperitoneal B and visceral C of the adipose tissue of three patients)

^{1}H NMR spectra of different fat samples have a similar qualitative view as well. The differences between them are reflected on the integral values of the characteristic peaks which in a specific spectral region can be assigned to certain structural elements and one can determine the amount of different acyl groups in fat. Figure 3 represents the ^{1}H NMR spectra of representative fat samples obtained from the subcutaneous (A) layer of two patients. Evidently, both ^{1}H NMR spectra contain very similar chemical shifts. The chemical shifts and peak assignments of all samples are shown in Table 1 [24]. The integral values of the characteristic peaks observed in the ^{1}H NMR spectra of adipose tissue samples are summarized in Table 2. As seen, the changes in the integral values of chemical shift at 0.85–1.0 ppm are negligible. Consequently, this peak attributable to the hydrogen in the methyl group is not valuable for the characterization of adipose samples. The next most intensive peak in the ^{1}H NMR spectra (1.2–1.5 ppm; -(CH_2)n-) varies significantly depending on the analysed specimen. This could be associated with the presence of variety of fatty acids in different adipose samples. Similar conclusions might be made analysing the changes of the chemical shift located at 1.5–1.8 ppm. Interestingly, the integral values of 1.9–2.2 ppm peaks are the highest for the samples taken from the C (visceral) layer. The intensity of the 2.2–2.5 ppm shift is the highest for patients 4 and 5. It means that the adipose tissue samples taken from these patients have increased the amount of allylic (-CH_2-CH=CH-)

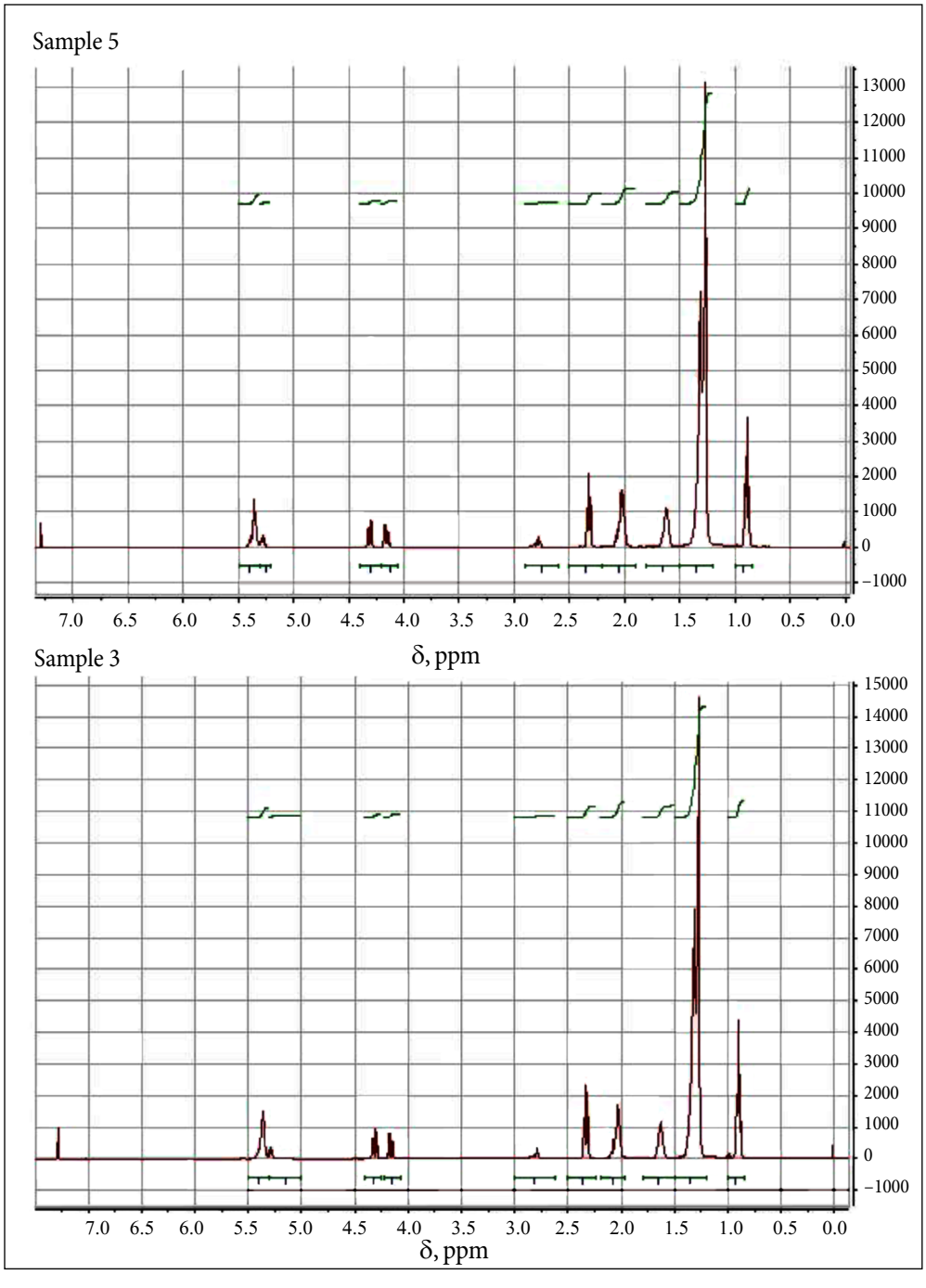

Fig. 3. ^{1}H NMR spectra of the adipose tissue taken from different patients

and acyl (-CH_2-COOH) groupings. From the results presented in Table 2 we could also conclude that the chemical shifts observed at 2.6–2.9, 4.06–4.2 and 4.2–4.4 ppm are not essential and could be omitted for future characterization of such spectra. The last two peaks attributable to the hydrogen in -CH-OCOR glycerol (β position) and -CH=CH- vinyl should be taken into account, since in samples 2 and 4 they have a specific character. Finally, the ^{1}H NMR spectra of adipose tissue samples taken from all patients are rather similar. Thus, according to the results the disease symptoms observed for these patients might be also similar. However, some characteristic features in each ^{1}H NMR spectrum could be also identified.

Table 1. **Chemical shifts and peak assignment of ^{1}H NMR spectra**

Signal	δ, ppm	Proton
1.	0.95	-CH_3 methyl
2.	1.2	-(CH_2)n- all fatty acids
3.	1.6	-OCO-CH_2-CH_2- β-methylene
4.	2.02	-CH_2-CH=CH- allylic
5.	2.2	-CH_2-COOH acyl
6.	2.76	-CH=CH-CH_2-CH=CH bis-allylic
7.	4.16–4.36	-CH_2-OCOR glycerol (α position)
8.	5.36	-CH-OCOR glycerol (β position)
9.	5.4	-CH=CH- vinyl

The fatty acid composition of adipose tissue was determined by using statistical methods. Based on the integral values of the ^{1}H NMR spectra the composition of adipose tissue was estimated on two classes of fatty acids: unsaturated and saturated. For the chemometric equations the following notation was adopted [25]: *n, s* represent the molar ratio of unsaturated and saturated acids; *x* represents the number of double bonds from the polyunsaturated fatty acids; I_1, I_2, I_3, etc. represent the integral values of the signals; *k* is a coefficient which correlates the signal integral with the number of protons [6, 7]. The molar ratio of unsaturated fatty acids is $n = (I_4)/(4 \cdot k)$. The molar ratio of saturated fatty acids is $s = 1 - n$. The number of double bonds obtained is $x = I_9/2 \cdot k \cdot n$; $k = 3 \cdot I_7/4$. Based on chemometric equations, the composition of fat samples from 5 different patients from layers A, B and C was calculated in terms of mono-unsaturated, poly-unsaturated and saturated fatty acids. The results are presented in Fig. 4 and Table 3. According to [26], we can make an assumption that patients with metabolic disorder have lower level of polyunsaturated and high level of mono-unsaturated fatty acids in the adipose tissue. These our results show that patients 1, 2 and 5 have similar fatty layers, but patients 3 and 4, possibly, have a different pathology.

For the determination of the amount of metals in the adipose samples (Na, K, Mg, Ca, Cr, Mn, Fe, Cu, Zn and Ni) an AAS analysis method was applied. The results for the determination of selected elements in different layers of the adipose tissue obtained from different patients are summarized in Table 4. Evidently, the concentration of sodium and potassium

Table 2. **The integral values of the characteristic peaks observed in the ^{1}H NMR spectra of adipose tissue samples**

Sample	Chemical shift δ, ppm	Layer	The integral values of the peaks	Layer	The integral values of the peaks	Layer	The integral values of the peaks
1	0.85–1.0	A	4.60	B	4.59	C	4.61
	1.2–1.5		30.90		31.04		30.79
	1.5–1.8		3.35		3.62		3.58
	1.9–2.2		4.39		4.34		4.52
	2.2–2.5		3.11		3.11		3.10
	2.6–2.9		0.53		0.56		0.50
	4.06–4.2		1		1		1
	4.2–4.4		1		1		1
	5.0–5.3		0.39		0.40		0.38
	5.3–5.5		2.66		2.68		2.74
2	0.85–1.0	A	4.65	B	4.61	C	4.61
	1.2–1.5		31		30.87		30.76
	1.5–1.8		3.28		3.29		3.36
	1.9–2.2		4.30		4.22		4.37
	2.2–2.5		3.13		3.11		3.13
	2.6–2.9		0.46		0.49		0.49
	4.06–4.2		1		1		1
	4.2–4.4		1		1		1
	5.0–5.3		0.47		0.45		0.41
	5.3–5.5		2.51		2.52		2.61
3	0.85–1.0	A	4.62	B	4.53	C	4.55
	1.2–1.5		30.77		30.36		30.41
	1.5–1.8		3.27		3.36		3.35
	1.9–2.2		4.34		4.29		4.48
	2.2–2.5		3.10		3.09		3.12
	2.6–2.9		0.64		0.67		0.64
	4.06–4.2		1		1		1
	4.2–4.4		1		1		1
	5.0–5.3		0.42		0.41		0.41
	5.3–5.5		2.72		2.27		2.81
4	0.85–1.0	A	4.47	B	4.62	C	4.62
	1.2–1.5		30.69		30.56		30.72
	1.5–1.8		3.47		3.22		3.29
	1.9–2.2		5.79		4.71		4.54
	2.2–2.5		3.21		3.14		3.16
	2.6–2.9		0.59		0.55		0.60
	4.06–4.2		1		1		1
	4.2–4.4		1		1		1
	5.2–5.3		0.60		0.57		0.51
	5.3–5.5		2.41		2.53		2.72
5	0.85–1.0	A	4.55	B	4.56	C	4.56
	1.2–1.5		30.18		30.46		30.09
	1.5–1.8		3.26		3.36		3.30
	1.9–2.2		4.26		4.36		4.54
	2.2–2.5		3.11		3.20		3.11
	2.6–2.9		0.51		0.60		0.54
	4.06–4.2		1		1		1
	4.2–4.4		1		1		1
	5.0–5.3		0.48		0.45		0.47
	5.3–5.5		2.52		2.65		2.71

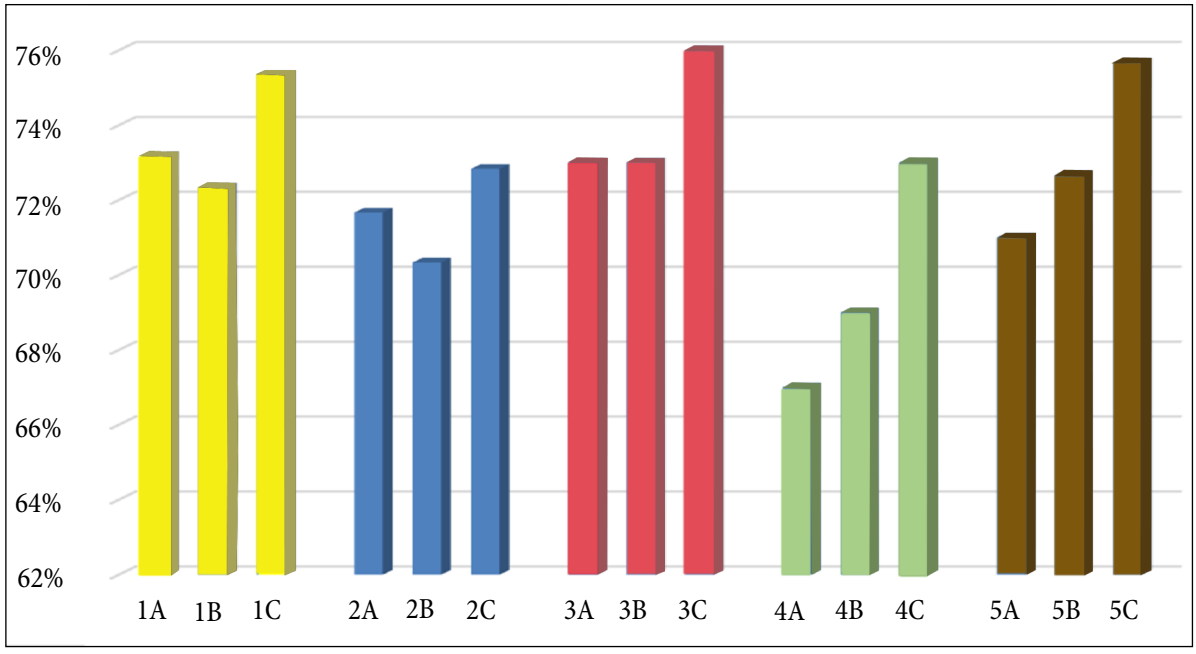

Fig. 4. Composition of polyunsaturated fatty acids in different patient's layers: subcutaneous A, preperitoneal B and visceral C

Table 3. **Fatty acids composition determined from ^{1}H NMR**

Sample	*x*, % mol	*n*, %	*s*, %
1A	1.214612	73	27
1B	1.240741	72	28
1C	1.217778	75	25
2A	1.167442	72	28
2B	1.194313	70	30
2C	1.185355	73	27
3A	1.260274	73	27
3B	1.275229	73	27
3C	1.25	76	24
4A	1.12782	67	34
4B	1.106796	69	31
4C	1.186207	73	28
5A	1.183099	71	29
5B	1.215596	73	27
5C	1.193833	76	24

in the adipose tissue is much higher in comparison with other elements. As seen, the level of sodium and potassium varies in the fat samples of different patients. The concentration of potassium in the adipose tissue of patients 4 (1 480 μg/g) and 5 (1 334 μg/g) is about 2–3 times higher than in fat samples from other three patients. The concentration of sodium is almost 2 times higher in the adipose tissue of patients 2 (1 173 μg/g) and 3 (1 032 μg/g). The concentration of magnesium is higher in samples 1 and 3, amount of calcium is very high in sample 1 (267 μg/g). On the other hand, rather low concentration of calcium was determined in sample 2 (33 μg/g). Evidently, sample 4 contains higher amount of copper. Interestingly, chromium was found only in the adipose of patients 2 (in all A, B and C layers) and 3 (only in layer A). The concentrations of Fe and Zn do not vary significantly in the adipose tissue samples from different patients. Moreover, Mn and Ni were not detected in all analysed samples of the adipose tissue. The relative standard deviation (RSD) values obtained for the determination of metals in the adipose from the obese patients (6.4–10.3%) indicate a high degree of homogeneity, which could be expected for adipose samples. Moreover, the values obtained are not unusual for such type of analysis and can be considered as suitable for routine analysis.

No doubt, the results obtained show various distributions of different metals in the adipose tissue of patients with different metabolical state. We may assume that the results of distribution of potassium, calcium, copper and chromium in adipose tissue layers in obese patients are promising for further medical observation. The change of these metals concentrations in the adipose tissue, however, might be the sign or the possible reason of appearance of symptoms of the abovementioned diseases. Finally, the initial observations show such a tendency that higher concentration of metals prevails in layer B of the adipose tissue. However, the distribution of metal levels in adipose tissue layers is chaotic and does not serve very important information.

The morphology of the adipose samples was investigated by scanning electron microscopy. It is interesting to note that the main morphological features of the adipose tissue taken from adipose tissue layers A, B and C of an individual patient are very similar. The scanning electron micrographs of the adipose tissue obtained from different layers of patient 1 are shown in Fig. 5 indicating surface similarity of the specimens. The microstructure of sample 1 obtained from adipose tissue layers A, B and C is characterized by a number of planar

Table 4. **Results obtained for the determination of average amount of metals in the adipose tissue from obese patients**

Sample	Metal	Layer	Amount, µg/g	Layer	Amount, µg/g	Layer	Amount, µg/g
1	Na	A	268	B	404	C	159
	K		160		216		287
	Mg		12.8		16.9		14.4
	Ca		97		73		97
	Fe		19.3		36.8		57.4
	Zn		2.8		4.0		4.4
	Cu		0.26		0.99		0.48
	Cr		–		–		–
2	Na	A	260	B	548	C	365
	K		117		180		153
	Mg		9.9		11.1		8.9
	Ca		11.39		18.86		2.83
	Fe		28.5		53.1		18.6
	Zn		2.1		6.3		2.8
	Cu		0.50		0.51		1.05
	Cr		3.71		6.34		1.78
3	Na	A	256	B	498	C	278
	K		139		177		119
	Mg		7.8		30.6		13.3
	Ca		8.24		41.60		17.95
	Fe		24.9		29.0		18.8
	Zn		2.5		2.7		2.0
	Cu		0.23		0.20		0.56
	Cr		1.55		–		–
4	Na	A	175	B	198	C	271
	K		334		482		664
	Mg		5.9		11.2		8.7
	Ca		18.6		17.3		7.1
	Fe		30.8		26.6		24.5
	Zn		3.2		3.6		24.5
	Cu		0.42		0.52		2.44
	Cr		–		–		–
5	Na	A	195	B	311	C	143
	K		456		584		294
	Mg		8.5		6.5		3.9
	Ca		21.4		20.5		13.5
	Fe		22.7		32.2		16.8
	Zn		4.6		4.8		3.2
	Cu		0.26		0.52		0.65
	Cr		–		–		–

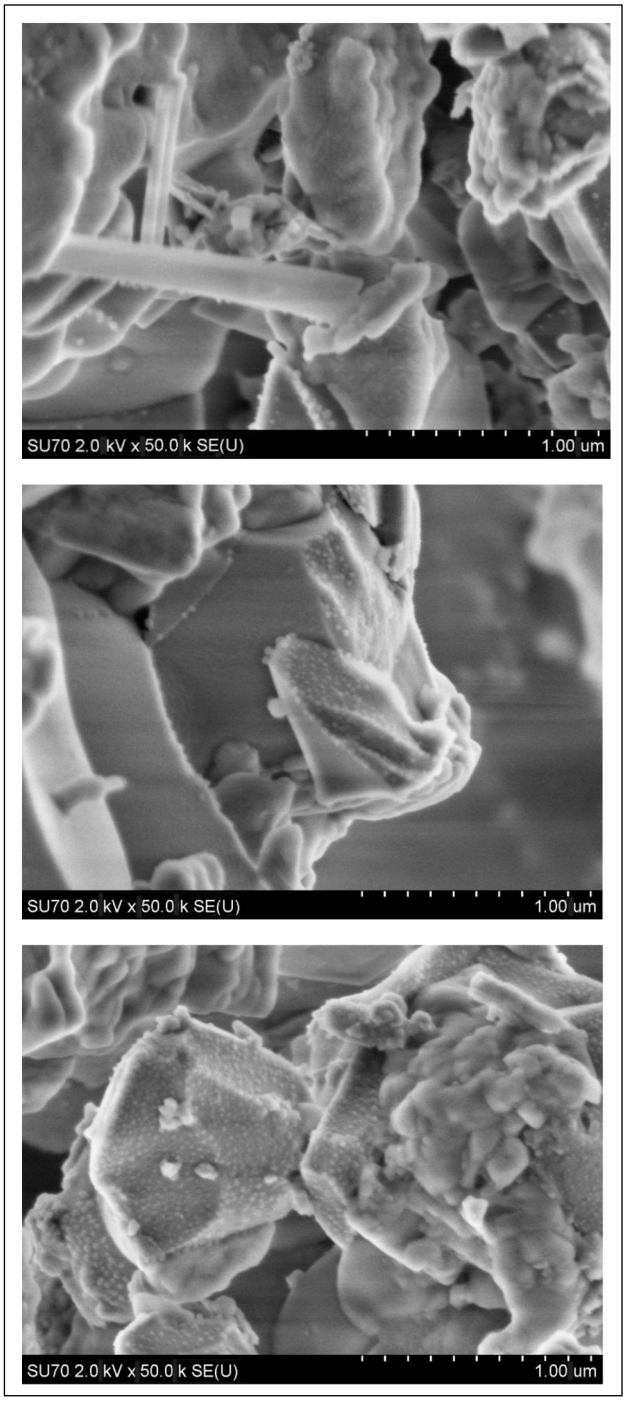

Fig. 5. SEM micrographs of the adipose tissue obtained from subcutaneous A (top), preperitoneal B (middle) and visceral C (bottom) layers of patient 1

particles 1–1.5 µm in size. However, some rod- and/or stick-like and spherical individual particles could also be seen. The adipose material shows a rather open structure and a large surface area with no microscopic evidence for the existence of pores or interparticle voids. Since the microstructure of the adipose tissue taken from different layers is almost analogous, further SEM experiments were performed only with the samples from the preperitoneal (B) layer.

Figure 6 shows the SEM micrographs of the adipose tissue samples taken from patients 2, 3, 4 and 5. No progressive changes in the morphology of samples 2 and 3 were observed. The SEM micrographs of these samples show that the adipose tissue is composed of irregularly shaped 200–400 nm in size particles which are closely connected to each other forming hard agglomerates. However, the microstructure of the adipose tissue obtained from patient 4 is quite different. As seen, the sample morphology is consisting of a single cloudy material but not of particulate matter. The existence of a continuous network of particles is evident. Only some additional very small nanoscaled separate particles could

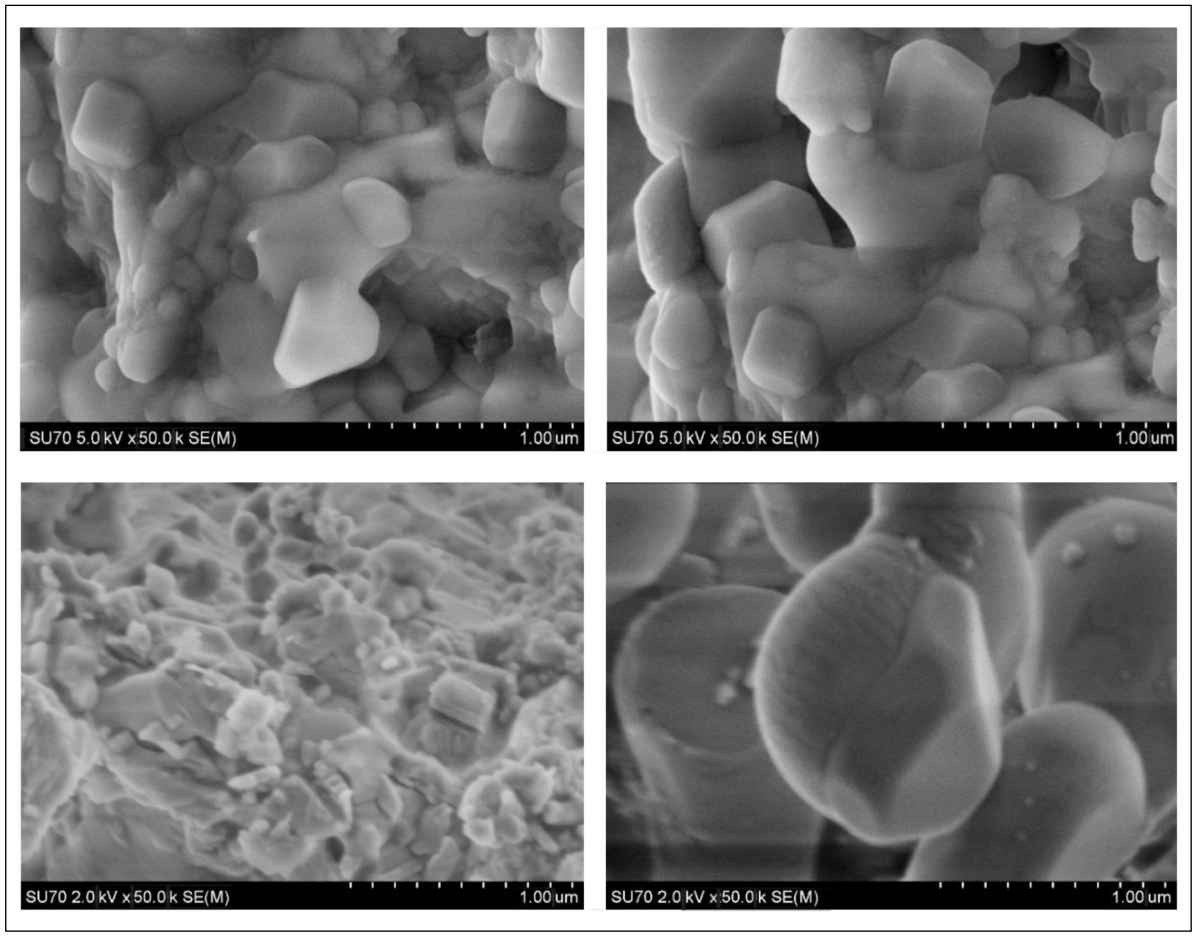

Fig. 6. SEM micrographs of the adipose tissue obtained from the layer preperitoneal B of different patients: 2 (top, left), 3 (top, right), 4 (bottom, left) and 5 (bottom, right)

be seen on the surface of the adipose tissue. Moreover, pores and voids can also be seen, which result probably from the different natural condition of the adipose tissue. It can be also seen from Fig. 6 that sample 5 is composed of spherical particles less than 1 μm in size. The SEM micrograph also revealed the formation of a very homogeneous and uniform surface of particles. Thus, careful morphological observations revealed individual surface morphology of some adipose tissue samples taken from different patients.

CONCLUSIONS

We demonstrated for the first time, to the best of our knowledge, the application of analytical techniques, such as Fourier transform infrared spectroscopy (FTIR), nuclear magnetic resonance (NMR) spectroscopy, scanning electron microscopy (SEM) and metal content determination using atomic absorption spectroscopy (AAS) for the characterization of the adipose tissue from obese patients. According to the FTIR analysis results it was concluded that the main functional groupings in the adipose tissue of different obese patients were the same, however, the real chemical composition of the samples obtained from the subcutaneous, preperitoneal and visceral layers of the adipose tissue from different patients was different. Therefore, FTIR spectroscopy could be used for the analysis and direct qualitative characterization of the adipose tissue composition. The specific features of ^{1}H NMR spectra of adipose tissue samples could be used to predict different pathologies of obese persons. The results of the elemental analysis showed various distribution of different metals in the adipose tissue of differently obese patients. It could be concluded that the results of distribution of potassium, calcium, copper and chromium in adipose tissue layers of people with overweight are promising for further medical observation. Also, careful morphological observations using SEM measurements revealed individual surface morphology of adipose tissue samples taken from different patients. From the obtained results we concluded that such characterization of the adipose tissue is an essential step for possible prediction of the appearance of symptoms of different diseases, since recent studies have emphasized a close relationship between adipose tissue properties and development of fat distribution and metabolism, and different diseases [27]. Obviously, further investigation is needed to prove the impact of chemical composition, structural and morphological

features on adipose tissue activity and their relationship with a disease stage. Of course, medical conclusions could be made only after careful and systematic investigation of numerous patients with obesity and different comorbidities.

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RIEBALINIO AUDINIO STRUKTŪROS IR SUDĖTIES ANALIZINIS APIBŪDINIMAS: NAUJAS POŽIŪRIS DIAGNOZUOJANT ŽMOGAUS APYKAITOS SUTRIKIMUS

S a n t r a u k a

Nutukusių žmonių riebalinio audinio pavyzdžiai buvo tirti infraraudonosios spektroskopijos (FTIR), branduolio magnetinio rezonanso (BMR) spektroskopijos, skenuojančios elektroninės mikroskopijos (SEM) ir atominės absorbcinės spektroskopijos (AAS) metodais. Gauti rezultatai suteikė informacijos apie riebalinio audinio sluoksnių cheminę ir elementinę sudėtį, sandarą bei mikrostruktūrą. Pirmą kartą parodyta, kad šie tyrimo metodai yra labai svarbūs norint nustatyti žmogaus riebalinio audinio specifinius bruožus. Gauti rezultatai leido daryti išvadą, kad žmogaus riebalinio audinio trijų sluoksnių išsamus apibūdinimas yra esminis etapas įvairių ligų simptomams identifikuoti.