Microvesicular steatosis : a missed item in the management of nonalcoholic fatty liver disease?

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Abstract

Background **: Nonalcoholic fatty liver disease (NAFLD) is among the most common causes of chronic liver disease and cirrhosis. In NAFLD, histological course of steatosis is usually macrovesicular (MacroS), but it may be accompanied by varying degrees of microvesicular steatosis (MicroS). Thus, in this study, we aimed to evaluate the prevalence and significance of MicroS in subjects with NAFLD.**

Methods **: A retrospective analysis of clinical and laboratory data of patients with histologically proven NAFLD was performed. The liver biopsy specimens which stained with hematoxylin eosin, reticulin, and Masson's Trichrome stains were evaluated by single expert liver pathologist. Scoring and semiquantitative assessment of steatosis and NAFLD severity was done according to Kleiner scale known as NAFLD activity score (NAS). Grading for steatosis, steatosis type, zonal distribution of steatosis and other histological findings were also determined.**

Results **: The prevalence of MicroS among the study population (n= 191) was 30.4%. There was no difference regarding the demographic and biochemical parameters between patients with or without MicroS. On the other hand, the prevalence of ballooning injury and megamitochondria were higher in patients with MicroS (p= 0.019 and p= 0.036, respectively). There was a significant association of MicroS with ballooning injury (OR 2.65, 95% CI= 1.26-5.55 ; p= 0.005) and the presence of megamitochondria (OR 3.72, 95% CI= 1.00-13.72 ; p= 0.037).**

Conclusion **: MicroS is common in patients with NAFLD and is associated with early histological findings in this clinically relevant condition. Further longitudinal studies are needed to characterize the role of MicroS in the natural history of NAFLD.** (Acta gastroenterol. belg., **2020**, 83, **565-570**).

Keywords : microvesicular steatosis, macrovesicular steatosis, ballooning injury, megamitochondria, nonalcoholic fatty liver disease.

Introduction

Nonalcoholic fatty liver disease (NAFLD) is one of the most common causes of chronic liver disease and encompasses a spectrum of conditions associated with lipid deposition in hepatocytes. It ranges from nonalcoholic fatty liver (NAFL-isolated steatosis alone) to nonalcoholic steatohepatitis (NASH—fatty changes with inflammation and hepatocellular injury or fibrosis), to advanced fibrosis and cirrhosis (1,2). Though it varies among geographical regions, the estimated prevalence of NAFLD has been reported as 20-30% among normal population and up to 70–80% of subjects with obesity and/or type 2 diabetes (T2D) (3,4). It is typically associated with T2D, hypertension, obesity, or dyslipidemia and now considered to be the hepatic component of the metabolic syndrome (MetS) (5,6).

In NAFLD, hepatic steatosis is usually macrovesicular form (MacroS), referring to hepatocytes with a single large intracytoplasmic fat droplet or smaller well defined droplets expanding the cell and displacing the nucleus to the cell periphery (7). In some of the cases, microvesicular steatosis (MicroS) which is characterized by distended hepatocytes with foamy appearing cytoplasm and small lipid vesicles (less than 1μm in diameter) may also be discernible (8,9). Little is known about the pathogenesis of MicroS, but in many instances the primary defect could be a mitochondrial dysfunction. In MicroS, transfer of electrons to oxygen molecules generates reactive oxygen species, thus producing superoxide anions and hydrogen peroxide which induces mitochondrial swelling and leads to the formation of megamitochondria (10-14). There is also very scarce data regarding the prevalence and significance of MicroS in NAFLD (8,9). In this study, we aimed to investigate the prevalence of MicroS and its association with clinical and laboratory parameters and also histological findings in subjects with NAFLD.

Methods

We retrospectively analyzed our pathology database at our center (Gulhane Medical School, Ankara-Turkey) and identified all adult patients diagnosed with the pathology diagnosis of steatosis, steatohepatitis, or fatty liver. An extensive review of the patients′ medical records including all notes from clinic visits, laboratory and imaging data and liver biopsy reports was performed.

The Institutional Ethics Committee approved the study protocol, and the study was conducted in accordance with the Declaration of Helsinki. All patients had given consent for participation in medical research.

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Submission date : 02/12/2019
Acceptance date : 04/06/2020

Characteristics of study population

One hundred and ninety-one subjects with biopsy-proven NAFLD were included in the present study. Inclusion criteria were : persistently (at least 6 months) elevated aminotransferases, ultrasonographic presence of bright liver without any other liver or biliary tract disease and liver histology compatible with a diagnosis of NAFLD. Subjects with the following conditions or diseases were excluded : alcohol consumption ≥20 g/day in the last year, a positive test for hepatitis B surface antigen, hepatitis C antibody and other causes of liver disease, presence of T2D, morbid obesity (body mass index : BMI ≥40 kg/m²), and any other major diseases, including generalized inflammation or advanced malignant diseases, exposure to occupational hepatotoxins or drugs known to be steatogenic or to affect glucose and lipid metabolism.

Clinical examination and laboratory analyses

BMI was calculated as body weight divided by height squared (kg/m²), obesity defined as a BMI of at least 30 kg/m² and BMI between 25 and 29.9 kg/m² as overweight. The waist circumference (WC) was measured midway between the lowest rib margin and the iliac crest in a standing position by the same examiner.

Fasting blood samples were obtained from the antecubital vein, and serum/plasma samples were separated for the analysis of the biochemical parameters without freezing. Fasting plasma glucose (FPG), alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT), triglyceride (TG), total cholesterol (TC) and high-density lipoprotein cholesterol (HDL-C) levels were measured by enzymatic colorimetric methods with an Olympus AU2700 (Beckman Coulter, USA) auto analyzer using commercially available reagents. Low-density lipoprotein cholesterol (LDL-C) was calculated by the Friedewald formula (15). The Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) was calculated according to the formula of fasting glucose (mg/dL) x fasting insulin (μU/mL)/405 (16).

Liver histology

In more than 90% of cases included the liver biopsy was performed due to persistent elevation of liver enzymes in patients with confirmed fatty infiltration of the liver detected on imaging studies. The liver biopsy was performed to confirm the diagnosis of NAFLD after appropriate exclusion of liver disease of other etiology, such as alcohol-induced or drug-induced liver disease, autoimmune or viral hepatitis, and cholestatic or metabolic/genetic liver disease.

The liver biopsy specimens were evaluated by single expert liver pathologist who was unaware of the subject's identity, group and all clinical information. Liver biopsy preparations stained by Hematoxylin and eosin (H&E) were examined under light microscopy by using high-power fields (x400) and presence of megamitochondria was evaluated at x1.000 magnification (Figure-1A and 1B). Masson's trichrome staining was used for evaluation of liver fibrosis. Histological features of samples were interpreted as outlined by Kleiner et al. (17).

MacroS was graded 0–3 based on the percentage of fat containing hepatocytes : 0= <5% ; 1= 5-33% ; 2= 34–66% ; 3= >66%. Small lipid vesicles (less than 1μm in diameter) providing hepatocytes foamy appearing cytoplasm with the centrally located nucleus were considered positive for MicroS. Lobular inflammation was graded 0-3 based on inflammatory foci per each magnification field (0= none ; 1= 1–2 ; 2= ≤4 ; 3= >4). Hepatocellular ballooning was defined by H&E staining showing enlarged cells with rarefied cytoplasm and by changes in the cytoskeleton (Figure-1C). It was graded as none (grade-0), few ballooning cells (grade-1), and many ballooned cells (grade-2). The presence of steatosis according to zones was categorized as (A) zone-3 steatosis, (B) panacinar steatosis, and (C) azonal steatosis. NAFLD activity score (NAS), which is the sum of steatosis (scale from 0 to 3), lobular inflammation (scale from 0 to 3), and hepatocellular ballooning (scale from 0 to 2), according to Kleiner et al. (17). Fibrosis staging was graded on a scale from 0 to 4, according

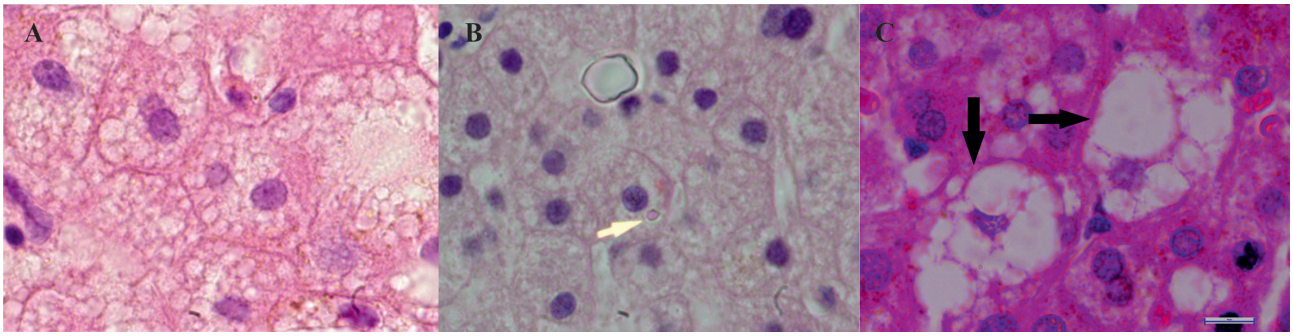

Figure 1. — A . Foamy appearance of hepatocytes cytoplasm in microvesicular steatosis. (H&E x1000). B : Megamitochondria in microvesicular steatosis (arrow). (H&E x1000). C : Hepatocyte ballooning (arrow) (H&E x1000).

Table 1. — **NAFLD histological scoring system**

Parameter	Score	Definition
Steatosis	0 1 2 3	<5% 5-33% >33-66% >66%
Lobular inflammation	0 1 2 3	None 1-2 foci per 200 x field ≤4 foci per 200 x field >4 foci per 200 x field
Ballooning	0 1 2	None Few ballooning cells Many ballooned cells
Fibrosis	0 1 2 3 4	None Portal/perisinusoidal Perisinusoidal and portal/periportal Septal or bridging fibrosis Cirrhosis
NAS	≤2 3-4 ≥5	Non-NASH Borderline NASH NASH

NAS, which is the sum of steatosis, lobular inflammation and hepatocellular ballooning, according to Kleiner et al. Fibrosis staging was graded on a scale from 0 to 4, according to the classification of Brunt et al. ; NAFLD : Nonalcoholic fatty liver disease; NASH : nonalcoholic steatohepatitis ; NAS : NAFLD activity score.

to the classification of Brunt et al. (7) (Table 1). Other histological parameters like portal inflammation, Mallory-Denk body, glycogenated nucleus, Councilman Body and megamitochondria were evaluated as "yes" or "no" (0= no, 1= yes). NASH (steatosis with inflammation and hepatocyte ballooning, often with fibrosis) was defined as a NAS ≥5, borderline NASH (steatosis with minimal inflammation and hepatocyte ballooning) as a NAS of 3 or 4, and non-NASH (steatosis in the absence of inflammation and ballooning hepatocyte degeneration) as a NAS ≤2.

Statistical analyses

Statistical analyses were made using the Statistical Package for Social Science 15.0 software (SPSS, Chicago, IL., USA). Categorical variables were expressed as number (%) and continuous variables were expressed as mean ± standard deviation or median (minimum-maximum). One sample Kolmogorov Smirnov test was used for the evaluation of distribution. Comparisons between the two groups were made using the Student's t-test or Mann-Whitney U test as appropriate. Categorical variables were compared using the χ^2 test. Chi-squared and Fisher's exact test correlation were used to examine the relationship between MicroS and other parameters. Results were expressed by odds ratios (ORs) and the corresponding 95% confidence interval (CI). P values less than 0.05 were considered statistically significant.

Results

Overall, MicroS was present in 30.4% of the study participants. Table 2 shows the anthropometric and laboratory data of the subjects with (n= 58) and without (n= 133) MicroS. There was no statistically significant difference in age, BMI, WC, glucose, lipid parameters, fasting insulin and HOMA-IR indexes between two groups.

The histopathological findings in liver biopsy

Table-2. — **Clinical and biochemical characteristics of the patients with and without MicroS**

	Without MicroS (n= 133)	With MicroS (n= 58)	P value
Age (years)	32.54 (20-53)	32.00 (21-45)	0.458
BMI (kg/m²)	28.30 (23.0-36.9)	28.33 (21.6-35.5)	0.211
WC (cm)	100.75 (86-128)	100.44 (86-120)	0.71
Smoker (%)	25.6	37.9	0.84
SBP (mmHg)	117.3 (±10.1)	122.4 (±11.6)	0.652
DBP (mmHg)	66.8 (±10.5)	65.3 (±9.6)	0.84
FPG (mg/dL)	94.7 (70-119)	91.91 (71-122)	0.085
Insulin (µU/L)	15.56 (4.90-53.78)	15.11 (5.85-47.19)	0.479
HOMA-IR	4.19 (1.25-12.88)	3.87 (0.94-10.63)	0.077
TC (mg/dL)	211.49 (80-328)	211.07 (102-338)	0.71
TG (mg/dL)	210.95 (48-774)	203.91 (44-617)	0.565
LDL-C (mg/dL)	134.56 (23-226)	133.14 (34-221)	0.761
HDL-C (mg/dL)	40 .86 (28-65)	41.78 (27-57)	0.39
AST (IU/L)	50.26 (15-118)	55.09 (32-139)	0.88
ALT (IU/L)	109.03 (19-286)	109.47 (54-201)	0.417
GGT (IU/L)	64.86 (19-160)	82.78 (27-455)	0.87

SBP and DBP values are expressed as mean and standard deviation. P values obtained by Chi-square tests for age, BMI and smoker rate, Student's t-test or Mann-Whitney U test for other parameters. Micro : Microvesicular steatosis ; BMI : body mass index ; WC v: waist circumference ; SBP : systolic blood pressure ; DBP : diastolic blood pressure ; HOMA-IR : homeostatic model assessment for insulin resistance ; TC : total cholesterol ; TG: triglyseride ; LDL-C : low-density lipoprotein cholesterol HDL-C : high-density lipoprotein cholesterol ; AST : aspartate aminotransferase ; ALT : alanine aminotransferase ; GGT : gamma-glutamyltransferase.

Table-3. — **Histopathological findings in patients with and without MicroS**

	Without MicroS n= 133 (%)	With MicroS n =58 (%)	P value*
Steatosis			
0	5 (3.7)	3 (5.17)	
1	37 (27.8)	15 (25.8)	0.258
2	58 (43.6)	22 (37.9)	
3	33 (24.8)	18 (31.0)	
Ballooning			
0	2 (1.5)	0 (0)	
1	112 (84.2)	40 (69)	0.019
2	19 (14.3)	18 (31)	
Lobular inflammation			
0	4 (3)	0 (0)	
1	63 (47.4)	26 (44.8)	
2	58 (43.6)	31 (53.4)	0.239
3	8 (6)	1 (1.7)	
Fibrosis			
Absent	108 (81.2)	45 (77.6)	
Present	25 (18.8)	13 (22.4)	0.565
NAS	4.41	4.74	0.14
Portal inflammation			
Absent	50 (37.6)	14 (24.1)	
Present	83 (62.4)	44 (75.9)	0.07
Megamitochondria			
Absent	129 (97)	52 (89.7)	0.036
Present	4 (3)	6 (10.3)	
Mallory-Denk bodies			
Absent	122 (91.7)	50 (87.7)	
Present	11 (8.3)	7 (11.3)	0.387
Councilman's bodies			
Absent	124 (93.2)	49 (84.5)	0.057
Present	9 (6.8)	9 (15.5)	
Glycogenated nucleus			
Absent	68 (51.1)	22 (37.9)	
Present	65 (48.9)	36 (62.1)	0.093
Steatosis zone			
Zone 3	95 (71.4)	46 (79.3)	
Panacinar	13 (9.8)	6 (10.3)	0.345
Azonal	25 (18.8)	6 (10.3)	

*Mann-Whitney U test : MicroS: Microvesicular steatosis ; NAS : NAFLD activity score

specimens of the subjects with and without MicroS are shown in Table 3. Because of the relatively small number of subjects with fibrosis in study population, we assessed the patients with different degrees of hepatic fibrosis in one group. Eventually, patients were categorised into two groups based on the presence or absence of liver fibrosis. The prevalence of ballooning injury and the presence of megamitochondria were significantly higher in subjects with MicroS (p= 0.019 and p= 0.036, respectively). However, other histopathological findings revealed no difference between two groups.

The patients were divided into three groups according to the NAS score ; NAFL, borderline NASH and NASH. For statistical purposes, we included patients with borderline NASH (n= 75) and NASH (n= 25) in one large group that was positive for NASH (n= 100), age 32.57±6.49 years. The NAFL group included 91 patients, age 32.16±6.49 years. As expected there was a significant difference regarding the presence of lobular and portal inflammation, and also NAS score between two groups. On the other hand, no significant difference was found regarding the presence of MicroS, ballooning injury and megamitochondria. There was a significant association of MicroS with ballooning injury (OR 2.65, 95% CI= 1.26-5.55 ; p= 0.005) and the presence of megamitochondria (OR 3.72, 95% CI= 1.00-13.72 ; p= 0.037). However, no relationship was found between MicroS and NAS score, diagnosis of NASH and other histological parameters including lobular or portal inflammation and fibrosis.

Discussion

There are three main histopathological features in NAFLD, which are accumulation of fatty acid content greater than 5% of the hepatocytes, ballooning degeneration, and lobular or portal inflammation (7). Although MacroS is the most common form of hepatic steatosis, it may be accompanied by MicroS in some patients with NAFLD. In literature, there is limited data about the prevalence and significance of MicroS in NAFLD. In their study, Tandra et al., reported the prevalence of MicroS in 10% of subjects with NAFLD (8). In a study by Noureddin et al., compared to nonelderly patients with NAFLD, elderly patients (≥65 year) had a higher prevalence of MicroS (10.1% and 14.8%, respectively) (11). In another study performed in a pediatric population, MicroS was present in 19% of the patients with NAFLD (9). However, in the present study we found higher MicroS prevalence rate (30.4%) in a well-characterized population with NAFLD. As can be seen, there are significant differences between studies in terms of the frequency of MicroS in NAFLD. In the study by Tandra et al., histopathological examination methods of biopsy specimens (using x4 and if necessary x10 magnification) were suggested a factor affecting the detection rate of MicroS. (8). On the other hand, there was no information about the histopathological methods for examination in other two studies (9,11). In our study, histopathological examination of all samples was performed under the light microscope by using x400 magnification. Moreover, presence of megamitochondria was evaluated at x1000 magnification. In light of these data, we suggest that the variation in MicroS detection rates reported in the literature could be at least partly related to result from different pathologic interpretation of liver biopsy specimens.

The presence of MacroS in NAFLD is thought to be a good prognostic factor by itself in the long term and rarely results in fibrosis and cirrhosis (8). On the other hand, diffuse MicroS in liver such as acute fatty liver in pregnancy, Reye's syndrome and the intake of certain drugs/toxins may lead to hepatic failure and encephalopathy (10). Furthermore, compared to MacroS, MicroS has been suggested to be an independent predictor of advanced liver injury in NASH (8). Ballooning degeneration is characterized by hepatocyte swelling, arising from excess accumulation of microvesicular fat

and bile acids. It is mostly seen in perivenular area close to hepatocytes with steatosis and coexists with inflammation and pericellular fibrosis (18,19). It is basically defined as an indicator of hepatocellular damage, likewise apoptosis and necrosis (20). It was also suggested to be an important finding in the differentiation of NAFL and NASH in the presence of lobular inflammation (21,22). Mitochondrial changes are reported to be an important finding of progressive liver disease in NAFLD (13). It was demonstrated that, defect in β-oxidation leads to accumulation of fatty acids in the cytosol resulting in formation of megamitochondria (23). Megamitochondria is also associated with NASH more than NAFL and characterized by the loss of crista and presence of para-crystalline inclusions.

To the best of our knowledge, there are only few studies investigating the relationship of MicroS with other histological findings in NAFLD. In the study by Tandra et al., the presence of MicroS was associated with higher grades of steatosis, ballooning injury, presence of Mallory-Denk bodies and megamitochondria, higher NAS scores, more advanced fibrosis, and diagnosis of borderline or definite NASH (8). In studies by Ikura and Caldwell, it was reported that membranes of ballooning hepatocytes containing MicroS are the initial point of oxidative stress and this oxidative stress and consequent lipid peroxidation seem to play a pivotal role in liver injury (14,23). Moreover, the coexistence of MicroS and ballooning injury is thought to be an important factor in the development of hepatocyte damage caused by oxidative stress. In clinical studies performed on patients with NASH, megamitochondria was determined in 5-15% of the subjects with azonal distribution (12,14). In the study by Carter-Kent et al., biopsies with mixed steatosis were approximately 20 times more likely to have megamitochondria than those with MacroS alone (9). In the present study, the prevalence of ballooning degeneration and megamitochondria were significantly higher in patients with MicroS compared to those without MicroS. In addition, there was a significant association of MicroS with ballooning degeneration and the presence of megamitochondria. Accordingly, our findings about the relationship between MicroS and other histological findings seem to be consistent with current literature. However, unlike the study by Tandra et al., we could not find any relationship of MicroS with NAS scores, diagnosis of NASH and fibrosis (8). This can be explained by younger age of our patients, absence of other fibrosis-related factors (T2D, morbid obesity, etc.) and limited number of patients with advanced fibrosis. Thus, age and accompanying metabolic disorders are well known factors associated with advanced liver injury in patients NAFLD (10,24).

The current study has several limitations that could be addressed. Firstly, in order to prevent any interference of confounding factors for histopathological findings, we studied a specifically selected group having no additional disorders such as T2D. For this reason, the findings obtained are not representative for all subjects with NAFLD. Secondly, although no significant difference in both sexes was found regarding the prevalence of MicroS in literature (8), all participants in our study were men, and it remains to be determined whether these results are similar in women. Thirdly, the main strength of our work is that histological examination was done by one expert liver pathologist using H&E stain under light microscopy at higher magnifications. However, it should be noted that, because of their similar histological appearance, differentiation may be difficult between MicroS and cytoplasmic ballooning. Therefore, it could be better to apply histochemical staining with Oil Red O or immunohistochemical staining with Cytokeratin18 Antibody for this purpose. Lastly, due to the cross-sectional nature of this investigation, our results do not imply causation and longitudinal studies are needed to ascertain causality.

Conclusion

The findings of the present study suggest potential links between MicroS and early histological features of liver injury in NAFLD. Therefore, longitudinal studies are needed to address the role of MicroS in mediating cellular injury and disease progression in NAFLD.

Conflict of interest

None.

Funding

None

Ethical approval

The Institutional Ethics Committee approved the study protocol, and the study was conducted in accordance with the Declaration of Helsinki.

Competing interest

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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