Концентрации адипокинов в сыворотке крови у больных сахарным диабетом 2 типа: взаимосвязи с распределением, гипертрофиеи и васкуляризацией подкожной жировой ткани

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Организации

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Структурированная аннотация

Обоснование. Дисфункция жировой ткани (ЖТ) играет важную роль в развитии метаболических нарушений при ожирении и сахарном диабете 2 типа (СД2). Роль распределения, гипертрофии и васкуляризации ЖТ в нарушении секреции адипокинов нуждается в уточнении.

Цель. Определить взаимосвязи между концентрациями адипокинов в сыворотке крови, массой и распределением ЖТ, диаметром адипоцитов и васкуляризацией подкожной ЖТ у больных СД2.

Материалы и методы. Обследовано 125 пациентов с СД2, включая 82 с ожирением. Контролем являлись 30 здоровых лиц, сопоставимых по полу и возрасту. Концентрации лептина, резистина, висфатина, адипсина и адипонектина в сыворотке крови натощак определяли с помощью мультиплексного анализа. Массу и распределение ЖТ оценивали с помощью двухэнергетической рентгеновской абсорбциометрии. Образцы подкожной ЖТ (ПЖТ) были забраны из околопупочной области с помощью ножевой биопсии у 25 пациентов и у 15 лиц, погибших от внешних причин. Кровеносные и лимфатические сосуды в ПЖТ типировали с помощью иммуногистохимии и, используя антитела к CD34 и к подопланину соответственно. Оценивали объемную, численную плотность и ультраструктуру кровеносных и лимфатических сосудов, а также диаметр подкожных адипоцитов.

Результаты. Пациенты с диабетом, по сравнению с контролем, имели значительно более высокие уровни лептина, резистина, адипсина и висфатина (все р≤0,0003). Уровень адипонектина не показал различий. Концентрации лептина, резистина, висфатина, адипсина и адипонектина прямо коррелировали с массой ЖТ на бедрах. Уровни лептина и адипсина, кроме того, положительно коррелировали с массой ЖТ на туловище и в центральной области живота. Концентрация лептина, но не других адипокинов, была ассоциирована с гипертрофиеи подкожных адипоцитов. В ПЖТ больных СД наблюдались уменьшение объемной плотности микрососудов (p=0,01) и увеличение объемной и численной плотности лимфатических сосудов (p=0,02 в обоих случаях). В лимфатических капиллярах выявлено набухание цитоплазмы, митохондрий, цистерн гранулярной эндоплазматической сети и сниженное содержание микропиноцитарных везикул. Резистин и висфатин показали обратные ассоциации с объемной плотностью сосудов.

Заключение. Эндокринная дисфункция ЖТ у больных СД2, проявляющаяся увеличением концентрации лептина, резистина, висфатина и адипсина в сыворотке крови, ассоциирована с...
Serum adipokine concentrations in patients with type 2 diabetes: the relationships with distribution, hypertrophy and vascularization of subcutaneous adipose tissue

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ABSTRACT
BACKGROUND: Adipose tissue (AT) dysfunction plays an important role in metabolic disorders in obesity and type 2 diabetes. The role of distribution, hypertrophy and vascularization of AT in adipokine secretion disturbances remain to be clarified.
AIMS: To determine the relationships between serum concentrations of adipokines and the mass and distribution of AT, diameter of adipocytes and vascularization of subcutaneous AT in patients with type 2 diabetes.
MATERIALS AND METHODS: A total of 125 patients were examined, including 82 subjects with obesity. Thirty healthy individuals, matched by sex and age, were acted as control. Concentrations of leptin, resistin, visfatin, adipin and adiponectin in fasting serum were determined using multiplex analysis. Mass and distribution of AT was assessed by dual-energy X-ray absorptiometry. Samples of SAT were obtained from umbilical region using a knife biopsy in 25 patients and in 15 individuals who died in accidents. Blood and lymphatic vessels in SAT were revealed with immunohistochemistry, using antibody to CD-34 and podoplanin respectively. The volume and numerical density, ultrastructure of blood and lymphatic vessels, and mean diameter of subcutaneous adipocytes were evaluated.
RESULTS: Patients with diabetes, as compared to control, had significantly higher levels of leptin, resistin, adipin and visfatin (all p<0.001). Adiponectin showed no differences. Concentrations of leptin, resistin, visfatin, adipin and adiponectin correlated positively with gynoid fat mass. Additionally, leptin and adipin showed positive correlations with truncal and central abdominal fat mass. Concentration of leptin, but not other adipokines, was associated with hypertrophy of subcutaneous adipocytes. A decrease in volumetric density of microvessels(p=0.01) and increase in volume and numerical density of lymphatic vessels (both p=0.02) was observed in subcutaneous AT from diabetic subjects. The swelling of cytoplasm, mitochondria, cisterns of granular endoplasmic reticulum and reduced content of micropinocytotic vesicles was revealed in lymphatic capillaries. Resistin and visfatin showed inverse associations with density of microvessels.
CONCLUSION: Endocrine dysfunction of AT in patients with type 2 diabetes, manifested by elevation of serum concentrations of leptin, resistin, visfatin and adipin, is associated with mass and distribution of AT, hypertrophy of subcutaneous adipocytes and vascularization abnormalities of subcutaneous AT.

KEYWORDS
diabetes; obesity; adipose tissue; adipokines; blood vessels; lymphatic vessels

Adipose tissue (AT) plays an important role in the regulation of energy homeostasis. Adipokines secreted by AT regulate the energy balance by participating in the control of appetite and metabolic activity of other organs, such as skeletal muscle and liver [1, 2]. AT hormonal dysfunction plays an
important role in the development of metabolic disorders in obesity and type 2 diabetes mellitus (DM2). In this case, changes in adipokine secretion are associated with the formation of insulin resistance, chronic inflammation, and metabolic disorders [3, 4]. The mechanisms of AT redistribution in obesity and DM2 remain the subject of intensive research. More and more data indicate that AT redistribution in different fat depots is associated with a change in its regulatory functions [1, 5]. The adipogenesis process is associated with a change in the expression of a large number of genes, including those associated with AT distribution and adipocyte hypertrophy [6]. It was found that in patients with obesity, DM2 and metabolic syndrome, AT distribution is associated with the blood concentration of AT hormones and adipocytokines [7–9]. An important element of AT remodeling is the formation of adipocyte hypertrophy. A change in the number and size of adipocytes is accompanied by a change in adipokine secretion and the metabolic function of AT [1]. Angiogenesis is an aspect of AT remodeling which is interesting and underexplored yet. It is known that DM is characterized by complex and sometimes multidirectional changes in angiogenesis in various vascular pools [10]. Expansion of AT in obesity requires compensatory growth (neogenesis) of blood vessels. Inadequate angiogenesis is supposed to contribute to the development of AT dysfunction and insulin resistance [11]. With obesity, the volume density of blood vessels in the AT decreases. It has been hypothesized that the resulting AT hypoxia is one of the causes of impaired secretory function of adipocytes [12]. Peptide products of AT secretion (adipokines) can enter the bloodstream not only through the blood capillaries, but also through the lymphatic system roots. It was revealed that the concentrations of some adipokines (in particular, leptin) in lymph flowing from the subcutaneous tissue (SCT) are significantly higher than those in blood plasma [13]. Changes in the lymphatic collectors of AT and their relationship with adipocyte dysfunction in DM patients have not been studied. Thus, taking into account the characteristics of the distribution, morphology and vascularization of AT can be important for understanding the patterns of development of AT dysfunction in DM2. In previous studies, the main focus was on changes in the hormonal function of visceral AT in obesity and DM2, and the role of changes in subcutaneous tissue (SCT) was less studied.

AIM

We aimed to determine the relationship between serum adipokine concentrations, weight and distribution of AT, adipocyte diameter, density and morphological aspects of blood and lymphatic vessels of SCT in DM2 patients.

METHODS

STUDY DESIGN

One-stage comparative non-interventional single-site study. Figure 1 presents schematically the study design.
Рис. 1. Дизайн исследования.

<table>
<thead>
<tr>
<th>Пациенты СД 2 типа (n=125)</th>
<th>Type 2 DM patients (n=125)</th>
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<tbody>
<tr>
<td>Лица без СД и ожирения (n=30)</td>
<td>Persons without DM or obesity (n=30)</td>
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<tr>
<td>Общеклиническое обследование для оценки соответствия критериям включения и не включения</td>
<td>General clinical examination to assess compliance with inclusion and exclusion criteria</td>
</tr>
<tr>
<td>Образцы ПЖК лиц, погибших от внешних причин (n=15)</td>
<td>Samples of SCT of persons who died from external causes (n=15)</td>
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<tr>
<td>Определение концентраций адипокинов в сыворотке крови натощак</td>
<td>Determination of fasting serum adipokine concentrations</td>
</tr>
<tr>
<td>Исследование КСТ и распределения ЖТ по областям</td>
<td>Study of BC and AT distribution by regions</td>
</tr>
</tbody>
</table>
| Ножевая биопсия ПЖК; морфометрический анализ подкожных адипоцитов; иммуногистохимическое типирование, определение объемной плотности и структурных особенностей кровеносных и лимфатических сосудов в ПЖК | Scalpel biopsy of SCT; morphometric analysis of
**INCLUSION CRITERIA**

The main group of patients was composed of DM2 patients, men and women aged 40–70 years, with DM duration of more than one year. The exclusion criteria were secondary forms of obesity, a history of bariatric surgery on the gastrointestinal tract, congestive cardiac failure (functional class 3 or 4 according to NYHA), chronic renal failure stage 4–5; treatment with glucagon-like peptide-1 analogues, anorectics, thiazolidinediones over the past year. The control group consisted of individuals without DM and obesity, who were selected using the same exclusion criteria as in the formation of the main group.

**CONDITIONS OF THE STUDY**

Patients were enrolled at the clinic of the Research Institute of Clinical and Experimental Lymphology, a branch of the Federal Research Center, Institute of Cytology and Genetics, Siberian Branch of the Russian Academy of Sciences (RICEL, branch of the FRC, SB RAS).

**STUDY DURATION**

The patients were enrolled in the study in 2014-2017.

**DESCRIPTION OF MEDICAL INTERVENTION**

The study participants were DM2 patients (125 people); individuals without DM and obesity (30 people) constituted the control group for clinical and biochemical research methods. All DM patients who participated in the study underwent a general clinical examination in the endocrinology department of the clinic in accordance with the Algorithms for the provision of specialized care for DM patients [14]. The protocol of examination of the control group individuals included the history taking, analysis of anthropometric data, a general analysis of blood, urine, a biochemical blood test, and determination of the level of glycated hemoglobin (HbA1c). In all DM patients and those in the control group, the concentrations of adipokines in fasting blood serum were determined, the study of body composition (BC) and AT distribution in regions was conducted. A subgroup of 25 patients was formed of the 125 DM patients included in the study, for a morphological study of SCT. The subgroup was created by sequential selection, based on a ratio of 1:5. The patients included in this subgroup underwent scalpel biopsy of the SCT in the paraumbilical region. Given the invasiveness of the study, the biopsy of the SCT was not performed in non-diabetic individuals. The control for morphological studies consisted of samples of SCT taken at the Office of forensic medical examination from persons who died from external causes. The protocol of the morphological study of SCT included morphometric analysis of subcutaneous adipocytes, immunohistochemical typing, determination of volume density and ultrastructural aspects of blood and lymph vessels. In DM patients who underwent the biopsy, an analysis of the conjugacy of the serum level of adipokines with the weight, distribution of AT and morphological aspects of SCT, was performed.

**PRIMARY STUDY OUTCOME**

The study evaluated the parameters, namely fasting serum concentrations of leptin, resistin, visfatin, adipisin and adiponectin; total weight of AT, weight of AT in the trunk, in the central region of the abdomen and on the hips; the mean diameter of SCT adipocytes; volume and numerical density, diameter of blood and lymph vessels in the subcutaneous AT; the degree of conjugation between these signs.

**ANALYSIS IN SUBGROUPS**

The parameters evaluated in the study were compared in DM2 patients and in the control group. Among the DM2 patients, subgroups with obesity and without obesity were analyzed.
METHODS OF OUTCOME REGISTRATION

Determination of serum adipokine concentrations. Blood samples were taken from the ulnar vein after an 8-hour fasting. Serum was frozen after separation and stored at −80°C until analysis. Serum leptin, resistin, visfatin, adipsin and adiponectin concentrations were determined using a Bio-PlexPro™ multiplex assay using the Bio-Plex Protein Assay System (Bio-Rad, USA) and commercially available kits (No. 171-07001M and No. 171-A7002M, Bio-Rad, USA). The method was used to conduct a simultaneous study of the concentrations of molecules in one sample of the biological material. The study was conducted in accordance with the instructions of the kit manufacturer.

The study of BC was conducted using dual-energy X-ray absorptiometry using a LunarProdigy apparatus and the Body Composition program (GE, USA). The total weight of AT, the weight of body fat, the ratio of the AT weight in the central region of the abdomen and on the hips was determined. According to the settings of the Body Composition program, when measuring fat in the central abdomen (Android fat), we studied the area bounded below by the pelvic section, above, by a line passing above the pelvic section by 20% of the distance between the pelvic and neck sections, and at the sides, by hand sections. When measuring the AT weight on the hips (gynoid fat), we evaluated the area bounded from above by a line passing below the pelvic section 1.5 times the height of the android fat region described above, and from the sides, by the sections of the legs. The height of the gynoid fat region is normally about 2 times greater than the area of fat deposition in the abdominal central region (Fig. 2).

![Fig. 2. Schematic representation of the assessment of the adipose tissue distribution by regions in the Body Composition program. 1 – android fat area; 2 – gynoid fat area.](image)

Biopsy of the SCT. Samples of SCT in DM patients were taken from the paraumbilical region using a scalpel biopsy in the supine position, after processing the surgical field and local anesthesia (lidocaine 2%). A skin incision was made conventionally with a scalpel up to 0.5 cm, 2 cm lateral to the umbilical ring. In the presence of insulin-induced lipohypertrophy in these areas, the sample was taken at a distance of less than 1 cm from the edge of the lipohypertrophy site. Tissue samples were taken with a volume of 2–3 cm³, which corresponded to approximately 3–5 g of AT. After the biopsy, intradermal sutures and aseptic dressing were applied to the skin. SCT samples of persons, taken to the Office of forensic medical examination, who died from external causes (road injury) we used as a control. During the forensic examination of these individuals, no signs of chronic diseases or poisoning were revealed. The samples were taken according to the same methodology as in DM patients, within 2 hours after death.
Morphological studies of AT. SCT samples were fixed in a 4% formaldehyde solution, processed according to standard histological procedures, and embedded in paraffin. The average adipocyte diameter in the SCT samples was determined using the open Image J platform (https://imagej.net) using the algorithm presented by SD Parlee et al. [15].

For immunohistochemical staining of the vessels, sections of AT were dewaxed in a series of lowering concentration alcohols, rehydrated, and subjected to antigen revelation in a microwave oven in a citrate buffer solution (pH 6.0). Then, after blocking the non-specific binding, the sections were hybridized with primary rabbit monoclonal antibodies to CD34 [EP373Y] (ab81289, Abcam, UK) or murine monoclonal antibodies to podoplanin [D2-40] (MON-RTU1092, Monosan, Netherlands) overnight at +4°C and further with the corresponding secondary polyclonal goat antibodies to rabbit IgG (ab205718, Abcam, UK) or to mouse IgG (ab6789, Abcam, UK) conjugated with horseradish peroxidase for 1 hour at +25°C. After hybridization, the sections were washed, contrasted with a hematoxylin solution, dehydrated and enclosed in a mounting medium. Images were obtained with a LEICA DME light microscope (Leica Microsystems, Germany).

Blood vessels were identified as structures stained positive to CD34. Lymphatic vessels were identified as structures with positive intense linear immunohistochemical staining for podoplanin (D2-40). Slides from the same block were stained with hematoxylin and eosin to estimate the number of blood microvessels. Any brown stained endothelial cell or cell cluster was considered a single vessel. The numerical and volume density of blood and lymph vessels was measured over 0.75 mm² of sample area using the Image J software (https://imagej.net).

To assess ultrastructural changes in the lymphatic and blood vessels, half-thin sections 1 μm thick were prepared, stained with toluidine blue, studied under a light microscope, and the necessary areas were selected for examination using an electron microscope. Ultrathin sections 35–45 nm thick were obtained from the selected material on an LKB-8800 ultratome, contrasted with a saturated aqueous solution of uranyl acetate, lead citrate, and studied using a JEM 1010 electron microscope (Japan).

ETHICAL CONSIDERATIONS
The study protocol was approved by the Ethics Committee of the Research Institute of Clinical and Experimental Lymphology, a branch of the Institute of Cytology and Genetics, SB RAS (protocol No. 110 dated 20.05.2014). All patients gave written informed consent to participate in the study.

STATISTICAL ANALYSIS
The principles of calculating the sample size: the sample size was not preliminarily calculated.
Methods of statistical data analysis. Statistical processing was performed using the STATISTICA 10 program (StatSoft, Inc, 2011, USA). The data obtained were checked for normal distribution using the Kolmogorov – Smirnov criterion. Given that the distribution of most of the signs studied was different from normal, non-parametric statistics methods were applied. Intergroup differences were evaluated using the Mann – Whitney test. The relationship between the signs was evaluated using the Spearman correlation analysis. The critical level of significance in testing statistical hypotheses was taken equal to 0.05. Data are presented as medians, 25–75 percentiles.

RESULTS

OBJECTS (PARTICIPANTS) OF THE STUDY
The study included 125 DM2 patients, 48 men and 77 women, aged 43 to 70 years (median 62 years). DM duration ranged from 1 year to 36 years (median 13 years). The level of HbA1c ranged from 5.5% to 13% (median 8.2%). All the patients received sugar-lowering drugs, most of them received insulin therapy (n=103) in combination with oral sugar-lowering drugs, including metformin (n=81), sulfonylurea (n=25) or dipeptidyl peptidase-4 inhibitors (n=7). A combination of metformin and sulfonylurea was given to 14 patients, and 7 patients received metformin monotherapy. Complications of DM and comorbid conditions included hypertension (n=121), chronic kidney disease (CKD) stages 1–3 (n=103), and diabetic retinopathy (n=100), coronary heart disease (n=51), macroangiopathy of the lower extremities (n=19), non-alcoholic fatty liver disease (n=103), and neuropathy (n=123).

The patients examined included 82 obese patients, 22 patients had normal body mass index (BMI), 21 patients were overweight (BMI 25–29.9 kg/m²). In a statistical analysis, patients with normal body mass and overweight patients were considered as one subgroup. The clinical characteristics of the examined patients with and without obesity are presented in Table 1. The patients with obesity, in comparison with patients with normal body mass and overweight patients, had a higher HbA1c level and lower cholesterol of high density lipoproteins (HDL). Age, duration of DM, and other metabolic indicators were not distinguished between the groups.

Table 1. Clinical characteristics of DM2 patients with and without obesity
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Signs

|Patients with BMI <30 kg/m² (n=43) | Patients with BMI > 30 kg/m² (n=82) |
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<td>Age, years</td>
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<td>BMI, kg/m²</td>
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<td>DM duration, years</td>
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<td>WC/HC index</td>
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<td>HbA₁c, %</td>
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<td>Total cholesterol, mmol/l</td>
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<td>HDL cholesterol, mmol/L</td>
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<td>Uric acid, mmol/l</td>
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<td>Estimated GFR, ml/min/1.73 m²</td>
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<td>UACR, mg/mmol</td>
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Notes: data are presented as medians (25th; 75th percentiles). BMI – body mass index; HC – hip circumference; WC – waist circumference; LDL – low density lipoproteins; HDL – high density lipoproteins; GFR – glomerular filtration rate (according to the formula CKD-EPI, 2009); UACR – urinary albumin to creatinine ratio.

The control group consisted of 30 individuals without DM and obesity, 5 men and 25 women, aged 41 years to 70 years.

**PRIMARY STUDY RESULTS**

**Serum adipokine concentrations and their relationship with AT distribution in DM2 patients**

In all the patients included in the study, the concentration of AT hormones in the blood serum were determined. Compared with the control, DM patients had significantly higher levels of leptin (median 21.4 and 12.3 pg/ml, respectively, p=0.01), resistin (8.1 and 2.8 pg/ml, p <0.0001), adipin (1,848 and 1,203 pg/ml, p <0.0001), visfatin (3.6 and 1.9 pg/ml, p=0.0002). Adiponectin concentrations did not show significant differences (8711 and 8647 pg/ml, p=0.96). Levels of leptin, resistin, adipin, and visfatin were significantly higher in DM patients and those with obesity compared with patients with a BMI lower than 30 kg/m². Adiponectin concentrations did not differ between the groups (Table 2).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control group (n=30)</th>
<th>DM2 patients</th>
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<tbody>
<tr>
<td>Leptin</td>
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<td>Resistin</td>
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<td>Visfatin</td>
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<td>Adipin</td>
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<td>Adiponectin</td>
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Notes: the data are presented as medians (25th; 75th percentiles). P – significance of differences between subgroups of DM patients; Pk – significance of differences with the control group. BMI – body mass index, DM2 – type 2 diabetes mellitus.

All the regulators studied, with the exception of adiponectin, had positive associations with BMI (for leptin r=0.43, p <0.0001; for resistin r=0.43, p <0.0001; for adipin r=0.29, p=0.001; for visfatin r=0.31, p=0.0004), waist circumference (WC; for leptin r=0.34, p=0.001; for resistin r=0.33, p=0.001;
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for adipsin r=0.35, p=0.0007; for visfatin r=0.30, p=0.004), and hip circumference (HC; for leptin r=0.50, p <0.0001; for resistin r=0.26, p=0.01; for adipsin r=0.27, p=0.01; for visfatin r=0.26, p=0.01).

Significant correlations between adipokine concentrations and the WC/HC ratio were observed only in men (for leptin r=0.53, p=0.0001; for resistin r=0.41, p=0.004; for adipsin r=0.40, p=0.005; for visfatin r=0.39, p=0.007). We have not revealed any correlations between the level of these adipokines and the parameters of carbohydrate and lipid metabolism. Adiponectin levels negatively correlated with triglyceride concentration (r=-0.37, p=0.0002) and positively correlated with HDL cholesterol (r=0.33, p <0.001). There was a weak positive correlation between the level of visfatin and the concentration of uric acid (r=0.26, p=0.003).

The total weight of AT, the weight of AT in the trunk, and the weight of AT in the central region of the abdomen and on the hips were expected and appeared to be greater in obese patients compared with patients with normal body weight and overweight patients (Table 3). Levels of leptin, resistin and adipsin showed direct correlations with the total weight of AT, while the levels of leptin and adipsin were positively correlated with the weight of AT in the trunk and in the abdominal central region. The concentration of visfatin directly correlated with the weight fraction of AT in the body. All the adipokines studied showed a relationship with the weight of AT in the hips (Table 4).

Table 3. Parameters of body composition in DM2 patients with and without obesity and in individuals of the control group.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control group (n=30)</th>
<th>DM2 patients</th>
<th>P</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>BMI &lt;30 kg/m²</td>
<td>BMI &gt; 30 kg/m² (n=82)</td>
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<td>AT weight, kg</td>
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<td>AT weight, %</td>
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<td>AT weight in the trunk, kg</td>
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<tr>
<td>AT weight in the central region of the abdomen (android fat), kg</td>
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<td>AT weight on the hips (gynoid fat), kg</td>
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</table>

Notes: data are presented as medians (25th; 75th percentiles). P – significance of differences between subgroups of DM patients; Pk – significance of differences with the control group. AT – adipose tissue, DM2 – type 2 diabetes mellitus.

Table 4. Correlations between serum adipokine concentrations and body composition parameters in DM2 patients

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Leptin</th>
<th>Resistin</th>
<th>Visfatin</th>
<th>Adipsin</th>
<th>Adiponectin</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT weight, kg</td>
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<tr>
<td>AT weight, %</td>
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<tr>
<td>AT weight in the trunk, kg</td>
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<tr>
<td>AT weight in the central region of the abdomen (android fat), kg</td>
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<tr>
<td>AT weight on the hips (gynoid fat), kg</td>
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</tbody>
</table>

Notes: Spearman’s rank correlation coefficients are presented, * p <0.05. AT – adipose tissue.

**Adipocyte diameter, density, and morphological aspects of blood and lymph vessels in SCT in DM2 patients**

Morphological studies of SCT were performed in 25 DM2 patients, 7 men and 18 women. In this group, 12 patients were obese, 3 patients had normal body weight, and 10 were overweight. The control consisted of SCT samples from 15 individuals, 5 men and 10 women aged 43 years to 71 years, who died from external causes and did not have obesity or DM2.

In DM2 patients, the average subcutaneous adipocyte diameter ranged from 139 to 432 μm, while in the control, it was from 17 to 241 μm. The median of the indicator in DM patients was 3.8 times greater than in individuals without obesity and DM (281 and 73 μm, respectively, p=0.0005)
Blood and lymphatic vessels in the SCT were detected in the intralobular loose fibrous connective tissue, while the lymphatic vessels, as a rule, accompanied the blood vessels (Fig. 3A, 3B). Overgrowths and protrusions of the luminal and basal surfaces of the endothelial cells of the lymphatic capillaries were noted. An electron microscopic examination in the lymphatic capillaries of DM patients revealed swelling of the cytoplasm, mitochondria, cisterns of the granular endoplasmic reticulum and a decrease in the content of micropinocytotic vesicles. Contacts between endothelial cells of the type of superposition and interdigitation were noted, but no predominance of any type of contact was revealed (Fig. 4B). Cytoplasm swelling was also observed in the cytoplasm of endotheliocytes of blood capillaries, and the volume density of organelles was reduced (Fig. 4D). A distinguishing characteristic of the blood vessel endotheliocytes in DM patients was the predominance of luminal micropinocytotic vesicles over basal ones and a decrease in the proportion of cytoplasmic micropinocytotic vesicles.

Morphometric analysis revealed a decrease in the volume density of blood microvasculature in SCT in DM patients as compared with the control. The numerical density of microvessels did not demonstrate significant differences. Volume and numerical density of lymphatic microvessels, on the contrary, were statistically significantly increased in DM patients (Fig. 3C, 3D). The relationship between adipocyte hypertrophy and changes in SCT vascularization was observed. The diameter of the subcutaneous adipocytes inversely correlated with the volume density of the microvessels (r=-0.42, p=0.007) and directly correlated with the volume density of the lymphatic vessels (r=0.4, p=0.01).
Fig. 3. Visualization and density of lymphatic vessels in subcutaneous tissue in DM2 patients and in the control. A – the normal lymphatic vessel; B – lymphatic vessels in a DM2 patient. Immunohistochemical staining for a marker of endotheliocytes of the lymphatic vessels, podoplanin. Magnification x400; C – volume density of lymphatic vessels, LDVv,%; D – the numerical density of the lymphatic vessels, LVDq, n; * – p <0.02 compared with the control group.
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Fig. 4. Ultrastructural organization of the endothelial cells of the lymphatic and blood vessels of the adipose tissue of DM2 patients. A – normal endothelium of the lymphatic capillary; B – endothelium of the lymphatic capillary of a patient with DM2 and obesity. Overgrowths and protrusions of the luminal and basal surfaces of the endothelial cell of the lymphatic capillary. Contact with an adjacent endothelial cell (arrows); C – normal endothelium of the blood capillary; D – endothelium of the blood capillary of a patient with DM2 and obesity. Swelling of the endotheliocyte cytoplasm, a decrease in the content of cytoplasmic organelles and vesicles. Electron microscopy. Magnification x10,000.

Analysis of the conjugacy of adipokine concentrations and morphological parameters of SCT in DM2 patients
The average adipocyte diameter in DM patients correlated positively with the level of leptin (r=0.42, p=0.03), the remaining adipokines did not show significant associations with this parameter. In a rank correlation analysis, the inverse relationships of resistin and visfatin concentrations with the volume and quantitative density of microvessels were revealed (for resistin r=−0.52, p=0.008 and r=−0.54, p=0.005, respectively; for visfatin r=−0.44, p=0.03 in both cases). In addition, visfatin was directly correlated with the volume density of lymphatic microvessels (r=0.44, p=0.03). Concentrations of other adipokines did not show significant relationships with vascular density in SCT.

ADVERSE EVENTS
Three patients had the formation of subcutaneous hematomas after a biopsy of the SCT. The hematomas resolved within 3–7 days.

DISCUSSION
SUMMARY OF THE PRIMARY RESULT OF THE STUDY
AT dysfunction in DM2 patients is manifested by an increase in serum concentrations of adipokines, namely leptin, resistin, visfatin and adipin. The severity of AT dysfunction is associated with aspects of AT distribution, hypertrophy of subcutaneous adipocytes, decrease in the volume density of blood vessels, and an increase in the volume density of the SCT lymphatic vessels. The levels of leptin, resistin, visfatin, adipin and adiponectin correlate directly with the AT weight in the hips, and the levels of leptin and adipin are also positively associated with the weight of AT in the trunk and in the central abdomen. The concentration of leptin, but not of other adipokines, positively correlates with the diameter of subcutaneous adipocytes. The levels of resistin and visfatin demonstrate inverse associations with the volume density of SCT microvessels.

DISCUSSION OF THE PRIMARY RESULT OF THE STUDY
The AT ability to change the metabolism of energy substrates in the after-meal state, during fasting and physical exertion, including through regulatory effects on muscles and the liver through the secretion of adipokines, is an essential element in adapting energy metabolism to changing life conditions. In
turn, a decrease in “metabolic flexibility” of AT is combined with resistance to insulin and DM2 [16]. Disorders of AT regulatory function in DM2 are closely related to the process of AT remodeling which includes changes in the AT weight, distribution and morphology. In this paper, we analyzed the clinical, biochemical, and morphological characteristics of AT remodeling in DM2 patients. The data obtained indicate that production disorders of various adipokines are associated to varying degrees with an increase in the mass of AT, the aspects of its distribution, hypertrophy of subcutaneous adipocytes, and changes in SCT vascularization. Five adipokines were selected to study the AT function, namely leptin, resistin, visfatin, adipsin and adiponectin. The choice of adipokines was determined by their influence on metabolic processes and sensitivity to insulin, as well as the predominant secretion of various fat depots. In DM2 patients, a significant increase in serum concentrations of leptin, resistin, visfatin and adipsin was recorded. The data obtained are generally consistent with the results of previous works, which showed an increase in the concentration of leptin, resistin, and visfatin in DM2 patients [7, 8, 17, 18] and does not confirm data on a decrease in the level of adipsin in this disease [18]. Changes in adipokine production in DM2 patients were expectedly associated with the severity of obesity. All studied regulators, with the exception of adiponectin, showed positive relationships with BMI, WC and HC. It is known that AT is the main source of leptin, resistin, visfatin, and adipsin and almost the only source of adiponectin. At the same time, the molecules studied were associated to varying degrees with the weight of AT and its distribution. The levels of leptin, resistin and adipsin showed direct correlations with the total weight of AT, and the levels of leptin and adipsin, in addition, were positively correlated with the weight of AT in the trunk and in the central abdomen. Obviously, the different nature of the associations of adipokine levels with BC parameters demonstrates special characteristics of the secretion of these molecules by different fat depots. In recent years, morphological aspects of AT remodeling in obesity and its associated conditions have been of great interest. In particular, the relationship between AT dysfunction and adipocyte hypertrophy is discussed. It is known that SCT has a limited ability to expand due to precursor cells; it responds to the excess supply of energy substrates by adipocyte hypertrophy. Enhanced lipolysis in hypertrophic adipocytes leads to a massive influx of free fatty acids into the bloodstream, ectopic deposition of fat, chronic inflammation, and insulin resistance [19]. A significant increase in the average diameter of subcutaneous adipocytes in the DM patients examined by us indicates a predominantly hypertrophic type of their obesity. The recorded correlation between the average diameter of the adipocyte and the level of leptin confirms the importance of adipocyte hypertrophy in the development of AT dysfunction.

The processes of angiogenesis and lymphangiogenesis are significant in remodeling of AT in obesity. The role of changes in angiogenesis in the formation of endocrine adipocyte dysfunction represents an interesting and still scantily studied issue. Gealekman et al. showed in their work that SCT has a greater ability to expand its capillary network than visceral AT, but this ability decreases with morbid obesity. At the same time, a decrease in the SCT angiogenic ability correlates with insulin resistance [20]. According to experimental data, a high-fat diet reduces the density of capillaries in the subcutaneous and visceral AT. Aerobic exercise, on the contrary, increases vascularization [21]. In this study, we recorded a decrease in the volume density of the microvasculature network in the SCT in DM2 patients. This is consistent with data on impaired angiogenic ability of AT cells in DM [22]. An electron microscopic examination of the endothelial cells of the AT blood vessels revealed cytoplasmic edema and a decrease in transendothelial transport. It can be assumed that decrease in volume density and changes in the ultrastructure of blood vessels can contribute to the development of AT hypoxia. There is evidence that AT hypoxia is associated with mitochondrial adipocyte dysfunction, overproduction of reactive oxygen metabolites, impaired differentiation of preadipocytes into adipocytes, inhibition of macrophage migration from AT, and a change in their secretory phenotype [12]. A cascade of molecular signals triggered by hypoxia is involved in the development of fibrosis, AT inflammation, and insulin resistance [23]. A likely consequence of these disorders is AT regulatory dysfunction. This concept is supported by the inverse correlations recorded by us between the serum levels of resistin and visfatin and the density of the SCT blood vessel network. The role of the lymphatic system in the development of AT dysfunction in diabetes has not been previously studied. In this study, we recorded an increase in the density of the network of lymphatic microvessels in SCT in DM2 patients. Compared to changes in the density of blood vessels, the density of lymphatic vessels changed in the opposite way, which may indicate known differences in the regulation of angiogenesis and lymphangiogenesis, including in DM [24]. It is known that in the postnatal period, activation of lymphangiogenesis accompanies tumor and inflammatory processes. It can be assumed that AT inflammation in obesity promotes the activation of lymphangiogenesis. In mice fed a high-fat diet, perilymphatic inflammatory infiltration of AT was described [25]. It should be
noted that the lymphatic collectors function during inflammation is not limited to the transport of antigens and immunocompetent cells, as endothelial cells of the lymphatic capillaries are able to regulate the activity and phenotype of immunocytes. Under various conditions, lymphangiogenesis can both inhibit and aggravate the course of inflammatory processes [26].

Experimental and clinical studies have recorded a decrease in the drainage and transport function of the AT lymphatic system in obesity [25, 27, 28]. Indirectly, dysfunction of the lymphatic microvessels of the SCT in the patients examined by us is evidenced by changes in the cytoplasm and intracellular organelles (mitochondria, granular endoplasmic reticulum) of endotheliocytes identified by us through electron microscopy. Previous studies have shown a relationship between impaired lymphatic drainage and fat accumulation in obesity in mice [27, 29]. It is known that impaired lymphatic outflow in patients with congenital or acquired lymphedema is also associated with activation of adipogenesis [30]. It has been suggested that decrease in the ability of the lymphatic system to eliminate macromolecules from the interstitial space in patients with obesity may be a link between obesity and inflammation of the AT [28]. However, in this study, we were not able to record any relationship between the density of the SCT lymphatic vessels and the concentrations of adipokines in blood. The question on the role of the lymphatic system in the formation of AT dysfunction in DM requires further research.

STUDY LIMITATIONS

The one-stage (cross-sectional) design of the study does not enable to determine unambiguously the causal relationships between the signs. The methodological approach to assessing AT distribution, used in the work, did not enable to differentiate subcutaneous and visceral fat depots. A relatively small sample size of patients, who underwent biopsy, requires caution in interpreting the results of morphological studies. The inclusion in the study of a large number of patients receiving insulin therapy could lead to a systematic data offset regarding the general population of DM2 patients.

CONCLUSION

The study of the processes of remodeling and regulatory dysfunction of AT is of great importance for decoding the mechanisms of insulin resistance and DM2 formation. In this work, we studied changes in serum concentrations of several adipokines, namely leptin, resistin, visfatin, adipisin and adiponectin in DM2 patients and determined their relationships with the characteristics of the distribution, hypertrophy, and vascularization of SCT. An increase in the concentrations of leptin, resistin, visfatin and adipisin indicates endocrine dysfunction of AT. It was revealed that in DM2 patients, the concentrations of leptin, resistin, visfatin, adipisin and adiponectin correlate directly with the weight of AT in the hips, and the levels of leptin and adipisin, in addition, demonstrate associations with the weight of AT in the trunk and in the central abdomen. The hypertrophic type of obesity in DM2 patients is associated with hyperleptinemia.

We have shown for the first time that hypertrophy of subcutaneous adipocytes in DM2 patients is combined with a decrease in the volume density of blood vessels and an increase in the volume and number density of lymphatic vessels in the SCT. These changes may indicate multidirectional changes in the intensity of angiogenesis and lymphangiogenesis processes. Concentrations of resistin and visfatin showed an inverse relationship with the volume density of microvessels. In the vessels themselves, ultrastructural changes in endotheliocytes were revealed, more pronounced in the lymphatic capillaries, which indicate indirectly a disorder of microcirculation and lymphatic drainage. Study of the relationship between adipo-, angio- and lymphangiogenesis and regulatory adipocyte dysfunction, and development of approaches to correction of the AT endocrine function by influencing its vascularization represent promising tasks for future research.

ADDITIONAL INFORMATION

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Contribution of authors. V.V. Klimontov conducted the study concept and design, performed analysis of results, wrote the text; D.M. Bulumbaeva collected clinical data, performed morphometry, statistical processing and analysis of the results, wrote the text; N.P. Bgatova created the study concept and design, performed analysis of the morphological data; Yu.S. Taskaeva performed immunohistochemical examination of blood vessels; N.B. Orlov studied the level of adipokines; O.N. Fazullina performed the BC study; M.Yu. Soluyanov performed biopsy of subcutaneous tissue in DM patients; S.V. Savchenko performed biopsy of subcutaneous tissue in patients of the control group; V.I.
Konenkov created the concept and design of the study, interpreted the results. All the authors made a significant contribution to the research and preparation of the article, read and approved the final version of the article before publication.

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