UNIVERSITATEA DE MEDICINĂ ȘI FARMACIE
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THE CORRELATION BETWEEN PRE-ADIPOCYTE DIFFERENTIATION AND THE METABOLIC SYNDROME IN OBESE BARIATRIC PATIENTS

PhD. THESIS ABSTRACT

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2019

The thesis includes a general part structured in 7 chapters, totaling 42 pages and the personal part consisting of 6 chapters totaling 81 pages, 95 figures and 21 tables, 360 bibliographic references and one appendix.

The table of contents and abbreviations are kept as found in the doctoral thesis, and the figures and tables selected for the abstract keep the numbering within the thesis.

Keywords: obesity, metabolic syndrome, adipose tissue, mesenchymal stem cells, adipogenic differentiation, in vitro cell cultures.

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ABBREVIATIONS

WHO – World HealthOrganization

BMI – Body Mass Index

MetS – Metabolic syndrome

HOMA-IR – Homeostatic Model Assessment for InsulinResistance

ASCs – Adiposederived stem cells

RYGB – Gastric by-passRoux-en-Y

LGS – LaparoscopicGastricSleeve

DMEM – Dulbecco'sModifiedEagle Medium

FBS – Bovine fetal serum

IDF – International DiabetesFederation-

MHO – Metabolic HealthyObese-

SVF – Stromal Vascular Fraction-

WAT – White AdiposeTissue

CRP – C Reactive Protein

AMPK – adenosine monophosphate

DAPI-4′,6 – diamidino-2-fenilindol

PPAR-γ – peroxisome proliferator-activated receptor gamma

STUDY BACKGROUND AND AIMS

Obesity is a major public health problem in modern society, and the prospects are extremely worrying given the increasing trend of incidence for this disease in both the adult and pediatric populations. The medical implications are mainly related to the morbid-mortality of cardio-vascular cause associated with obesity through the demonstrated association with the metabolic syndrome, representing an accumulation of risk factors, which include high blood pressure, dyslipidemia and type 2 diabetes mellitus.

This doctoral research was built around an extremely current concern to identify early markers of metabolic alteration in obese patients, starting from the concept of "adiposopathy" which defines a complex pathophysiological alteration of adipose tissue, which precedes and determines by endocrine-related and immunologicalmechanisms the development of the metabolic syndrome. The identification of this etiopathogenic source for the metabolic disturbances associated with obesity is extremely promising for research, revealing very complex and versatile mechanisms and differentiation pathways for mature adipocytes starting from multi-potent mesenchymal precursors.

For obese patients who accumulate cardiovascular risk factors clustered within the notion of metabolic syndrome, the hypothesis of a limited capacity of adipogenic tissue expansion, altered adipogenic differentiation capacity and thus a limitation of the capacity to store excess lipid in fat deposits which causes their accumulation at the visceral level or high ectopic locations was formulated (Heilbronn L et al., 2011).Adipocyte hypertrophy, inflammation in adipose tissue and adipocyte dysfunction may be possible manifestations or mechanisms behind this storage disorder. These adipocyte functions may have a genetic determinism. However, the exact mechanism of the onset of metabolic disorders in obese patients has remaines unclear.

The inter-individual variability, regarding the metabolic response to excess weight, is incompletely elucidated and capable of offering new pharmacological targets for the early control of the metabolic dysfunctions

associated with obesity. In this direction, the complex regulation of the adipogenesis transcription pathway and auto- paracrine communication through the various adipokines is a modern and revealing approach to understanding and addressing the metabolic syndrome associated with obesity.This thesis follows the changes in adipose tissuebiological pathways changes that lead to metabolic complications in obesity.

Through this doctoral paper we set out to pursue a series of study objectives such as:

- Evaluation of the metabolic profile and serum level of adipokines: adiponectin and leptin in obese patients and evaluation of the particularities of these dosages for the subgroups of obese patients with or without associated metabolic syndrome.

- Evaluation of the morphology and area of adipocytes in subcutaneous adipose tissue of obese patients in relation to their metabolic profile;

- Identifying the particularities of adipogenesis in obese patients and demonstrating the major impact of the adipocyte precursor cell capacity (mesenchymal type) to generate new mature adipocytes, capable of efficiently storing lipids in the determinism of metabolic disorders associated with obesity;

-Opening up new perspectives in understanding the process of adipogenesis and the changes that occur in obesity, with the potential of a pharmacological target that would allow the control of the extremely serious metabolic complications associated with obesity, which have become a major public health problem globally.

MATERIALS AND METHODS

The present doctoral thesis was organized in the form of 3 studies, including:

Study No.1 - prospective clinical study presenting clinical-anthropometric data, extended biochemical and hormonal profile focused on the dosage of leptin, adiponectin and leptin / adiponectin ratio in the serum of obese patients addressed for bariatric surgery in relation to the metabolic syndrome defined by the criteria IDF.The study included 106 obese patients**.**Clinical and biological parameters studied:

1) Clinical and anthropometric evaluation includes: Waist measurement (cm), Body weight (kg), BMI calculation (kg / m2). 2) Paraclinical evaluation: biochemical, hormonal, inflammatory and nutritional profile. Assessment of biochemical parameters in patients' serum at the time surgery .Hormonal balance: TSH, fT4, fT3 (thyroid hormonal profile), Morning cortisol (9am), Insulinemia, C Peptide .3) Metabolic status assessment indices as Insulin resistance (HOMA-IR) - The HOMA index is calculated from the basal glucose and insulin levels by the formula: HOMA-IR = (insulin (μU / mL) x blood glucose (mg / dL)) / 405.Based on the lipid fractions dosed for our group of obese patients, the following indices were calculated: Total Cholesterol / HDL - Cholesterol (Castelli Index), Triglycerides / HDL Cholesterol (Reaven Index).4) Dosage of adiponectin and leptin by ELISA technique:**Study No. 2** is a histological study that included 23 obese patients for whom subcutaneous adipose tissue samples were collected, embedded in paraffin, sectioned and stained hematoxylin-eosin and the obtained images were evaluated by automated optical microscopy and subsequently analyzed with dedicated software. Ţhe obtained adipocyte area was analyzed in relation with the metabolic syndrome defined by the IDF criteria.Following the images evaluation ,histology images of the subcutaneous adipose tissue were obtained for the study group (Figure 9.5).

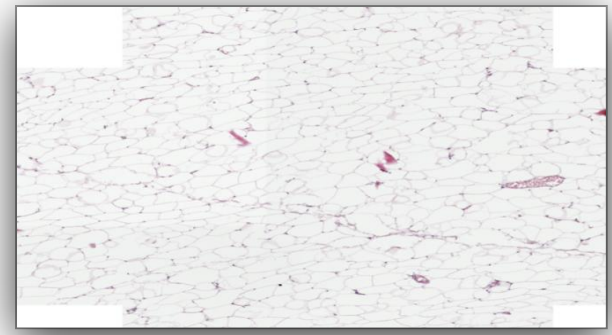


Fig. 9.5. Adipose tissue section with area of 380 µm2, high resolution acquisition with20x Air lens.

Based on the obtained images, the automated measurement of the adipocyte dimensions was made using the Adiposoft analysis software and thus, in Excel format, the estimated area of the analyzed adipocytes, the mean and the standard deviation calculated for the respective values, for each analyzed section are obtained. An average value of the subcutaneous adipocyte area is obtained for each patient included in the study, which will be introduced in the statistical analysis in correlation with the clinical and paraclinical parameters.

Study No. 3 is an experimental study, which involved evaluating the in vitro differentiation capacity of adipocyte mesenchymal precursors isolated from subcutaneous adipose tissue of obese, adipogenic patients and their relationship with metabolic syndrome. There were analyzed 23 subcutaneous adipose tissue samples: 20 samples from obese patients and 3 subcutaneous adipose tissue samples from normal-weight patients, respectively.

Multipotent mesenchymal stem cells were isolated from subcutaneous adipose tissue samples, and their proliferation and differentiation were followed in vitro to mature adipocytes in adipogenic culture medium. The data obtained from the obese patients compared to the normoponderal (control) patients but

also between the obese patients with associated metabolic syndrome and those without metabolic impairment were evaluated.

Isolation and proliferation of mesenchymal stem cells derived from adipose tissue: Mesenchymal cells derived from adipose tissue (Adipose Derived Stem Cells-ASCs) will be isolated according to a specific working protocol, described in the literature (Karen M. Lyons-UCLA, 2008).

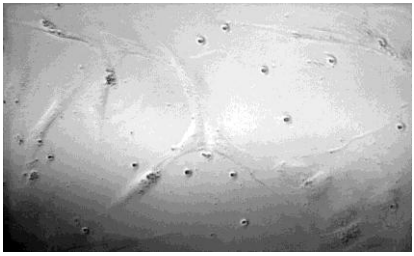
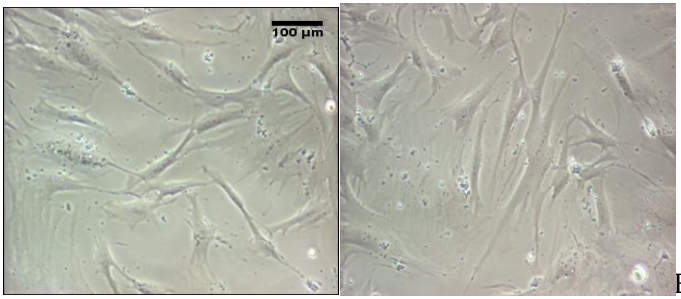


Fig. 9.13. Isolated mesenchymal stem cell (Day 3) from subcutaneous adipose tissue of obese patients.

The small number of cells at incubation and their dispersion in the culture well led to a slow growth and proliferation compared to the literature data (Mok PL, 2008), about 4 weeks were necessary to obtain 80-90% confluence .

A 

B

Fig. 9.14. Proliferation protocol for ASCs isolated from subcutaneous adipose tissue. A. Tracking the ASCs proliferation Day 6-brightfield mode microscopy ("light field") -20x; B. Tracking the ASCs proliferation Day 9 - brightfield mode microscopy ("light field") -20x

Adipogenic differentiation of confluent ASCs: The progressive accumulation of lipids is observed using the optical microscope starting with Z15 of the adipogenic differentiation protocol in the form of refractory inclusions of variable dimensions with the tendency to group in the form of "clusters" as can be seen in figure 9.20. Lipid accumulation involves visualization in the cell culture of lipid inclusions, usually visualized by means of phase contrast optical microscopy (PH2). The complete differentiation cycle was completed after 21 days. The implementation and reproducibility of this protocol are the essential elements of our study and represent an important starting point for further research in this field.

A B

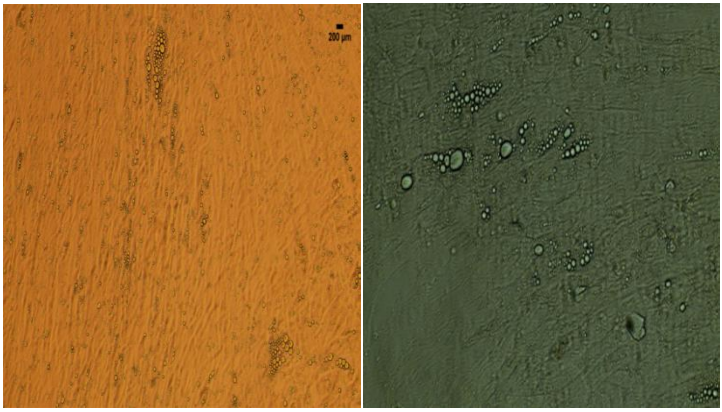


Fig. 9.20. Monitoring at the optical microscope of lipid inclusions in the culture of adipogenic differentiated ASCs. A) Adipogenesis protocol - brightfield mode optical microscopy ("light field") - Day 14; B) Adipogenesis protocol - phase contrast optical microscopy-PH2-Day 16;

Statistical analysis for the 3 studies was performed using SPSS version 20.0 and included comparisons between two or more categories of variables (t-student test, Mann-Whitney, ANOVA), correlation analysis (Pearson or Spearman, respectively), bivariate, multiple and hierarchical regression

analysis. Significance threshold was set at $p < 0.05$. Data are expressed as mean ± standard error.

RESULTS

Study No.1 - Clinical, biochemical and hormonal study of obese patients for bariatric surgery.Prospective analysis (2015-2017)

1) Comparative assessment on the criterion of metabolic syndrome in the group of obese patients.

Parameters	**Lot MetS (n=64)**	**Lot nonMetS (n=42)**	***p* value**
Age (years)	43,21±9,95	40,31±11,23	0,143
Female patients number (%)	43 (67,6%)	38 (89,8%)	**0,004**
BMI, kg/m^2	43,02±8,37	38,68±9,85	**0,012**
Morning Glycemia, mg/dl	123,57±47,24	91,72±11,40	**0,001**
Insulinemia, µUI/ml	26,04±18,36	16,47±9,74	**0,001**
HbA1c, %	6,16±0,88	4,80±1,58	**0,001**
HOMA-IR	9,40±7,00	4,43±3,72	**0,001**
Total Cholesterol , mg/dl	223,23±41,26	202,31±32,12	**0,006**
LDLc, mg/dl	148,62±32,73	136,36±29,65	**0,050**
HDLc, mg/dl	43,16±8,58	50,41±9,56	**0,001**
Triglycerides, mg/dl	208,20±99,33	106,06±38,74	**0,001**

Table 10.2. Comparative evaluation of the 2 subgroups of obese patients according to the IDF criteria for MetS

* marking the parameters where statistically significant differences were obtained.

2) Comparative statistical evaluation. Metabolic syndrome criteria for glucidic metabolism parameters.

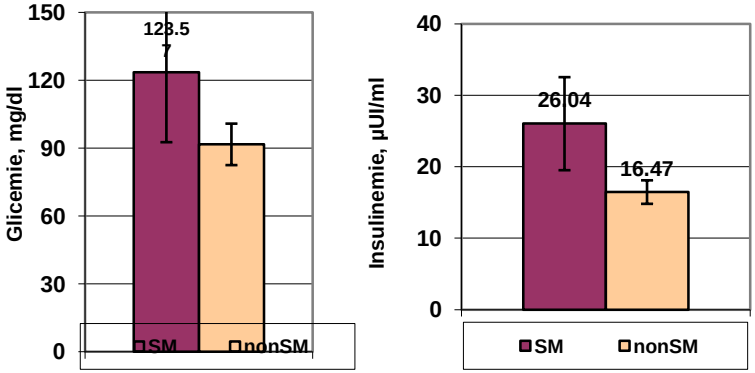


Fig. 10.5. Mean values of glucose metabolism parameters compared to study groups

Thus, a significantly higher mean level for insulinemia (30.01 vs 16.8; p = 0.001), HOMA-IR (9.39 vs 4.48; p <0.001) is noted. With a sensitivity of 83% and a specificity of 60%, HOMA-IR can be a good predictor for metabolic syndrome (AUC = 0.768; 95% CI: 0.655-880), cut-off value = 3.35. (Fig. 10.17)

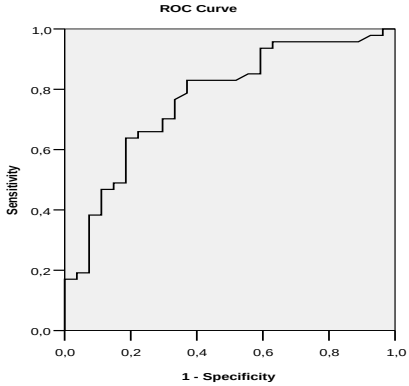


Fig. 10.17. The sensitivity / specificity balance of HOMA-IR in the determinism of the metabolic syndrome

By drawing the ROC curve it is noted that the leptin / adiponectin ratio, with a probability of 65.8%, can be a good predictor in the determinism of the metabolic syndrome (AUC = 0.658; 95% CI: 0.437-0.699) (Figure 10.31)

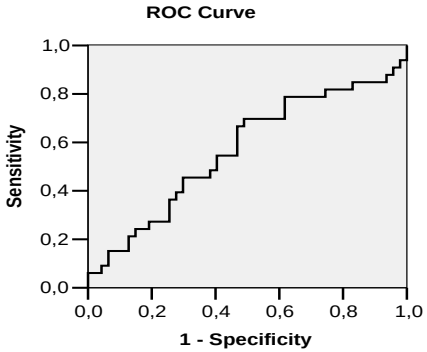


Fig. 10.31 The sensitivity / specificity balance of the leptin / adiponectin ratio in the determinism of the metabolic syndrome.

Study No.2. Histological evaluation of subcutaneous adipose tissue sections - adipocyte area correlations

The mean area of subcutaneous adipocytes was significantly higher in obese patients with metabolic syndrome (3200 vs. 1289 µm2; p = 0.001) compared to those who did not meet this criteria.

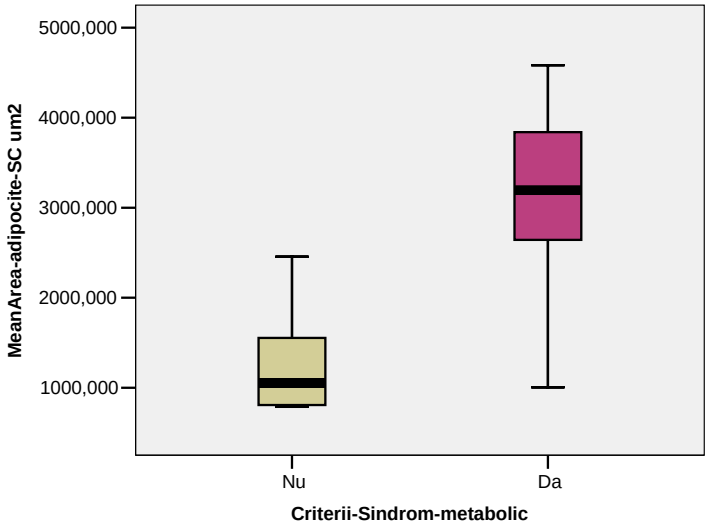


Fig. 10.38. The average level of the adipocytes area depending on the presence of the metabolic syndrome

In 57.5% of patients, the increased level of the mean area of subcutaneous adipocytes correlated with a higher level of BMI (r = + 0.575; R2 = 0.3308; p = 0.004) as indicated in Figure 10.43.

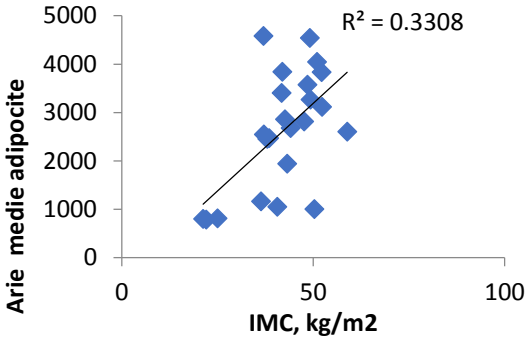


Fig. 10.43. Correlation of the mean level of subcutaneous adipocytes with obesity

The correlation between mean adipocyte area and HOMA-IR was direct, with moderate intensity (r = +0.493; p = 0.017), similar to that obtained for C Peptide (r = +0.622; p = 0.002) as indicated in Figure 10.45.

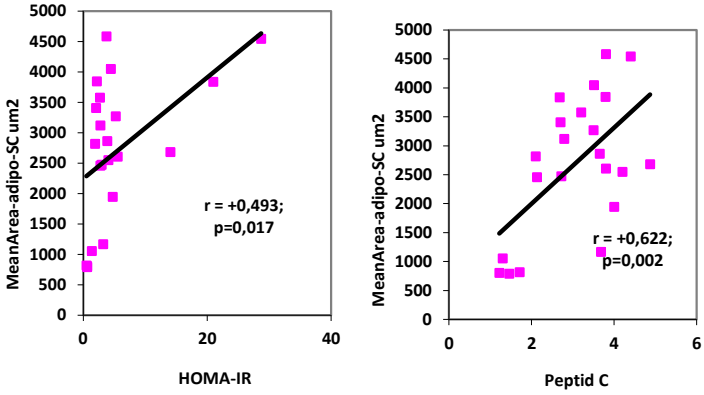


Fig. 10.45. Correlation of mean area of adipocytes with HOMA-IR and peptide C

Over 60% of patients associated higher values of leptin / adiponectin ratio with higher values of mean adipocyte area (r = +0.602; p = 0.002) (Fig. 10.46).

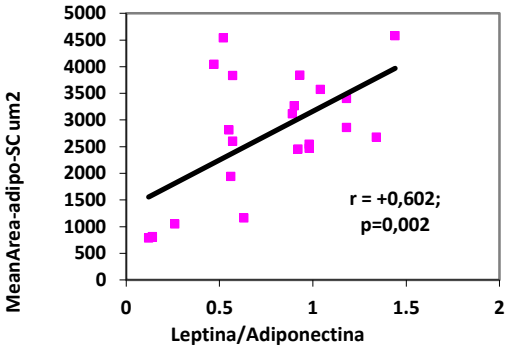
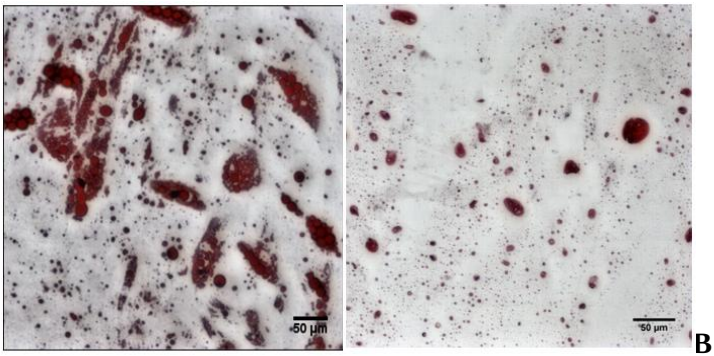


Fig. 10.46. Correlation between mean adipocyte area and Leptin / Adiponectin ratio

Study No.3-Results of the experimental evaluation of the adipogenic differentiation capacity of subcutaneous ASCs



A **B**

Fig. 10.51**.** Comparative assessment of the accumulation of specific lipid pigment (Oil Red O)A. adipogenic differentiated wells for ASCS - control lot - normal-weight patients – microscopybrightfield ("light field") 20x; B. Adipogenic differentiation for ASCs - lot of obese patients - brightfieldmicroscopy ("light field") 20x

a) Descriptive analysis of lipid accumulation evaluated by Oil red O absorbance for the studied group:

Individual values of lipid accumulation ranged from 0.410 to 0.820, the mean level being significantly lower in patients with metabolic syndrome (0.461 vs. 0.668; p = 0.001), especially in the female sex (Table 10.19, Figure 10.56).

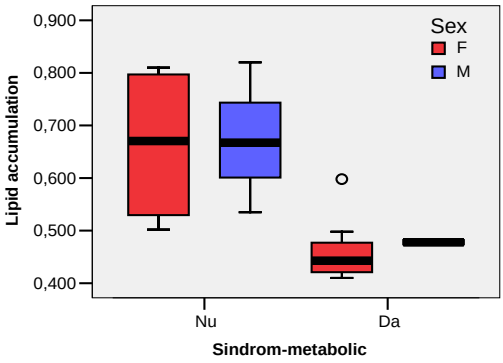


Fig. 10.56. Average values of Oil Red absorbance by sex compared to study groups

b) Lipid accumulation for obese patients with associated metabolic syndrome compared to "metabolic healthy obese":A significantly lower level of lipid accumulation (0.46 vs 0.67; p = 0.001) is objectified in patients with metabolic syndrome compared with obese patients without associated metabolic syndrome.

Parameter	Metabolic healthy obese-Non MetS N=4	Obese patients with associated metabolic syndrome N=16	T-test (Sig) p<0.05
Lipid accumulation-absorbance value	0.67±1.23	0.46±0.59	0.001

Table 10.20. Comparative analysis according to the metabolic status of obese patients for the value of lipid accumulation.

By drawing the ROC curve, it is found that Oil Red absorbance can be a good predictor for the metabolic syndrome (AUC = 0.973; IC95: 0.913-1.033) (Figure 10.57).

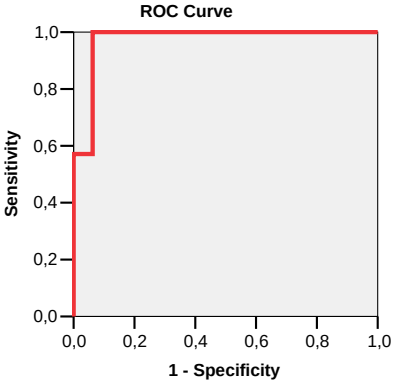


Fig. 10.57. Oil Red absorbance - a predictor of the syndrome

DISCUSSIONS

Study No. 1 - Clinical, biochemical and hormonal study of obese patients for bariatric surgery. Prospective analysis (2015-2017): As obesity has lately become a health problem with global epidemic dimensions, identifying the subgroup of patients at significantly higher risk of developing severe metabolic complications is essential for focusing on weight loss and controlling risk factors in a target sub-population. This can be practically and economically an important step towards personalizing obesity management (Stefan N, 2018).

Excess adiposity is associated with various disorders. Metabolic syndrome, insulin resistance, type 2 diabetes, high blood pressure, dyslipidemia, atherosclerosis and cardiovascular disease are major consequences of obesity (WHO Obesity Fact sheet, 2015), (Mokdad AH, 2001).The data published by

Karelis AD et al. 2004 and Brochu (2001) identified a proportion of 20-30% of "metabolically healthy" obese, while Karelis (2005) detected a greater proportion of about 40% of the group of obese patients studied.

Since the early 2000s, adiponectin has begun to be considered a valuable biomarker for metabolic pathology, with an inverse correlation with both visceral adiposity and basal blood glucose, triglycerides, insulinemia or blood pressure (Ryo M, 2004). Thus, results similar to those obtained in our study were published by Kotani et al. (Kotani K, 2011) and showed higher levels of LAR in patients with metabolic syndrome or Ayina et al (Ayina CNA, 2017), which demonstrated statistically significant correlations between LAR and metabolic syndrome or HOMA-IR. In our study, the correlation between adiponectin and HOMA-IR was indirect and reduced in intensity, but given the small group of patients included in the study it can be estimated that for a larger group of obese patients this relationship should be maintained.

Study No.2 Histological evaluation of subcutaneous adipose tissue fragments: Adipocyte morphology appears in the literature as an individual characteristic with increased inter-population variability. The area of subcutaneous adipocytes has also been associated with insulin resistance in non-diabetic patients, independent of BMI (Lundgren, 2007), but also with blood glucose levels (Hoffstedt, 2010; Yang, 2012). It is known to alter the secretory profile of hypertrophic adipocytes, by increasing leptin production and reducing the level of adiponectin (Meyer LK, 2013). Both BMI and metabolic syndrome criteria, HOMA-IR or serum adipokine levels are not ideal parameters in the early assessment of metabolic changes in obese patients and therefore the evaluation of morphological and functional changes of adipocytes with subcutaneous localization becomes an important hypothesis in early identification. of metabolic dysfunctions associated with obesity. Thus, the size of subcutaneous adipocytes is thought to be an important parameter, and the phenotype of the hypertrophic, large-area adipocytes is associated with higher values of HOMA-IR (Skurk T, 2007), but also of hsCRP as a marker of chronic inflammation.

In this thesis, we support the hypothesis that the increased size of adipocytes, but also the low capacity for proliferation or hyperplasia, are factors directly related to the metabolic abnormalities of the adipose tissue but also to the metabolic peculiarities at the systemic level, so that a separate category of individuals can be identified. prone to metabolic complications of obesity.

Adipocyte size is considered an important marker for assessing the metabolic risk associated with obesity, tracking the quality of fat tissue more than the quantity. Recent studies have shown that reduced adipocyte volume is associated with improved insulin sensitivity after weight loss. Also, the association between increased adipocyte volume and metabolic syndrome is established (Andersson DP, 2014), with adipocyte size being demonstrated as a predictor for the risk of developing type 2 diabetes (Lonn M, 2010).

Study No.3-Results of the experimental evaluation of the adipogenic differentiation capacity of subcutaneous ASCs: The development of insulin resistance and its complications known as metabolic syndrome are associated with the abdominal type distribution of adipose tissue and have as a physiopathological mechanism the predominance of their adipocyte hypertrophy and proliferation. hyperplastic. This particularity seems to be an essential element in the pathogenesis of the metabolic syndrome and has been intensively studied in the last decade. Numerous factors of adipocyte microenvironment have been evaluated as having a potential role in inducing this tendency towards hypertrophy and accumulation of lipids (Symonds ME, 2012).

Regarding the adipogenic function of the mesenchymal precursors isolated from the subcutaneous adipose tissue level, the estimation of lipid accumulation is according to our experimental data, in close correlation with the peripheral insulin resistance. Thus, the tendency towards massive lipid loading depends on the precursors of these adipocytes obtained in vitro under identical environmental conditions and this transcriptional pathway of adipogenesis may be the starting point for the pathophysiological cascade of metabolic pathology in obese patients. Our experimental study has shown a decrease the ability of adipogenic differentiation for ASCs isolated from obese versus normal-weight patients in the control group and the lipid accumulation in the mature adipocytes obtained was significantly correlated with the level of insulinemia but also with the leptin / serum adiponectin ratio.

Although there is a growing concern for the adipocyte differentiation process from mesenchymal precursors, there are still few studies to establish the

relationship with insulin resistance and metabolic syndrome for the human model. Our recently published study evaluated the ability of adipogenic differentiation of ASCs collected from obese grade III patients for bariatric surgery in which a significant reduction in the ability to generate new adipocytes was demonstrated compared with the control group of normoponderal patients.

CONCLUSIONS

1.Evaluation of lipid profile in obese patients is shown to provide valuable predictors for metabolic syndrome, in our study both serum triglyceride levels and LDL cholesterol, total cholesterol and total cholesterol / HDL cholesterol ratio were found to be reliable predictors of metabolic syndrome.

2.For the Leptin / Adiponectin Serum (LAR) ratio although slightly lower values were detected in patients with metabolic syndrome without obtaining statistical significance, however, by tracing the ROC curve it is noted that LAR can be a good predictor of the syndrome metabolic.

3. The average area of subcutaneous adipocytes was significantly associated with both the level of peptide C and the HOMA-IR index, demonstrating the close correlation between adipocyte morphology and insulin resistance syndrome, as an essential pathogenic mechanism in type 2 diabetes.

4. The correlations obtained between the level of absorption of the lipid pigment and the insulin resistance index (HOMA-IR), the peptide C but also with the parameters of the lipid metabolism support the importance of this marker of evaluation of the adipocyte differentiation as an integrated element of the pathophysiology of the metabolic syndrome in obesity.

5. In our study, the particularities of the metabolic and endocrine profile of the obese patients, characterized by the association of the metabolic syndrome criteria, but also with experimental aspects regarding the histology of the adipose tissue, the area of the subcutaneous adipocytes in these patients, as well as the disturbances of the process, were highlighted. of adipogenesis demonstrated by the in vitro cell culture study of the adipogenic differentiation

capacity of mesenchymal precursors in subcutaneous adipose tissue of obese patients.

PERSPECTIVES. ELEMENTS OF ORIGINALITY OF THE THESIS

Progress in deciphering the pathophysiological mechanisms of the metabolic syndrome associated with obesity brings to the fore the adipose tissue and the particularities related to the expansion, the storage capacity of the fatty acids resulting from the positive energy balance but also disturbances in the endocrine activity of the adipose tissue.

We intend to expand this research direction in the future to identify specific molecular markers within the signaling pathways of adipogenesis, which will be valuable predictors for the metabolic evolution of obese patients, thus better stratifying the type of intervention. therapeutic and possibly indication for bariatric surgery.

The results of this research open the horizon of broader fundamental research approaches in the field of obesity and metabolic syndrome in trying to find new pharmacological targets that target the pathway of adipogenesis as a prime element in the cascade of metabolic dysfunctions associated with excess weight.

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