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Diabetic and dyslipidaemic morbidly obese exhibit more liver alterations compared with healthy morbidly obese

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ABSTRACT

Background & aims: To study the origin of fat excess in the livers of morbidly obese (MO) individuals, we analysed lipids and lipases in both plasma and liver and genes involved in lipid transport, or related with, in that organ.
Methods: Thirty-two MO patients were grouped according to the absence (healthy: DM−DL−) or presence of comorbidities (dyslipidemic: DM−DL+; or dyslipidemic with type 2 diabetes: DM+DL+) before and one year after gastric bypass.
Results: The livers of healthy, DL and DM patients contained more lipids (9.8, 9.5 and 13.7 times, respectively) than those of control subjects. The genes implicated in liver lipid uptake, including *HL*, *LPL*, *VLDLr*, and *FAT/CD36*, showed increased expression compared with the controls. The expression of genes involved in lipid-related processes outside of the liver, such as *apoB*, *PPARα* and *PGC1α*, *CYP7a1* and *HMGCR*, was reduced in these patients compared with the controls. *PAI1* and *TNFα* gene expression in the diabetic livers was increased compared with the other obese groups and control group. Increased steatosis and fibrosis were also noted in the MO individuals.
Conclusions: Hepatic lipid parameters in MO patients change based on their comorbidities. The gene expression and lipid levels after bariatric surgery were less prominent in the diabetic patients. Lipid receptor overexpression could enable the liver to capture circulating lipids, thus favouring the steatosis typically observed in diabetic and dyslipidaemic MO individuals.

1. Introduction

It has become increasingly clear that visceral fat deposition, which is common in severe obesity, is associated with triacylglyceride (TAG) accumulation in the liver, even in the absence of significant alcohol consumption [1–3].

The term NAFLD (non-alcoholic fatty liver disease) includes a spectrum of fatty liver diseases ranging from simple steatosis to

Abbreviations: DM−DL−, "Healthy" obese patients, or patients without type 2 diabetes or dyslipidaemia; DM−DL+, Dyslipidemic obese patients; DM+DL+, Obese patients with type 2 diabetes and dyslipidaemia; *HL*, Hepatic lipase; *VLDLr*, Very-Low-Density Lipoprotein receptor; *FAT/CD36*, Fatty Acid Translocase or Cluster of Differentiation 36; LDLr, Low-Density Lipoprotein receptor; *apoB*, Apolipoprotein B; *PPARα*, Peroxisome Proliferator-Activated Receptor alpha; *PPARα*, Peroxisome Proliferator-Activated Receptor gamma Coactivator 1-alpha; CYP7a1, Cholesterol 7 Alpha-Hydroxylase; *HMGCR*, 3-Hydroxy-3-Methylglutaryl-CoA Reductase; *PAI1*, Plasminogen Activator Inhibitor of Type 1; *TNFα*, Tumour Necrosis Factor-alpha; ATGL, Adipose Tissue Glycerol Lipase; SCARB1, Scavenger Receptor Class B, Member 1; CPT1a, Carnitine Palmitoyltransferase 1a; UCP2, Uncoupling Protein 2; iNOS2, Inducible Nitric Oxide Synthase 2; eNOS3, Endothelial Nitric Oxide Synthase 3; IL6, Interleukin-6; TAGs, Triacylglycerides; NAFLD, Non-alcoholic fatty liver disease; IR, Insulin resistance; NASH, Non-alcoholic liver steatohepatitis; MO, Morbidly obese; BMI, Body Mass Index; DM, Type 2 diabetes mellitus; DL, Dyslipidaemia; RYGBP, Roux-en-Y gastric bypass; HTA, Hypertension; HOMA-IR, Homeostasis Model Assessment of Insulin Resistance; PLs, Phospholipids; TC, Total cholesterol; cLDL, Low-Density Lipoprotein Cholesterol; cHDL, High-Density Lipoprotein Cholesterol; NEFA, Non-esterified fatty acid; AST, Aspartate transaminase; ALT, Alanine transaminase; GGT, gamma-glutaryl transferase; CRP, C-reactive protein; KBs, Ketone bodies; ApoA1, Apolipoprotein A1; HSL, Hormone-sensitive lipase; QMs, Chylomicrons; SAT, Subcutaneous adipose tissue; VAT, Visceral adipose tissue.

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steatohepatitis (NASH) and cirrhosis [4]. The more progressive forms of NAFLD have been related to metabolic syndrome and obesity [5,6]. The epidemic of obesity has increased the prevalence of NAFLD, and it is already the most common liver disorder in developed countries. Morbid obese patients have a high proportion of NAFLD [7].The coexistence of metabolic syndrome and NAFLD has made insulin resistance central to the pathogenesis of these disorders. The metabolic consequence of insulin resistance is impaired hepatic glucose output and abnormal lipid handling. In the face of continued metabolic insults the normal hepatic regulatory mechanism gets overwhelmed and fat accumulates in the hepatocytes. The subsequent fate of steatotic hepatocytes depends on the capacity of additional factors such as adipocytokines and toxicity induced by the free fatty acids themselves to induce inflammatory response [8]. NASH is characterised by hepatocyte injury, inflammation and fibrosis, which can lead to cirrhosis, liver failure and hepatocellular carcinoma [9]. It is associated with increased serum levels of hepatic enzymes [10], the activities of which are strongly influenced by the plasma *PAI1* levels in both hyper- and normolipidaemic subjects [11].

Furthermore, obese subjects exhibit increased liver lipoprotein lipase (*LPL*) activity [12]. *LPL* is an extra-hepatic enzyme that limits uptake of circulating TAGs into tissues. Hence, its activity could enable the liver to accumulate circulating TAGs, leading to steatosis [12]. Moreover, morbidly obese (MO) patients exhibit increased local hepatic lipase (*HL*) activity and mRNA expression, favouring cholesterol uptake by the liver and its re-exportation to steroidogenic organs [13].

It is unclear why some obese people develop IR and type 2 diabetes mellitus (DM), whereas others with the same BMI do not [14,15]. However, the absence of DM or dyslipidaemia (DL) is not sufficient to define "healthy obesity", at least in the context of the MO [16]. In fact, marked deficiencies in several haematological parameters have been observed in "theoretically healthy obese" individuals [16]. Additionally, patients with dyslipidaemia and type 2 diabetes exhibit decreases in adipose tissue lipase expression and activity, which are not observed in "healthy" MO or normal-weight patients [17].

Finally, although surgery is considered the most effective treatment for obesity, its effects on NAFLD and lipid metabolism are variable and procedure dependent [18,19]. Following gastric bypass, important improvements in the lipid profile and IR have been reported [12]. However, the effects of surgery on lipid metabolism enzymatic activity in the liver have not been studied in depth. Furthermore, whether differences in surgery-induced alterations in liver function are dependent on the presence or absence of comorbidities has not been assessed.

In this study, changes in lipid levels and lipase activities (*LPL* and *HL*) in the liver and plasma were studied in "healthy" and unhealthy (those with DL or both DM and DL) MO patients who underwent bariatric surgery and received follow-up for 1 year during the weight loss period. Additionally, the expression levels of genes related to lipid metabolism, inflammation and oxidative stress were studied and correlated with plasma and tissue liver injury markers.

2. Materials and methods

2.1. Patient selection and samples extraction

Thirty-two MO patients (23 women and 9 men) between 21 and 61 years of age who underwent Roux-en-Y gastric bypass (RYGBP) surgery were enrolled and received follow-up at the Hospital de la Vall d'Hebron in Barcelona, Catalonia, as described elsewhere [20]. The subjects presented the necessary indications for bariatric surgery: BMI >40 or >35 kg/m^2, with at least one comorbidity (including hypertension (HTA), DM, DL, obstructive sleep apnoea, or weight-induced rheumatologic disease). The diagnostic criteria used for DM, HTA, and metabolic syndrome are detailed in the National Cholesterol Education Program [21]. None of the diabetic or dyslipidaemic patients were being treated with anti-diabetic or anti-hyperlipidaemic drugs, respectively, before or after bariatric surgery. The study protocol was accepted by the hospital ethics committee. The protocol conformed to the Declaration of Helsinki, and all subjects provided written informed consent to participate.

Patients were considered to be "healthy" morbidly obese patients when we apply the most restrictive criteria of Wildman et al. [22]. Thus for DM, the threshold was for fasting plasma glucose ≥100 mg/dL or medically diagnosed DM; the criteria for HTA was: systolic and diastolic blood pressure (SBP and DBP, respectively), SBP ≥ 130 mmHg and DBP ≥ 85 mmHg, and the criteria for DL was: TG ≥ 150 mg/dL, cLDL ≥110 mg/dL and cHDL <40 and 50 mg/dL for men and women, respectively, or medically diagnosed DL.

The patients were divided into three groups according to their hospital medical diagnosis of DL and/or DM as follows: 10 "healthy" obese patients (DM−DL− group, 6 women and 4 men); 15 obese patients with DL (DM−DL+ group, 11 women and 4 men); and 7 obese patients with DL and DM (DM+DL+ group, 6 women and 1 man).

Blood samples were obtained under fasting conditions between 8:00 and 10:00 a.m. on the day of RYGBP and at 1, 6 and 12 months after surgery (labelled 1, 6 and 12 M in the Graphs and Tables). An index biopsy from 24 patients was taken at the time of bariatric surgery (Roux-en-Y gastric bypass), with a Hepafix needle. In those patients a follow-up biopsy was obtained 16.3 ± 3 months (range: 12–18 months) after bariatric surgery as a percutaneous biopsy using a same kind of needle. All biopsies were at least 2 cm in length and contained at least eight portal tracts. The anaesthetic procedures were standardised in both elective surgery and biopsy procedures. In biopsy procedures, 1% Scandicain was used. Epinephrine was avoided. Liver samples were quickly minced, frozen in liquid nitrogen, and stored at −80 °C for further analysis.

The control group (labelled as C in the Graphs and Tables) included 22 euthyroid, normal-weight, normotensive, non-diabetic patients (12-h fast) who underwent elective cholecystectomy at the same time that a blood sample was obtained. Liver biopsy was obtained from only seven of these patients.

2.2. Anthropometric and body composition measurements

Body weight, height, and waist circumference were measured according to standardised procedures [23]. The body fat percentage and amounts of total, subcutaneous and visceral fat were calculated as described previously [24].

2.3. Measurements of plasma and liver parameters

The leptin, ghrelin, adiponectin, insulin, glucose, homeostasis model assessment of insulin resistance (HOMA-IR), and plasma and liver lipid (TAG, PL, TC, cLDL, cHDL and NEFA) levels were determined as previously described [25,26]. DNA in the liver biopsies was quantified via the fluorimetric method [27]. The AST, ALT and GGT levels were measured enzymatically at the hospital's routine chemistry laboratory. The alkaline phosphatase and bilirubin levels were measured using a Beckman Coulter AU5400/2700 analyser (Brea, USA). The glycerol level was determined via the enzymatic method [28]. The CRP level was determined using an immunometric/turbidimetric assay performed with a METROLAB 2300 autoanalyser (RAL, Laboratory Techniques, Spain). The *PAI1* level was measured by enzyme-linked immunosorbent assay (ELISA) [29]. Ketone body (KB) levels were indirectly determined by quantification of b-hydroxybutyrate via an enzymatic method [30].

2.4. Liver lipid extraction

We used a method for extracting a small amount of tissue that has been previously described by our group [31]. Lipid extracts were analysed as described [12].

2.5. Assays of LPL and HL activities in plasma and liver

Lipase assays involving the use of TAGs containing radiolabeled acyl chains are highly specific and sensitive [32]. In our study, the *LPL* and *HL* activities were assessed as described previously [33,34] with minor modifications [13].

2.6. Histological analysis of liver

Haematoxylin-eosin- and trichrome-stained samples from all liver biopsies (obtained pre- and post-surgery) were reviewed by a pathologist without knowledge of the clinical data. The samples were classified according to the criteria of Brunt [35]. The following parameters were graded in the biopsies: a) steatosis: 0–3; b) hepatocyte ballooning: 0–3; c) lobular inflammation: 0–3; and d) portal inflammation with or without different fibrosis stages: 0–4.

2.7. Total RNA and cDNA preparation and PCR analyses

Total RNA was extracted from 15 to 25 mg of human liver biopsy sample with Tripure Isolation Reagent (Roche, USA). First-strand complementary DNA (cDNA) was synthesised from 0.4 μg total RNA using random primers and TaqMan high-capacity cDNA reverse transcription reagents (Applied Biosystems, USA). To perform real-time PCR, TaqMan low-density array cards were used (Applied Biosystems, USA). A 100-ng aliquot of cDNA was mixed with TaqMan Gene Expression MasterMix (Applied Biosystems, USA) and applied to the card. Gene-specific primers and probe mixtures were subsequently placed on the card. Relative mRNA levels were evaluated using the ΔΔCt method. Details about the genes used in this study are provided in Supplemental Table 1.

2.8. Statistical analysis

The results are reported as the mean ± SEM. Significant differences among the mean values for the control (C), obese (OB) and 12 months (M) (in some cases, 1 M and 6 M were added) after surgery (weight loss) groups were assessed using the non-parametric Kruskal–Wallis test, and individual comparisons were made using Dunn's post-test. Significant differences between the "healthy" MO (DM − DL −), dyslipidaemic (DM − DL +) and diabetic and dyslipidaemic (DM + DL +) individuals at different times after surgery (weight loss effect) were assessed by two-way ANOVA (comorbidities and surgery effect, respectively) and the Bonferroni post-test. For histological comparisons pre-surgery and post-surgery, paired t-tests were used and confirmed with Wilcoxon signed rank tests. Correlations between independent variables were determined by Pearson's correlation coefficient. Statistical comparisons were considered significant at a $p < 0.05$.

All statistical analyses were computed using GraphPad Prism version 5.0 software for Windows (GraphPad Software, San Diego CA, USA, www.graphpad.com).

3. Results

3.1. Clinical characteristics of patients (Table 1)

The "healthy" obese patients exhibited 13 and 17.5% more total and subcutaneous fat, respectively, compared with the DM + DL + obese individuals. Additionally, the DM − DL + and DM + DL + individuals had respectively 69 and 61% more leptin, 72 and 69% more TAG, 41 and 27% more TC, 44 and 26% more cLDL, 17 and 19% more apoA1 and 33% more *apoB* than the "healthy" obese individuals. The "healthy" obese group exhibited an HOMA-IR level (6.92 ± 1.37 au) compared with the DL group. The leptin/adiponectin ratios (IR marker) [36] were 0.83, 2.81, 3.59 and 3.59 in the control, "healthy", dyslipidaemic and diabetic obese individuals, respectively. One year after surgery, these ratios were decreased to 0.26, 0.48 and 0.44, in the "healthy", dyslipidaemic and diabetic obese individuals, respectively. Reductions in both body weight (53, 47 and 44 kg for the "healthy", DM − DL + and DM + DL + groups, respectively) and BMI (19, 18 and 17 points, respectively) were observed at one year after surgery. Additionally, the fat content, waist circumference, and leptin, TAG, NEFA, TC, cLDL (*apoB* was also reduced), insulin, glucose, CRP, *PAI1* and HOMA-IR levels were reduced. The adiponectin and ghrelin levels increased significantly and proportionally to the weight loss, and the lipid profile improved, with increases in cHDL and ApoA1. It should be noted that some parameters such as TC, LDL, HDL and apo A1, after a year of surgery, are higher, but did not become significant difference, in the DM + DL + group than in the DM − DL + group and, in this higher than in the DM − DL.

3.2. Biochemical liver parameters (Table 2)

The amount of lipids per cell was 10-fold in the "healthy" and dyslipidaemic obese individuals and 14-fold in the DM patients compared with the controls. These amounts were proportional with the observed plasma lipid level. The "healthy" obese patients exhibited 2-fold more *LPL* and *HL* activities, between 7- and 10-fold in the TAG and lipid levels, an 18-fold in the PL level, a 28-fold in TC and 30-fold in the NEFA and CRP levels than control individuals. These parameters are similar to those observed in the patients with dyslipidaemia, but the levels were even higher in the obese individuals with DM. The increased *LPL* activity in both the "healthy" and dyslipidaemic individuals was directly related with increased liver enzyme mRNA levels. The increased *HL* activity in the obese individuals was related to increases in both the TC and PL levels in the liver.

3.3. Other plasma parameters related to hepatic metabolism (Table 3)

The "healthy", DL and DM obese subjects exhibited 3.3-, 3.5- and 3.6–fold increases, respectively, in the glycerol and NEFA levels compared with the controls with similar plasma profiles after surgery, secondary to adipose tissue TAG hydrolysis via hormone-sensitive lipase (HSL) [24]. The glycerol and NEFA levels remained elevated at one year after surgery given that HSL activity remained elevated, as we have recently demonstrated [24]. The concentration of plasma KBs was increased in the obese individuals at one month after surgery. Moreover, the ketotic ratio (KB:NEFA), a non-invasive in vivo measurement that provides insights into the direct ketotic activities of hormones independent of their lipolytic effects [37], was also increased after one month but returned to baseline at one year after bariatric surgery.

3.4. Histological liver results (Tables 4 and 5)

Diabetic MO patients exhibited clear increasing trends in the levels of the assessed parameters, as measured by Brunt's index [35], compared with the "healthy" and dyslipidaemic individuals, especially steatosis and fibrosis (Table 4). The decrease in liver damage following bariatric surgery was significant in all cases; however, this decrease was considerably more pronounced in the patients with steatosis and ballooning. In general, there were a larger proportion of non-responder with respect to liver histology in DM − DL + and DM + DL + groups than in "healthy" DM − DL − group following bariatric surgery. Table 5 shows scoring for the grade and stage of NASH in liver biopsies performed at surgery and during follow-up. None of the second biopsies revealed progression of grade or stage of liver disease. One year after the surgery, only 2 patients (one in DM − DL + group and 1 more in DM + DL + group) presented NAFLD (pre- vs. post-surgery, $p < 0.0001$).

Fibrosis (Table 5) score improved overall by two stages in 1 patient with DM − DL + and by one stage in 9 patients (4 patients in DM − DL − group, 2 in DM − DL + and 3 in DM + DL +). In 13 patients, fibrosis remained stable and we didn't observe any patient with worsening of liver fibrosis (Table 5).

Table 1
Clinical and metabolic characteristics of morbidly obese patients in each group before and after bariatric surgery.

	PLASMA					Anova-2, p value	
Parameters	Control (n = 22)	Time	DM−DL− (n = 10)	DM−DL+ (n = 15)	DM+DL+ (n = 7)	Comorbidities effect	Surgery effect
Body weight (Kg)	–	OB	136.7 ± 6.4	131.8 ± 4.7	118.0 ± 7.4	0.0004	<0.0001
		12 M	83.8 ± 4.9	84.8 ± 5.3	74.4 ± 4.4		
			p < 0.0001	p < 0.0001	p < 0.0001		
BMI (Kg/m2)	–	OB	49.4 ± 1.9	49.8 ± 1.3	47.37 ± 1.24	ns	<0.0001
		12 M	30.9 ± 1.7^{ooo}	31.7 ± 1.5^{ooo}	30.1 ± 1.6^{ooo}		
			p < 0.0001	p < 0.0001	p < 0.0001		
Total fat (Kg)	–	OB	81.2 ± 5.8	82.3 ± 4.6	71.0 ± 4.6	0.0103	<0.0001
		12 M	35.1 ± 3.3^{ooo}	37.7 ± 3.9^{ooo}	31.0 ± 3.2^{ooo}		
			p < 0.0001	p < 0.0001	p < 0.0001		
SAT (Kg)	–	OB	62.4 ± 5.3	60.5 ± 4.3	51.5 ± 4.0	ns	<0.0001
		12 M	30.3 ± 5.0^{oo}	27.2 ± 3.7^{ooo}	29.1 ± 3.1^{o}		
			p = 0.0006	p = 0.0001	p = 0.0069		
VAT (Kg)	–	OB	21.3 ± 2.4	22.6 ± 2.4	19.4 ± 2.5	ns	0.0001
		12 M	6.7 ± 1.2^{oo}	9.4 ± 1.7^{oo}	8.3 ± 2.3^{o}		
			p = 0.0002	p = 0.0048	p = 0.0207		
Waist (cm)	–	OB	134.8 ± 3.9	136.7 ± 3.7	138.1 ± 5.0	ns	<0.0001
		12 M	95.2 ± 4.0^{ooo}	99.0 ± 4.3^{ooo}	102.7 ± 9.5^{o}		
			p < 0.0001	p < 0.0001	p = 0.0068		
Leptin (ng/mL pl.)	15.0 ± 0.9	OB	26.1 ± 5.7	44.1 ± 6.9$^{\&,cc}$	42.0 ± 6.8$^{\&,c}$	0.0024	<0.0001
		12 M	4.8 ± 1.2^{oo,ccc}	10.7 ± 2.6^{ooo}	7.9 ± 2.7^{ooo,c}		
			p = 0.0040	p < 0.0001	p = 0.0002		
Ghrelin (pg/mL pl.)	132.0 ± 0.3	OB	83.5 ± 13.3^{cc}	48.7 ± 5.8$^{\&,ccc}$	86.0 ± 16.8$^{+,c}$	ns	0.01
		12 M	124.9 ± 23.9^{o}	122.5 ± 22.5^{oo}	122.4 ± 21.6^{o}		
			p = 0.0141	p = 0.0005	p = ns		
Adiponectin (μg/mL pl.)	18.1 ± 2.3	OB	9.3 ± 1.1^{ccc}	12.3 ± 1.7^{c}	11.7 ± 3.0	ns	<0.0001
		12 M	18.6 ± 3.2	22.3 ± 2.5^{o}	17.9 ± 1.9		
			p = ns	p = 0.0150	p = ns		
TAG (mg/dL pl.)	89.0 ± 9.0	OB	103.4 ± 8.2	177.6 ± 33.9$^{\&,c}$	174.8 ± 14.5$^{\&\&,ccc}$	<0.0001	<0.0001
		12 M	78.9 ± 11.2	99.8 ± 7.5^{o}	102.3 ± 13.9		
			p = 0.0083	p = 00.111	p = 0.0334		
NEFA (mM pl.)	0.32 ± 0	OB	0.55 ± 0.07^{c}	0.60 ± 0.05^{ccc}	0.60 ± 0.09^{c}	ns	<0.0001
		12 M	0.42 ± 0.05	0.56 ± 0.07^{cc}	0.55 ± 0.07^{c}		
			p = 0.0003	p < 0.0001	p = ns		
TC (mg/dL)	164.0 ± 3.0	OB	174.3 ± 5.2	245.4 ± 18.2$^{\&\&\&,ccc}$	220.7 ± 9.1$^{\&\&,ccc}$	<0.0001	<0.0001
		12 M	132.9 ± 10.2^{oo,c}	142.1 ± 18.2^{o}	175.0 ± 5.5^{o}		
			p < 0.0001	p = 0.0183	p = 0.0158		
cLDL (mg/dL)	105.0 ± 4.0	OB	109.2 ± 3.4	157.1 ± 10.9$^{\&\&,ccc}$	137.5 ± 7.2$^{\&,ccc}$	<0.0001	<0.0001
		12 M	75.0 ± 7.1^{oo,c}	96.9 ± 6.1^{o}	98.5 ± 5.0^{o}		
			p < 0.0001	p = 0.0236	p = 0.0319		
cHDL (mg/dL pl.)	74.0 ± 4.0	OB	44.7 ± 2.1^{ccc}	53.0 ± 5.3^{cc}	48.2 ± 3.4^{ccc}	0.0002	<0.0001
		12 M	42.4 ± 2.8^{ccc}	51.5 ± 2.8^{ccc}	56.0 ± 2.2^{ccc}		
			p = 0.0001	p = 0.0019	p = 0.0091		
apoA1 (mg/dL pl.)	197.5 ± 6.4	OB	167.5 ± 4.1^{cc}	195.4 ± 8.5	198.5 ± 8.4	0.0003	<0.0002
		12 M	159.1 ± 6.5^{**,ccc}	197.4 ± 10.6	207.9 ± 9.0		
			p < 0.0001	p < 0.0001	p < 0.0001		
apoB (mg/dL pl.)	68.4 ± 4.9	OB	69.6 ± 1.3	92.7 ± 4.3^{ccc}	92.4 ± 6.4^{ccc}	0.0004	<0.0003
		12 M	52.1 ± 2.7^{oo,cc}	70.6 ± 4.4**	68.7 ± 3.0**		
			p = 0.0003	p = ns	p = ns		
Insulin (UI/L pl.)	11.0 ± 1.0	OB	27.7 ± 5.2^{cc}	22.8 ± 5.3	23.4 ± 4.0^{c}	ns	<0.0001
		12 M	9.2 ± 1.4^{oo}	7.8 ± 1.1^{oo,c}	8.7 ± 1.5^{o}		
			p = 0.0017	p = 0.0028	p = 00.109		
Glucose (mg/dL pl.)	72.0 ± 2.1	OB	99.8 ± 2.9^{ccc}	104.0 ± 4.4$^{\&\&\&,ccc}$	164.1 ± 22.3$^{\&\&,cc}$	<0.0001	<0.0001
		12 M	83.9 ± 2.5^{oo,cc}	87.7 ± 2.1^{oo,ccc}	95.0 ± 6.1^{o,cc}		
			p = 0.0007	p = 0.0032	p = 0.0079		
HOMA (a.u.)	2.0 ± 0.1	OB	6.9 ± 1.4^{cc}	5.7 ± 1.5	10.2 ± 2.8^{c}	ns	<0.0001
		12 M	1.8 ± 0.5^{oo}	1.6 ± 0.3^{oo}	2.2 ± 0.6^{o}		
			p = 0.0023	p = 0.0018	p = 0.0121		
CRP (mg/L pl.)	6.9 ± 0.9	OB	18.0 ± 2.6^{cc}	22.4 ± 2.9^{ccc}	21.9 ± 4.0^{cc}	0.0041	<0.0001
		12 M	3.1 ± 0.8^{ooo,cc}	5.3 ± 1.1^{ooo}	3.3 ± 1.0^{oo,c}		
			p = 0.0002	p < 0.0001	p = 0.0004		
PAI1 (ng/mL pl.)	76.2 ± 11.0	OB	156.8 ± 28.1^{c}	158.2 ± 24.5^{c}	160.0 ± 40.4	ns	<0.0001
		12 M	39.3 ± 11.8^{o,c}	32.0 ± 7.7^{ooo,cc}	34.5 ± 12.3^{o,c}		
			p = 0.0066	p = 0.0006	p = 0.0331		

The data are expressed as the means ± SEM. Abbreviations: SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue; TAG, triacylglycerides; NEFA, non-esterified fatty acid; TC, total cholesterol; cHDL, HDL cholesterol; cLDL, LDL cholesterol; HOMA-IR, homeostasis model assessment-insulin resistance; a.u., arbitrary units; CRP, C-reactive protein; *PAI1*, plasminogen activator inhibitor 1; OB and 12 M, OB− and 12 M−, and OB+ and 12 M+, obese and 12 months after surgery in the DM−DL−, DM−DL+ and DM+DL+ groups, respectively. The (o) symbol indicates the differences between obese and 12 months after surgery in each group; the (&) symbol indicates the differences between the obese in each group versus the healthy group (DM−DL−); the (*) symbol indicates the differences between 12 and 6 months after surgery in each group; the (+) symbol indicates the differences between DM−DL+ and DM+DL+; the (c) symbol indicates the differences between each time and obese group vs. control (lean) group. One symbol, p < 0.05; two symbols, p < 0.01; three symbols, p < 0.001; ns, non-significant.

Table 2
LPL and *HL* activities and lipid parameters on morbidly obese liver patients in each group before and after bariatric surgery.

	LIVER					Anova-2, p value	
Parameters	Control (n = 7)	Time	DM−DL− (n = 9)	DM−DL+ (n = 14)	DM+DL+ (n = 6)	Comorbidities effect	Surgery effect
LPL (mU/g tissue)	36.8 ± 2.1	OB	78.7 ± 10.8^{c}	97.4 ± 8.6^{cc}	72.7 ± 17.6	ns	ns
		12 M	68.7 ± 17.3	67.7 ± 19.9	67.0 ± 10.3		
			p = ns	p = 0.0295	p = ns		
HL (mU/g tissue)	153.2 ± 9.3	OB	303.7 ± 26.3^{cc}	253.2 ± 20.1^{c}	231.5 ± 31.3^{c}	ns	<0.0001
		12 M	146.8 ± 16.7^{oo}	142.6 ± 30.4^{o}	115.5 ± 6.2^{o}		
			p = 0.0036	p = 0.0081	p = 0.0430		
Lipid (mg/g tissue)	24.0 ± 2.0	OB	201.1 ± 25.2^{ccc}	178.1 ± 40.0^{ccc}	244.4 ± 38.7^{c}	0.0087	0.0001
		12 M	27.5 ± 4.6^{ooo}	74.0 ± 27.2^{o,c}	69.9 ± 1.4^{o,c}		
			p < 0.0001	p = ns	p = 0.0065		
Lipid (mg/mg DNA)	10.8 ± 2.0	OB	105.9 ± 18.7^{cc}	102.7 ± 24.4^{c}	148.0 ± 23.2^{ccc}	0.0049	0.0017
		12 M	19.7 ± 5.0^{oo}	49.3 ± 16.9^{o}	55.8 ± 9.7^{o,c}		
			p = 0.0009	p = ns	p = 0.0007		
TAG (mg/mg DNA)	6.4 ± 1.6	OB	44.0 ± 8.6^{cc}	40.0 ± 10.4	57.6 ± 9.8^{ccc}	0.0219	0.0532 (ns)
		12 M	9.6 ± 1.2^{oo}	26.9 ± 10.2	34.7 ± 14.9		
			p = 0.0040	p = ns	p = 0.0226		
TC (mg/mg DNA)	0.44 ± 0.08	OB	12.5 ± 2.3^{c}	13.3 ± 3.2^{c}	19.0 ± 2.5^{cc}	0.0095	<0.0001
		12 M	2.2 ± 0.5^{o,c}	3.5 ± 1.9^{o}	2.4 ± 1.2^{oo}		
			p = 0.0070	p = ns	p = 0.0008		
NEFA (mg/mg DNA)	0.23 ± 0.08	OB	6.8 ± 1.4^{cc}	8.5 ± 1.0^{ccc}	8.5 ± 1.2^{ccc}	<0.0001	ns
		12 M	4.7 ± 1.1^{c}	10.9 ± 0.3^{c}	6.4 ± 0.9^{c}		
			p = 0.0508	p = ns	p = 0.0021		
PL (mg/mg DNA)	2.43 ± 0.45	OB	42.59 ± 8.97^{cc}	48.04 ± 12.25^{cc}	79.35 ± 8.92^{ccc}	0.0026	<0.0001
		12 M	5.70 ± 1.52^{o}	8.32 ± 2.63^{oo}	9.05 ± 0.87^{ooo}		
			p = 0.0143	p = 0.0475	p = ns		
CRP (mg/mg DNA)	0.09 ± 0.05	OB	2.78 ± 0.51^{cc}	6.02 ± 1.32^{c}	3.44 ± 1.14	0.0396	0.0003
		12 M	0.0 ± 0.0^{ooo}	0.41 ± 0.19^{o}	0.21 ± 0.21		
			p = 0.0003	p = 0.0108	p = 0.0419		

The data are expressed as the means ± SEM. Abbreviations: *LPL*, lipoprotein lipase; *HL*, hepatic lipase; mU, nmol/min; PL, phospholipid. The (o) symbol indicates the differences between obese and 6 or 12 months after surgery in each group; the (c) symbol indicates the differences between each time and obese group vs. control (lean) group. One symbol, p < 0.05; two symbols, p < 0.01; three symbols, p < 0.001; ns, non-significant.

3.5. Liver gene expression (Supplemental Table 1, and Figs. 1 to 3)

1. Genes with increased expression

Both the *LPL* and *FAT/CD36* (Supplemental Table 1, and Fig. 2, top left panel) genes were over-expressed (2-fold compared with the control) in the DM−DL+ and DM+DL+ groups. At one year after bariatric surgery, *LPL* mRNA was detected in only 8 patients, including 3 in the "healthy" group, 4 in the DM−DL+ group and 1 in the DL+DM+ group. Moreover, slight elevations in the liver *HL*, *VLDLr* (Supplemental Table 1, and Fig. 1, top left panel) and *SCARB1* (Supplemental Table 1, and Fig. 1, middle right panel) expression levels were observed.

In the 3 obese groups, the *TNFα* (Supplemental Table 1, and Fig. 2, bottom left panel) and *PAI1* (Fig. 2, bottom right panel, and Supplemental Table 1) expression levels were between 40 and 100% greater than the normal control levels. However, after surgery, *TNFα* expression tended to decrease in the DL and DM groups compared with the obese basal level, but its expression remained higher than that in the control group. Additionally, *PAI1* expression decreased by 30 to 50% compared with the control group. Increased/decreased *PAI1* gene expression in the obese individuals pre- and post-surgery, respectively, corresponded with the plasma *PAI1* levels observed before and after surgery (Table 1). The expression level of interleukin-6 (*IL6*, Supplemental Table 1) was perfectly correlated with those of plasma *PAI1* (r = 0.83, p < 0.0001) and *TNFα* (r = 0.48, p = 0.0009) in the obese individuals.

2. Genes with reduced expression

The enzyme adipose triglyceride lipase (*ATGL*, Supplemental Table 1) is also present in the liver. The *ATGL mRNA* level was decreased by 30% in the healthy obese group compared with the control group, and it was reduced by 23 and 18%, respectively, in the obese individuals with comorbidities. After surgery, the *ATGL* mRNA level exhibited an increasing trend, with restoration to the normal level. Liver *apoB* expression (Supplemental Table 1, and Fig. 1, bottom left panel) exhibited a significant decrease in the "healthy" individuals (p < 0.05 vs. control) compared with the other 2 groups. At one year after surgery, a clear increase in its expression was observed in the "healthy" and dyslipidaemic patients but not in the diabetic patients. The obese patients exhibited significantly reduced *LDLr* (Supplemental Table 1, and Fig. 1, middle left panel) expression compared with the controls (in most cases, its expression decreased to less than 50% of the control level, both before and after surgery; Fig. 1). Surgery tended to significantly increase *LDLr* expression, and this increase was more marked in the "healthy" (p = 0.0004) and diabetic patients (p = 0.0201). The de novo cholesterol synthesis regulatory enzyme *HMG-CoA reductase* (Supplemental Table 1, and Fig. 1, top right panel) exhibited significantly decreased expression in the "healthy" obese individuals (50% decrease, p < 0.01 vs. control), but this reduction was not as notable in the other two obese groups (Fig. 1). At one year after surgery, its expression tended to return to the baseline level in the patients, with the exception of the dyslipidaemic obese patients. Additionally, the *SCARB1*, *HMGCR*, *LDLr* and, *apoB* (Fig. 1) expression profiles were similar in all obese groups. Cholesterol 7 alpha-hydroxylase (*CYP7A1*) expression (Supplemental Table 1, and Fig. 1, bottom right panel) was significantly reduced in all three obese groups compared with the control group ("healthy" obese = p < 0.001; DM−DL+ p < 0.001; DM−DL+ p < 0.05; Fig. 1). At one year after surgery, its level remained decreased by 50% in all groups.

In the 3 obese groups, *PPARα* and *PGC1α* expression (Supplemental Table 1, and Fig. 2, middle right and left panel, respectively) was reduced compared with the control group. After surgery, the *PPARα* level increased to above the control level in all three MO groups. However, after surgery, the *PGC1α* level increased in the "healthy" MO group compared with the other MO groups.

Fig. 3 summarises the liver gene expression in the MO patients in the DM+DL+ obese.

4. Discussion

This study is the first to report that 1) hepatic lipid parameters in MO patients change based on their comorbidities; 2) laboratory parameters

Table 3
Additional plasma parameters in morbidly obese patients in each group before and after bariatric surgery.

			PLASMA			Anova-2, p value	
Parameters	Control (n = 22)	Time	DM−DL− (n = 10)	DM−DL+ (n = 15)	DM+DL+ (n = 7)	Comorbidities effect	Surgery effect
AST (UI/L)	24.0 ± 1.4	OB	23.1 ± 2.0	20.8 ± 1.6	22.5 ± 2.4	ns	0.0002
		1 M	38.8 ± 4.7^{o}	33.2 ± 3.2^{oo}	30.8 ± 4.9		
		6 M	20.3 ± 2.4**	21.3 ± 1.8**	17.2 ± 1.6*		
		12 M	22.2 ± 4.7*	21.8 ± 2.2**	18.7 ± 2.5		
			p = 0.0022	p = 0.0012	p = 0.0365		
ALT (UI/L)	26.0 ± 1.5	OB	34.4 ± 4.9	29.9 ± 3.6	33.0 ± 5.3	ns	<0.0001
		1 M	64.6 ± 10.3^{o}	51.5 ± 6.5^{oo}	39.8 ± 9.4		
		6 M	22.5 ± 4.8***	22.9 ± 2.6***	15.9 ± 4.3*		
		12 M	20.3 ± 5.3***	22.4 ± 2.8***	17.9 ± 2.0		
			p = 0.0004	p < 0.0001	p = 0.0136		
AST/ALT	0.94 ± 0.10	OB	0.77 ± 0.08	0.75 ± 0.06	0.72 ± 0.07	ns	0.0007
		1 M	0.70 ± 0.09	0.68 ± 0.05	0.85 ± 0.14		
		6 M	1.07 ± 0.12	0.98 ± 0.06*	1.32 ± 0.28		
		12 M	1.23 ± 0.16*	1.02 ± 0.08**	1.05 ± 0.09		
			p = 00.112	p = 0011	p = ns		
GGT (UI/L)	32.0 ± 1.8	OB	28.4 ± 2.5	47.4 ± 7.8	41.2 ± 8.2	ns	0.0008
		1 M	32.1 ± 4.4	42.3 ± 8.3	25.5 ± 5.7		
		6 M	16.2 ± 1.8**	20.3 ± 3.2^{oo,*}	15.5 ± 1.8^{o}		
		12 M	17.4 ± 3.2	17.9 ± 2.3^{oo,*}	14.1 ± 2.6^{oo}		
			p = 0.0023	p = 0.0005	p = 0.0039		
Alkalin phosphatase (UI/L)	58.2 ± 8.2	OB	76.7 ± 5.7	84.9 ± 5.6	68.8 ± 8.1	<0.0001	ns
		1 M	79.0 ± 5.5	99.2 ± 6.8	72.3 ± 4.5		
		6 M	90.6 ± 9.3	93.1 ± 4.6	79.7 ± 6.5		
		12 M	91.8 ± 8.2	94.9 ± 5.4	75.9 ± 5.7		
			p = ns	p = ns	p = ns		
Esterified bilirubin (mg/dL)	0.07 ± 0.00	OB	0.20 ± 0.03	0.21 ± 0.01	0.22 ± 0.03	<0.0001	ns
		1 M	0.30 ± 0.04^{c}	0.27 ± 0.02^{c}	0.24 ± 0.03		
		6 M	0.30 ± 0.03^{c}	0.23 ± 0.03	0.26 ± 0.02^{c}		
		12 M	0.28 ± 0.03	0.27 ± 0.04^{c}	0.31 ± 0.04^{cc}		
			p = 0.0082	p = 0.0279	p = 0.0045		
Total bilirubin (mg/dL)	0.51 ± 0.03	OB	0.44 ± 0.08	0.42 ± 0.04	0.47 ± 0.09	ns	ns
		1 M	0.62 ± 0.08	0.61 ± 0.06	0.55 ± 0.06		
		6 M	0.69 ± 0.09	0.55 ± 0.07	0.66 ± 0.07		
		12 M	0.66 ± 0.09	0.71 ± 0.15	0.74 ± 0.11		
			DM−DL− p = ns	DM−DL+ p = ns	DM+DL+ p = ns		
Glycerol (mg/dL)	0.54 ± 0.11	OB	1.76 ± 0.14^{ccc}	1.88 ± 0.15^{ccc}	1.96 ± 0.16^{ccc}	<0.0001	0.0194
		1 M	1.96 ± 0.25^{ccc}	2.00 ± 0.14^{ccc}	2.13 ± 0.23^{ccc}		
		6 M	1.51 ± 0.13^{cc}	1.60 ± 0.15^{ccc}	1.35 ± 0.26		
		12 M	1.51 ± 0.19^{cc}	1.49 ± 0.12^{ccc}	1.51 ± 0.25^{c}		
			p < 0.0001	p < 0.0001	p < 0.0001		
NEFA (mM)	0.47 ± 0.04	OB	0.55 ± 0.07	0.60 ± 0.05	0.60 ± 0.09	0.0083	<0.0001
		1 M	0.96 ± 0.10^{oo,ccc}	1.06 ± 0.11^{ooo,ccc}	0.84 ± 0.12^{cc}		
		6 M	0.66 ± 0.10	0.65 ± 0.05***	0.57 ± 0.06		
		12 M	0.42 ± 0.05***	0.56 ± 0.07***	0.55 ± 0.07		
			p < 0.0001	p < 0.0001	p = 0010		
KB (mM)	0.08 ± 0.03	OB	0.08 ± 0.02	0.07 ± 0.02	0.05 ± 0.01	<0.0001	<0.0001
		1 M	0.95 ± 0.21^{o,ccc}	0.74 ± 0.17^{ooo,ccc}	1.72 ± 0.48^{oo,ccc}		
		6 M	0.39 ± 0.17*	0.16 ± 0.02^{o,cc}	0.57 ± 0.06		
		12 M	0.11 ± 0.05*	0.11 ± 0.01**	0.11 ± 0.03		
			p = 0.0002	p < 0.0001	p = 0.0003		
KB/NEFA	0.20 ± 0.08	OB	0.13 ± 0.03	0.10 ± 0.02	0.09 ± 0.02	<0.0001	<0.0001
		1 M	0.96 ± 0.21^{o,ccc}	0.71 ± 0.13^{ooo,ccc}	1.77 ± 0.48^{c,o}		
		6 M	0.45 ± 0.14	0.25 ± 0.02^{o,c}	0.28 ± 0.06		
		12 M	0.22 ± 0.09	0.20 ± 0.03	0.22 ± 0.07		
			p = 0.0004	p < 0.0001	p = 0.0051		

The data are expressed as the means ± SEM. Abbreviations: AST, aspartate transaminase; ALT, alanine transaminase; AST:ALT, ratio of AST and ALT activities; GGT, γ-glutaryl transferase; NEFA, non-esterified fatty acid; KB, ketone bodies; KB:NEFA, ratio of KB and NEFA; OB, 6 M and 12 M, obese, 6 and 12 months after surgery in the DM−DL−, DM−DL+ and DM+DL+ groups, respectively. The (o) symbol indicates the differences between obese and 1, 6 or 12 months after surgery in each group; the (*) symbol indicates the differences between 1 month and 6 or 12 months after surgery in each group; the (c) symbol indicates the differences between each time or obese group versus control (lean) group. One symbol, p < 0.05; two symbols, p < 0.01; three symbols, p < 0.001; ns, non-significant.

related to liver damage underestimate its severity; 3) liver damage is worse in patients with dyslipidaemia or in those with diabetes and dyslipidaemia; 4) cholesterol metabolism and NEFA levels are significantly altered in patients with diabetes and dyslipidaemia; 5) bariatric surgery typically restores various plasma and liver parameters, but its effectiveness depends on the number of comorbidities present; and 6) early changes (at one month) in hepatic parameters measured in the plasma after surgery can indicate systemic metabolic modifications.

4.1. Lipid metabolism

The three types of MO patients exhibited profoundly altered cholesterol and lipid metabolism, especially when DL and DM were present.

The diabetic obese individuals exhibited greater alterations in the lipid parameters even though they had less total subcutaneous and visceral fat, and this finding was even observed in those with a reduced BMI. The *apoB*:*apoA-I* atherogenesis ratio [38] was 0.47 in these individuals compared with the "healthy" obese (0.42) and control individuals

Table 4
Histological scores from Brunt's index for the 24-paired liver biopsies before and after bariatric surgery.

		BRUNT's index													
		DM − DL −				DM − DL +				DM + DL +				Anova-2, p value	
		0	1	2	3	0	1	2	3	0	1	2	3	Comorbidities effect	Surgery effect
Feature before surgery	Steatosis	0	5	2	1	0	6	3	2	0	1	2	2	ns	
	Balloon	3	1	4	0	5	4	2	0	2	1	1	1	ns	
	Lobinfl	0	7	1	0	1	10	0	0	0	4	1	0	ns	
	Portinfl	0	8	0	0	1	9	1	0	0	4	1	0	ns	
	Fibrosis	0	8	0	0	1	5	5	0	0	2	1	2	0.0395	
Feature a year after surgery	Steatosis	8	0	0	0	10	1	0	0	4	1	0	0	ns	0.0001
		p = 0.0008				p = 0.0001				p = 0.0111					
	Balloon	8	0	0	0	10	1	0	0	5	0	0	0	ns	0.0021
		p = 0.0148				p = 0.0107				p = ns					
	Lobinfl	6	2	0	0	5	6	0	0	3	2	0	0	ns	0.0004
		p = 0.0062				p = 0.0379				p = 0.0161					
	Portinfl	5	3	0	0	1	10	0	0	0	5	0	0	0.0065	0.0280
		p = 0.0112	p = ns	p = ns											
	Fibrosis	4	4	0	0	3	5	3	0	1	2	1	1	ns	0.0159
		p = 0.0331				p = ns				p = ns					

Each column in each group indicates the number of patients with each grade for the following categories: steatosis, hepatocyte ballooning (*balloon*), lobular inflammation (*lobinfl*), portal inflammation (*portinfl*) and fibrosis. The results of the two-way ANOVA (anova-2) are presented in the right columns. The p value under each group of patients and feature, in the lower part of Table, is the result of compare before and a year after surgery.

(0.35). Moreover [39], the controls exhibited a TC:cHDL ratio of 2.22, while this ratio was 3.90 in the "healthy" obese patients and 4.58 in the diabetic patients. Thus, modifications in the plasma lipid parameters could be attributed to liver impairment. These alterations in liver were accompanied by larger hepatic cells (less DNA/g liver: 2.1 ± 0.1 mg/g in DM − DL −, 1.9 ± 0.1 mg/g in DM − DL + and, 1.7 ± 0.1 mg/g in DM + DL +) and lipid-laden cells (see Table 2). The presence/increment of *LPL* activity and the increment of *HL* activity could to contribute to worsen the liver conditions (see below correlation between *LPL* and *HL* and histological features) in morbid obese patients, as we could seen by the concentration of total lipid, TAG, TC, NEFA and PL. In fact, DM + DL + are less able to recover after surgery, because those mentioned lipid were higher not only vs. DM − DL +, but also vs. healthy and control patients. In our previous studies [12,13] and those of other authors [40,41] it was mentioned this possibility.

4.2. Liver lipid accumulation

The increases in the liver lipid levels were consistent with the hepatic steatosis observed in all patients, and they were greater in the individuals with DL or DL and DM.

The presence of excess lipids (especially NEFAs and lipids derived from TAG hydrolysis, DAGs and ceramides) results in liver inflammation [39], and these increases were found to be correlated with elevated tissue and plasma CRP levels and increased *TNFα*, *IL6* and *PAI1* expression. Recent evidence indicates that sphingolipid metabolism is altered in obese individuals, suggesting a common pathway that links both excessive nutrient intake and inflammation with increased metabolic and cardiovascular risks [42]. The liver phospholipid increases observed in our patients, including the "healthy" and dyslipidaemic individuals and especially the diabetic patients, were potentially related to increased ceramide synthesis.

Uncontrolled lipolysis in the adipose tissue of obese individuals due to peripheral IR provides an increased and continuous flow of NEFAs to the liver [43]. Our results are consistent with previous reports of elevated HSL activity [24] in both subcutaneous and visceral adipose tissues. We observed increased liver NEFA levels that were potentially attributed to facilitated diffusion by *FAT/CD36* carriers resulting from hydrolysis of TAGs in QMs and of VLDL by *LPL* and the action of *HL* on HDL2. Phospholipids are captured by *HL*, whereas cholesterol is captured via binding to lipoproteins or remnants containing apoE, which mediates *VLDLr*, *HL* and *SCARB1* expression (*LDLr* is not involved, given its decreased expression). Therefore, the increased liver lipid levels in the diabetic MO individuals can be explained by differences in plasma enzymatic activity and gene expression.

Additionally, *VLDLr* overexpression and increased *FAT/CD36* expression via *TNFα* liberation are strongly related to liver steatosis and inflammation [44]. We propose that *LPL* in the liver could act as another

Table 5
Scoring for the grade and stage of Non-alcoholic steatohepatitis (NASH) for the 24-paired liver biopsies before and after bariatric surgery.

	Score												Anova-2, p value	
	DM − DL −				DM − DL +				DM + DL +				Comorbidities effect	Surgery effect
Before surgery	0	1	2	3	0	1	2	3	0	1	2	3		
Grade	0	5	2	1	0	6	3	2	0	1	2	2	ns	
%	0	63	25	13	0	55	27	18	0	20	40	40		
Stage	0	8	0	0	1	5	5	0	0	2	1	2	0.0395	
%	0	100	0	0	9.1	45	45	0	0	40	20	40		
A year after surgery														
Grade	8	0	0	0	10	1	0	0	4	1	0	0	ns	<0.0001
%	100	0	0	0	91	9,1	0	0	80	20	0	0		
	p = 0.0008				p = 0.0001				p = 0.0111					
Stage	4	4	0	0	3	5	3	0	1	2	1	1	ns	0.0159
%	50	50	0	0	27	45	27	0	20	40	20	20		
	p = 0.0112				p = ns				p = ns					

Each column in each group indicates the number of patients with each grade and stage with the corresponding percentages before and a year after surgery. The results of the two-way ANOVA (anova-2) are presented in the right columns. The p value under each group of patients and feature, in the lower part of Table, is the result of compare before and after surgery.

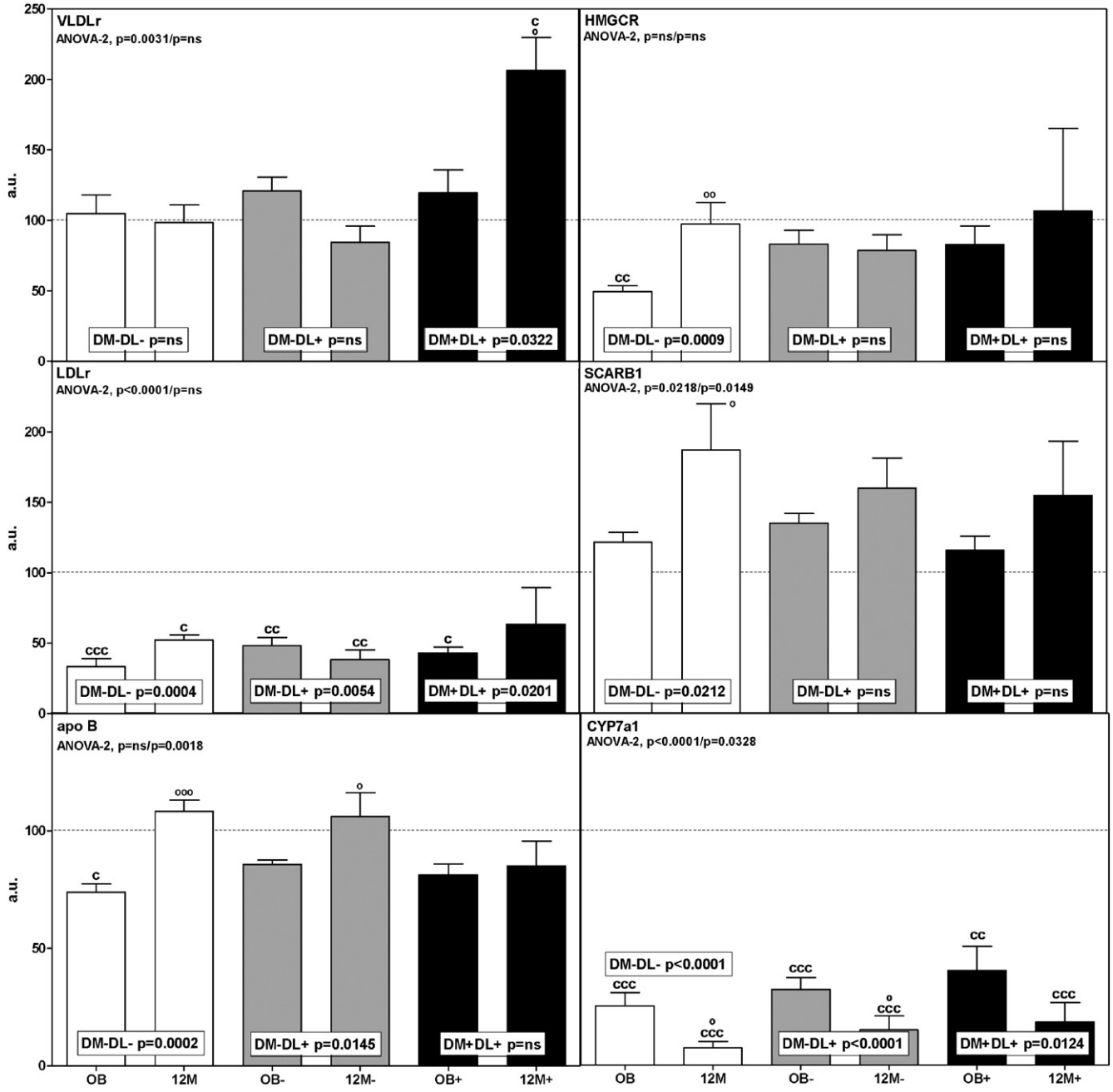

Fig. 1. *VLDLr*, *HMGCR*, LDLr, SCARB1, *apoB* and, *CYP7a1* expression in the liver. Relative mRNA levels were evaluated using the ΔΔCt method. The results are expressed as the means ± SEM vs. 100% in the control group (dotted line). For gene abbreviations, see Supplemental Table 1. The DM−DL− group is presented as white bars. The DM−DL+ group is depicted as grey bars, and the DM+DL+ group is presented as black bars. Two-way ANOVA (anova-2) results are presented in left corner in each graph; the first number is the result of the comorbidity effects, and the second number represents the surgery effects. Statistical results in each group vs. the control are presented as the white square in each group of the bar. Abbreviations: OB and 12 M, obese, and 12 months after surgery in the DM−DL−, DM−DL+ and DM+DL+ groups, respectively; a.u., arbitrary units. The (o) symbol indicates the differences between obese and 12 months after surgery in each group; the (c) symbol indicates the differences between each time vs. the control (lean) group. One symbol, $p < 0.05$; two symbols, $p < 0.01$; ns, non-significant.

factor to promote lipid accumulation and the subsequent onset/progression of NAFLD. In fact, *TNFα* promotes *LPL* liver expression [45]. Moreover, *LPL* overexpression in mice causes hepatic steatosis and insulin resistance [40]. *LPL* mRNA has been reported to be present in the human fatty livers of IR subjects [12]. However, the correlation between *LPL* expression and its local enzymatic activity has not been elucidated.

Our results are consistent with previous reports suggesting that NEFAs in hepatocytes can be oxidised or converted to KBs [46], especially during the first month after surgery. When the liver is no longer steatotic, e.g., after surgery, *LPL* expression is not detected. Consequently, increased liver *LPL* expression could act as a protective mechanism by redirecting lipid accumulation to the liver, ameliorating the effects of plasma hyperlipidaemia on other tissues.

4.3. Lipid metabolite destinations within the liver

Hepatic steatosis could develop through any combination of increased liver free fatty acid (FFA) uptake and storage as TAG, increased de novo lipogenesis, decreased fatty acid oxidation, and decreased secretion of TAG as VLDL [47–49]. Evidence from Morris et al. [50] and others [51,52] suggests that elevated mitochondrial number and function increase fatty acid oxidation, which may play a protective role by

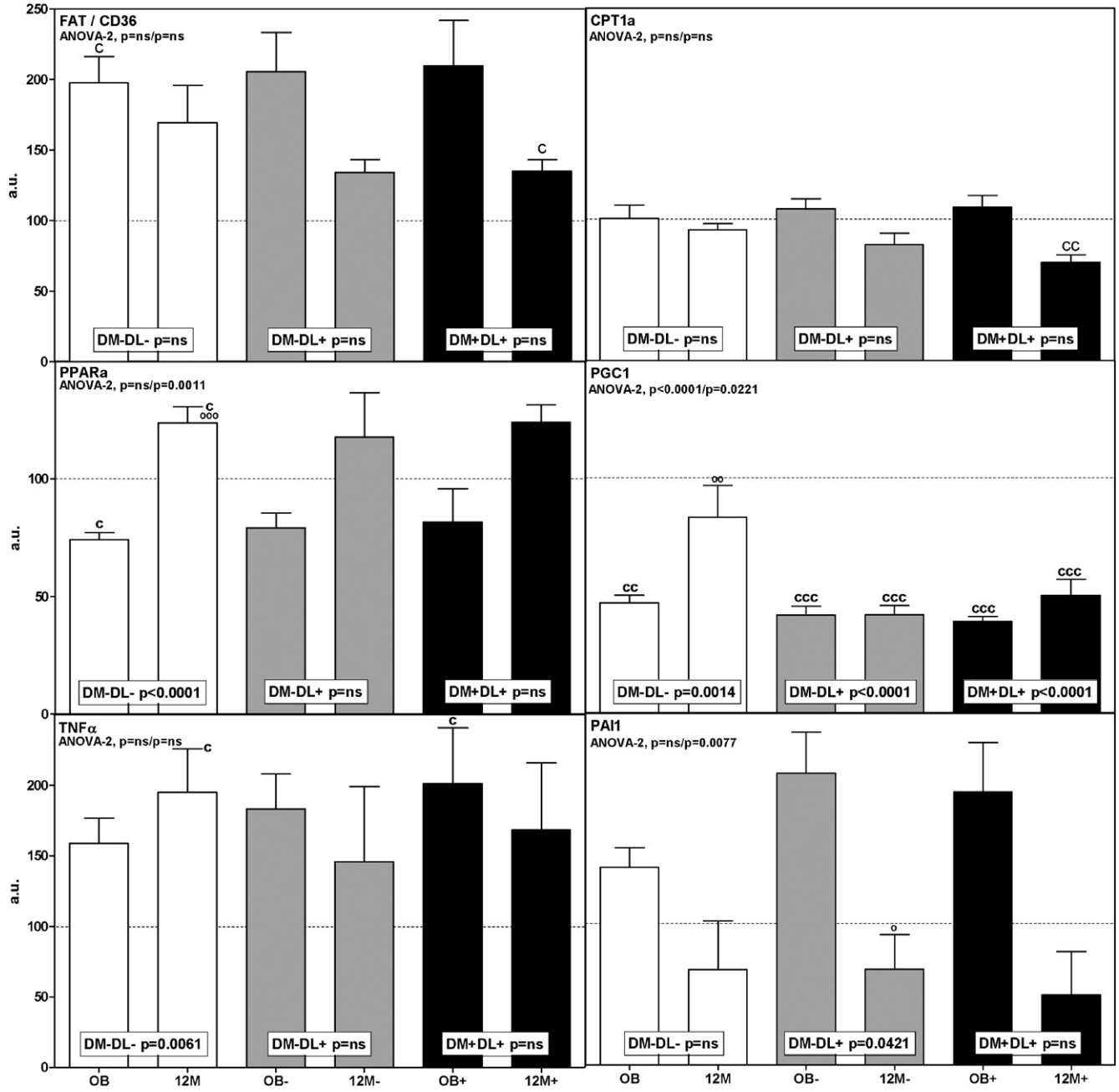

Fig. 2. *FAT/CD36, CPT11α, PPARα PPARα, TNFα* and, *PAI1* expression in the liver. Relative mRNA levels were evaluated using the ΔΔCt method. The results are expressed as the means ± SEM vs. 100% in the control group (dotted line). For gene abbreviations, see Supplemental Table 1. The DM−DL− group is presented as white bars. The DM−DL+ group is depicted as grey bars, and the DM+DL+ group is presented as black bars. Two-way ANOVA (anova-2) results are presented in left corner in each graph; the first number is the result of the comorbidities effects, and the second number represents the surgical effects. Statistical results in each group vs. control are presented as the white square in each group of bar. Abbreviations: OB and 12 M, obese, 6 and 12 months after surgery in the DM−DL−, DM−DL+ and DM+DL+ groups, respectively; a.u., arbitrary units. The (o) symbol indicates the differences between obese and 12 months after surgery in each group; the (c) symbol indicates the differences between each time vs. control (lean) group. One symbol, $p < 0.05$; two symbols, $p < 0.01$; three symbols, $p < 0.001$; ns, non-significant.

reducing hepatic TAG accumulation. However, recently, has been described that despite similar mitochondrial content, obese humans with or without NAFLD had 4.3- to 5.0-fold higher maximal respiration rates in isolated mitochondria than lean persons. NASH patients featured higher mitochondrial mass, but 31%–40% lower maximal respiration, which associated with greater hepatic insulin resistance, mitochondrial uncoupling, and leaking activity [53]. On the other hand, some authors described Increased VLDL-TG in type 2 diabetic men is caused by greater VLDL-TG secretion and less so by lower VLDL-TG clearance [54]. In addition, with liver fatty acid uptake, gene expression of hepatic lipase (*HL*) and liver lipoprotein lipase (*LPL*) are higher in obese subjects with NAFLD than subjects without NAFLD, suggesting that FFA released from lipolysis of circulating TAG also contribute to hepatocellular FFA accumulation and steatosis [13,41,49].

NEFA β-oxidation was decreased in the patients with NAFLD, possibly due to inhibition caused by the effects of increased malonyl-CoA on CPT1a [55]; however, this hypothesis was not supported by our results. Alternatively, increased UCP2 [56] has been reported to improve the elimination of excess NEFAs, and it was found to be slightly increased in the MO individuals in our study. Moreover, the direction of acetyl-CoA towards ketogenesis was highly increased in our patients, mainly after the fifth month post-surgery, when a great quantity of fat was

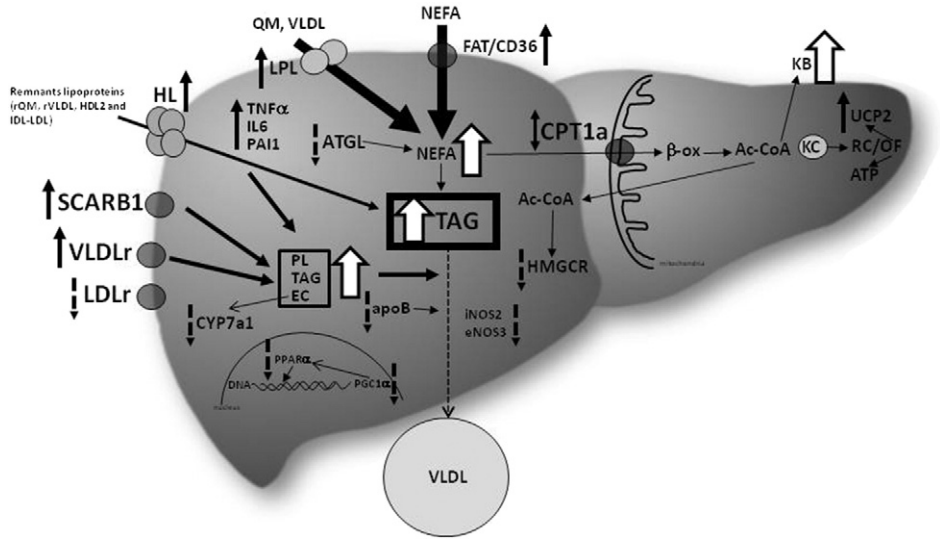

Fig. 3. Scheme of liver gene expression in the DM + DL + obese group. For gene abbreviations, see Supplemental Table 1. The upward arrow by a gene's name indicates increased expression, whereas dotted downward arrows indicate decreased expression for comparisons between the DM + DL + obese group vs. control (see Supplemental Table 1). The up and down arrows (CPT1a) indicate no difference vs. control. The wide upward arrows by the KB, NEFA, PL, TAG and EC compounds indicate that these compounds are increased in the liver (see Results section in the text). Other abbreviations: β-oxidation (β-ox); acetyl CoA (Ac-CoA); Krebs cycle (KC); respiratory chain/oxidative phosphorylation (RC/OF); adenosine tri-phosphate (ATP); chylomicron or remnants (QM or rQM); very low density lipoproteins or remnants (VLDL or rVLDL); high, intermediate and low density lipoproteins (HDL2, IDL and LDL, respectively); very low and low density lipoprotein receptor (*VLDLr* and LDLr).

mobilised. The direction of acetyl-CoA towards ketogenesis seems logical because the metabolic pathway for cholesterol synthesis or elimination via synthesis of bile salts was inhibited by decreased *HMGCR* expression, possibly due to the presence of excess cholesterol and CPY7a1 expression in the liver, as we have observed.

Nine of the 19 studied genes (*ATGL*, *LDLr*, *apoB*, *HMGCR*, *CYP7A1*, *PPARα*, *PGC1α*, *iNOS2* and *eNOS3*) exhibited decreased expression in the three types of MO individuals. This expression pattern was more pronounced in the "unhealthy" MO patients, especially in the presence of DM.

The plasma CRP and *PAI1* levels are not useful for identifying these changes considering that they were very similar in all three groups of MO patients. Moreover, the hepatic CRP level, as well as parameters commonly used to determine liver damage, such as AST, ALT, AST/ALT (a ratio of lower than 1 has been proposed by some authors [57] to be indicative of liver pathology), GGT, and alkaline phosphatase, was correlated with the severity of liver damage in the obese diabetics. Conversely, other studies have suggested that the TAG, HDL, AST and ALT levels (but not the *TNFα*, leptin or adiponectin level) are useful for differentiating between severely obese individuals without NAFLD and those with NASH [51]. Plasma *PAI1* protein and liver *PAI1* expression was found to be strongly correlated with inflammation in the 3 groups of MO patients and to decrease sharply at 12 months after surgery. Therefore, the DM + DL + patients exhibited a positive correlation ($r = 0.953$; $p = 0.012$) between *PAI1* expression and liver inflammation. Nevertheless, this correlation was not observed in the other obese groups. From our point of view, the hepatic lipase (*HL*) and liver lipoprotein lipase (*LPL*) analysis could cover a large part of the histological features that we can be observed in a liver biopsy. For example, the *LPL* correlated with liver steatosis ($r = 0.921$; $p = 0.027$) in "healthy" patients (DM − DL −) and, with liver ballonization ($r = 0.953$; $p = 0.047$) and fibrosis ($r = 0.991$; $p = 0.009$) in diabetic and dyslipidaemic group (DM + DL +). While *HL* correlated with portal inflammation ($r = 0.713$; $p = 0.021$) in dyslipidaemic group (DM − DL +).

4.4. Liver lipid levels and bariatric surgery

The metabolic effects of bariatric surgery were more pronounced in the "healthy" patients compared with the dyslipidaemic or diabetic patients. At one year after bariatric surgery, recovery of many of the previously altered biochemical parameters was observed in both the plasma and liver samples.

Consistent with previous reports [13], we observed drastic reductions in the TAG and TC levels in both the liver and plasma following bariatric surgery; however, these reductions were more marked in the present study compared with other reports [58]. These reductions in hepatocyte TAG levels were accompanied by reduced lipid levels (NEFAs and PLs). Additionally, the lipid per DNA (or per liver cell) level decreased by 81% at one year after bariatric surgery in the "healthy" obese individuals and by 50 and 60% in the DM − DL + and DM + DL + patients, respectively. After surgery, a trend of normalisation of *LDLr* expression (as has been observed by other authors [59] in monocytes collected from individuals after completing a weight reduction programme), *apoB*, *HMGCR*, *PPARα* and *PGC1α* as well as the reduced expression of *CYP7A1*, *FAT/CD36*, *IL6* and *PAI1*. However, the reductions in the TAG levels occurred in the absence of concomitant decreases in the plasma NEFA levels (without reduced liver uptake of NEFAs). In contrast, *LPL* activity and expression in the liver was significantly reduced after bariatric surgery, consistent with the decrease in hepatic TAGs. We reported good individual correlation between liver *LPL* activity and the degree of liver damage, both before and after bariatric surgery [12].

The mechanism(s) underlying the relationship between hepatic steatosis and insulin resistance remain unknown. However, other factors associated with steatosis, such as inflammation, circulating adipokines, endoplasmic reticulum stress, and unidentified lipid metabolites, can affect insulin sensitivity but are not necessarily directly correlated with intrahepatic triglyceride levels [48]. This observation could explain why the insulin resistance observed in our patients was decreased by up to 57% at only 1 month after surgery, whereas the FFA, glycerol and KB levels exhibited peak plasma concentrations at this time point. The CRP level after one month remained similar to that observed in the obese individuals, but the leptin and *PAI1* levels were decreased by 36%. These results suggest that IR is not necessarily associated with the plasma FFA concentration, as other authors have suggested [60].

In conclusion, we observed an increase in the hepatic lipid level and increased expression of genes involved in lipid accumulation in the

"healthy" MO patients and MO dyslipidaemic patients with or without diabetes; however, only a limited number of individuals were examined in this study. Increased *LPL* and *HL* activities in the livers of obese individuals, together with the increased expression of key receptors, such as *FAT/CD36*, could contribute to the accumulation of liver fat and subsequent steatosis. Thus, quantitative measurements of liver *LPL* and *HL* activities and fat content are important for evaluating the roles of liver fat in insulin resistance, obesity, and type 2 diabetes and for correctly diagnosing and reducing the severity of liver damage.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbacli.2015.12.002.

Statement of human rights and informed consent

All procedures were performed in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (59th WMA General Assembly, Seoul, October, 2008). Informed consent was obtained from all patients included in the study.

Declaration of interest

The authors have declared that no conflicts of interest exist. The authors who have taken part in this study do not have any relationships with the drug manufacturers involved either in past or present research, and they did not receive funding from the manufacturers to carry out their research.

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Authors' contributions

Study concept and design: Baena-Fustegueras, Lecube, Fort, Vargas, Peinado-Onsurbe. Acquisition of data: Pardina, Ferrer, Rossell. Analysis and interpretation of data: Pardina, Ferrer, Rossell, Baena-Fustegueras, Lecube, Fort, Vargas, Peinado-Onsurbe. Drafting of the manuscript: Balibrea, Peinado-Onsurbe. Critical revision of the manuscript for important intellectual content: Baena-Fustegueras, Balibrea, Caubet, Fort, González, Vilallonga, Vargas, Lecube, Peinado-Onsurbe. Statistical analysis: Baena-Fustegueras, Pardina, Ferrer, Rossell, Peinado-Onsurbe. Study supervision: Baena-Fustegueras, Balibrea, Caubet, Fort, González, Vilallonga, Vargas, Lecube, Peinado-Onsurbe.

Transparency Document

The Transparency document associated with this article can be found, in the online version.

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