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Liver changes in Wilson's disease: the full spectrum. A report of 127 biopsies from 43 patients

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Abstract. – OBJECTIVE: Wilson's Disease (WD) is an autosomal recessive copper overload. Several mutations of the copper pump named ATP7B have been involved. WD is difficult to diagnose mainly because of its heterogeneity of presentation. The histologic spectrum is wide and not specific, ranging from very mild changes to severe disease. The histological picture of WD may overlap different conditions, including ALD, NAFLD, viral hepatitis or autoimmune liver disease.

PATIENTS AND METHODS: We describe our experience on WD based on a single-center series of liver biopsies. One hundred twenty-seven liver samples from 43 Sardinian WD patients were reviewed. The most reported pattern was steatohepatitis, accounting 82/127 biopsies (64.6%), followed by hepatitis in 25 biopsies (19.7%), and steatosis in 20 biopsies (15.7%).

RESULTS: As for the elementary lesions, inflammation, steatosis, glycogenated nuclei and ballooning were the most frequent, being found in 107, 102, 90 and 86 biopsies out of the 127. Notably, all these lesions showed a predominant periportal location. There was no significant difference in the diagnostic pattern or in each elementary lesion between the biopsies performed at presentation and those performed during the follow-up. Lipogranuloma (significantly more numerous in the follow-up biopsies) and fibrosis (likewise significantly progressed in follow-up biopsies) were the only exceptions.

CONCLUSIONS: Our data confirm the variability of the histological pattern in WD. However, the preferential localization of steatosis and balloon cells in periportal zone can be a useful clue for the diagnosis of WD.

Key Words:
Wilson disease, Liver, Histopathology, Steatosis, Fibrosis, Ballooning.

Introduction

Wilson's disease (WD) is an inherited, autosomal recessive disorder of copper metabolism that, when early diagnosed and properly treated, has a favorable prognosis[1]. The implicated gene is localized at the 13q14.3 and encodes for the transmembrane protein, ATP7B. This protein is located in the Golgi apparatus and in the endosomal vesicles, depending on the changes in intracellular copper concentration[2]. A large number of mutations, more than 500, have been detected in WD patients. These mutations can be scattered over the 17 exons of the ATP7B gene. The worldwide most common mutation, which is found in the majority of WD carriers, is H1069Q. However, this mutation is not present in all countries, including patients from Lebanon[3]. A worldwide prevalence of 1:30000-1:100000 of WD is often reported. In the population from Sardinia, an island of the Mediterranean Sea,

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WD shows a relatively high prevalence (about 1:8700)[4]. Molecular analyses often fails to identify the most frequent mutations of this disease in the Sardinian patients, since the most common change is represented by a 15 nucleotides deletion in the promoter zone, the 5' UTR, of the WD gene[5]. Mutations in the regulatory elements of ATP7B may be detected in WD carriers from other geographical areas. Yet, variations in the promoter zone are uncommon in WD, except in Sardinian patients[6]. ATP7B dysfunction leads to copper storage inside the hepatocytes, because of the inability of ATP7B to transport copper atoms into biliary canaliculi[7]. Copper overload is not restricted to liver cells. Besides, frequently it is also found in many cells of the central nervous system[8]. In WD the content and distribution of other trace metals throughout the brain has been recognized[9]. The liver cell damage in WD patients is caused by the perpetual oxidative stress, following the intracellular copper overload. Excess free copper ions trigger peroxidative damage by production of reactive oxygen species (ROS), affecting hepatocytic degeneration and liver cell death[10-12].

The histological picture of liver biopsies in WD may be markedly different from one patient to the next[13,14]. Steatosis, Mallory-Denk bodies, lipogranulomas and glycogenated nuclei have been reported as characteristic findings of WD. This histological picture often mimics alcoholic liver disease (ALD) and non-alcoholic fatty liver disease (NAFLD)[15]. In other patients, the histological picture may overlap that typical of chronic active viral hepatitis or autoimmune liver disease[16-18]. Histochemical stains for copper may be useful in the diagnosis of copper overload in WD, even though negativity for copper stains cannot exclude the diagnosis of WD. This situation is a consequence of the uneven distribution of copper overload throughout the liver[19]. Pilloni et al[20] carried out on a large series of liver biopsies from WD patients, clearly shown that Timm's silver stain is the most sensitive method for revealing copper overload in WD. In clinical practice, in order to increase the diagnostic value of histochemistry, multiple copper stains are required. These histochemical stains include rhodamine, orcein and rubeanic acid methods. The latter, when performed on microwave-treated sections, represents a very sensible and quick method for the detection copper granules[21]. Moreover, transmission electron microscopy represents another useful tool in the diagnosis of WD, since electron microscopy allows the detection of characteristic mitochondrial changes. Mitochondrial changes in shape, electron density of the matrix and enlarged or narrow cristae have been reported. These mitochondrial changes are generally considered typical of WD-related liver disease although they are not specific[22,23].

The aim of our study was to analyze the hepatic elementary histological lesions in a large series of Sardinian WD patients, in order to better define the diagnostic criteria and help the pathologist in the interpretation of liver biopsy in WD carriers.

Patients and Methods

We examined 127 liver biopsies from 43 patients with an established diagnosis of WD, *via* clinical, biochemical or genetic methods, according to the Leipzig score[24]. Among the 43 patients analyzed, 22 were males and 21 females. The patient's age ranged from 1 year up to 52 years. The median age at presentation was 16 years old.

Fine liver biopsies from WD patients were obtained by the Menghini technique under ultrasound guidance. The biopsies were collected between 1980 and 2018 in the Pathology Unit of the University Hospital of Cagliari. Liver biopsies were subdivided into two groups: group 1 including 43 biopsies obtained at presentation in the absence of any therapy and group 2 including 84 subsequent biopsies obtained during the follow up. The therapy included restriction of dietary copper intake, lifelong medical therapy with a standard regime of penicillamine, trientine, and/or zinc sulfate. Each biopsy was formalin-fixed, and paraffin embedded. Four-micron-thick sections were stained with hematoxylin & eosin, silver stain and immune-stained for keratin 7. All the specimens were contemporarily examined at the multi-heads' microscope by two pathologists (DF, GF) and a detailed histological report was prepared describing all elementary lesions. Steatosis was considered present when more than 5% of hepatocytes were involved. Steatosis was evaluated as mild (between 5% and 33%) moderate (between 33% and 66%) and severe (more than 66%). Steatosis topographic location was also recorded. Inflammation was graded according to the number of foci at 200 HPF in the acinus and to the density of inflammatory cells in portal space as follows. Grade 0 when no lobular foci nor portal inflammation were found. Grade 1 when less than 2 foci and/or mild inflammation

Table I. Steatosis.

	Present		Absent	
Steatosis	102	80.3%	25	19.7%
Microvesicular	9	7.1%		
Macrovesicular	34	26.8%		
Micro- and macrovesicular	59	46.5%		

in portal space were observed. Grade 2 when 3 or 4 foci and mild or moderate inflammation in portal spaces were present. Grade 3 when more than 4 foci and moderate inflammation in portal space were detected. Cell type composition of inflammation was also recorded.

Staging of fibrosis was based on the evaluation of the presence of sinusoidal (present or absent), peri-terminal vein (present or absent), and peri-portal fibrosis, bridging fibrosis and cirrhosis. Only biopsies with at least 10 complete portal space were included in the fibrosis evaluation. While those biopsies that did not fulfill the criteria were considered not valuable for fibrosis staging, even though they were analyzed for other features.

Moreover, all the elementary lesions previously described in WD (hepatocyte ballooning with/without Mallory-Denk bodies, glycogenated nuclei, lypogranuloma) were evaluated. Any adjunctive lesion was recorded when present. Finally, in each biopsy a diagnostic pattern conclusion was performed according to the following definitions. Steatosis pattern when steatosis was the only feature. Hepatitis when inflammation without significant steatosis was found. Steatohepatitis when both features were observed.

Statistical analysis was used to compare the features found in the first 43 biopsies in the absence of any therapy versus the 84 subsequent biopsies obtained during the patient's follow up. *p*-value was calculated with one-tailed and two-tailed Fisher's tests, Pearson's chi-squared test and Yates's chi-squared test. The difference between the means were considered to be statistically significant if $p<0.05$. No adjustments for multiple comparisons were made. Analyses were performed by an informatic engineer (PP).

Results

The histological picture of the 127 liver biopsies of the 43 patients with WD evidenced a marked inter-individual variability. Steatosis was the most common lesion detected in 102 out of 127 liver biopsies (80.3%) (Figure 1A, B). A mixed micro- macro-vesicular steatosis was observed in the majority of cases (59/102).

The severity of steatosis was not related to the age of patients ($p = 0.42$).

In 55 biopsies all acinar zones were involved by steatosis, while in the remaining 47 it was mainly periportal (zone 1).

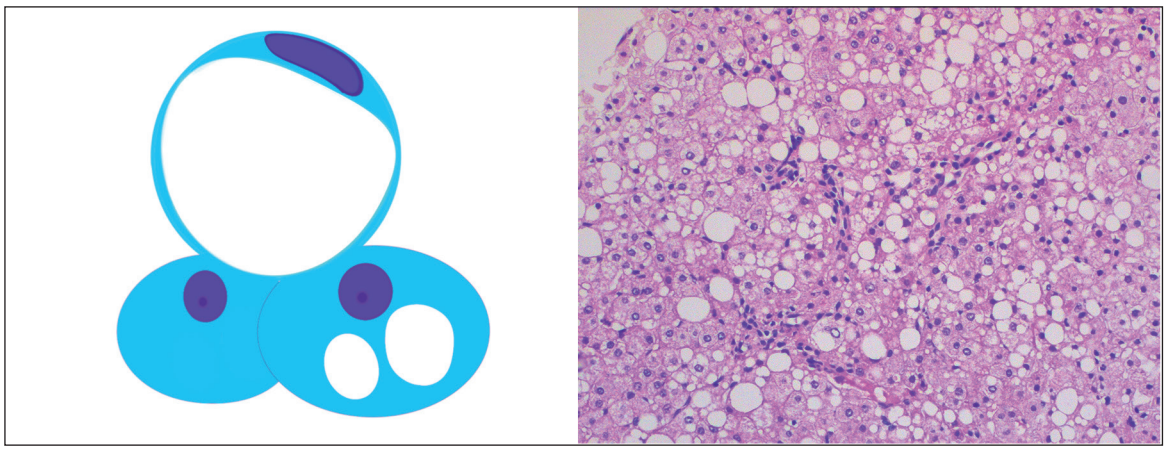

Figure 1. Schematic representation **(A)** and histological picture **(B)** of diffuse micro- macro- vesicular steatosis in Wilson disease (H&E; magnification 10×).

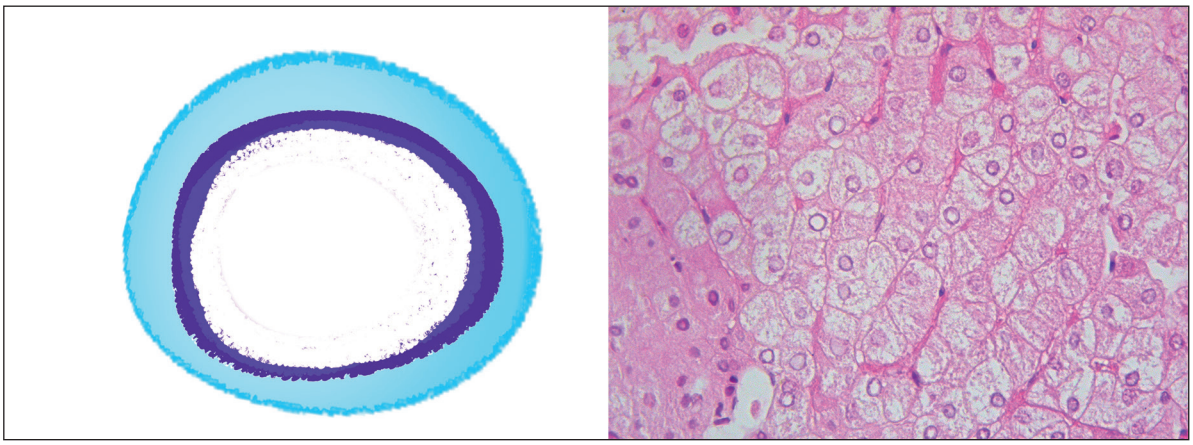

Figure 2. Schematic representation **(A)** and histological picture **(B)** of glycogenated nuclei (H&E; magnification 40×).

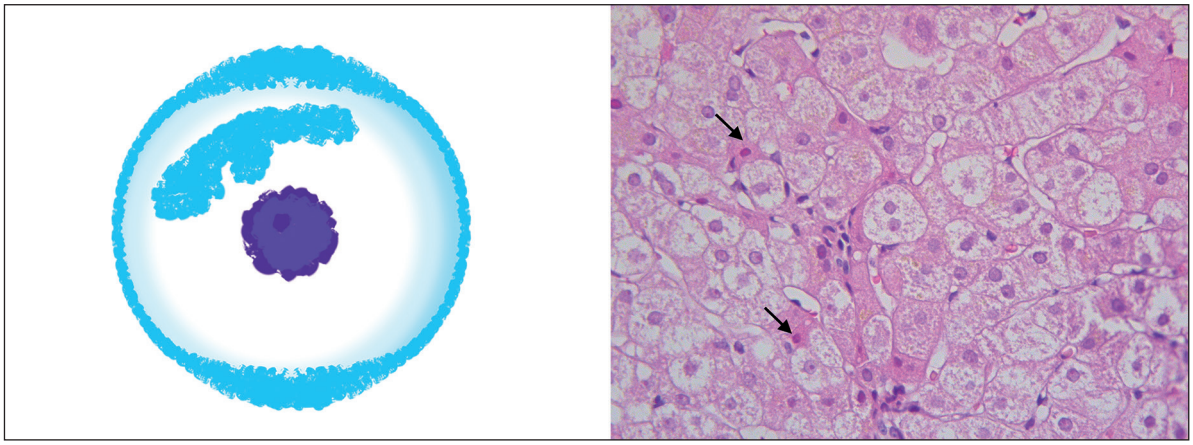

Figure 3. Schematic representation **(A)** and histological picture **(B)** of hepatocytic ballooning. Scattered apoptotic cells (*arrows*) are also present (H&E; magnification 40×).

Glycogenated nuclei were observed in 90 biopsies (Figure 2A, B). They were more frequent in the periportal areas, although occasionally detected in all acinar zones.

Balloon hepatocytes were observed in 86 biopsies (Figure 3A, B). They were always found in the periportal zones and were absent in the centrilobular areas. In a minority of cases, 29 out of 127 biopsies, Mallory-Denk bodies were found inside the cytoplasm of periportal balloon cells.

In 20 out of 127 (15.7%) biopsies there was not inflammation (grading 0). In the 107 remaining cases, the inflammation involved both the portal spaces and the intra-acinar zones (Figure 4). In the majority (70/127), grade 1 of both portal and lobular inflammation was observed. Grade 2 was

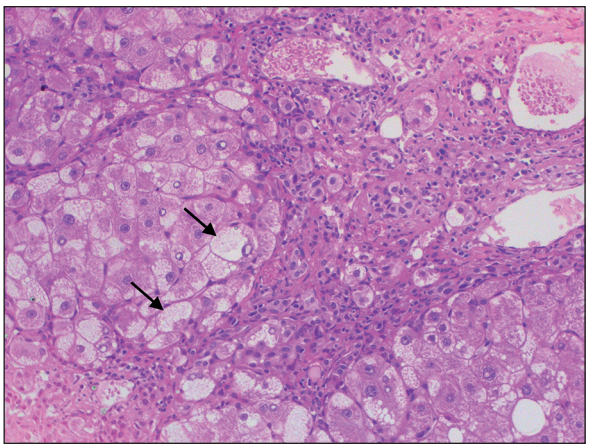

Figure 4. Portal and periportal inflammation in Wilson disease. Periportal balloon cells are also found (*arrows*) (H&E; magnification 20×).

Table II. The main features of the histological picture.

Features	Number	%
Ballooning	86	67.7%
Mallory-Denk bodies	29	22.8%
Glycogenated nuclei	90	70.9%
Regenerative nuclear changes	40	31.5%
Ductular reaction	35	27.6%
Groundglass cytoplam	10	7.9%
Inflammation	107	84.3%
Grading 0	20	15.7%
Grading 1	70	55.1%
Grading 2	33	26.0%
Grading 3	4	3.1%
Lymphomonocytes	29	22.8%
Lymphomonocytes and neutrophils	73	57.5%
Lymphomonocytes, histiocytes and neutrophils	1	0.8%
Lymphomonocytes and eosinophils	3	2.4%
Lymphomonocytes, neutrophils and plasma cell	1	0.8%
Portal	107	84.3%
Lipogranulomas	45	35.4%

scored in in 33 biopsies and grade 3 only in 4 cases. In 29 biopsies only mononucleated cells were detected. In 73 biopsies scattered neutrophils were seen associated with the mononucleated infiltrate. Eosinophils and plasma cells were very rare (3 cases with eosinophils: 1 case with few plasma cells).

Forty-five biopsies showed lipogranuloma. Regenerative nuclear changes, including slight poly-dimensional nuclei and bi-nucleated hepatocytes, were shown in 40 biopsies (31.5%). In 35 biopsies (27.6%) ductular reaction was observed by immunostaining for keratin 7. Ten biopsies were characterized by hepatocytes with ground glass cytoplasm (Table II).

Fibrosis was not valuable in 15 biopsies, because of the inadequate number of complete portal spaces. Sixty-four biopsies showed bridging fibrosis. Forty-one biopsies exhibited peri-portal fibrosis. Four biopsies displayed periterminal vein fibrosis, while two biopsies were characterized by cirrhosis (Table III) (Figure 5A, B and Figure 6).

The most reported pattern conclusion was steatohepatitis, accounting 82 biopsies, followed by hepatitis in 25 biopsies and steatosis in 20 biopsies.

The comparison of all the observed features did not show any statistical significative differences between the two groups of biopsies, that is from the one of the first biopsy and the second of subsequent biopsies after therapy (Table IV). The only exception were lipogranulomas, which were found higher in number in the subsequent biopsies than in the first one, and fibrosis, which was more advanced in the subsequent biopsies than in the first one. The two patients with cirrhosis did not undergo any further fine-needle liver biopsy.

Discussion

In the present study, we analyzed the histological picture of the liver in a large series of biopsies from patients affected by Wilson's disease, with the aim of identifying the most typical elementary lesions of this congenital disorder of copper metabolism. The clinical presentation and the follow-up were homogeneous in our WD patient population. The most frequent symptoms and signs were related with liver function and chronic hepatitis without any meaningful clinical differences among the patients. Acute liver failure, noteworthy neurological and psychiatric manifestations were evidenced.

Table III. Fibrosis staging.

Staging	Number	%
Absent	1	0.8%
Peri–terminal vein fibrosis	4	3.1%
Peri-portal fibrosis	41	32.3%
Bridging fibrosis	64	50.4%
Cirrhosis	2	1.6%
Not valuable	15	11.8%

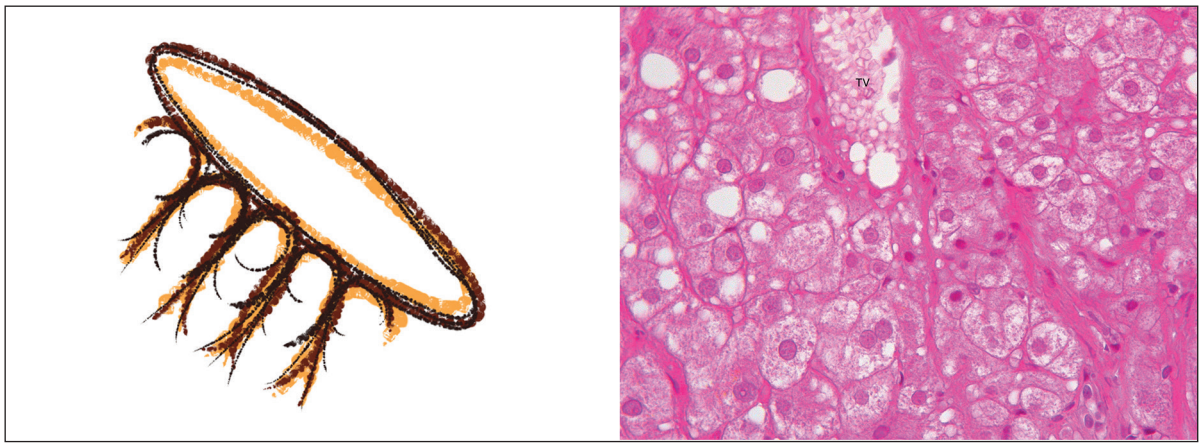

Figure 5. Schematic representation **(A)** and histological picture **(B)** of periterminal chicken wire fibrosis (zone 3). TV= terminal vein.

Our results clearly evidence a marked interindividual variability regarding the histological picture of the liver in WD patients. This variability showed a huge range. In one side there was the steatosis pattern, which was characterize by steatosis and/or glycogenated nuclei. Then, another pattern was steatohepatitis, in which the presence of balloon cells and Mallory-Denk bodies were similar to what is normally found in ALD or in NASH. The further pattern was hepatitis, where inflammation was alike to that usually observed in viral hepatitis. The marked variability of the multiple histological patterns here described confirms previous reports on the interindividual variability of the morphological patterns of WD-related liver disease[25] and lays stress on the complexity of performing the diagnosis of WD when only based on morphological data.

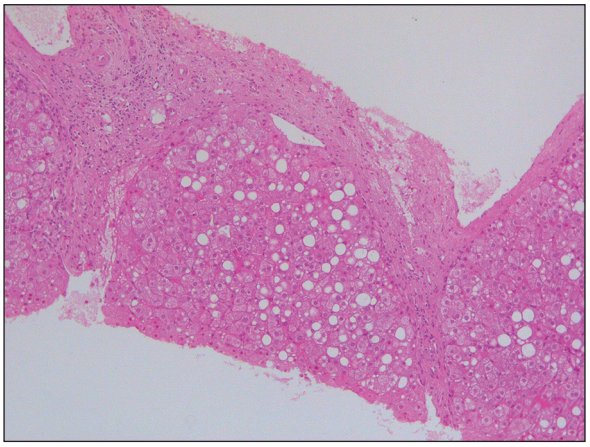

Figure 6. Bridging fibrosis in Wilson Disease (H&E; magnification 5×).

The liver histological picture in WD was the main focus of our study. Regarding the frequency of liver changes, the association of steatosis and glycogenated nuclei represented the most typical feature of WD, being detected in the vast majority of cases. The two features were observed respectively in 102 and 90 biopsies out of 127 biopsies analyzed. We did not observe steatosis in 25 biopsies.

Macrovesicular steatosis, with large and medium-sized lipid droplets, mixed with microvesicular steatosis, with small-sized lipid droplet, represented the most frequent feature of WD patients in this study. As for the predominant localization of steatosis in the different acinar zones, the most frequent picture was a diffuse pattern, characterized by the involvement of the vast majority of hepatocytes. In the remaining cases, steatosis was mainly found in the periportal areas, suggesting that in WD acinar zone 1 represents the initial site of fat droplet accumulation. Then, the progressive recruitment of zone 2 and 3 hepatocytes follows. The process ends with diffuse steatosis in the advanced phases of WD-related liver disease. This finding accounts for the central role of copper overload-related oxidative stress in the pathogenesis of liver disease in WD carriers, since copper storage is mainly localized in periportal hepatocytes. In other words, given that periportal hepatocytes represent the typical site of copper storage in WD[20], the finding of a preferential periportal steatosis reinforces the hypothesis that fatty changes are strictly related to the oxidative stress caused by free copper storage in periportal hepatocytes[21].

Table IV. Comparison between first biopsy and subsequent biopsies after therapy.

Features	First biopsy		Subsequent biopsies	
Steatosis	**34**	**79.1%**	**68**	**81.0%**
Microvesicular	4	9.3%	5	6.0%
Macrovesicular	15	34.9%	19	22.6%
Micro- and macrovesicular	15	34.9%	44	52.4%
Balloning	33	76.7%	53	63.1%
Mallory-Denk bodies	6	14.0%	23	27.4%
Glycogenated nuclei	30	69.8%	60	71.4%
Regenerative nuclear changes	11	25.6%	29	34.5%
Ductular Reaction	11	25.6%	24	28.6%
Groundglass cytoplam	3	7.0%	7	8.3%
Inflammation	**39**	**90.7%**	**68**	**81.0%**
Grading 0	4	9.3%	16	19.0%
Grading 1	24	55.8%	46	54.8%
Grading 2	13	30.2%	20	23.8%
Grading 3	2	4.7%	2	2.4%
Lymphomonocytes	11	25.6%	18	21.4%
Lymphomonocytes and neutrophils	26	60.5%	47	56.0%
Lymphomonocytes, hystiocytes and neutrophils	0	0.0%	1	1.2%
Lymphomonocytes and eosinophils	1	2.3%	2	2.4%
Lymphomonocytes, neutrophils and plasmacells	1	2.3%	0	0.0%
Portal	39	90.7%	68	81.0%
Lipogranulomas	9	20.9%	35	41.7%
Fibrosis	**33**		**79**	
Absent	0	0.0%	1	1.2%
Peri–terminal vein fibrosis	3	7.0%	1	1.2%
Peri-portal fibrosis	11	25.6%	30	35.7%
Bridging fibrosis	17	39.5%	47	56.0%
Cirrhosis	2	4.7%	0	0.0%
Not valuable	10	23.3%	5	6.0%

From a practical point of view, a liver picture characterized by steatosis and glycogenated nuclei makes the differential diagnosis with ALD, NAFLD/NASH and diabetic liver disease very difficult, and often impossible, when only based on morphology[26]. The only differential finding between WD and ALD or NAFLD is represented by the preferential localization of steatosis in the acinar zone 1 in WD, whereas in ALD and NAFLD steatosis typically originates in the pericentral areas (acinar zone 3), where oxidative stress and oxidative cellular damage originate[27,28]. In clinical practice, the detection in a liver biopsy of a preferentially periportal steatosis, in the absence of a significant steatosis of zone 3 hepatocytes, should be considered a finding against the diagnosis of ALD or NAFLD and should induce to consider other possible etiologies, including Wilson's disease. However, when the biopsy came from the liver of a child, as it often happen, unfortunately, this clue is not helpful, since in children NAFLD liver steatosis may show preferential periportal localization[29,30]. In this situation, the third feature associated with the diagnosis of WD in liver biopsies in this study is represented by hepatocellular ballooning, that becomes the most important evidence. Despite this finding was reported in the histological pattern of both WD and NAFLD/NASH[31], in our study, it appeared always localized in the periportal zone and it was never observed in periterminal zone (zone 3). The preferential localization of balloon cells in zone 1 may support the hypothesis that, in WD, ballooned hepatocytes represent a morphological sign of copper toxicity, being copper mainly stored in periportal hepatocytes[32]. From a practical point of view, the preferential localization of balloon cells in zone 1 hepatocytes in WD may represent a relevant tool in the differential diagnosis with NAFLD/NASH, in which ballooned hepatocytes are typically restricted to the periterminal areas (zone 3)[33].

Even though Mallory-Denk bodies have been reported as a typical feature of WD[34], in this study they were not detected in a large number of liver biopsies. Mallory-Denk bodies were observed in 29 out of 127 (22.8%) biopsies. Moreover, when present, Mallory-Denk bodies were few, scattered

and mainly localized in the periportal areas. The pathogenesis and the clinical significance of Mallory-Denk bodies in Wilson's disease has not been clarified yet. In recent years, the finding of reactivity of Mallory-Denk bodies for p62, a protein involved in oxidative stress that binds to ubiquitinated proteins, suggested that Mallory-Denk bodies might represent an agglomerate of misfolded proteins induced by Cu-induced oxidative stress[35]. According to this hypothesis, the detection of Mallory-Denk bodies in WD might indicate high levels of hepatocellular copper toxicosis, leading to protein misfolding, ending with liver cell death.

The comparison between the two groups revealed that the treatment and the disease progression did not modify the main morphological picture. Either in the first biopsy and in the subsequent biopsies after therapy interindividual variability was observed. The histological picture ranged from steatosis to a steatohepatitis and to hepatitis pattern. The statistical analysis evidenced differences only in the number of lipogranumas and fibrosis progression in the second group. However, this data can be easily explained by the unavoidable disease progression that neither the treatment and diet can prevent[36].

Conclusions

The histopathological analysis of liver biopsy in a subject affected by Wilson's disease can give very important data regarding a potentially reversible condition[25,37]. In a percentage of patients, WD may evolve towards a severe liver disease, ending with cirrhosis and liver transplant[38,39]. In fact, under the definition of WD-related liver disease, a spectrum of different liver diseases may be included, ranging from simple, benign and possible reversible steatosis to steatohepatitis with progressive liver fibrosis that may end with cirrhosis and hepatocellular carcinoma (HCC). Our data confirm the marked variability of the histological pattern in WD, ranging from simple steatosis to steatohepatitis and hepatitis, with overlapping features with viral and autoimmune hepatitis. Moreover, this study indicates some histological changes which may be helpful in the differential diagnosis between WD and ALD or NAFLD. The finding of a preferential localization of steatosis and balloon cells in periportal hepatocytes, according with our study, should be considered a clue in clue in order to suspect WD. Given the absence of any specific histological liver changes in WD a strict correlation between histological, laboratory and clinical data remains mandatory, so as to rich this complex diagnosis.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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