4.3.2 Human Tissues

Enrolled patients

10 subjects were recruited: 3 of them obese diabetic, 4 obese normoglycaemic and 3 post-obese subjects undergone to abdominoplasty intervention.

	n°	F; M	age (years)	BMI (kg/m²)	glycaemia	HbA1c
obese diabetic	3	1; 2	55,5±6,5	42,49±6,45	13,23±5,31	84,3±33,6
obese normoglycaemic	4	2; 1	46,25±7	45,29±1,65	5,1±0,5	-
Post obese	3	3; 0	43,6±7,6	25,34±1	5,1±0,5	-

Table 4.2: anthropometric, clinical and biochemical characteristics of enrolled patients analyzed in MS and in western blot.

Western Blot analysis

In order to demonstrate the presence of AGEs products in the human AT, preliminary analyses in western blot were done. A set of AT was separated in 1D-SDS gel and AGEs were detected using anti-AGEs antibody after transferring to a nitrocellulose membrane.

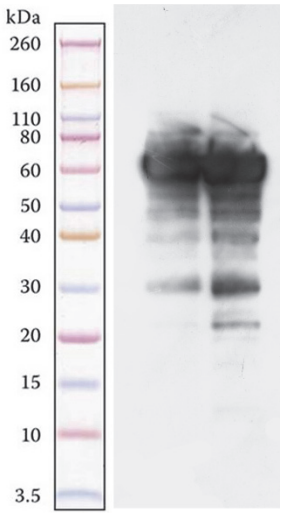

Figure 4.34: Representative 1D western blotting images of AGEs modified AT proteins. In the figure are represented the bands of AGEs proteins of two SAT from post obese/weight lost patients. At left is reported the reference weight standards.

SAT lysates from post-obese subjects were loaded onto a pre-cast NuPAGE® 10% Bis-Tris 1.0 mm mini-gel until the dye front reached the end of the gel; then the proteins were immunoblotted on a nitrocellulose membrane. Next, using an anti-AGEs antibody, the AGEs modified proteins were detected, following the protocol presented in Materials and Methods. The western blot for AGEs modified proteins shows several bands, the major one with a molecular weight of around 60 kDa, probably it is albumin, and many other are visible at lower weights; indicating that many protein species were potentially AGEs modified (fig. 4.34). The patterns of AGEs staining were similar in all the post-obese AT samples but have had variation in intensity. The results of the current preliminary test might demonstrate the probable presence of AGEs modified proteins in AT.

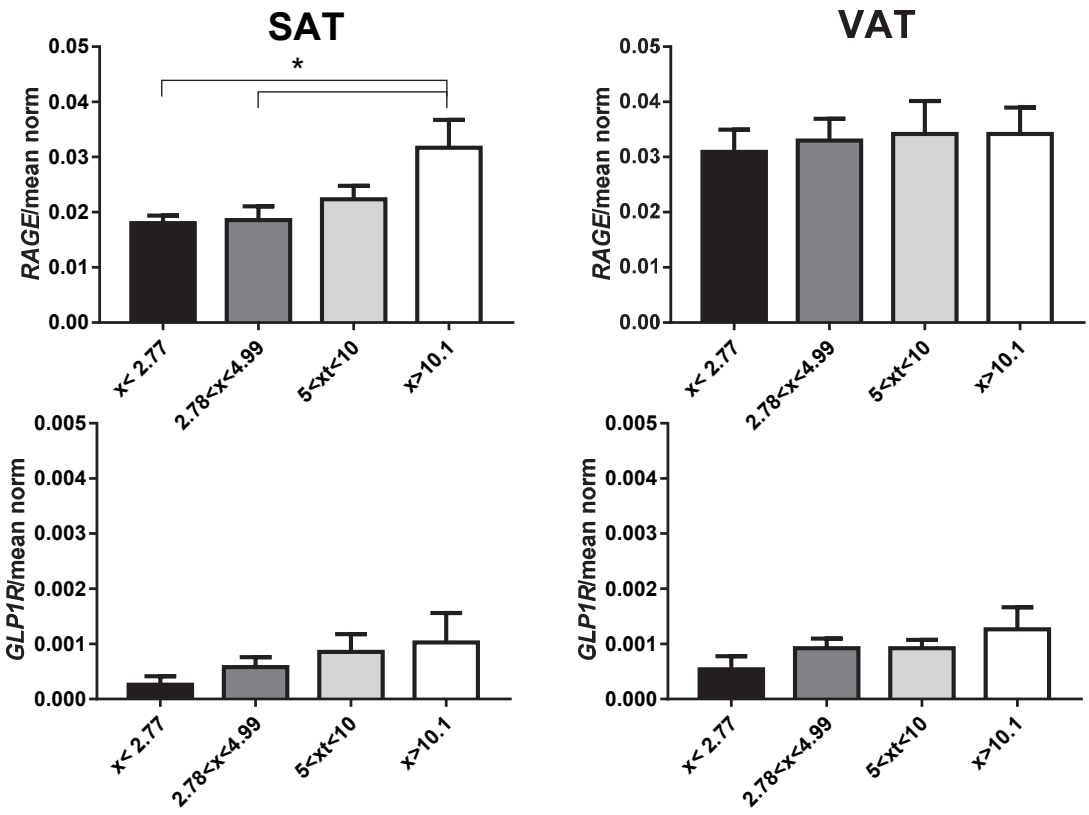

*Figure 4.40: Gene expression profile for: RAGE and GLP1R subdivided by HOMA-IR categories. Mann-Whitney nonparametric test. *p<0.05*

In SAT of diabetic subjects, the expression of *RAGE* displays (fig. 4.39) a significant increase that could be correlated with an altered glucose tolerance. On the contrary, in VAT the expression shows a quite flat trend, with only a slight increase in pre-diabetic and diabetic subjects. Reorganizing and re-processing data for the HOMA -IR index (fig. 4.40) in the subcutaneous tissue, it seems to appear an even more clear correlation of *RAGE* expression with insulin resistance. Nevertheless, in the visceral tissue the expression appears constant among the categories of subjects.

The expression for *GLP1R* (fig. 4.39) in SAT proves to be lacking in a specific trend, with a slight increase in prediabetic and a decrease in the diabetic. On the opposite, in VAT it shows a little but significant increase in expression in diabetic VAT. Reorganizing and re-processing data for the HOMA -IR index (fig. 4.40), *GLP1R* displays both in SAT and in VAT a slight increase of the expression, that it is linked to the increasing of HOMA-IR.

The expression of *DPP4* shows no quantitative differences among the three groups of subjects; on the other hand, a considerable difference between the SAT and VAT depots can be observed. In particular, VAT got nearly threefold of expression of SAT.

Finally, the trend of *LEP* suggests a possible link with the glycaemic state and metformin treatments. Pre-diabetic subjects show a significant increase in expression compared with the control group. Whereas, obese diabetic shows a smaller rising in expression.

4.3.3 Exploratory studies

For a long time, many investigators focused their attention on the role of leptin in the pathogenesis of obesity. Leptin is an "anti-obesity" hormone produced mainly by AT that is released into the circulatory system and plays a central role in the regulation of metabolism, energetic balance, immune and inflammatory response, haematopoiesis, angiogenesis, bone formation, and wound healing (1).

Since the glycation can alter the functionality of the protein, the possibility that leptin could be glycated and thereby change its biologic activity, contributing to the leptin resistance mechanisms was investigated. Previous studies using ESI-MS in the diabetic subjects was successfully used to detect and characterize glycated insulin in plasma from patients with type 2 diabetes. AGEs precursors, i.e. MGO, can glycate insulin to form insulin-MGO adducts and in turn changing the insulin structure, thereby abrogating its physiological function. (58) Taking into account that leptin is the most abundant protein in the obese, on the basis of this evidence, it was decided to analyze its *in vitro* glycation.

and after insulin stimulus IF staining was performed, focusing on the possible alterations of the trafficking of the glucose transporter. Furthermore, to investigate if AGEs may directly interfere with the trafficking of GLUT4, a co-staining for AGEs and GLUT4 were performed in order to verify the hypothesis that glycation of GLUT4 could result in a functional impairment, because of the well-known effect of glycation on both structure and protein function. Despite a probable co-localization of the two proteins have been found, further investigations with co-immunoprecipitation and cell membranes fractionating are mandatory to confirm these interesting suggestions.

5.2 HUMAN TISSUES

A major confounder in studies on IR or T2DM is obesity, and it is well-known that obesity and diabetes have multi-levels associations. (165). At this issue was relevant to ask whether the identifies alterations are influenced by a hyper-glycated status.

Lipidome analysis revealed an obesity-related specific signature in both subcutaneous and visceral adipose tissue depots. (166) The expansion of AT in response to a positive energy balance is challenging for adipocytes in the maintenance of membrane integrity and functionality. The growth and the enlargement of adipocytes require that more phospholipids have to be incorporated into cell membranes. The lipid metabolism and the immune response are highly integrated; indeed, the accumulation of harmful lipids may interfere with the immune regulation in multiple tissues, causing a vicious cycle of immune-metabolic dysregulation. The tight correlation between glucose metabolism, inflammation, FFA and metabolic syndrome is well known. (167, 168),(31, 169-171) A selective enrichment of specific triglycerides, glycerol-phospholipids, and sphingolipids in the AT of obese subjects were observed. In the particular case of acquired obesity, an increased proportion of palmitoleic and arachidonic acids in AT was discovered. (166, 172) Thus, the relative proportion of 71 lipid species ranging from 300Da to 1100Da was enquired, focusing the attention on the difference among obese normoglycaemic, obese diabetic and weight-loss/post obese. The most intriguing lipid is the *m/z* 881.781, seemingly a triglyceride POO (C52:2), that seems increased in AT of obese diabetic subjects. At present, we do not know whether and how, the lipids composition might be influenced by a glycation-inducing environment. Moreover, what could be the functional aspects of these modified in cell lipids remains to be discovered.

It was thought that among the pathological alterations present in the AT of obese subjects with T2DM those related to chronic exposure to a glycation-inducing environment could have a great

importance in the development of the so-called adiposophaty. In order to investigate these aspects, the expression of *GLP1, RAGE, DPP4* and *LEP* in both SAT and VAT of 64 patients were collected. Obese diabetic subjects were all treated with metformin, a well-known first line anti-hyperglycaemic agent mostly acting on hepatic glucose production and increasing glucose utilization. Metformin activates AMP-activated protein kinase which is a major cellular regulator of lipid and glucose metabolism. Moreover, metformin induces GLP1 release from intestinal L cells, and also GLP1R expression on pancreatic β-cells, is associated with amelioration of low-grade tissue inflammation in the adipose tissue of obese animals. (173) In addition, previous studies indicate that it could dose-dependently inhibit the AGEs-induced apoptosis and the inflammatory and fibrotic reactions in tubular cells probably by reducing ROS generation via down-regulation of *RAGE* expression through AMP-activated protein kinase activation. (174-176) Metformin could also reduce leptin level in morbidly obese individuals and this fact has been proven in several studies, suggesting that the, even small, anorectic effects of metformin could be potentially mediated via an increase in the central sensitivity to leptin. (159, 177) At the contrary, others studies have shown opposite results. (178)

Our results (fig. 4.39) lead to hypothesize that a glycation-inducing environment, in particular in VAT, could result in a stimulation of leptin gene expression. This effect could be observed very early in the progression from obesity to diabetes and starting from the condition of prediabetes. In metformin-treated obese diabetic patients, the effect of glycation on leptin could be dampened by the pharmacological action of the drug. The increased expression of leptin, a common feature in obesity, could be further increased through a glycation-inducing environment thereby prompting the already present leptin resistance.

Using the western blot analysis (fig. 4.34) we were able to confirm the presence of AGEs withing AT. These products can crosslink with both intracellular and extracellular proteins inducing dysfunction. Moreover, binding to their receptor they can prompt the expression of *RAGE* itself. (76, 80, 179). On the basis of our experimental evidence, it is possible to suggest that although the slight increase of *RAGE* expression begins in VAT starting from the pre-diabetic condition and that, metformin could limit this upward trend. SAT, on the other hand, shows a great increase only in the obese diabetic patients (fig. 4.39) leading to suggest that this depot could be less susceptible than VAT to the glycation-inducing environmental. This is the reason why the rising of *RAGE* expression is delayed.

In literature, has also been reported that metformin can increase the *GLP1R* expression on islet cells (173), but so far no data are available about the effects of metformin on *GLP1R* expression in AT. In VAT of treated obese patients, a significant increased *GLP1R* expression was detected. (fig.

4.39) Our experimental conditions do not allow to distinguish if obese diabetic VAT *GLP1R* expression is due to the glycation-inducing environment or to metformin therapy. However, our data performed on 3T3-L1 (fig. 4.14) strongly suggest that the glycation-inducing environmental could be the cause of the increased *GLP1R* expression. Further studies are needed to clarify this open question.

DPP4 is an enzyme that inactivates GLP1. The levels of GLP1 can be enhanced for their beneficial effects by inhibiting the action of DPP4 through a DPP4 inhibitor, resulting in enhanced levels of endogenous secretion of GLP1. Previous studies (180, 181) revealed that serum levels of DPP4 are independently associated with various metabolic parameters and that AGEs could up-regulate its expression. On the contrary, in our analyses, the expression of *DPP4* in AT does not seem to be impaired either by glycation-inducing-environment nor by treatment with metformin. However, a considerable difference between the SAT and VAT depots can be observed, confirming that VAT consistently displays a higher expression than SAT. (182)

Therefore, the increased insulin resistance of AT under conditions of augmented glycation recognizes, in addition to the activation of RAGE, some other possible intracellular pathophysiological mechanisms competitors. Our finding may provide new insights into the alteration of incretin/RAGE/AGEs axis driven by glycated proteins derived from diets or/and chronically hyperglycemic condition.

Further studies will be needed to determine the molecular pathways involved in AGEs accumulation effects and for comprehend the real impact of glycation on physiological system in order to develop innovative strategies to reduce and/or retard those effects and in this way, understand how these relationships might be manipulated to restore metabolic health.