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Gene expression of paired abdominal adipose *AQP7* and liver *AQP9* in patients with morbid obesity
Relationship with glucose abnormalities

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Abstract

The trafficking of glycerol from adipose and hepatic tissue is mainly mediated by 2 aquaporin channel proteins: *AQP7* and *AQP9*, respectively. In rodents, both aquaporins were found to act in a coordinated manner. The aim was to study the relationship between adipose *AQP7* and hepatic *AQP9* messenger RNA expression and the presence of glucose abnormalities simultaneously in morbid obesity. Adipose tissue (subcutaneous [SAT] and visceral [VAT]) and liver biopsies from the same patient were obtained during bariatric surgery in 30 (21 male and 9 female) morbidly obese subjects. Real-time quantification of *AQP7* in SAT and VAT and hepatic *AQP9* gene expression were performed. A 75-g oral glucose tolerance test was performed in all subjects. The homeostasis model assessment of insulin resistance and lipidic profile were also determined. Visceral adipose tissue *AQP7* expression levels were significantly higher than SAT *AQP7* ($P = .009$). Subcutaneous adipose tissue *AQP7* positively correlated with both VAT *AQP7* and hepatic *AQP9* messenger RNA expression ($r = 0.44$, $P = .013$ and $r = 0.45$, $P = .012$, respectively). The correlation between SAT *AQP7* and liver *AQP9* was stronger in intolerant and type 2 diabetes mellitus subjects ($r = 0.602$, $P = .011$). We have found no differences in compartmental *AQP7* adipose tissue distribution or *AQP9* hepatic gene expression according to glucose tolerance classification. The present study provides, for the first time, evidence of coordinated regulation between adipose aquaglyceroporins, with a greater expression found in visceral fat, and between subcutaneous adipose *AQP7* and hepatic *AQP9* gene expression within the context of human morbid obesity.

1. Introduction

Adipose tissue has a major role as an energy storage organ where many metabolic changes occur in response to the whole-body energy balance. Under lipogenic conditions, insulin increases glucose transport into the cell; and glucose is converted to glycerol-3-phosphate. Likewise, lipoprotein lipase activated by insulin recruits fatty acids from circulation into the adipocytes; and both are esterified into triglyceride (TG). Conversely, under lipolytic conditions, catecholamines stimulate adrenergic receptors that translocate hormone-sensitive lipase, which is a key enzyme in hydrolyzing TG to free fatty acids (FFA) and glycerol. Free fatty acids and glycerol are used for thermogenesis and gluconeogenesis, respectively. In fasting states, gluconeogenesis from the liver is the main source of plasma glucose [1]; and about 22% of total glucose production comes from glycerol in humans [2], the adipose tissue being the main source of plasma glycerol. Trafficking of glycerol from

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adipose and hepatic tissue is mainly mediated by 2 aquaporin channel proteins: *AQP7* and *AQP9*, respectively. *AQP7* is the sole described channel that permits the exit of glycerol from adipocytes, and plasma glycerol is introduced into the hepatocytes by the *AQP9* [1,3]. Both aquaporins act in a coordinated manner. In animal studies, feeding state reduces the messenger RNA (mRNA) expression of the adipose *AQP7* and results in a reduction of glycerol release from adipocytes [4]. Feeding also reduces liver *AQP9* mRNA expression and glycerol-induced gluconeogenesis [1]. However, obese and insulin-resistant *db*/*db* mice show increased *AQP7* and *AQP9* mRNA levels (in mesenteric fat and liver, respectively), despite hyperinsulinemia [1]. Increased glycerol release from adipocytes in parallel with increased gluconeogenesis induced by the high glycerol levels in portal vein results in hyperglycemia through the pathologic induction of liver *AQP9* [1].

Adult *AQP7*-knockout animals develop obesity by increasing hypertrophic adipocytes in epididymal white adipose tissue with higher intracellular glycerol content compared with wild-type mice [5]. Moreover, *AQP7*-knockout mice develop severe insulin resistance associated with obesity. These observations have led us to propose *AQP7* as a new factor influencing not only glycerol but also glucose metabolism.

Studies on *AQP7* in human obesity are scarce and have shown differences according to the source of the adipose tissue. In this regard, lower *AQP7* mRNA levels have been described in subcutaneous adipose tissue (SAT) samples from subjects with severe obesity than those obtained in lean subjects [6-8]. By contrast, higher *AQP7* mRNA expression has been found in visceral adipose tissue (VAT) from massively obese subjects [9]. *AQP7* mRNA levels in type 2 diabetes mellitus (T2D) subjects were similar to those in nondiabetic controls in both adipose depots [6,9]. With regard to *AQP9* gene expression in the liver, it appears to be down-regulated by insulin, potentially via an "insulin-responsive element" in the *AQP9* promoter. In this sense, there is only 1 study showing a down-regulation of this gene in liver biopsies obtained from obese T2D patients [9].

The close regulatory mechanism depicted by these 2 aquaporins and their role in glycerol and glucose metabolism would suggest a need to increase our knowledge of its behavior in human obesity. To shed light on this issue, we have studied paired *AQP7* mRNA expression in adipose (SAT and VAT) tissue and *AQP9* mRNA hepatic expression in biopsies from a cohort of morbidly obese patients. In addition, the relationship between *AQP7* and *AQP9* mRNA expression and the presence of glucose abnormalities were also investigated.

2. Methods and materials

We recruited 30 consecutive morbidly obese subjects of Caucasian origin who underwent gastric bypass surgery at the University Hospital Vall d'Hebron (Barcelona, Spain). All patients met the eligibility criteria established by the guidelines of the National Institutes of Health Consensus Conference [10]. The preoperative evaluations included assessment by an endocrinologist, a psychiatrist, and a pneumonologist to identify and treat all comorbid medical conditions before operation. None of the subjects presented evidence of metabolic disease other than obesity, diabetes, and dyslipidemia. No T2D patients were receiving glitazone treatment. All hypolipidemic and oral hypoglycemic agents were stopped at least 72 hours before the surgical procedure.

Before the surgical procedure, a 75-g oral glucose tolerance test was performed on those patients in whom diabetes was not previously diagnosed; and patients were classified according to American Diabetes Association criteria [11].

The ethics committee approved the study, and informed consent was obtained from all enrolled patients.

2.1. Anthropometric measurements

Height was measured to the nearest 0.5 cm and body weight to the nearest 0.1 kg. Body mass index was calculated as weight (kilograms) divided by height (meters) squared. Waist circumference was measured midway between the lowest rib margin and the iliac crest.

2.2. Histologic studies of liver

Hematoxylin-eosin and trichrome stains of all liver biopsies were reviewed by a pathologist without knowledge of the clinical data and were then classified according to the criteria of Brunt [12]. The following parameters were graded in the biopsies: (*a*) steatosis, 0 to 3; (*b*) hepatocyte balloonization, 0 to 3; (*c*) lobular inflammation, 0 to 3; and (*d*) portal inflammation, with or without different fibrosis stages, 0 to 4.

2.3. Collection and processing of samples

All patients had fasted overnight, at least 12 hours before undergoing the surgical procedure. Two experienced surgeons in abdominal surgery performed all the laparoscopic Roux-en-Y gastric bypass procedures. Blood samples were collected before the surgical procedure from the antecubital vein, 20 mL of blood with EDTA (1 mg/mL) and 10 mL of blood in silicone tubes. Fifteen milliliters of collected blood was used for the separation of plasma. Plasma and serum samples were stored at −80°C until analytical measurements were performed. Five milliliters of blood with EDTA was used for the determination of glycated hemoglobin (HbA_{1c}).

During the surgical procedure, adipose tissue samples from SAT and VAT were obtained, as well as a liver biopsy from the same patient included in the study. Adipose tissue samples were washed in phosphate-buffered saline 1×, immediately frozen in liquid nitrogen, and stored at −80° C. The liver biopsies were collected in an RNA preservative solution (RNAlater; Sigma-Aldrich,

St Louis, MO). After RNA later solution was removed, the samples were immediately frozen in liquid nitrogen and were stored at −80° C.

2.4. Analytical methods

Glucose, cholesterol, and TG plasma levels were determined in a Hitachi 737 autoanalyzer (Boehringer Mannheim, Marburg, Germany) using the standard enzyme methods. High- and low-density lipoprotein (LDL) cholesterol was quantified after precipitation with polyethylene glycol at room temperature (PEG-6000). Plasma insulin was determined by radioimmunoassay (Coat-A-Count Insulin; Diagnostic Products, Los Angeles, CA) in all subjects of the study, except in T2D patients treated with insulin. The HbA_{1c} was measured by chromatography microcolumn (IsoLab, Akron, OH). Plasma high-sensitivity C-reactive protein was determined by a highly sensitive immunonephelometry kit (Dade Behring, Marburg, Germany). Plasma glycerol levels were analyzed using a free glycerol determination kit, a quantitative enzymatic determination assay (Sigma-Aldrich). Intra- and interassay coefficients of variation were less than 6% and less than 9.1%, respectively. Nonesterified free fat acid (NEFA) serum levels were determined in an Advia 1200 autoanalyzer (Siemens, Munich, Germany) using an enzymatic method developed by Wako Chemicals (Neuss, Germany). Assay sensitivity was 0.01 mEq/L, and the inter- and intraassay coefficients of variation were lower than 8%.

The homeostasis model assessment of insulin resistance was calculated as [glucose (milligrams per deciliter) × insulin (micro–international units per liter)]/405 [13].

2.5. Gene expression relative quantification

Four hundred to 500 mg of frozen adipose tissue and 200 mg of frozen liver tissue were homogenized with an Ultra-Turrax 8 (Ika, Staufen, Germany). Total RNA was extracted using an RNeasy Lipid Tissue Midi Kit (QIAGEN Science, Hilden, Germany) for adipose tissues and an RNeasy Midi Kit (QIAGEN) for hepatic biopsies, duly following the manufacturer's instructions. Total RNA was treated with 55 U RNase-free DNase (QIAGEN) before column elution to avoid contamination with genomic DNA.

A total of 1.5 μg of RNA was reverse transcribed to complementary DNA (cDNA) using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) in a final volume of 20 μL.

Real-time quantitative polymerase chain reaction was performed with 1 μL of cDNA on a 7900HT Fast Real-Time PCR System using Taqman Assays (Applied Biosystems). Three replicate reactions per sample and gene were performed. SDS software 2.3 and RQ Manager 1.2 were used to analyze the results with the comparative C_t method ($2^{-\Delta\Delta Ct}$). Data were expressed as an n-fold difference relative to the calibrator (a mix of 3 tissues). The C_t values for each sample were normalized with the geometric mean of 2 endogenous controls: *PPIA* (cyclophilin A) and *ACTB* (β-actin).

2.5.1. Statistical analysis

Statistical analysis was performed using the SPSS/PC+ statistical package (version 15 for Windows; SPSS, Chicago, IL). For clinical and anthropometric variables, normally distributed data are expressed as mean value ± SD; and for variables with no Gaussian distribution, values are expressed as median (75th percentile). For statistical analysis of expression variables that did not have a Gaussian distribution, values were logarithmically or inversely transformed.

Differences in clinical/laboratory parameters or expression variables between groups were compared by using analysis of variance with a post hoc Scheffe correction. Interactions among factors as well as the effects of covariates and covariate interactions with factors were assessed by general linear model univariate analysis. Associations between quantitative variables were evaluated by Pearson or Spearman (for non-Gaussian–distributed variables) correlation analysis. Correction for confounding and interacting variables was performed using a stepwise multiple linear regression analysis. Results are expressed as multiple correlation coefficient (R). Statistical significance occurred if a computed 2-tailed probability value was < .050.

3. Results

3.1. Aquaporin expression levels according to glucose tolerance status

Clinical and laboratory variables of the study participants are summarized in Table 1. The T2D obese patients were significantly older than normoglycemic (NG) subjects (50.14 ± 6.01 vs 41.08 ± 5.90 years, P = .018); and therefore, this was taken into account in the statistical analysis. Impaired glucose tolerance (IGT) and T2D subjects showed significantly increased fasting glucose levels compared with NG subjects. Low total and LDL cholesterol levels in the T2D patients were mainly due to the high percentage (43%) of preoperative statin treatments. Circulating serum glycerol and NEFA levels were not significantly different between the studied groups (Table 1).

We did not find any statistical difference in mRNA expression of *AQP7* in adipose tissue (SAT and VAT) or *AQP9* in the liver according to glucose tolerance classification, despite controlling for age (Table 1). Subjects with IGT or T2D showed a strong positive correlation between subcutaneous adipose *AQP7* and *AQP9* mRNA levels (r = 0.602, P = .011) (Fig. 1).

3.1.1. Aquaporin expression in the entire obese cohort

When the whole sample was considered (n = 30), *AQP7* expression levels were compared in both adipose depots and analyzed by sex using a univariate general linear model. The results showed that *AQP7* levels differed between adipose depots (P = .009, with a partial η^2 of 11.5%, which reports

Table 1
Clinical, anthropometric, and analytical characteristics (units) and relative mRNA levels (arbitrary units) according to glucose tolerance classification

	NG (n = 12)	IGT (n = 11)	T2D (n = 7)
Anthropometric and analytical characteristics			
Age (y)	41.1 ± 5.9	48.2 ± 8.5	50.1 ± 6.0*
Female/male (n)	4/8	2/9	3/4
BMI (kg/m^2)	43.3 ± 5.1	44.5 ± 5.6	43.2 ± 4.1
Waist circumference (cm)	124.3 (133.6)	125.0 (128.5)	126.3 (147.1)
Antihypertensive treatment (%)	33,3	45,4	71
Hypolipidemic agents (%)	–	27,2	42,8
Oral hypoglycemic agents (%)	–	18	71
Fasting glucose (mg/dL)	89.5 (97.3)	110.5 (118.3)*	113.0 (163.0)†
Insulin (μIU/mL)	15,8 ± 7,4	20,6 ± 11,5	11,0 ± 7,5
HOMA-IR	3,48 ± 1,55	5,41 ± 3,10	4,25 ± 2,53
HbA_{1c} (%)	5.25 ± 0.34	5.76 ± 0.44	7.72 ± 2.28†‡
TG (mg/dL)	119.5 (200.3)	128.0 (155.8)	150.0 (178.0)
Cholesterol (mg/dL)	190.0 (212.0)	208.0 (243.3)	134.0 (153.0)*‡
HDL cholesterol (mg/dL)	43.0 ± 12.8	49.5 ± 7.3	39.1 ± 7.1
LDL cholesterol (mg/dL)	117.0 ± 25.8	134.3 ± 43.7	69.5 ± 14.1*§
Glycerol (μmol/L)	131.0 ± 51.0	109.8 ± 25.2	120.5 ± 24.5
NEFA (mmol/L)	0.64 ± 0.18	0.63 ± 0.14	0.79 ± 0.27
hs-CRP (mg/L)	0.47 (0.84)	1.30 (1.88)*	1.05(1.27)
Relative mRNA levels (arbitrary units)			
AQP7 SAT	1.02 ± 0.45	1.13 ± 0.27	0.83 ± 0.35
AQP7 VAT	1.33 ± 0.75	1.36 ± 0.61	1.30 ± 0.51
AQP9 liver	4.31 (7.75)	4.19 (5.67)	3.04 (11.15)

BMI indicates body mass index; HDL, high-density lipoprotein; hs-CRP, high-sensitivity C-reactive protein; HOMA-IR, homeostasis model assessment of insulin resistance.
Differences vs NG: *$P < .05$; $^{\dagger}P < .01$.
Differences vs IGT: $^{\ddagger}P < .05$; $^{\S}P < .01$.

the proportion of total variability attributable to depot origin) (Fig. 2) but not with sex. No differences attributable to sex were found for *AQP9* expression levels ($P = .158$).

The SAT and VAT *AQP7* adipose depots were positively correlated ($r = 0.449$, $P = .013$) (Fig. 3). No other clinical or analytical variables were found to be associated with *AQP7* adipose expression. Subcutaneous adipose tissue *AQP7* and hepatic *AQP9* showed a positive correlation ($r = 0.459$, $P = .012$). Hepatic *AQP9* expression also showed a negative correlation with plasma TG levels ($r = -0.399$, $P = .036$).

To test the strength of these associations, we constructed a linear regression model for each aquaporin to analyze the

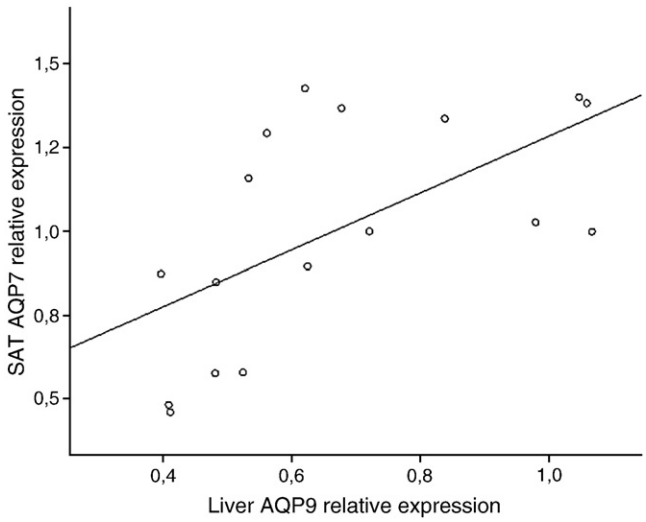

Fig. 1. Correlation between SAT *AQP7* and *AQP9* mRNA levels in IGT and T2D subjects (arbitrary units) ($r = 0.602$, $P = .011$).

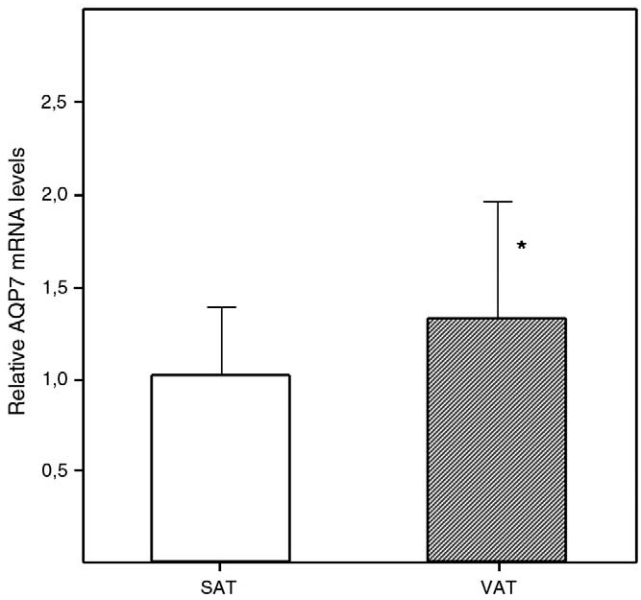

Fig. 2. Levels of expression of VAT *AQP7* relative to SAT *AQP7* (n = 30, mean ± SD; *$P = .009$).

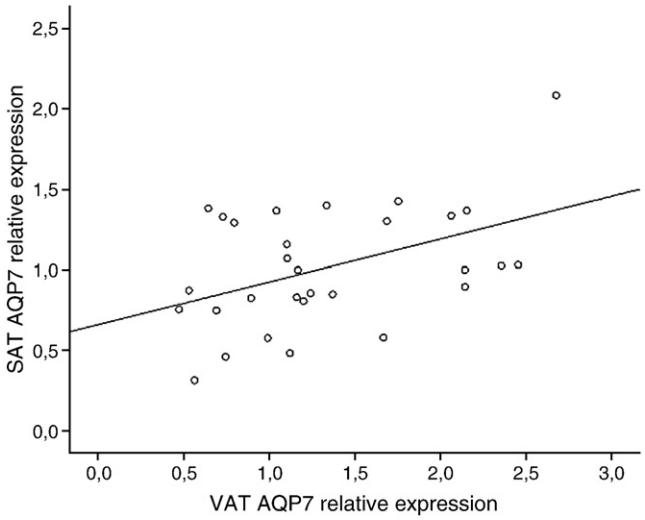

Fig. 3. Correlation between SAT and VAT *AQP7* mRNA levels (arbitrary units) ($r = 0.449$, $P = .013$).

main effects, as well as an interaction term for sex, age, and hypolipidemic or hypoglycemic treatment effects, for each aquaporin. Subcutaneous adipose tissue *AQP7* and VAT *AQP7* were positively related with SAT *AQP7* as dependent variable ($R = 0.508$, $P = .019$) and with VAT *AQP7* as dependent variable ($R = 0.627$, $P = .004$). Hepatic *AQP9* expression remained negatively associated with plasma TG concentration and with SAT *AQP7* expression ($R = 0.623$, $P = .008$ and $P = .010$, respectively).

We did not find any relationship between the hepatic mRNA levels of *AQP9* and the degree of hepatic steatosis or fibrosis.

4. Discussion

The present study provides, for the first time, evidence of a coordinated regulation between subcutaneous adipose *AQP7* and hepatic *AQP9* gene expression within the context of human morbid obesity. Moreover, SAT *AQP7* and hepatic *AQP9* mRNA levels are positively correlated. In addition, a negative association between liver *AQP9* mRNA expression and circulating TG levels is described independently of glucose metabolic status.

It has been suggested that adipose tissue has 2 functionally different preadipocytes. These 2 "pools" (VAT and SAT) of adipose tissue depots receive differential sympathetic innervations and have different enlargement rates with a characteristic release of hormones and metabolites specific for each tissue compartment [3]. Adipose tissue distribution depends on many factors, and sex is one of the main contributors for explaining body fat distribution. Thus, it is well known that estrogens increase the size and number of subcutaneous adipocytes and attenuate lipolysis [14]. In a previous report in which *AQP7* expression was analyzed in both SAT and VAT in a small cohort of morbidly obese subjects, sex dimorphism was suggested, showing increased expression levels in women [15]. However, in our study, we failed to find differences attributable to sex, in accordance with our previous observation in nonsevere obese subjects [6]. Likewise, visceral fat showed greater *AQP7* levels than SAT, confirming a previous observation in isolated visceral adipocytes in which *AQP7* was overexpressed in comparison with subcutaneous adipocytes [15]. Increased lipolytic activity of visceral adipocytes may be partly responsible for these differences. In comparison with subcutaneous fat, VAT is more sensitive to catecholamine-induced lipolysis and less sensitive to the antilipolytic effects of insulin. In fact, increased expression of lipoprotein lipase and hormone-sensitive lipase in visceral vs subcutaneous fat has been reported [16,17].

We have found no differences in compartmental *AQP7* adipose tissue gene expression according to glucose metabolic status. It may be worth considering that, in morbid obesity, the presence of insulin resistance does not influence the greatest expression of *AQP7* observed in VAT. In fact, this observation has been made for other lipolytic genes, like adipose triglyceride lipase, which catalyzes the initial step in TG hydrolysis and has a close regulation with insulin. This enzyme is not differentially expressed between visceral and subcutaneous fat, despite being related to insulin sensitivity independently of body fat mass and fat distribution [16].

Similar assumptions may be deduced from the analysis of hepatic *AQP9* expression in our morbidly obese cohort. Glucose tolerance distribution did not influence *AQP9* liver expression. However, the design of our study does not permit to discard an effect of insulin on human *AQP9* mRNA liver expression. In fact, most of our patients showed a notable degree of insulin resistance before surgery, which could contribute to explaining the absence of differences in *AQP9* gene expression observed after glucose tolerance classification. Furthermore, a close correlation between subcutaneous *AQP7* and hepatic *AQP9* expression was observed mainly in intolerant and T2D patients. *AQP7* and *AQP9* mRNA expression has been shown to be coordinately regulated by plasma concentrations of insulin in rodents, in accordance with nutritional condition, such as fasting and refeeding. These facts indicate the possible involvement of aquaglyceroporins in pathophysiologic glucose metabolism [1]. Our work supports these observations, showing a robust correlation between SAT *AQP7* and *AQP9* liver expression mainly in patients with an altered glucose metabolism. It remains to be determined whether AQP7 and AQP9 protein levels have similar profiles to mRNA because the present study was conducted only by examining mRNA levels.

Visceral adipose tissue has drainage over the portal venous system with a direct flow to the liver, and it is reasonable to assume that the main dependence for *AQP9*

expression comes from visceral fat. We have no satisfactory explanation for this preferential association between SAT and hepatic aquaglyceroporins; however, the strong relationship between SAT and VAT aquaporins (26% of SAT *AQP7* variability was explained by VAT *AQP7* levels) may be partly responsible for this association. We know that the transversal design of our study does not permit to infer mechanistic conclusions, and we are conscious that many other variables could be related with fat and hepatic aquaporin coordination. Therefore, one is tempted to speculate as to an alternative glycerol secretion channel in adipocytes, as has been suggested by many authors [18-20]. To date, 4 aquaglyceroporins have been described (*AQP3*, *7*, *9*, and *10*), with glycerol transport ability, close to diffusion rates and with a low conductance to water. Although the expression of *AQP3* in mice liver [21] and *AQP9* in pig adipose tissue [22] has been described, to our knowledge, in humans no other aquaglyceroporin in adipose tissue and liver than *AQP7* and *AQP9* has been described. Moreover, we have not found *AQP10* expression in adipose tissue (data not shown).

Measurement of circulating glycerol levels underestimated the glycerol released into the portal vein by adipose tissue before its clearance by the liver [23]. This could help interpret the absence of correlation observed between aquaglyceroporin expression in both adipose and hepatic tissues, and circulating glycerol levels, although the negative association between plasma TG levels and *AQP9* expression reveals an indirect role of *AQP9* in glycerol metabolism, in agreement with the metabolic findings described in *AQP9*-knockout mice [24]. Besides, FFA release into circulation by adipocyte lipolysis is also undervalued because of the reesterification of the FFA in adipose tissue. Thus, in humans, the recycling in this tissue has been estimated to be as high as 40% [25]. Finally, it is important to note that we did not find any relationship between *AQP9* expression in the liver and the degree of hepatic steatosis or fibrosis. Therefore, it seems that *AQP9* expression is not essential in regulating fatty liver deposits. In agreement with these findings, Rojek et al [24] found no apparent histologic abnormalities in the liver between control mice and *AQP9*-knockout mice.

One limitation to our study is the fact that protein levels were not measured, which would show whether they are correlated with gene expression.

In summary, we have found that glucose tolerance status does not seem to influence adipose *AQP7* and hepatic *AQP9* expression in morbidly obese patients. However, a close relationship between SAT *AQP7* and hepatic *AQP9* was found mainly in subjects with glucose metabolic abnormalities. Adipose aquaglyceroporins appeared to be regulated coordinately with a greater expression found in visceral fat in our morbidly obese cohort. More mechanistic studies are needed to further explore the implication and response of *AQP7* and *AQP9* in physiologic and pathologic glucose homeostasis.

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