Histological and immunohistochemical assessment of liver biopsies in morbidly obese patients

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Summary. Aims: To study liver lesions in morbidly obese patients who underwent liver biopsy at the time of bariatric surgery to define histological lesions, especially inflammatory infiltrates, diagnostic categories and the possible influence of gender in this respect. Methods and results: 110 biopsies (36 males-M- and 76 females -F-) were evaluated and categorized, according to the NAS (NAFLD -non alcoholic fatty liver disease- Activity Score) system and other criteria, as non-NAFLD (15.5%, F predominance), non-alcoholic steatohepatitis (NASH) (16.5%, M predominance), non-alcoholic hepatosteatosis (NAHS) (21%, F predominance) and, the most numerous group, NASH-borderline (NASH-BORD) (47%), with three subgroups, characterized by centrozonal lesions, portal area preferential involvement or affecting both areas. The predominant form of hepatocytosteatosis was mixed with a multivesicular component that was present in most cases with fibroinflammatory portal involvement. Nuclear glycogenosomes were found in greater number of biopsies in patients in the third and sixth decades. Portal inflammation was present in a large number of cases (M predominance); the application of immunohistochemical techniques (myeloperoxidase and CD68 antibodies) to evaluate lobular inflammation revealed “surgical hepatitis” in one third of the cases, and the presence of microgranulomas (CD68+) (M predominance), which were more abundant with increasing lesion severity. Conclusions: Portal inflammation and multivesicular hepatocytosteatosis are highly prevalent in morbidly obese patients. This study identifies a new subtype of NASH-BORD characterized by centrozonal and porto-periportal area involvement and the existence of liver biopsies without steatosis. CD68+ microgranulomas constitute an unequivocal marker of lobular inflammation in surgical biopsies and of lesion severity, which is gender-related.

Key words: Immunohistochemistry, Morbid obesity, Multivesicular hepatocytosteatosis, NAFLD, Portal inflammation

Introduction

Non-alcoholic fatty liver disease (NAFLD) has become the most prevalent form of liver disease in industrialized countries (Straub and Schirmacher, 2010; Tiniakos et al., 2010). One of the factors associated with this increase is obesity, which is an ongoing health problem, affecting not only the adult population but also (and disturbingly) children (Angulo, 2006; Ogden et al., 2006). Although imaging studies and laboratory-based tests accurately detect significant hepatosteatosis and/or advanced fibrosis, respectively, the diagnosis and characterization of NAFLD ultimately depend on histopathologic evaluation (Brun, 2009; Straub and Schirmacher, 2010).

Hepatocytosteatosis (HcS) is the hallmark of NAFLD. The minimum histological criterion for the diagnosis of NAFLD is the presence of fat (mainly triglycerides) in more than 5% of hepatocytes (Kleiner et al., 2005). HcS is commonly macrovesicular, although groups of hepatocytes with microvesicular HcS may also be observed (Brun, 2009; Brun and Tiniakos, 2010).

Abbreviations. Ab: antibodies; BMI: body mass index; BORD: borderline; F: females; HcS: hepatocytosteatosis; M: males; MDB: Mallory-Denk bodies; MPO: myeloperoxidase; NAFLD: Non-alcoholic fatty liver disease; NAS: NAFLD Activity Score; NAHS: non-alcoholic hepatosteatosis; NASH: Non-alcoholic steatohepatitis; PI: portal inflammation; PMN: polymorphonuclear leukocytes.
Liver histology in morbid obesity

Mallory’s hyaline or Mallory-Denk bodies (MDB), cytosolic hyaline inclusions of centrilobular distribution, are characteristic of alcoholic hepatitis and of non-alcoholic steatohepatitis (NASH) (Zatloukal et al., 2007; Brunt, 2009), the form of NAFLD that can progress to cirrhosis. In NASH, MDB are present in smaller quantities, or are rarely observed and more poorly conformed than in alcoholic hepatitis, and are difficult to detect with routine stains (Hübscher, 2006).

Another histological change observed in NAFLD is the presence of vesiculated nuclei due to nuclear glycogen accumulation (Tiniakos et al., 2010), or glycogenosomes, also present in diabetes (Abraham and Furth, 1994) and in childhood NAFLD (Schwimmer et al., 2005); this is a common physiological change in young people (Levene and Goldin, 2010).

Lobular inflammation, generally mild and either mixed or chronic type, with the formation of microgranulomas and lipogranulomas, is usually present in NAFLD (Brunt and Tiniakos, 2010). In biopsies obtained inter-operationally, there may be aggregations of polymorphonuclear leukocytes (PMN), often arranged around centrilocubilar veins or steatotic hepatocytes, a phenomenon that has been termed “surgical hepatitis” (Brunt, 2009). This alteration is different from “satellitosis”, which is characterised by groups of PMN around hepatocytes ballooned with MDB (Brunt et al., 1999; Bateman, 2007).

Portal inflammation (PI) is frequently observed in type 2 childhood NASH associated with fibrosis (Schwimmer et al., 2005) and in adult obese patients (Abrams et al., 2004; Liew et al., 2006). At one time, PI was not a feature used in calculating the principal numerical score to evaluate NAFLD (Kleiner et al., 2005). However, in studies of large series of patients, increased PI was found to be related to NASH and advanced fibrosis (Brunt et al., 2009; Rakha et al., 2010).

Gender and ethnic-related differences have been observed with respect to fat distribution, metabolic syndrome parameters and the severity of hepatic lesions in the obese, among others. In the latter respect, contradictory data have been published (Arun et al., 2006; Tiniakos et al., 2010; Hashimoto and Tokushige, 2011).

The aim of the present study was to characterise liver lesions, including portal inflammation and the phenotypes of the principal components of lobular inflammatory infiltrate, in a series of morbidly obese patients undergoing bariatric surgery, in order to define histological lesions and diagnostic categories, their relation with fibrosis, and the possible influence of gender in this respect, in this etiological group of patients with NAFLD.

Materials and methods

Subjects

Over a period of five years, morbidly obese patients [body mass index (BMI) ≥40 or ≥35 with clinical comorbidities] were enrolled prospectively and consecutively in the study. Criteria for exclusion were patients being treated with hepatotoxic or antineoplastic drugs, or with primary liver disorders other than fatty liver that could account for HcS. The maximal alcohol consumption of the study participants was 30 g per week in men and 20 g in women. All patients underwent bariatric surgery at the San Cecilio University Hospital (Granada, Spain). The ethics committee of the hospital approved the study, and all subjects provided written informed consent.

Biological samples

Liver biopsies were obtained at the moment of bariatric surgery, using a Tru-cut of 18-G. Only samples longer than 14 mm and containing at least 7 portal tracts on histological examination were included in the study (110 samples from a total of 193 liver biopsies) (Bravo et al., 2001; Riley and Rugiero, 2008).

Hepatic cylinders were immediately fixed in buffered formalin and embedded in paraffin; 4-μ sections were obtained and stained with hematoxylin-eosin, PAS-diastase, Gomori trichrome, Gordon-Sweet reticulin and Prussian blue (Perls), to assess cumulative hepatocyte changes and necroinflammatory, fibrotic or architectural modifications.

Histological study

We analysed the intensity, distribution (centrilobular, panzonal and azonal) and quality (macrovesicular, multivesicular or mixed) of the HcS. The term multivesicular HcS is used to refer to hepatocytes showing multiple small-medium sized vesicles without nuclear displacement to the cell periphery (similar to pure microvesicular HcS, but unlike the latter, identification is easy). This was assessed as present or absent.

In addition, we noted the presence or absence of lobular inflammation, PI, pericellular and portal fibrosis and of any other hepatocellular injury (MDB, nuclear glycogenation, megamitochondria, haemosiderin) -see also Immunohistochemical study-.

Four diagnostic categories were established (Table 1): a) Category 0 or non-NAFLD (biopsies without HcS and no injuries or reactive damage); b) Category 1 or NAFLD non-NASH or non-alcoholic hepatosteatosis (NAHS) (HcS >0 and NAS -NAFLD activity score- 1 or 2); c) Category 2 or NASH-BORD (NAS 3 or 4), which was subdivided into 2A-BORD1 (injuries in acinar zone 3), 2B-BORD2 (lesions in acinar zone 1), and 2C-BORD1+2 (overlap of the latter two); d) Category 3 or NASH (NAS 5 to 8) (Kleiner et al., 2005; Schwimmer et al., 2005; Brunt et al., 2009); this coincides with the histological diagnosis of steatohepatitis (varying degrees of HcS, ballooning and/or pericellular fibrosis in the perivenular area and lobular inflammatory infiltrate -Brunt et al., 1999).
Liver fibrosis was evaluated according to the criteria of Kleiner et al. (2005) as: stage 0 (no fibrosis), stage 1 (perisinusoidal or portal/periportal fibrosis) [1A-mild perisinusoidal, 1B-moderate perisinusoidal, 1C-portal/periportal], stage 2 (perisinusoidal and portal/periportal fibrosis), stage 3 (bridging fibrosis) and stage 4 (cirrhosis). Stage 1A and 1B were considered jointly, and distinct from Stage 1C (Bateman, 2007).

Immunohistochemical and quantitative study

To identify “surgical hepatitis” and thus to obtain correct scores for lobular inflammation, immunoperoxidase techniques were applied, using an automated system (Lab Vision Autostainer 720), introducing primary antibodies (Ab) into myeloperoxidase (MPO) (polyclonal Ab, prediluted, Master Diagnostica -MD-, Spain) and CD68 (monoclonal Ab 512H12, prediluted, MD). Anti-p62 Ab (Monoclonal Ab 3P62LCK, 1/500 dilution, BD Transduction) was also applied to locate MDB.

MPO and p62 were scored as absent or present. Lobular microgranulomas CD68 (+) were quantified using an Eyepiece (reticle 1x1 cm, divided into 100 squares each with an area of 1 mm²), adapted to the eyepiece of a conventional optical microscope. Observation was performed using the 40x lens, and an average of 20 fields (chosen randomly) were calculated, with the result being expressed as number/mm².

Statistical analysis

The results were analysed using the SPSS 15 statistical program. Quantitative variables are expressed as mean and standard deviation of the mean (x ± SD) and qualitative variables (with categories grouped into present or absent) by number and percentage. Bivariate analysis was performed using the Student t test for numerical variables and the Chi-Square (χ²) test for qualitative variables. ANOVA was carried out between a categorical variable (categorized into 3 or more strata) and a quantitative variable. The criterion for statistical significance was p≤0.05.

Results

The study included 110 patients with morbid obesity, 34 of whom were males (M) and 76, females (F). There were no significant differences in age between the sexes (40.44±1.63 years for M and 44.28±1.29 years for F). Fig. 1 shows the “age” variable transformed into decades (3rd decade: up to 30 years; 4th decade: 31-40 years; 5th decade: 41-50 years; 6th decade: over 50 years) and the distribution by gender. The patients’ BMI ranged between 37 and 80 kg/m² (mean value 51.70±0.77), with 51.27±1.25 in M and 51.89±0.96 in F.

Table 2 shows the main histological changes evaluated, the major diagnostic categories (non-NAFLD, NAFLD non-NASH or NAHS, NASH-borderline and NASH) and the stages of fibrosis, both for the whole series, and for each sex. In 17 cases (15.5% and F predominance) HcS was absent, and so they were categorized as non-NAFLD; the largest category (NASH-BORD) included almost half of the biopsies (47%), with similar percentages for M and F (Table 2).

When analyzing the subgroups included in the category NASH-BORD, the subgroup with portal/periportal area lesions (2B) was observed in 23 of the 52 cases (44%), a higher percentage than the other 2 subgroups (2A and 2C: 29% and 27%, respectively). A similar number of cases were observed for each of the different grades of HcS. According to the different diagnostic categories, patients with HcS grade 1 (31 cases) were mainly diagnosed as NAHS (74%), whereas patients with HcS grade 2 (32 cases) and grade 3 (30 cases) were diagnosed as NASH-BORD (84% and 57%, respectively) or NASH (16% and 43%, respectively) (p<0.0001, χ² test). The distribution pattern of HcS was

![Fig. 1. Distribution of patients by decades, for both sexes. There was a similar percentage of male and female patients in the 3rd and 5th decades, and a significant predominance of females in the others (p<0.007, χ² test).]
mainly centrilobular (38% and M predominance), and no cases of periportal HcS were observed. In many biopsies macrovesicular HcS was accompanied by multivesicular HcS (mixed HcS 74% - preferentially M -) (Fig. 2A), although in some cases the latter was the predominant form.

MDB were present in a few cases (16%), mainly in M (32% vs 9%), and mostly categorized as NASH or NASH-BORD. Their distribution was centrilobular and the intensity was mild in many cases, and so the use of immunohistochemical techniques detecting protein p62 (Fig. 2D) facilitated their identification.

Hepatocytes with nuclear glycogenosomes were found in over half of the biopsies (55%, with no gender predominance) and in all diagnostic categories (Fig. 2B), with a preferential periportal distribution; in most cases, the intensity was mild. Higher percentages of cases were observed in the third and sixth decades (75% in the 3rd decade, 34% in the 4th, 57% in the 5th and 70% in the 6th; \( p < 0.012; \chi^2 \) test).

Other hepatocellular injuries evaluated included megamitochondria, observed in 28% of the patients (mostly NASH or NASH-BORD). Siderosis was absent in all biopsies.

Lobular inflammation, chronic or mixed, was found in many cases (85% - M 91%, F 83%-), although in the majority with mild intensity. The use of immunoperoxidase techniques with CD68 Ab revealed a disperse pattern of Kuppfer cells in sinusoidal locations or in lobular accumulations (Fig. 2E), generally small.

![Fig. 2](https://example.com/fig2.png)

**Fig. 2.** Liver injury in NAFLD in morbidly obese patients. Hepatocytes with multivesicular steatosis (multiple small or medium-sized cytosolic vesicles without nuclear displacement to the periphery-arrow-), together with others with macrovesicular steatosis (A) and vesiculated/glycogenated nuclei (arrow) (B). Portal tract with chronic inflammatory infiltrate (C) (HE). The use of immunohistochemical techniques highlights Mallory-Denk bodies (anti-p62 Ab) in ballooned hepatocytes (D), lobular microgranulomas (anti-CD68 Ab) (E) and aggregations of polymorphonuclear leukocytes around steatotic hepatocytes (anti-MPO -myeloperoxidase- Ab) in “surgical hepatitis” (F) (Immunoperoxidase). A, D-F, x 400; B, x 200; C, x 100
and with a preferential centrizonal distribution. The quantification of microgranulomas highlights the scant presence of these cells in the mildest diagnostic categories, which increases with greater lesion severity. The average number of microgranulomas recorded was higher in M than in F (Fig. 3).

After the use of MPO Ab, in 36% of biopsies there was observed to be an accumulation of centrizonal PMN, often arranged around steatotic hepatocytes (Fig. 2F) or centrilobular veins (“surgical hepatitis”), which were not accounted for as lobular inflammatory response.

PL was present in 71% of cases (preferentially M), distributed in all diagnostic categories, either chronic (with the presence of CD68+ macrophages, isolated or in small clusters) or mild in most cases of NASH-BORD (Fig. 2C) and moderate in NASH, coexisting with fibrosis in the same location. In more than half of the biopsies categorized as non-NAFLD (59%) there was focal PL. PL was only observed in biopsies with fibrosis stages 1C, 2 and 3 (p<0.001, 0.010 and 0.023, respectively; $\chi^2$ test).

Regarding the stages of fibrosis (Table 2), the highest percentage of biopsies (41%, F predominance) revealed portal/peripoportal fibrosis (stage 1C), followed by pericellular and porto-peripoportal fibrosis (stage 2: 25.5% - M 35%, F 21% -) and mild pericellular fibrosis (stage 1A: 13.5% - M 9%, F 16% -). Stages 1B and 3 (bridging fibrosis) presented few cases (1% and 4.5%, respectively) and the remaining 14.5% had no fibrosis (stage 0 - F predominance -). No case of stage 4 (cirrhosis) was observed.

No significant differences were observed in lesion severity (diagnostic categories and stages of fibrosis) in the joint consideration of the patients’ age (decades) and gender; this could be influenced by the small number of cases in some of the groups.

**Discussion**

Obesity is a growing health problem in developed countries; it has been considered the epidemic of the XXI century (Angulo, 2006; Tiniakos et al., 2010). Examination of liver biopsies in patients who underwent liver biopsy at the time of bariatric surgery for the treatment of morbid obesity has been very helpful for understanding the natural history of NAFLD, and, moreover, that not all morbidly obese patients suffer NAFLD lesions (Ong et al., 2005), as was the case in 17 of the patients in our study, a majority of whom were women.

Since the introduction of semiquantitative assessment of histological lesions in NAFLD (Brunt et
al., 1999) in the form of the NAS scoring system (Kleiner et al., 2005), the description of these lesions has been standardised, especially with respect to NASH. The diagnosis of NASH, thus, requires the presence of various indicators of activity injury (HcS, ballooning, lobular inflammation), while fibrosis, a consequence of the above, is entered as a separate stage, as in chronic hepatitis.

Biopsies in children and adolescents present a distinct and frequently found histological NASH variant, characterized by HcS and inflammation and fibrosis in the portal area. In addition, another pattern has been described, with zone 1 and zone 3 lesions (overlapping pattern) (Schwimmer et al., 2005; Carter-Kent et al., 2009). On the other hand, recent studies have subdivided NASH-BORD (NAS 3–4) into two categories (Brunt et al., 2009), reflecting a zonal accentuation of lesions: zone 3 and zone 1 pattern. The latter manifestation was present in numerous cases in our series, compared with results from the above-mentioned studies.

The prevalence of NASH in morbid obesity is high, ranging between 26% (Ong et al., 2005) and 37% (Abrams et al., 2004; Liew et al., 2006; Machado et al., 2006); no conclusions can be drawn about its incidence in this study, since the inclusion of cases was dependent on the quality of the biopsies. Some publications have reported a higher prevalence of NASH in post-menopausal women (Hashimoto and Tokushige, 2011), and others, in males (Arun et al., 2006; Tiniakos et al., 2010), as was the case in our series. The more advanced stages of fibrosis, too, were most commonly found in males; this could be related to their greater amount of abdominal fat and to its etiopathogenic role in hepatic lesions (Arner, 2003; Angulo, 2006).

Macrovesicular or mixed HcS in NAFLD has been described (Brunt, 2009; Brunt and Tiniakos, 2010), the latter being comprised of hepatocytes with both large and small droplets. This was the predominant form observed in our series (74%). We use the term “multivesicular”, also referred to as small vacuole steatosis (Brunt, 2010), to indicate the presence of hepatocytes with multiple vacuoles of intermediate or small size, but easily identifiable and usually without peripheral nuclear displacement. By contrast, pure microvesicular HcS is also characterized by the formation of multiple vesicles, but these are much smaller and more difficult to identify, sometimes giving it a foamy or ballooned hepatocyte appearance, with an indented and centrally located nucleus (Brunt, 2009).

MDB are scarce and hard to detect using standard staining for NASH (Hübischer, 2006). They consist of aggregates of different proteins (keratins, heat shock proteins, ubiquitin, p62 protein, etc) (Zatloukal et al., 2007; Tiniakos et al., 2010) that can be used for immunolocalization. Despite the use of protein p62, we did observe MDB in a few cases in this series, a fact that has also been reported in other studies of the obese (Beymer et al., 2003), especially among males.

Hepatocyte nuclear glycogenation has been considered a feature of NAFLD and diabetes (Abrahams and Furth, 1994; Tiniakos et al., 2010), most frequently in children (Schwimmer et al., 2005). This was found to be present in over half of our biopsies (55%), with no significant differences between the sexes. Current publications indicate that a physiological change is common in young subjects and that in NAFLD it tends to increase with age (Levene and Goldin, 2010). This was indeed the case in our study, except in the group of patients under 30, who had a glycogenation rate as high as those aged over 60 (the factor of the patients’ relative youth, together with that of metabolic alterations, could act to enhance the above effect).

Lobular inflammation, chronic or mixed type, was found in many cases (85%), although in the majority with mild intensity. In NAFLD, there is a constant presence of microgranulomas (Brunt, 2009), accumulations of Kupffer cells that phagocytise hepatocyte debris (necrotic and apoptotic) (Tiniakos et al., 2010). Some immunohistochemical studies, using CD68 Ab, have reported a different pattern of expression in steatosis (disperse) and in NASH (centrizonal grouping) (Lefkowitch et al., 2002). Our results confirm the absence or scarcity of microgranulomas in Category 0, and maximum values in NASH, but also their presence in cases with NAHS or NASH-BORD, and with greater significance than that obtained in other quantitative studies of NAFLD (Fotiadu et al., 2010), as well as a greater quantification in M than in F in all diagnostic categories.

In biopsies performed during surgery for obesity, there may occur “surgical hepatitis” (Brunt, 2009), which was detected in one third of the cases in our series, after the utilisation of MPO Ab, and which were not accounted for as lobular inflammatory response. Moreover, this enabled us to differentiate it from true “satellitosis” (Brunt et al., 1999; Bateman, 2007).

PI is a frequent histopathological alteration in NAFLD (Brunt et al., 2009) and bariatric surgery for obesity has also highlighted its existence (Abrams et al., 2004, Liew et al., 2006). This lesion was present in a high percentage of cases (71%) in the present series, distributed throughout the different diagnostic categories, although the largest group was found in the NASH-BORD category (50%) and within the 2C subgroup; its presence was correlated with the stages of fibrosis.

PI in NAFLD has been interpreted in many different ways (Hübischer, 2006). It has also been observed in severe cases of NASH in both adults and children, or following treatment for obesity (medical or surgical), although its cause remains unknown (Brunt, 2009). Its presence in children, many of them obese (Schwimmer et al., 2005), and the high percentage recorded among the obese adults in our series suggests that it may be a feature that accompanies obesity. Its M predominance may also be influenced by mediators released from abdominal adipose tissue. In fact, more than half (59%) of our patients without HcS (category non-NAFLD) had
portal inflammation, albeit usually in a mild form. More than mild PI may be considered a marker of advanced disease (in both children and adults) (Brunt et al., 2009, Rakha et al., 2010).

For all these reasons, we believe that in biopsies of morbidly obese adults, the NAS score should be applied to define the existence or otherwise of NASH. We propose that three subgroups within NASH-BORD should be established, given the significant number of biopsies with lesional involvement in acinar zone 1. The study of larger series could better define the clinicopathologic features of these patients.

Since most of the patients considered in series of NAFLD children are obese (Schwimmer et al., 2005), it is not uncommon for morbidly obese patients, such as those studied here, to present similar lesions regarding the injury pattern of inflammation and/or portal fibrosis, as well as the small number of cases with MDB, although there are differences (centrizonal HcS and multivesicular type predominance, etc.).

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