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Occult hepatitis B virus infection predicts non-alcoholic steatohepatitis in severely obese individuals from Italy

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Abstract

Background & Aims: Obesity is associated with non-alcoholic fatty liver (NAFL), which may progress towards non-alcoholic steatohepatitis (NASH), cirrhosis and hepatocellular carcinoma (HCC). Occult hepatitis B virus infection (OBI) may contribute to hepatic damage in patients with chronic liver disease of different aetiologies (eg HCV, alcohol). However, information on the prevalence and clinical impact of OBI in obese individuals is lacking. The aims of this study were to investigate NASH prevalence and risk factors in obese people who underwent bariatric surgery.

Methods: Two-hundred and twenty-six subjects (160 females; mean age 42.9 years ±10.8 SD) without evidence of any further cause of liver disease consecutively underwent bariatric surgery in two Italian liver centers. During surgery, all patients underwent liver biopsy for histological evaluation and molecular studies. Liver DNA extracts were tested for PNPLA3, TM6SF2, MBOAT7, IRGM polymorphisms and for OBI. Univariate and multivariate analyses were used to identify predictors of NASH.

Results: Histology showed NASH in 115 (50.9%) and NAFL in 111 cases (49.1%). Twenty-nine/226 (12.8%) cases had OBI, 24 (82.8%) of whom had NASH and 5 (17.2%) NAFL, whereas among the 197 OBI-negative cases, 91 (46.2%) had NASH and 106 (53.8%) NAFL (P = .0002). Multivariate analysis showed that older age (P = .03, OR 1.034), alanine aminotransferase values (P = .005, OR 1.023), insulin resistance/diabetes (P = .02, OR 2.257), TM6SF2 polymorphism (P = .04, OR 3.168) and OBI (P = .004, OR 5.503) were independent predictors of NASH.

Conclusion: NASH is highly prevalent in obese individuals undergoing bariatric surgery. OBI is one of the strongest risk factors of NASH in these patients.

Abbreviations: ALT, alanine aminotransferase; Anti-HBc, antibody to HBV core antigen; ASH, alcoholic steatohepatitis; AST, aspartate aminotransferase; BMI, body mass index; CI, confidence interval; HBsAg, hepatitis B virus surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HOMA-IR, Homeostasis Model Assessment of Insulin Resistance; IRGM, immunity-related GTPase family M; MBOAT7, membrane bound O-acyltransferase domain-containing 7; NAFL, non-alcoholic fatty liver; NASH, non-alcoholic steatohepatitis; OBI, occult hepatitis B virus infection; OR, odds ratio; PLT, platelet; PNPLA3, patatin-like phospholipase domain-containing protein 3; SD, standard deviations; SNPs, single-nucleotide polymorphisms; TM6SF2, transmembrane 6 superfamily member 2; γGT, gamma-glutamiltranspeptidase.

KEYWORDS
diabetes, genetic polymorphisms, non-alcoholic fatty liver, obesity, occult HBV infection

1 | INTRODUCTION

Obesity is one of the most important risk factors for the development of cirrhosis and hepatocellular carcinoma (HCC) worldwide.[1-9] The vast majority of obese individuals show non-alcoholic fatty liver (NAFL).[7] Occurrence of non-alcoholic steatohepatitis (NASH) in NAFL patients is an important driver of hepatic damage towards the most severe clinical outcomes. However, the prevalence of NASH and, consequently, the factors that might predispose or favour its development in obese persons have not been sufficiently investigated so far. This knowledge gap is essentially due to the fact that the diagnosis of NASH is still essentially based on liver histological examination, whereas liver biopsy in obese subjects is an uncommonly performed procedure. All features of the metabolic syndrome (including insulin resistance and diabetes, hypertriglyceridaemia and hypercholesterolaemia and arterial hypertension) as well as particular genetic polymorphisms are recognized as potential risk factors of NASH occurrence in the general population.[10,11] It is likely that these mechanisms also play a role in the development of NASH associated with obesity, although it appears clear that other, still unrecognized factors may be involved. There is evidence that the occult hepatitis B virus (HBV) infection (OBI)—as it is defined the long-lasting intrahepatic persistence of HBV genomes in HBV surface antigen (HBsAg) negative individuals[12]—may contribute to the worsening of liver disease in individuals with alcoholic steatohepatitis (ASH) and cryptogenic liver disease as well as in subjects with hepatitis C virus (HCV) infection.[13-17] Actually, OBI has never been investigated as a possible risk factor of NASH in obese people.

The aims of this study were to evaluate the prevalence of—and the risk factors associated with—NASH in a large cohort of obese people from two distinct Italian geographical areas who underwent bariatric surgery.

Key points

- Prevalence and risk factors of non-alcoholic steatohepatitis (NASH) in obese subjects have not yet been sufficiently investigated, also because the histological evaluation of liver specimens remains uncommon in obese patients who usually do not undergo percutaneous liver biopsy.
- This study shows that the majority of obese patients undergoing bariatric surgery have NASH.
- Occult hepatitis B virus infection is a strong predictor of NASH in morbidly obese subjects.

2 | PATIENTS AND METHODS

2.1 | Patients

This prospective study involved two liver centres located, respectively, in the North-East (Trieste) and in the South (Messina) of Italy. Overall, 226 subjects [160 females, 66 males; mean age 42.9, ±10.8 standard deviations (SD)] with severe obesity who consecutively underwent bariatric surgery according to international criteria [ie body mass index (BMI) >40 or >35 when a major comorbidity (ie diabetes, obstructive sleep apnoea syndrome) co-existed] were included in the study (Table 1).

The only exclusion criteria were HBsAg and/or anti-HCV positivity and an intake of more than 30 or 20 gr/die of alcohol in male or female patients respectively.[18] In particular, 105 North-East Italian obese subjects consecutively underwent bariatric surgery at the Trieste University Hospital and 121 obese Sicilians underwent bariatric surgery at the University Hospital of Messina from January 2015 to December 2017 (Table 1). Antibody to HBV core antigen (anti-HBc) had been tested in 194 cases, 22 (11.3%) of whom were positive. Values of aspartate aminotransferase (AST), alanine aminotransferase (ALT), bilirubin, gamma-glutamiltranspeptidase (γGT), platelet (PLT) count as well as the presence of insulin resistance/diabetes and of hypercholesterolaemia and/or hypertriglyceridaemia (from now 'dyslipidaemia') before surgery were recorded for each patient.

Insulin resistance was defined by the Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) and calculated from fasting levels of glucose and insulin.[19] An HOMA-IR ≥2.5 identified insulin resistance.[20,21] Dyslipidaemia was identified according to the metabolic syndrome definition cut-offs of high-density lipoprotein cholesterol and triglycerides.[22,23]

All cases with frank diabetes and the majority of those with insulin resistance as well as patients with dyslipidaemia were under specific treatment.

One-hundred and eighty-three patients (81%) underwent laparoscopic sleeve gastrectomy, whereas 43 patients (19%) underwent laparoscopic Roux-en-Y gastric bypass. During surgery, a liver biopsy was performed in each patient and the specimen was divided to be in part sent to the pathology department for histology and in part immediately frozen and stored in liquid nitrogen until the time of the molecular analyses.

Histological evaluation was based on the Kleiner classification.[24] In accordance with international guidelines, the diagnosis of NASH was performed when steatosis, lobular inflammation and ballooning were contemporarily present, whereas NAFL was diagnosed when steatosis was present alone or together with either lobular inflammation or ballooning.[18,25] Moreover, fibrosis was staged from 0 (absent)

TABLE 1 Characteristics of 226 morbidly obese patients according to geographical origin

	All patients (n 226)	Patients from Messina (n 121)	Patients from Trieste (n 105)	P[a]
Mean age (±SD) (y)	42.9 (±10.8)	42 (±11.2)	44 (±10.3)	n.s. (.2)
Sex F/M	160/66	86/35	74/31	n.s. (.9)
AST mean (±SD) (U/L)	23.6 (±12.8)	22.8 (±12.6)	24.6 (±13)	n.s. (.3)
ALT mean (±SD) (U/L)	30.2 (±23)	30.3 (±23.1)	30.1 (±23)	n.s. (.9)
γGT mean (±SD) (U/L)	33.4 (±38.3)	29.4 (±33.5)	38.1 (±42.9)	n.s. (.09)
Bilirubin mean (±SD) (mg/dL)	0.5 (±0.3)	0.47 (±0.27)	0.53 (±0.25)	n.s. (.09)
PLT^3/mmc mean (±SD)	270.5 (±69.9)	276.8 (±71.3)	260.3 (±54.7)	n.s. (.06)
BMI mean (±SD)	44.3 (±6)	43.6 (±5.9)	45.1 (±6.1)	n.s. (.06)
Insulin resistance/ diabetes n (%)	153 (67.7%)	76 (62.8)	77 (73.3)	n.s. (.09)
Dyslipidaemia n (%)	93 (41.2%)	31 (25.6)	62 (59)	**<.0001**
NAFL/NASH	111/115	61/60	50/55	n.s. (.7)
No. of cases with fibrosis (%)	96 (42.5)	45 (37.2)	51 (48.6)	n.s. (.08)
No. of PNPLA3 rs738409 CC/CG/ GG	110/95/21	54/55/12	56/40/9	n.s. (.4)
No. of TM6SF2 rs58542926 CC/ CT/TT	204/21/1	114/7/0	90/14/1	n.s. (.08)
No. of MBOAT7 rs641738 CC/ CT/TT	67/113/46	36/59/26	31/54/20	n.s. (.9)
No. of IRGM rs10065172 CC/ CT/TT	169/53/4	86/33/2	83/20/2	n.s. (.3)
OBI positive n (%)	29 (12.8)	15 (12.4)	14 (13.3)	n.s. (.8)

[a]Patients from Messina vs patients from Trieste.

Statistically significant values are indicated in bold.

to 4 (cirrhosis), defining patients with clinically significant fibrosis those with a fibrosis stage from 2 to 4. Histological evaluation was performed at each centre by one expert pathologist. In 40 randomly selected cases (20 from each centre), the inter-observer agreement between pathologists was tested for fibrosis, steatosis, lobular inflammation and ballooning by using weighted kappa scores. The study was approved by the Ethics Committees of the Messina and Trieste University Hospitals, and conducted in accordance with the ethical principles of the Declaration of Helsinki. Written informed consent was obtained from all patients before performing any study procedure.

2.2 | HBV DNA analyses

DNA was extracted from each frozen liver specimen according to standardized methods. Part of each DNA sample was tested for OBI using procedures previously optimized and well-established in the Messina liver unit laboratory.[13,26,27] In brief, DNA was extracted from each specimen using Master-PureTM DNA Purification Kit (epicentre, Madison, WI, USA) and examined for the presence of HBV genomes by performing four different in-house nested-PCR amplification assays to detect preS-S, preCore-Core, Polymerase and X HBV genomic regions respectively. Appropriate negative and positive controls were included in each PCR experiment. In particular, the negative controls included in each test were (a) liver tissue DNA extracts from samples known to be negative for HBV DNA; (b) DNA-free reaction buffer; and (c) water. In addition, to eliminate false-negative results, beta-globin was used as a housekeeping gene. Moreover, direct sequencing of all amplicons confirmed the specificity of the reactions. Cases in which at least two different viral genomic regions were detected at nested-PCR analysis were considered OBI-positive.

2.3 | Genetic polymorphisms

Liver DNA extracts from each liver specimen were further examined to test the following four different single-nucleotide polymorphisms (SNPs): (a) the patatin-like phospholipase domain-containing protein 3 (PNPLA3) rs738409 C>G, (b) the transmembrane 6 superfamily member 2 (TM6SF2) rs58542926 C>T, (c) the membrane bound O-acyltransferase domain-containing 7 (MBOAT7) rs641738 C>T and (d) the immunity-related GTPase family M (IRGM) rs10065172 C>T. The choice to test these particular genetic variants was based on the well-established association with NAFL of PNPLA3, TM6SF2 and MBOAT7 SNPs,[28-30] and on recent reports showing an increased risk of developing steatosis associated with IRGM SNP in young obese individuals, in particular Italians.[31,32]

PNPLA3 rs738409, TM6SF2 rs58542926, MBOAT7 rs641738 and IRGM rs10065172 genotypes were determined by allelic discrimination using TaqMan reagents (Applied Biosystems) according to the manufacturer's protocols. Control samples of known genotypes were also included for each experiment in every 96-well plate (blank, homozygous wild-type, homozygous mutant and heterozygous). An additive genetic model to test the effects of the genetic variants was applied.

2.4 | Sample size calculation

With the analysis of NASH incidence in the population with severe obesity as a primary end point of the study, assuming—for sample size calculation—a population incidence of 35%[33] and an expected incidence in the study of about 45% (percentage derived by analysing the first 50 subjects included in the study), a statistical power of 80% and a two-sided significance level of 0.05, the minimum number of subjects for adequate study power is equal to 183. Thus, the overall sample size of 226 subjects achieved during the established enrolment time exceeded the study power.

2.5 | Statistical analysis

Continuous variables were expressed as mean ± SD or median and ranges as appropriate, and were compared by using the non-parametric Mann-Whitney test. Categorical variables were expressed in absolute values and percentage and were compared with the Chi Square test. The non-parametric approach was used when the continuous variables were not normally distributed, as verified by Kolmogorov-Smirnov test. Univariate logistic regression model was estimated in order to identify possible predictors of NASH and fibrosis. Stepwise multivariate logistic regression model was estimated to identify independent predictive factors of NASH and fibrosis.

Finally, the odds ratio (OR) and its 95% confidence interval (CI) of each NASH risk factor found to be significant at multivariate analysis (and evaluated both singly and combined each other) were calculated. Furthermore, the impact of OBI on each histological feature of liver damage (steatosis, lobular inflammation, ballooning and fibrosis) was assessed by ordinal regression analysis. Statistical analyses were performed using SPSS 22.0 for Window package. A *P*-value smaller than .05 was considered to be statistically significant.

3 | RESULTS

All patients included in the study had liver steatosis, and in particular 111/226 (49.1%) of them had NAFL and 115/226 (50.9%) had NASH (Table 2). Fibrosis was present in 96/226 (42.5%) cases. In particular, fibrosis was found in 2/111 (1.8%) NAFL patients (mild in 1 of the patients, severe in the other), and in 94/115 (81.7%) patients with NASH ($P < .0001$) (mild in 77, moderate in 11, severe in 4, whereas histology revealed the presence of cirrhosis in the remaining two

TABLE 2 Characteristics of 226 morbidly obese patients according to the presence of NAFL or NASH

	NAFL (111 patients)	NASH (115 patients)	*P*
Mean age (±SD) (years)	41.1 (±11.2)	44.7 (±10.1)	**.01**
Sex F/M	81/30	79/36	n.s. (.5)
AST mean (±SD) (U/L)	20.9 (±8.7)	26.3 (±15.3)	**.001**
ALT mean (±SD) (U/L)	24.9 (±16.5)	35.4 (±26.9)	**.0005**
γGT mean (±SD) (U/L)	25.5 (±16.9)	41.1 (±50.1)	**.002**
Bilirubin mean (±SD) (mg/dL)	0.5 (±0.2)	0.52 (±0.3)	n.s. (.6)
PLT^3/mmc mean (±SD)	274 (±70.1)	267.2 (±69.9)	n.s. (.5)
BMI mean (±SD)	43.2 (±5.6)	45.3 (±6.3)	**.009**
Insulin resistance/ diabetes n (%)	61 (55)	92 (80)	**<.0001**
Dyslipidaemia n (%)	42 (37.8)	51 (44.3)	n.s. (.3)
No. of cases with fibrosis (%)	2 (1.8)	94 (81.7)	**<.0001**
No. of PNPLA3 rs738409 CC/CG/ GG	56/46/9	55/48/12	n.s. (.8)
No. of TM6SF2 rs58542926 CC/ CT/TT	106/5/0	98/16/1	**.03**
No. of MBOAT7 rs641738 CC/CT/ TT	32/57/22	35/56/24	n.s. (.9)
No. of IRGM rs10065172 CC/ CT/TT	79/30/2	90/23/2	n.s. (.5)
OBI positive n (%)	5 (4.5%)	24 (20.9%)	**.0002**

Statistically significant values are indicated in bold.

patients), with an overall prevalence of clinically significant fibrosis of 8% (18/226 patients). The k inter-observer agreements for fibrosis, steatosis grade, lobular inflammation and ballooning in the 40 cases whose histological features have been revised by pathologists from both the centres were 0.88, 0.77, 0.62 and 0.57, respectively, similar to those reported in the literature.[24]

OBI was diagnosed in 29/226 cases (12.8%), 17 of whom were anti-HBc positive, 10 anti-HBc negative and two were not tested for this antibody.

The North-East and South of Italy subpopulations were comparable in all parameters evaluated with the exception of dyslipidaemia that was significantly more prevalent in subjects in the Trieste series (Table 1). In analogy, also NASH was equally distributed between the two subpopulations. Univariate analysis showed that patients with NASH were older (P = .01), had higher AST, ALT, γGT values (P = .002, P = .001 and P = .003 respectively), higher BMI (P = .01), a higher prevalence of insulin resistance/diabetes (P = .0001), and had a significantly higher prevalence of TM6SF2 genetic polymorphism (P = .01) compared to patients without NASH. Notably, a highly significant association was found between OBI and NASH (P = .001). All the other investigated parameters (including PNPLA3, MBOAT7 and IRGM SNPs) showed no difference between patients with or without NASH. Multivariate analysis evaluation showed that older age, ALT values, insulin resistance/diabetes, TM6SF2 polymorphism and OBI persisted as independent predictors of NASH, with OBI showing the highest OR (5.5, 95% CI 1.710-17.706) (Table 3). When the variables significant at multivariate analysis were combined, the contemporary presence of OBI and insulin-resistance/diabetes showed the highest OR for NASH (8.043, 95% CI 2.325-27.817, P = .001). Ordinal regression analysis showed that OBI had a significant impact on the severity of lobular inflammation (P = .001), ballooning (P = .0001) and fibrosis (P = .001), but not on steatosis (P = .5) (Figure 1). Furthermore, we found that the contemporary presence of portal inflammation and periportal fibrosis—a pattern frequently observed in HBsAg-positive patients as well as in the so-called 'type 2 NASH'—was more frequent in OBI-positive patients (9/29, 31%) than in OBI-negative individuals (29/197, 14.7%) (P = .03). Univariate analysis showed that patients with liver fibrosis were older (P = .02), had lower PLT count (P = .04), had higher AST, ALT, γGT values (P = .003, P = .009 and P = .001 respectively), had higher BMI (P = .002) as well as higher prevalence of insulin resistance/diabetes, of TM6SF2 genetic polymorphism and of OBI (P = .0001, P = .03 and P = .001 respectively). At multivariate analysis, γGT and BMI values, presence of insulin resistance/diabetes and OBI positivity continued to be independent predictors of fibrosis (P = .03, P = .02, P = .003 and P = .002 respectively), with OBI again showing the highest OR (5.7, 95% CI 1.860 - 17.219) (Table 4).

Uni- and multivariate analysis were performed to identify potential independent predictors of clinically significant fibrosis. Univariate analysis showed that patients with clinically significant liver fibrosis were more frequently males (P < .05), had higher AST, ALT and γGT values (P = .001, P = .03 and P < .05 respectively), had higher BMI (P = .004) and had lower PLT count (P = .0001). Multivariate analysis showed that higher γGT and BMI values, and lower PLT count were independent predictors of clinically significant fibrosis (P = .03, P = .002 and P = .0001 respectively) (Table 5).

4 | DISCUSSION

Fifty-one percent of the obese patients had NASH, and 42.5% had fibrosis. Importantly, fibrosis was almost invariably associated with NASH. Therefore, the histological evaluation showed that a

TABLE 3 Univariate and multivariate analysis of the predictors of NASH evaluated in 226 morbidly obese patients

	Univariate analysis			Multivariate analysis		
	OR	**95% CI**	***P***	**OR**	**95% CI**	***P***
Age	1.032	1.006-1.058	**.01**	1.034	1.003-1.067	**.033**
Sex	1.230	0.692-2.187	.5	//	//	//
AST	1.040	1.014-1.066	**.002**	0.995	0.949-1.043	.8
ALT	1.025	1.009-1.040	**.001**	1.023	1.007-1.040	**.005**
γGT	1.020	1.007-1.034	**.003**	1.009	0.993-1.025	.3
Bilirubin	1.480	0.555-3.946	.4	//	//	//
PLT	0.999	0.995-1.002	.5	//	//	//
BMI	1.062	1.014-1.113	**.01**	1.037	0.983-1.094	.2
IR/Diabetes	3.279	1.817-5.917	**.0001**	2.257	1.144-4.452	**.02**
Dyslipidaemia	1.309	0.769-2.227	.3	//	//	//
PNPLA3	1.147	0.768-1.714	.5	//	//	//
TM6SF2	3.610	1.310-9.951	**.01**	3.168	1.066-9.412	**.04**
MBOAT7	0.989	0.682-1.434	.9	//	//	//
IRGM	0.734	0.425-1.267	.3	//	//	//
OBI	5.591	2.050-15.250	**.001**	5.503	1.710-17.706	**.004**

Statistically significant values are indicated in bold.

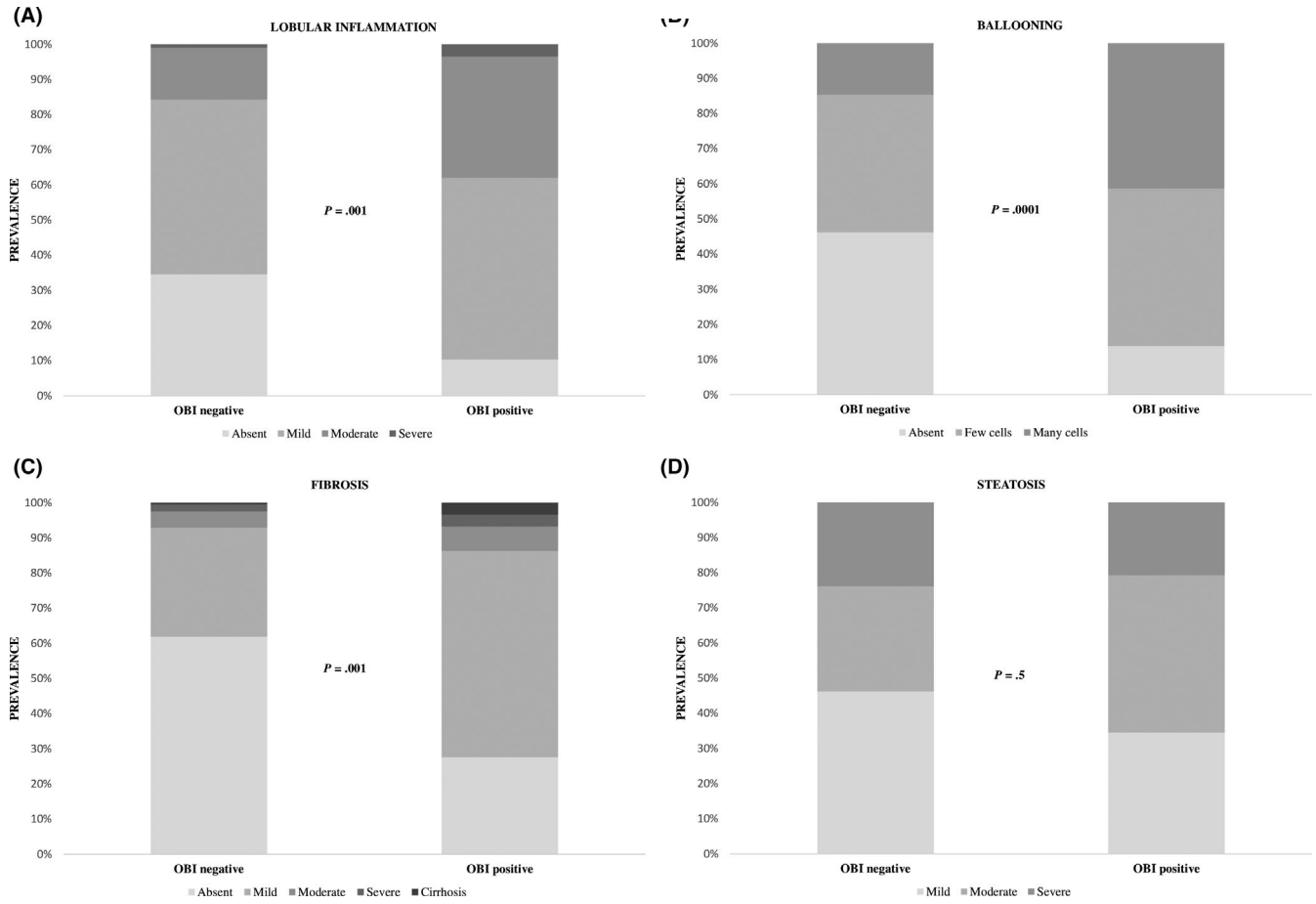

FIGURE 1 A-D, Severity of individual histological features of liver damage according to the presence of OBI

TABLE 4 Univariate and multivariate analysis of the predictors of liver fibrosis evaluated in 226 morbidly obese patients

	Univariate analysis			Multivariate analysis		
	OR	95% CI	*P*	OR	95% CI	*P*
Age	3.048	1.005-1.057	**.02**	1.018	0.983-1.055	.3
Sex	1.412	0.793-2.514	.2	//	//	//
AST	1.036	1.012-1.061	**.003**	0.997	0.952-1.045	.9
ALT	1.017	1.004-1.030	**.009**	1.005	0.980-1.031	.7
γGT	1.022	1.009-1.035	**.001**	1.015	1.001-1.028	**.03**
Bilirubin	2.144	0.788-5.832	.1	//	//	//
PLT	0.996	0.992-1.000	**.04**	0.998	0.992-1.003	.5
BMI	1.080	1.030-1.132	**.002**	1.065	1.009-1.125	**.02**
IR/Diabetes	4.350	2.270-8.337	**.0001**	3.058	1.470-6.365	**.003**
Dyslipidaemia	1.298	0.760-2.217	.3	//	//	//
PNPLA3	1.280	0.854-1.919	.2	//	//	//
TM6SF2	2.611	1.083-6.296	**.03**	2.546	0.905-7.163	.07
MBOAT7	0.829	0.568-1.210	.3	//	//	//
IRGM	0.618	0.347-1.100	.1	//	//	//
OBI	4.270	1.800-10.127	**.001**	5.660	1.860-17.219	**.002**

Statistically significant values are indicated in bold.

considerable portion of our morbidly obese patients had a pathological condition potentially predisposing to a progression towards the most severe forms of liver disease. These results strongly confirm that liver histology should be routinely investigated in subjects undergoing bariatric surgery, also considering that the intraoperative liver biopsy at the time of surgical operation is an easy and quick

TABLE 5 Univariate and multivariate analysis of the predictors of clinically significant liver fibrosis evaluated in 226 morbidly obese patients

	Univariate analysis			Multivariate analysis		
	OR	**95% CI**	***P***	**OR**	**95% CI**	***P***
Age	0.975	0.981-1.079	.2	//	//	//
Sex	2.649	1.001-7.008	**<.05**	0.801	0.202-3.180	.8
AST	1.047	1.019-1.076	**.001**	1.025	0.982-1.071	.3
ALT	1.018	1.002-1.033	**.03**	0.988	0.949-1.027	.5
γGT	1.008	1.002-1.016	**<.05**	1.011	1.001-1.021	**.03**
Bilirubin	2.916	0.624-13.634	.2	//	//	//
PLT	0.975	0.963-0.986	**.0001**	0.973	0.960-0.986	**.0001**
BMI	1.111	1.035-1.194	**.004**	1.147	1.051-1.252	**.002**
IR/Diabetes	1.737	0.551-5.476	.3	//	//	//
Dyslipidaemia	1.158	0.439-3.054	.8	//	//	//
PNPLA3	1.722	0.855-3.466	.1	//	//	//
TM6SF2	1.757	0.510-6.056	.4	//	//	//
MBOAT7	0.747	0.371-1.508	.4	//	//	//
IRGM	0.662	0.471-1.125	.9	//	//	//
OBI	2.091	0.638-6.855	.2	//	//	//

Statistically significant values are indicated in bold.

procedure, with minimal (if existing) patient risk.[34] The study population was composed of individuals coming from two quite distant geographical areas of our country, also showing some differences in lifestyles including dietary habits (ie higher alcohol consumption and diet richer in fat in North-Eastern Italians). However, apart from dyslipidaemia that was significantly more frequent in subjects from Trieste, all the other characteristics were fully comparable between the two subgroups of patients. Indeed, also NASH prevalence and the various predictors investigated were similarly present in Northern and Southern Italians, showing that our cohort was a quite homogeneous representation of Italian obese subjects.

As expected, NASH was significantly associated with older age and higher BMI as well as with impaired glucose metabolism, but not with dyslipidaemia (neither when hypercholesterolaemia or hypertriglyceridaemia were separately evaluated, data not shown). Furthermore, NASH was associated with higher ALT values at statistical analysis. Indeed, the majority of cases had ALT levels at the upper limit of the normal range or at borderline values, rendering valueless in clinical practice the evaluation of aminotransferase levels as a parameter to identify obese subjects with NASH. These results are quite similar to those reported in large French cohorts,[33,35] where—however—dyslipidaemia was more frequently found in patients with NASH than in those without, possibly reflecting a slightly different distribution of this metabolic risk factor in the Italian and French obese populations.

Steatosis was found in all patients of this series, whereas it was not revealed in about 10%-15% of obese patients in previous studies. This difference is likely a casual consequence of the relatively limited number of patients included in our study.

Based on genome-wide association studies, a number of genetic polymorphisms have recently been recognized as possible contributors to NASH development in the general population. We investigated the PNPLA3 I148M and the TM6SF2 E167K genetic variants, the two major inherited determinants of liver steatosis and progressive NASH identified so far in the general population.[36] In addition, we tested the MBOAT7 rs641738 polymorphism that also seems to increase the severity of the liver disease,[30,36] and the IRGM rs10065172 polymorphism recently found to be significantly associated with obesity and NAFL in children and adolescents.[31,32] Surprisingly, no association between NASH and PNPLA3 emerged from our analysis. Indeed, such polymorphism appears to be associated with the more advanced forms of NASH and liver disease,[28,36] thus one might hypothesize that the lack of association in our study could be dependent on the mild-moderate grade of liver disease in the patients at the time of surgery. In analogy, no association between NASH and MBOAT7 and IRGM genetic polymorphisms was found in our population. On the contrary, TM6SF2 E167K genetic variant was significantly associated with NASH at multivariate analysis, and it showed a trend (although without reaching a statistical significance) of association with fibrosis, thus suggesting that this SNP—which was reported to be associated with progressive NASH—might represent a valid biomarker of NASH in obese people. Indeed, PNPLA3, TM6SF2 and MBOAT7 polymorphisms have previously been investigated in large cohorts of severely obese Italian subjects,[37,38] being consistently associated with a more advanced liver disease, and particularly with significant hepatic fibrosis. Indeed, these differences might be due to the limited number of patients with significant fibrosis in this study.

The main finding of the study emerged by testing patient liver DNA extracts for OBI. The general prevalence (12.8%) was similar to that (16%) we had previously reported analysing a large cohort of Italians with normal liver histology who had undergone abdominal surgery for various non-liver related causes in the early 2000s.[39] However, statistical analysis clearly revealed that OBI is a very

strong predictor of NASH in obese individuals, with an OR > 5 and >8 when OBI was coupled with diabetes. One might summarize that the prevalence of OBI in obese people is similar to that found in the general population of individuals without liver disease, but when an obese individual has OBI there is a high risk of developing NASH. This study also showed that—apart from NASH—OBI was significantly associated with liver fibrosis. This association was not found when the analysis was focused on cases with advanced fibrosis that, however, were too few to allow reliable conclusions. Larger studies are needed to verify the possible impact of OBI on clinically significant fibrosis. Indeed, the association of OBI with the severity of the histological features of liver damage (and in particular with lobular inflammation, ballooning and fibrosis) strengthens the hypothesis that it may act as an important co-factor of hepatic disease worsening in morbidly obese subjects. Furthermore, the finding of OBI association with the so-called 'type 2 NASH' histological pattern, which has been associated with faster disease progression in NAFL patients,[40-42] is another indirect confirmation that OBI my act as a disease-worsening factor in these patients. The possible role of OBI as cofactor in facilitating or accelerating the processes of liver inflammation and worsening of the disease in case of concomitant major cause of liver damage (ie HCV, alcohol, etc) is a still highly debated and unresolved argument.[12] This study adds an important piece to the puzzle, identifying OBI as a potential risk factor for liver disease progression also in patients with hepatic steatosis. It is worth considering, however, that NASH is a frequent prerequisite of development of cirrhosis and HCC in NAFL patients including obese people, and a large body of evidence confirms that OBI may maintain all the pro-oncogenic properties of the HBsAg-positive HBV infection.[12,27] Therefore, the strong association we found between NASH and OBI in obese individuals may represent a warning, and this association encourages the design of further studies that may overtime provide an evaluation of the outcome of cases with the coexistence of OBI and NASH.

CONFLICT OF INTEREST

The authors declare no conflicts of interest that pertain to this work.

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REFERENCES

1. Dixon JB, Bhathal PS, O'Brien PE. Nonalcoholic fatty liver disease: predictors of nonalcoholic steatohepatitis and liver fibrosis in the severely obese. *Gastroenterology*. 2001;121:91-100.
2. Larsson SC, Wolk A. Overweight, obesity and risk of liver cancer: a meta-analysis of cohort studies. *Br J Cancer*. 2007;97:1005-1008.
3. Renehan AG, Tyson M, Egger M, Heller RF, Zwhalen M. Body-mass index and incidence of cancer: a systematic review and meta-analysis of prospective observational studies. *Lancet*. 2008;371:569-578.
4. Saunders D, Seidel D, Allison M, Lyratzopoulos M. Systematic review: the association between obesity and hepatocellular carcinoma - epidemiological evidence. *Aliment Pharmacol Ther*. 2010;31:1051-1063.
5. Welzel TM, Graubard BI, Zeuzem S, El-Serag HB, Davila JA, McGlynn KA. Metabolic syndrome increases the risk of primary liver cancer in the United States: a study in the SEER-Medicare database. *Hepatology*. 2011;54:463-471.
6. Sun H, Yang W, Tian Y, et al. An inflammatory-CCRK circuitry drives mTORC1-dependent metabolic and immunosuppressive reprogramming in obesity-associated hepatocellular carcinoma. *Nat Commun*. 2018;9:5214.
7. Brar G, Tsukamoto H. Alcoholic and non-alcoholic steatohepatitis: global perspective and emerging science. *J Gastroenterol*. 2019;54:218-225.
8. Sepulveda-Villegas M, Roman S, Rivera-Iñiguez I, et al. High prevalence of nonalcoholic steatohepatitis and abnormal liver stiffness in a young and obese Mexican population. *PLoS ONE*. 2019;14:e0208926.
9. Anstee QM, Reeves HL, Kotsiliti E, Govaere O, Heikenwalder M. From NASH to HCC: current concepts and future challenges. *Nat Rev Gastroenterol Hepatol*. 2019;16:411-428.
10. Younossi ZM. Non-alcoholic fatty liver disease - a global public health perspective. *J Hepatol*. 2019;70:531-544.
11. Sanyal AJ. Past, present and future perspectives in nonalcoholic fatty liver disease. *Nat Rev Gastroenterol Hepatol*. 2019;16:377-386.
12. Raimondo G, Locarnini S, Pollicino T, et al. Update of the statements on biology and clinical impact of occult hepatitis B virus infection. *J Hepatol*. 2019;71:397-408.
13. Pollicino T, Squadrito G, Cerenzia G, et al. Hepatitis B virus maintains its pro-oncogenic properties in the case of occult HBV infection. *Gastroenterology*. 2004;126:102-110.
14. Donato F, Gelatti U, Limina RM, Fattovich G. Southern Europe as an example of interaction between various environmental factors: a systematic review of the epidemiologic evidence. *Oncogene*. 2006;25:3756-3770.
15. Shi Y, Wu YH, Wu W, Zhang WJ, Yang J, Chen Z. Association between occult hepatitis B infection and the risk of hepatocellular carcinoma: a meta-analysis. *Liv Int*. 2012;32:231-240.
16. Squadrito G, Cacciola I, Alibrandi A, Pollicino T, Raimondo G. Impact of occult hepatitis B virus infection on the outcome of chronic hepatitis C. *J Hepatol*. 2013;59:696-700.
17. Covolo L, Pollicino T, Raimondo G, Donato F. Occult hepatitis B virus and the risk for chronic liver disease: a meta-analysis. *Dig Liver Dis*. 2013;45:238-244.
18. European Association for the Study of the Liver (EASL); European Association for the Study of Diabetes (EASD); European Association for the Study of Obesity (EASO). EASL-EASD-EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease. *J Hepatol*. 2016;64:1388-1402.
19. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia*. 1985;28:412-419.
20. Ascaso JF, Pardo S, Real JT, Lorente RI, Priego A, Carmena R. Diagnosing insulin resistance by simple quantitative methods in subjects with normal glucose metabolism. *Diabetes Care*. 2003;26:3320-3325.
21. Vardeny O, Gupta DK, Claggett B, et al. Insulin resistance and incident heart failure the ARIC study (Atherosclerosis Risk in Communities). *JACC Heart Fail*. 2013;1:531-536.
22. Alberti KG, Zimmet P, Shaw J; IDF Epidemiology Task Force Consensus Group. The metabolic syndrome – a new worldwide definition. *Lancet*. 2005;366:1059-1062.

23. Alberti KG, Zimmet P, Shaw J. Metabolic syndrome – a new world-wide definition. A Consensus Statement from the International Diabetes Federation. *Diabet Med*. 2006;23:469-480.
24. Kleiner DE, Brunt EM, Van Natta M, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology*. 2005;41:1313-1321.
25. Chalasani N, Younossi Z, Lavine JE, et al. The diagnosis and management of nonalcoholic fatty liver disease: practice guidance from the American Association for the Study of Liver Diseases. *Hepatology*. 2018;67:328-357.
26. Cacciola I, Pollicino T, Squadrito G, Cerenzia G, Orlando ME, Raimondo G. Occult hepatitis B virus infection in patients with chronic hepatitis C liver disease. *N Engl J Med*. 1999;341:22-26.
27. Saitta C, Tripodi G, Barbera A, et al. Hepatitis B virus (HBV) DNA integration in patients with occult HBV infection and hepatocellular carcinoma. *Liver Int*. 2015;35:2311-2317.
28. Sookoian S, Pirola CJ. Meta-analysis of the influence of I148M variant of patatin-like phospholipase domain containing 3 gene (PNPLA3) on the susceptibility and histological severity of nonalcoholic fatty liver disease. *Hepatology*. 2011;53:1883-1894.
29. Dongiovanni P, Petta S, Maglio C, et al. Transmembrane 6 superfamily member 2 gene variant disentangles nonalcoholic steatohepatitis from cardiovascular disease. *Hepatology*. 2015;61:506-514.
30. Mancina RM, Dongiovanni P, Petta S, et al. The MBOAT7-TMC4 variant rs641738 increases risk of nonalcoholic fatty liver disease in individuals of European descent. *Gastroenterology*. 2016;150:1219-1230.
31. Lin YC, Chang PF, Lin HF, Liu K, Chang MH, Ni YH. Variants in the autophagy-related gene IRGM confer susceptibility to non-alcoholic fatty liver disease by modulating lipophagy. *J Hepatol*. 2016;65:1209-1216.
32. Bellini G, Miraglia Del Giudice E, Nobili V, Rossi F. The IRGM rs10065172 variant increases the risk for steatosis but not for liver damage progression in Italian obese children. *J Hepatol*. 2017;67:653-655.
33. Bedossa P, Poitou C, Veyrie N, et al. Histopathological algorithm and scoring system for evaluation of liver lesions in morbidly obese patients. *Hepatology*. 2012;56:1751-1759.
34. Denzer U, Arnoldy A, Kanzler S, Galle PR, Dienes HP, Lohse AW. Prospective randomized comparison of minilaparoscopy and percutaneous liver biopsy: diagnosis of cirrhosis and complications. *J Clin Gastroenterol*. 2007;41:103-110.
35. Bedossa P, Tordjman J, Aron-Wisnewsky J, et al. Systematic review of bariatric surgery liver biopsies clarifies the natural history of liver disease in patients with severe obesity. *Gut*. 2017;66:1688-1696.
36. Eslam M, Valenti L, Romeo S. Genetics and epigenetics of NAFLD and NASH: Clinical impact. *J Hepatol*. 2018;68:268-279.
37. Dongiovanni P, Stender S, Pietrelli A, et al. Causal relationship of hepatic fat with liver damage and insulin resistance in nonalcoholic fatty liver. *J Intern Med*. 2018;283:356-370.
38. Pelusi S, Cespiati A, Rametta R, et al. Prevalence and risk factors of significant fibrosis in patients with nonalcoholic fatty liver without steatohepatitis. *Clin Gastroenterol Hepatol*. 2019;17:2310-2319.
39. Raimondo G, Navarra G, Mondello S, et al. Occult hepatitis B virus in liver tissue of individuals without hepatic disease. *J Hepatol*. 2008;48:743-746.
40. Schwimmer JB, Behling C, Newbury R, et al. Histopathology of pediatric nonalcoholic fatty liver disease. *Hepatology*. 2005;42:641-649.
41. Hübscher SG. Histological assessment of non-alcoholic fatty liver disease. *Histopathology*. 2006;49:450-465.
42. Brunt EM, Kleiner DE, Wilson LA, et al. Portal chronic inflammation in nonalcoholic fatty liver disease (NAFLD): a histologic marker of advanced NAFLD-Clinicopathologic correlations from the nonalcoholic steatohepatitis clinical research network. *Hepatology*. 2009;49:809-820.