Obesity Surgery, **17**, *155-161*

Influence of Liver Biopsy Heterogeneity and Diagnosis of Nonalcoholic Steatohepatitis in Subjects Undergoing Gastric Bypass

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Background: Nonalcoholic fatty liver disease (NAFLD) is a chronic condition that can progress to cirrhosis and hepatocellular cancer. The most progressive form of NAFLD is nonalcoholic steatohepatitis (NASH). Currently, the only method to diagnose NASH is with a liver biopsy; however. sampling error may limit diagnostic accuracy. We investigated the discordance of paired liver biopsies in individuals undergoing gastric bypass.

Methods: Two liver biopsies, composite size of ≥25 mm and ≥8 portal tracts (PTs), were obtained from the left lobe in 31 subjects. Group 1 included specimens at least 15 mm in length with ≥4 PTs compared to a second biopsy of at least 10 mm and ≥4 PTs (Group 2).

Results: The mean specimen size (number of PTs) for group 1 was 20.4±4.2 mm (11.7±5.5 PTs) and group 2 was 16.1±5.3 mm (8.2±4.1 PTs). Prevalence of NASH was 26% in Group 1 and 32% in Group 2. Sampling discordance was greatest for portal fibrosis (26%), followed by zone 3 fibrosis (13%) and ballooning degeneration (3%). The negative predictive values from Group 1 liver biopsies for NASH and portal fibrosis were only 83% and 67%, respectively.

Conclusions: The results demonstrate that significant sampling variability exists in class 2 and 3 obese individuals undergoing screening liver biopsies for NAFLD. The degree and histopathological discordance is dependent upon zonal location and types of injury. Nevertheless, a 25-mm biopsy specimen without zone 3 cellular ballooning or fibrosis appears adequate to exclude the diagnosis of NASH.

Key words: NAFLD, paired liver biopsy, sampling variability, bariatric surgery, morbid obesity, type 2 diabetes

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Abbreviations:
NIH, National Institutes of Health; **NAFLD**, nonalcoholic fatty liver disease; **FL**, fatty liver; **NASH**, nonalcoholic steatohepatitis; **IPF**, isolated portal fibrosis; **ATP III**, Third Report of the National Cholesterol Education Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults Treatment Panel; **AST**, aspartate aminotransferase; **ALT**, alanine aminotransferase; **TG,** triglycerides; **HDL**, high density lipoprotein; **OGTT**, oral glucose tolerance test

Introduction

The increasing prevalence of obesity (BMI ≥30 kg/m^2), years of life lost and cancer risks due to excessive weight have been recently highlighted.[1-3] The prevalence of individuals with class 3 or morbid obesity (BMI ≥40 kg/m^2) is approximately 5% and escalating at a greater rate than lower degrees of obesity.[4] The histological spectrum of obesity-induced liver injury, called *nonalcoholic fatty liver disease* (NAFLD), encompasses *fatty liver* (FL) with and without nonspecific inflammation and *nonalcoholic steatohepatitis* (NASH) which requires the presence of zone 3 hepatocellular ballooning or fibrosis.[5] In class 3 obese individuals, we have demonstrated the prevalence of NAFLD and suggested broadening the classification to include the presence of steatosis and portal/periportal fibrosis in the absence of zone 3 liver injury and labeled it "isolated portal fibrosis" (IPF).[6]

NASH is considered the most progressive manifestation of NAFLD.[7] Several studies have investigated the diagnostic utility of clinical and biochemical variables for NASH.[8-12] However, no combina-

tion of clinical or biochemical abnormalities can accurately differentiate the spectrum of NAFLD. Therefore, the diagnosis of NASH is based solely upon a liver biopsy which also provides grading and staging. As in other etiologies of liver disease, the sampling variability of a liver biopsy may significantly limit the diagnostic accuracy and/or staging of the disease.[13-17] To date, a report of paired percutaneous liver biopsies in subjects with elevated liver enzymes evaluated for NAFLD demonstrated significant sampling error.[18] A single small study in 10 subjects undergoing bariatric surgery also suggested NAFLD sampling heterogeneity when comparing biopsies from both lobes of the liver.[19] The aims of this study were to prospectively investigate paired liver biopsies obtained from class 2 and 3 obese individuals, to assess sampling variability and the adequate specimen size needed to accurately diagnose the spectrum of NAFLD.

Patients and Methods

The protocol was approved by the Human Investigative Committee of the University of Alabama at Birmingham.

Patient Selection

A prospective study was carried out in 58 consecutive subjects undergoing laparoscopic Roux-en-Y gastric bypass (LRYGBP) between January 2002 and September 2003 who agreed to have two liver biopsies performed. At the beginning of surgery, a needle (Bard Max-Core, Covington, GA), was used to obtain both liver biopsies from the left lobe. The liver biopsies were interpreted by two pathologists (referred to as P-1 and P-2), who were blinded to clinical data. The biopsy specimens were coded and provided in a random order to P-1 who interpreted each sample for the degree of steatosis (minimal ≤5%, mild 6-33%, moderate 34-66% and severe (67-100%); presence/absence of zone 3 hepatocellular ballooning and pericellular/perisinusoidal fibrosis and stage of fibrosis.[20] The fibrosis scoring was as follows: Stage 1a/b – zone 3 fibrosis only, Stage 1c – zone 1 fibrosis only, Stage 2 – fibrosis in both zones 1 and 3, Stage 3 – bridging fibrosis, and Stage 4 – cirrhosis. Thus, we modified our classification to assimilate the NASH Clinical Research Network as none (stage 0); mild/moderate (stages 1 and 2) and advanced (stages 3 and 4). These 3 groups, as well as the steatosis grades and presence/absence of hepatocellular ballooning, generated the sampling variability and kappa scores between the liver biopsy specimens. A second pathologist (P-2), blinded to P-1 interpretations, interpreted both liver biopsy specimens as a single composite diagnosis which was compared to the composite diagnosis from P-1 to calculate inter-observer variability.

The histologic spectrum of NALFD was classified as FL (steatosis ± inflammation), NASH (hepatocellular ballooning or ZF in either specimen or cirrhosis) and IPF (steatosis and portal fibrosis without zone 3 liver injury). The higher stage of fibrosis in either biopsy specimen defined the composite diagnosis (e.g. if ZF was stage 2 in LBx1 and stage 3 in LBx2, it was considered 'stage 3' and interpreted as advanced fibrosis). The total sample size had to be ≥25 mm and have 8 portal tracts (PTs) comprising the composite diagnosis. A minimum of 15 mm and 4 PTs were considered an adequate tissue sample (Group 1) which was compared to the second sample of at least 10 mm and ≥4 PTs (Group 2).

Common causes of liver diseases such as alcohol, viral hepatitis, autoimmune disease and medications were excluded as previously described.[6] ATP III criteria was used to define the metabolic syndrome.[21] Diabetes was defined if the fasting blood glucose level was ≥126 mg/dl, a 2-hour oral glucose tolerance test (OGTT) was >200 mg/dl or subjects were taking diabetic medications. An OGTT glucose level ≥140 mg/dl and ≤200 mg/dl was classified as impaired glucose tolerance (IGT).

Statistical Analysis

Statistical analysis was performed using SPSS (V11.5) for Windows. Data were analyzed as both continuous and categorical variables. Continuous variables were expressed as mean ± standard deviation (SD). The categorical variables and the histopathological features were described as percentages. The agreement between histological spectrums for dichotomous values (e.g. presence or absence of NASH) was decided by Kappa coefficient (κ) and ordinal values (e.g. stages of PF and

ZF). Interpretation of κ agreements are classified as: merely by chance to poor (0-0.2): agreement, fair (0.2-0.4): moderate (0.4-0.6): good (0.6-0.8): very good to perfect (0.8-1). $P<0.05$ was considered statistically significant.

Results

A total of 58 consecutive patients with paired biopsies were collected, and 31 individuals fulfilled the above criteria to be included in the study. Therefore, we investigated a total of 62 liver biopsies (31 patients). Group 1 included the larger paired specimen (≥15 mm of tissue and ≥4 PTs) compared to Group 2 (≥10mm and ≥4 PTs).

General

Our cohort consisted of 27 (87%) females, 4 males (13%), and 28 (90%) non-Hispanic whites (Table 1). Mean age was 38±9.3 years and BMI was 48±6.2 kg/m^2. Overall, ALT (mean 26.3± 19.1 IU/L) and AST (mean 22.2 ± 9.8 IU/L) levels were elevated in 26% and 3% of subjects, respectively. Hyperglycemia and hypertension (ATP III criteria) was noted in 41.9% and 58.1% subjects, respectively; type 2 diabetes was present in 25.8%. Mean FBS by OGTT was 106.0±10 mg/dl; mean serum triglyceride level was 141.9±64.1 mg/dL, elevated in 54.8%; and mean HDL was 41.0±11.0 mg/d, low in 77.4% of subjects. The metabolic syndrome was present in 80.6% of the cohort.

Table 1: General and biochemical aspects of the cohort

Variables (N=31)	Mean ± SD / %
Age (yrs)	38 ± 9.3
Race (% W)	90
Gender (% F)	87
BMI (kg/m^2)	48 ± 6.2
AST (IU/L)	22.2 ± 9.8
AST % elevated	3
ALT (IU/L)	26.3 ± 19.1
ALT % elevated	26
Triglycerides (mg/dl)	141.9 ± 64.1
HDL (mg/dl)	41.0 ± 11.0
Fasting blood sugar (mg/dl)	106.0 ± 10.0
DM %	25.8
Hyperglycemia ATP III	41.9
Hypertension %	58.1
MS %	80.6

AST, aspartate aminotransferase; ALT, alanine aminotransferase; DM, diabetes; MS, metabolic syndrome.

Sampling Variability of Paired Biopsies

Liver Tissue Sample Size

In Group 1, the mean LBx size was 20.4±4.2 mm (range 15-30 mm) and the mean number of PTs was 11.7±5.5 (range 4-22). In Group 2, the mean LBx size was 16.1±5.3 mm (range 10-29 mm) and PTs 8.2±4.1 (range 4-19). Taken together, the mean composite biopsy was 34.4±6.6 mm (range 27 mm-59 mm) and number of PTs was 20.4±8.2 (range 8-41). Of our 62 biopsies 66.1% were ≥15 mm and 31% were ≥20 mm. Fifty (81%) of the liver biopsy specimens had ≥6 PTs and 67% had ≥11 PTs. Of the 41 samples having ≥15 mm of tissue, 20 (48.8%) samples had ≥11 PTs, and in the 19 samples with ≥20 mm of tissue, 10 (53%) had ≥11 portal tracts.

Spectrum of NAFLD

Overall, the prevalence of FL, IPF and NASH, as interpreted by P-1, was 35%, 32% and 26% in Group 1 compared to 23%, 38% and 32% in Group 2, respectively (Table 2). The grade of steatosis was only discordant on five biopsies, yielding a concordance of 83.9%. A diagnosis of FL was made in 11 patients (35%) in Group 1 and in 7 patients (23%) in Group 2 (κ=0.69).

The composite prevalence of zone 3 ballooning injury was only noted in 5 subjects (16.1%). Hepatocellular ballooning was identified in 4 biopsies from Group 1 compared to 5 biopsies from Group 2 (κ= 0.87), contributing to a concordance of 97%. If ballooning was present on the largest tissue specimen, it was also noted in the smaller biopsy in each of the 4 patients. Importantly, if hepatocellular ballooning was not present on the larger biopsy, it was identified in the smaller sample in only 1 of 27 patients, yielding a concordance of 92.3%. The smaller biopsy sample that detected hepatocellular ballooning was 10 mm in length compared to the larger sample of 18 mm in patient #2 (Table 3). The sensitivity of a liver biopsy specimen ≥15 mm for the diagnosis of NASH was 83%, and the negative predictive value was 92.3%.

Table 2. Histological features and sampling variability of NAFLD

Histological findings	P-1 N = 31		Sampling variability (discordance: Lbx1 & 2)		Sampling Agreement
	Lbx1 n (%)	Lbx2 n (%)	n	%	κ
Steatosis (presence / absence)					
Normal (1-5%)	3 (10)	2 (6)	5	16	0.88
Mild (5-33%)	19 (61)	20 (65)			
Moderate (33-66%)	8 (26)	8 (26)			
Severe (66-100%)	1 (3)	1 (3)			
Fatty Liver	11 (35)	7 (23)	6	19	0.69
Zone 3 Ballooning	4(13)	5 (16)	1	3	0.87
Zone 3 fibrosis (presence/absence)	6 (19)	8 (26)	4	13	0.63
Mild/Moderate	5 (16)	7 (23)	4	13	0.65
Advanced (bridging/cirrhosis)	1 (3)	1 (3)			
NASH (zone 3 ballooning and/or fibrosis)	8 (26)	10 (32)	4	13	0.69
Portal Fibrosis (presence/absence)	16 (52)	18 (58)	8	26	0.48
Mild/Moderate	16 (52)	18 (58)	8	26	0.48
Advanced (bridging/cirrhosis)	0 (0)	0 (0)			
Isolated Portal Fibrosis	10 (32)	12 (38)	6	19	0.58

P-1, pathologist 1; Lbx1, Liver biopsy 1; Lbx2, liver biopsy 2; κ, kappa score.

Table 3. Histological properties of discordant samples

Patient No.	Group # (mm, PT)	Grade of Steatosis	Grade of Ballooning	Fibrosis		Fibrosis Stage
				Zone 3	Portal	
1	1 (17, 5)	1	0	none	none	0
	2 (10, 7)	1	0	**Present**	Present	**2**
2	1 (18, 16)	2	0	none	Present	**1c**
	2 (10, 9)	2	**1**	none	none	0
6	1 (16, 6)	1	0	none	none	0
	2 (11, 5)	1	0	none	Present	**1c**
7	1 (28, 6)	1	1	none	Present	**1c**
	2 (12, 5)	1	1	none	none	0
9	1 (21, 11)	0	0	none	none	0
	2 (12, 7)	1	0	**Present**	Present	2
11	1 (18, 6)	2	0	none	Present	**1c**
	2 (13, 4)	2	0	none	none	0
15	1 (20, 5)	2	0	Present	Present	2
	2 (15, 14)	3	0	Present	Present	2
18	1 (16, 9)	2	0	none	none	0
	2 (13, 6)	2	0	none	Present	**1c**
21	1 (17, 5)	0	0	**Present**	Present	2
	2 (15, 15)	1	0	none	Present	1c
24	1 (20, 8)	3	0	none	none	0
	2 (11, 6)	2	1	Present	none	**1a**
26	1 (21, 14)	1	0	none	none	0
	2 (15, 13)	1	0	none	Present	**1c**
28	1 (21, 9)	1	0	none	none	0
	2 (11, 8)	0	0	none	none	0

Bold characters indicate discordance.

The composite prevalence of zone 3 fibrosis (Z3F) was 29%. Z3F was identified in 6 patients on the largest sample compared to 8 patients on the smaller sample (κ=0.63), with a concordance of 90%. When Z3F was present on the larger sample, it was also noted on the smaller sample in 5 of 6 patients (83% concordance). However, if Z3F was not present in the larger sample, it was noted on the smaller biopsy in 3 of 25 patients (12% discordance) and all three samples were <15 mm. The 4 biopsies from LBx1 that failed to detect fibrosis were all <25 mm in length (range 17-21 mm). However, the specimens that missed the fibrosis were significantly larger (18.8±2.1; 95% CI 15.5-22.0 vs 12.0±2.2; 95% CI 8.6-15.5; *P*=0.004, respectively) than the 4 smaller specimens that identified fibrosis (range 10-15 mm). The discrepancies in overall staging were from none to mild fibrosis.

The composite prevalence of NASH, defined by hepatocellular ballooning or Z3F, was 35%. NASH was diagnosed in 8 patients (26%) on LBx1 and in 10 patients (32%) on LBx2 (κ=0.69). Overall, the sensitivity of a biopsy >15 mm for the diagnosis of NASH (ballooning or Z3F) was 73% and the negative predictive value was 83%.

The composite prevalence of portal fibrosis (PF) was 68%. PF was identified in 16 patients (52%) on LBx1, and in 18 patients (58%) on LBx2, yielding the lowest concordance (74%) and sampling agreement (κ=0.48). There were 8 discordant cases in detecting the presence/absence of PF. The mean number of portal tracts (PTs) in the biopsies that did not identify fibrosis (7.88±3.5, 95% CI 4.9-10.7) was not significantly different compared to the mean number of PTs in the biopsy samples that did identify portal fibrosis (8.25±3.9, 95% CI 4.9-11.5, *P*=NS). PF was identified in 4 patients with fewer PTs compared to the second biopsy (Table 3). For example, patient #26 had 14 portal tracts on LBx1 that missed the presence of PF. Similarly, patient #6 had one less portal tract on LBx2 that detected PF. The PF stage was discordant in 8 patients (26%) (stages 0-1, n=5 (16.1%); stages 1-2, n=3 (9.6%); κ=0.48). The sensitivity of a biopsy >15 mm with at least 6 PTs for the presence of PF was 81%, with a negative predictive value of only 67%. The diagnosis of IPF was noted in 10 (32%) and 12 (38%) patients on adequate and ideal biopsies respectively and was altered in 6 patients (FL-PF, n=4; PF-NASH, n=2) with a moderate agreement (κ=0.58).

Overall, the stages of NAFLD, as defined by the NIH, were discordant as follows: Stage 0→1c = 6/31 (19% discordance), Stage 0→1a = 1/31 (3% discordance), Stage 0→2 = 2/31 (6%), and Stage 1c→2 1/31 (3%).

Inter-observer Variability

The composite biopsy findings by both pathologists were compared to each other by combining the interpretations on both biopsies. Table 4 demonstrates the inter-observer diagnostic variability with respect to the composite scores for grades of steatosis, zone 3 liver injuries, fibrosis and the spectrum of NAFLD. The grading of steatosis had a fair agreement (κ=0.34), whereas the diagnosis of NASH (ballooning only), portal fibrosis and IPF were moderately agreed upon (κ=0.52, κ= 0.60 and κ=0.54, respectively). These results are similar to a previous study by Younossi and colleagues.[22]

Discussion

The main finding in this study is that significant liver biopsy sampling heterogeneity is present in morbidly obese individuals with NAFLD. However, the degree of sampling variability is dependent upon the histologic characteristics that define NAFLD. Hepatic fibrosis, specifically portal fibrosis, has a greater degree of sampling error than zone 3 fibrosis which demonstrated more heterogeneity than hepatocellular ballooning. The diagnosis of NASH based only upon hepatocellular damage demonstrates a higher agreement than the presence of zone 3 fibrosis with or without cellular injury.

The concordance for NASH was 97% for ballooning alone and yielded a negative predictive value of 96.3%. Thus, a liver biopsy length of 15 mm appears adequate to assess the presence or absence of ballooning. However, it is important to remember that calculated predictive values (negative and positive) are significantly dependent upon the prevalence of the disease. Therefore, the lower the prevalence, the higher the negative predictive value, and this must be taken into consideration and limits generalization to other cohorts. In our study, the prevalence of ballooning injury was only 16%, thereby contributing to

Table 4. Interobserver variability

Histological features (N=31)	P-1 composite scores		P-2 composite scores scores		Inter-observer Agreement
	n	%	n	%	κ
Steatosis (presence/absence)	29	94	23	74	0.10
Normal (1-5%)	2	6	8	26	0.34
Mild (5-33%)	20	64	11	35	
Moderate (33-66%)	7	23	9	29	
Severe (66-100%)	2	6	3	10	
NASH (Hepatocellular Ballooning)	5	16	8	26	0.52
Zone 3 fibrosis					
Mild/moderate	8	26	6	19	0.32
Advanced	1	3	0	0	
Portal Fibrosis (presence/absence)	21	68	24	74	0.60
Mild/Moderate	21	68	23	74	0.54
Advanced (bridging/cirrhosis)	0	0	1	3	
Isolated Portal Fibrosis	10	32	12	38	0.58

P-1, pathologist 1; P-2, pathologist 2; κ, kappa score.

our very high negative predictive value. In comparison, the study from Ratziu et al[18] demonstrated a significantly lower negative predictive value (78%) for ballooning hepatocytes in the setting of a much higher prevalence (63%) of ballooning injury in their cohort. The two cohorts in these studies are significantly different; in addition to the BMI, the majority of our subjects had normal liver function tests and all underwent screening liver biopsies, whereas the latter study investigated individuals with elevated enzymes with a presumptive diagnosis of NASH.

The concordance for zone 3 fibrosis was 87%, less than cellular injury in the same location but better than fibrosis located in the portal region. This is similar to the 80% concordance noted by Ratziu and colleagues.[18] To our knowledge, the sample size needed to adequately detect fibrosis in zone 3 has not been established. In the present study, zone 3 fibrosis was 100% concordant in biopsies that were 25 mm in size, suggesting that this may represent the minimum specimen length necessary to mitigate against sampling variability regarding fibrosis around the central lobule. The prevalence of NASH was 35.5% when defined by either ballooning or zone 3 fibrosis and yielded a negative predictive value of 88%. Taken together, a morbidly obese subject can be counseled that a 25-mm liver biopsy without zone 3 ballooning injury or fibrosis essentially excludes the diagnosis of NASH whereas a smaller biopsy may miss the diagnosis of NASH.

As in many other etiologies of liver injury that result in portal fibrosis, significant sampling heterogeneity is also present in NAFLD. In the present study, we show a discordance of 26% in detecting portal fibrosis. A recent study presented by Janiec et al[19] in 10 morbidly obese individuals noted a discordance of 20%. However, they compared paired biopsies taken from the right and left lobes of the liver, and biopsies from different lobes have been previously demonstrated to display variability,[23] limiting comparisons to biopsy heterogeneity within the same lobes of the liver. Nevertheless, the authors concluded that a biopsy sample ≥10 mm in length with ≥10 portal tracts could be considered acceptable. As in other viral hepatitis studies, portal fibrosis might be attributed to the number of portal tracts as opposed to biopsy length. A study by Colloredo et al[24] suggested that 11 portal tracts are needed to stage chronic hepatitis C virus (HCV) and noted that only 38% of biopsies with at least 15 mm of tissue had the requisite number of portal tracts. That paper also suggested that 20 mm is necessary to obtain the requisite 11 portal tracts (94% of samples). In comparison, our study demonstrated that only 53% of the liver biopsy specimens >20 mm had 11 or more portal tracts. It is unclear whether this variance is attributable to the different etiologies (obesity and viral hepatitis)

of portal fibrosis. Also in our cohort, 25% of the biopsies that had >11 portal tracts did not detect portal fibrosis, and the mean number of portal tracts that identified fibrosis was not significantly different compared to the biopsies that did not identify fibrosis.

Overall, the prevalence of portal fibrosis in our cohort was 68% and the negative predictive value was only 62.5%. Although our study does not identify the minimum number of portal tracts necessary for the detection of fibrosis in NAFLD, the likelihood of detecting portal fibrosis improves when more portal tracts are present. Clinicians should be aware of these limitations when counseling their patients.

In conclusion, we have found that in morbidly obese individuals undergoing screening liver biopsies that sampling error is present but varies significantly, depending upon the histological characteristics of NAFLD. NASH, the progressive manifestation of NAFLD, can be reasonably excluded when the liver biopsy specimen is 25 mm in length.

The clinical tissue repository is supported in part by a grant from Tycohealthcare, Norwalk, CT.

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(Received August 2, 2006; accepted October 3, 2006)