Study of Biochemical and Clinical Markers in Steatohepatitis Related to Obesity

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Nonalcoholic fatty liver disease (NAFLD) is highly associated to obesity and comprises several liver diseases, from simple steatosis to steatohepatitis (NASH) with increased risk of developing progressive liver fibrosis, cirrhosis and hepatocellular carcinoma. Liver biopsy is the gold standard in diagnosing the disease, but it cannot be used in a large scale. The aim of the study was the assessment of some non-invasive clinical and biological markers in relation to the progressive forms of NAFLD. We performed a prospective study on 64 obese patients successively hospitalised for bariatric surgery in our Surgical Unit. Patients with history of alcohol consumption, chronic hepatitis B or C, other chronic liver disease or patients undergoing hepatotoxic drug use were excluded. All patients underwent liver biopsy during sleeve gastrectomy. NAFLD was present in 100% of the patients: hepatic steatosis (38%), NASH with the two forms: with fibrosis (31%) and without fibrosis (20%), cumulating 51%; 7 patients had NASH with vanished steatosis. NASH with fibrosis statistically correlated with metabolic syndrome (p = 0.036), DM II (p = 0.01) and obstructive sleep apnea (p = 0.02). Waist circumference was significantly higher in the steatohepatitis groups (both with and without fibrosis), each 10 cm increase increasing the risk of steatohepatitis (p = 0.007). The mean values of serum fibrinogen and CRP were significantly higher in patients having the progressive forms of NAFLD. Simple clinical and biological data available to the practitioner in medicine can be used to identify obese patients at high risk of NASH, aiming to direct them to specialized medical centers.

Keywords: Noninvasive Markers, NAFLD, Steatohepatitis, Obesity, Inflammatory markers

Nonalcoholic fatty liver disease (NAFLD) is highly associated with obesity, metabolic syndrome (MS) and type II diabetes (DM II) and defines a spectrum of liver disease ranging from simple steatosis to steatohepatitis (NASH) - the progressive form of the disease; the latter may exhibit varying degrees of hepatic fibrosis and may progress to cirrhosis and end stage liver disease; it also associates an increased risk of developing hepatocellular carcinoma [1]. NAFLD affects 15% of the non-obese population and 65% of grade I and II obese people; a higher percentage of the disease (more than 85%) is found in morbidly obese people, making it one of the most important modern public health issues in the world. The prevalence of the progressive form of NAFLD (NASH) ranges between 20 and 40% in obese patients [2], which should be worrying for public health systems [3].

Hepatic biopsy is the *gold* diagnostic method in NAFLD as it remains nowadays the only way to differentiate between the simple and the progressive forms of the disease, which involves lobular inflammation, hepatocyte balooning, accompanied or not by hepatic fibrosis. Though, it can not be used for the screening of NAFLD not only because it is an invasive, expensive, requiring qualified staff and risk-taking method [4] but also because it should be applied to a very large number of patients, given that NAFLD is a pandemic condition. Therefore, continuous and intense efforts are being made to identify noninvasive markers that could participate in NAFLD diagnosis. Among the directions with increasing interest in their use as non-invasive markers are the elastometry tests which seem to be more and more reproducible and reliable nevertheless highly accepted by patients [5, 6].

The aim of the study was to evaluate to what extent simple and available clinical and biological data can be used as noninvasive markers of the progressive form of NAFLD in obese patients.

Experimental part

Materials and methods

We performed a prospective study on 64 obese patients successively hospitalised for bariatric surgery in our Surgical Unit between November 2014 and November 2016. We included only patients aged over 18 years with medical indication of bariatric surgery. Exclusion criteria: patients with history of alcohol consumption (over 20g/day in women and over 30g/day in men), chronic hepatitis B or C, other chronic liver disease or patients undergoing hepatotoxic drug use. We also excluded patients with renal, endocrine or other chronic diseases that could affect metabolic or cardiovascular functions. All patients signed the Informed Consent approved by the Ethics Committee of the University of Medicine and Pharmacy Gr. T. Popa Iasi, according to the requirements of the Declaration of Helsinki and in accordance to some published models [7-9]. All patients underwent full clinical evaluation including personal and medical history and complete clinical examination. Anthropometric measurements were noted: BMI (body mass index) was calculated by the formula G (kg)/T^2 (cm) and waist circumference was measured in centimeters, halfpoint between the last rib and iliac spines. Routine and special biological tests were measured from the venous blood samples colected after a fasting night. CRP (C reactive protein) with normal values ranging between 0 and 0.50 mg/dL and serum fibrinogen with normal values ranging between 200 and 400 mg/dL were considered to evaluate the inflammatory status of the patients. The lipid metabolism was assessed by serum values of cholesterol, tryglicerides, HDL Chol and LDL Chol. The presence of MS, OSA (obstructive sleep apnea),

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hypertension and DM II was noted. All patients included in our study underwent liver biopsy during bariatric surgery consisting of laparoscopic sleeve gastrectomy. The biopsy specimens measured at least 1.4/0.4 cm and were evaluated by an experienced anatomopathologist who had no access to the clinical or biological data of the patients; hepatic tissue was fixed in buffered formalin and stained with hematoxylin-eosin and van Gieson. NAFLD in its various forms was diagnosed using Kleiner score [10]. The necroinflammatory activity score of NAFLD was also evaluated using the score system developed by Brunt [11].

The database was completed using Microsoft Excel 2013 version and the statistically analysis was performed in SPSS V.19.0. Continuous variables were expressed using mean, median and standard deviation (SD) values. Significance level (p-value) was considered to be 0.05 (5%) with 95% probability (confidence interval). The t-Student test, the ANOVA test or chi-square test were used to verify the statistical significance. We also calculated the odd ratio (OR-the chances of those exposed to present a certain feature are OR higher than the chances of the unexposed ones).

Results and discussions

The age of the patients in our study ranged between 18 and 60 years, with an average of 41.33 years ± 11.9 SD. The mean BMI was 45.06 kg/m^2± 6.67 DS, ranging between 35 and 67 kg/m^2. The male patients were significantly fewer (23.4%) than the females. We noted the presence of MS in 46.9% of our patients. DM II was present in 18.8% of cases, arterial hypertension in 32.8%, dyslipidemia in 92 % and OSA in 65.6 % of cases.

Histopathological evaluation of liver biopsies revealed the presence of NAFLD in 100% of the patients, at it follows: hepatic steatosis (38%), steatohepatitis in which we noted two forms: with fibrosis (31%) and without fibrosis (20%), cumulating 51%; 11% of cases (7 patients) had NASH with *vanished steatosis* as the histopathological examination revealed specific NASH changes (lobular inflammation, hepatocyte balooning with or without fibrosis) in the absence of macrovesicular steatosis, the condition for defining steatohepatitis. No necroinflammatory activity was noted in 50% of patients (NAS = 0), 43.75% of patients had mild necroinflammatory activity (NAS = 1) and 6.25% had moderate activity (NAS = 2). Liver fibrosis was present in 25 patients (39%), 18 patients had mild fibrosis, 4 medium fibrosis and 3 severe fibrosis; none of the patients had liver cirrhosis.

BMI was higher in the steatohepatitis group compared to the simple steatosis group (which we considered the basis of comparison because it is the benign form of the disease) but the results did not show statistical significance. In contrast, waist circumference was significantly higher in the steatohepatitis groups (both with and without fibrosis) compared to the hepatic steatosis group (table 1).

In patients diagnosed with MS, the proportion of histopathological form of NASH with fibrosis was nearly double (65%) compared to the proportion of simple steatosis (33.3%), statistically confirmed by the chi-square test (λ^2 = 4.385, p = 0.036). Data analysis also confirmed (p = 0.023) that the proportion of patients with NASH was about 3 times higher compared to patients with simple steatosis (45.5% versus 16.7%) in hypertensive patients. Regarding dyslipidemia, frequency analysis did not reveal statistically significant differences except for the comparison between the NASH with fibrosis group and simple steatosis group, the elevated levels of LDL Col correlating positively with the presence of NASH with fibrosis (λ^2 = 4.227, p = 0.04). We noted the statistically significant positive association between DM II and NASH with fibrosis when comparing this group with the group of patients dignosed with hepatic steatosis alone (λ^2 = 6.229, p = 0.01). Regarding OSA, our data proved that its presence was statistically associated with NASH, either with fibrosis (p = 0.01) and without fibrosis (p = 0.02).

Assessing the risk of waist circumference increase (in 10 cm thresholds) and BMI increase on the occurrence of specific liver disease within NAFLD, the results showed that each 10 cm increase in WC increased the risk of steatohepatitis 2.64 times (p = 0.007). The risk of NASH with fibrosis was increased by 3.7 times (p = 0.04) by the presence of MS and 6.8 times (p = 0.02) by the DM II; hypertension was associated with an increased risk of NASH (OR = 4.1, p = 0.02) and NASH with fibrosis (OR = 4.0, p = 0.04). Also, the presence of OSA was statistically associated with a significant increase in risk of NASH with fibrosis (OR = 9.4, p = 0.03) and simple NASH (OR = 5.5, p = 0.02), as showed in table 2.

Regarding the serum inflammatory markers, we firstly observed that the mean value of serum Fb was normal in patients with steatosis (379.83 mg/dL ± 43.89 SD) and pathologically increased in patients with NASH (421 mg/dL ± 44.84 SD), either in case of histopathological form with fibrosis (423.55 mg/dL ± 47.00 SD) or without fibrosis (417.08 mg/dL ± 42.86 SD) and in the NASH with *vanished steatosis* group (403.43 mg/dL ± 52 SD). Also, the mean value of serum CRP was normal (0.47 mg/dL ± 0.45 SD) in patients diagnosed with the benign form of NAFLD (steatosis) and pathologically increased in patients with NASH (1.07 mg/dL ± 0.94 SD), either with fibrosis (1.10 mg/dL ± 0.96SD) or without fibrosis (1.03 mg/dL ± 0.95 SD) and in the NASH with *vanished steatosis* group (1.48 mg/dL ± 1.08 SD). As presented in table 3, we found that the mean values of serum Fb and CRP were significantly higher in patients diagnosed with the progressive forms of NAFLD, namely NASH (both with and without fibrosis), but also in patients with NASH with fibrosis, when considered separately.

The risk analysis statistically confirmed that the increased serum value of Fibrinogen and CRP increases the risk of NASH and the specific subgroup of NASH with fibrosis (table 4).

Waist circumference	Mean	C.I. 95%		SD	Min.	Max.	p
		Min.	Max.				
Steatosis	125.29	117.84	132.75	17.657	89	148	
NASH	139.09	133.54	144.64	15.643	100	159	***0.003***
Simple NASH	136.92	126.93	146.91	16.530	103	159	***0.05***
NASH + fibrosis	140.50	133.34	147.66	15.306	100	158	***0.004***
NASH "vanished steatosis"	134.29	119.29	149.28	16.214	8	44	0.23

Table 1
DIFFERENCES IN ANTHROPOMETRIC INDEX MEAN VALUES ACCORDING TO THE CONSTITUTED LIVER DISEASES IN NAFLD

Risk factor	NAFLD	OR	95% C.I.		p
			Min.	Max.	
Waist Circumference – each 10 cm increase	Simple steatosis	0.63	0.45	*0.88*	***0.007***
	NASH	2.64	1.14	*2.36*	***0.007***
	Simple NASH	1.51	0.74	3.08	0.25
	NASH with fibrosis	1.40	0.92	1.77	0.82
	NASH "vanished steatosis"	1.51	0.73	3.08	0.25
Increasing in obesity grade (II-III)	Simple steatosis	1.06	0.27	4.08	0.93
	NASH	0.90	0.22	3.61	0.88
	Simple NASH	1.20	0.11	12.88	0.88
	NASH with fibrosis	1.11	0.098	1.83	0.92
	NASH "vanished steatosis"	1.20	0.11	12.88	0.8
Metabolic syndrome	Simple steatosis	0.409	0.143	1.172	0.09
	NASH	2.714	0.909	8.105	0.07
	NASH with fibrosis	3.714	1.063	12.975	***0.04***
	Simple NASH	1.714	0.431	6.826	0.44
	NASH "vanished steatosis"	1.500	0.268	8.383	0.64
DM II	Simple steatosis	0.307	0.060	1.576	0.15
	NASH	4.397	0.468	9.831	0.12
	NASH with fibrosis	6.828	1.228	7.952	***0.02***
	Simple NASH	1.735	0.474	6.350	0.40
	NASH "vanished steatosis"	4.805	0.458	50.375	0.19
Hypertension	Simple steatosis	0.271	0.078	0.938	0.3
	NASH	4.167	1.166	4.890	***0.02***
	NASH with fibrosis	4.091	1.020	6.403	***0.04***
	Simple NASH	4.286	0.928	9.796	0.06
	NASH "vanished steatosis"	2.000	0.282	14.198	0.48
OSA	Simple steatosis	0.127	0.040	0.406	0.1
	NASH	7.500	2.235	9.165	***0.01***
	NASH with fibrosis	9.444	2.151	11.475	***0.03***
	Simple NASH	5.556	1.200	5.712	***0.02***
	NASH "vanished steatosis"	10.000	1.030	97.044	***0.04***

Table 2
ANALYSIS OF CLINICAL FACTOR RISKS ON NAFLD

Serum Inflammation Marker	NAFLD	Mean	C.I. 95%		SD	Min.	Max.	p
			Min.	Max.				
Fibrinogen (mg/dl)	Steatosis	379.83	361.30	398.37	43.89	288.00	460.00	
	NASH	421.00	405.10	436.90	44.84	298.00	500.00	***0.002***
	NASH with fibrosis	423.55	401.55	445.55	47.00	298.00	493.00	***0.003***
	Simple NASH	417.08	391.18	442.98	42.86	359.00	500.00	***0.01***
	NASH "vanished steatosis"	403.43	355.34	451.52	52.00	315.00	482.00	**0.8**
CRP (mg/dl)	Steatosis	0.47	0.28	0.66	0.45	0.20	2.30	
	NASH	1.07	0.74	1.40	0.94	0.28	4.60	***0.02***
	NASH with fibrosis	1.10	0.65	1.55	0.96	0.36	4.60	***0.01***
	Simple NASH	1.03	0.45	1.60	0.95	0.28	3.20	**0.06**
	NASH "vanished steatosis"	1.48	0.48	2.48	1.08	0.46	3.00	***0.05***

Table 3
INFLAMMATORY SERUM MARKERS IN NAFLD

Risk factor	NAFLD	OR	C.I. 95%		p
			Min.	Max.	
Increased Fibrinogen value	Steatosis	0.994	0.977	1.010	0.40
	NASH	1.022	1.007	1.038	***0.05***
	NASH with fibrosis	1.023	1.002	1.044	***0.03***
	NASH "vanished steatosis"	1.013	0.99	1.035	0.23
Increased CRP value	Steatosis	0.883	0.436	1.791	0.73
	NASH	8.849	1.501	52.158	***0.01***
	NASH with fibrosis	3.698	0.917	14.916	***0.05***
	NASH "vanished steatosis"	5.618	1.306	24.159	***0.02***

Table 4
RISK ANALYSES OF INCREASED OF SERUM INFLAMMATORY MARKERS FOR NAFLD

Referring to the natural history of NAFLD we must take into account all pathological forms comprised in the spectrum of this disease, from liver steatosis to steatohepatitis the progressive and aggressive form of the disease, accompanied or not by liver fibrosis. The occurrence of hepatic fibrosis reaches 40-50% in patients with NASH and the results in our study support this data. This has a great importance, as a recent study of 619 patients followed for an average of 12.6 years shows that liver fibrosis, independent of the severity of any other liver histological modification and independent of the non-alcoholic fatty liver disease activity score, is the most important histopathological finding associated with an increased rate of general and hepatic mortality, with an increased likelihood of developing a liver complication [12]. Our results concerning the prevalence of NASH are concordant with literature data showing that up to 59.1% of NAFLD patients have the progressive form of liver disease [13]. Cases when the histopathological examination revealed important changes such as lobular inflammation, hepatocyte ballooning, accompanied or not by hepatic fibrosis but lacking macrovesicular liver steatosis, should be considered, especially when it comes to patients with

an increased risk of NAFLD due to obesity, MS or type II DM. These patients were considered as NASH with *vanished steatosis* in agreement with other researchers in NAFLD, finding patients with the same histopathological condition [14]. It is important to underline that patients with this special liver histopathology type associated abnormal mean values of inflammatory serum markers (CRP and fibrinogen).

Anthropometric indices are valuable in medical practice primarily because of their ease of use. As our data also show, waist circumference, the index that reflects excessive visceral fat, may be a good predictor of NASH in obese patients. In our study, the mean waist circumfference was significantly higher in NASH patients (with and without fibrosis) comparing with simple steatosis patients. The link between inflammatory hepatic changes (lobular inflammation and hepatocyte ballooning) and the excess of visceral adipose tissue can be explained by at least two pathological mechanisms: the ability of excess visceral fatty tissue to secrete proinflammatory factors (IL-6, IL-8, TNFα, leptin) and the hepatic lipotoxicity caused by the increased free fatty acids flux directly into the liver following increased lipolysis in excess of visceral fat tissue affected by insulin resistance [15]. Nevertheless, both visceral and non-visceral fatty tissue accumulation are a common pattern in patients with NAFLD [16].

The results of our study also showed significant association between the progressive form of NAFLD (NASH) and the obesity associated comorbidities. OSA and hypertension were significantly associated with an increased risk of NASH with fibrosis but also with an increased risk for overall NASH, while type II DM and MS significantly increased the risk of NASH with fibrosis. These results can be found in the previously published literature [17-21] and underline once again the importance of not only evaluating the obese patient from all points of view but of evaluating NAFLD as a disease pathologically connected with all obesity comorbidities. The importance of comorbidity assessment is very important as in obese patients there are two categories described, the metabolically healthy and metabolically unhealthy [22-27].

The inflammatory status in obese patient has some special features, as there are no autoimmune diseases or infectious pathologies involved. Our data confirmed first of all that in our patients, diagnosed with obesity and NAFLD, there were important imbalances in inflammatory status, quantified by serum values of CRP and fibrinogen. When compared to the benign form of NAFLD (steatosis), the high values of mean serum inflammatory markers correlated with its progressive forms (NASH with and without fibrosis). More, the increased values of serum CRP associated a significantly increased risk of NASH (OR = 8.84, p = 0.01) and NASH with fibrosis (OR = 3.69, p = 0.05) and it seems that the CRP value is a more important marker for the NASH prediction than the serum fibrinogen value. A study of 100 patients with histopathologically certified NAFLD underlines the importance of PCR assessment as a marker of steatohepatitis and, moreover, advanced liver fibrosis in patients with NASH [23]. Not the least, NAFLD and the metabolic driven comorbidities pose serious problems related to the adherence and compliance of patients therefore would continue being a serious public health issue [28]. The results of our study support the fact that the obese patients are in a state of chronic inflammation; more importantly, they show that the pathological changes of inflammatory serum markers mirror to some extent the status of hepatic impairment within NAFLD.

Conclusions

Diagnosing the degree of hepatic impairment in NAFLD prior to the severe hepatic fibrosis development has a major importance, but liver biopsy can not be used in NAFLD screening. Anthropometric indices as well as clinical data such as the presence of MS, DM II and OSA can be used to select a target group to be screened for NAFLD. Waist circumference, more than BMI, reflects the possible presence of NASH. Inflammatory markers such as serum CRP and fibrinogen values can serve as simple, reliable, noninvasive diagnostic markers for NASH, the progressive form of NAFLD. Simple clinical and biological data available to the practitioner in medicine can be used to identify obese patients at high risk of NASH, aiming to direct them to specialized medical centers.

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