



# Effect of Body Mass Index, Metabolic Health and Adipose Tissue Inflammation on the Severity of Non-alcoholic Fatty Liver Disease in Bariatric Surgical Patients: a Prospective Study

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## Abstract

**Background** Non-alcoholic fatty liver disease (NAFLD), driven by the obesity epidemic, has become the most common form of liver disease. Despite this, there is controversy regarding the prevalence and severity of NAFLD in obesity. Obesity-related factors, such as increasing adiposity, metabolic disease and inflammation, may influence prevalence. We therefore prospectively measured NAFLD prevalence in obesity and studied factors associated with NAFLD.

**Materials and methods** We recruited consecutive bariatric patients. Intraoperative liver biopsies were taken. The liver, adipose tissue and serum were collected to measure inflammation. Adipocyte cell size was measured. NAFLD severity was correlated to body mass index (BMI), metabolic health and adipose characteristics.

**Results** There were 216 participants; BMI  $45.9 \pm 8.9$  kg/m<sup>2</sup>, age  $44.4 \pm 12.1$  years, 75.5% female. Overall NAFLD prevalence was 74.1%, with 17.1% having non-alcoholic steatohepatitis (NASH) and/or steatofibrosis. Odds of NASH/steatofibrosis increased independently with BMI category (odds ratio (OR) 2.28–3.46, all  $p < 0.05$ ) and metabolic disease (OR 3.79,  $p = 0.003$ ). These odds markedly increased when both super obesity (BMI > 50) and metabolic disease were present (OR 9.71,  $p < 0.001$ ). NASH/steatofibrosis prevalence was significantly greater with diabetes, hypertension and dyslipidemia. Although greater visceral adipocyte hypertrophy was evident in NASH/steatofibrosis, there was no significant association between adipose inflammation and NASH/steatofibrosis.

**Conclusion** NAFLD remains endemic in obesity; however, NASH/steatofibrosis are less common than previously reported. Worsening obesity and metabolic disease increase odds of NAFLD independently, with substantially compounded effect with both. These observations may help with risk stratification in obese populations. We were unable to delineate clear associations between adipose inflammation and NASH/steatofibrosis in this obese population.

**Trial Registration** Australian Clinical Trials Registry ([ACTRN12615000875505](https://www.anzctr.org.au/Trial/Registration/Trial.asp?id=12615000875505)).

**Keywords** Bariatric surgery · Obesity · Non-alcoholic fatty liver disease · Metabolic syndrome · Inflammation

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## Introduction

Non-alcoholic fatty liver disease (NAFLD) is now the most common form of chronic liver disease [1]. Increasing obesity and metabolic disease has fuelled rapidly increasing rates of NAFLD. The significance of NAFLD is that it may progress to its more severe form, non-alcoholic steatohepatitis (NASH) and steatofibrosis [2]. Importantly, these more severe forms of NAFLD are most strongly associated with liver-related and overall mortality [3]. Due to its close association with obesity, having a clear understanding of the clinical associations and pathophysiology of NASH and steatofibrosis is of substantial importance to bariatric surgeons.

Despite its importance, the precise prevalence of NASH in the presence of severe and morbid obesity remains unclear. This is largely due the paucity of robust non-invasive tests for NASH, which necessitates liver biopsy for accurate study of prevalence [4]. Previous studies have reported high rates of NAFLD of up to 90% in obesity, with 25–56% NASH and 11% advanced fibrosis [5–7]. In contrast, Lassailly et al. [8] recently reported a NASH prevalence of only 7.3% in a well-documented series of 1489 consecutive bariatric surgical patients with routine intraoperative biopsies. Similarly, modest rates were found in the Longitudinal Assessment of Bariatric Surgery (LABS) Study, which reported 16.2% of patients with definitive NASH [9]. The exact reasons for this wide variation in reported prevalence in obese populations are unclear; however, differences in obesity-related characteristics may contribute.

Current understanding of NAFLD pathophysiology implicates obesity-related metabolic disease as a key mediator of NAFLD. Previous studies show that metabolic disease, including visceral obesity, dyslipidemia, insulin resistance and hypertension, affects 42.5% of patients with NAFLD and 70.7% of those with NASH [10]. At the opposite end of the spectrum is the phenomenon of metabolically healthy obese (MHO) [11]. This term broadly describes the phenotype where clinical obesity is present, in the absence of metabolic consequences [12]. Some argue that the MHO phenotype affords a similar metabolic risk profile to lean individuals [13, 14].

On a pathophysiological level, obesity-related inflammation, so-called meta-inflammation, may influence the prevalence of NAFLD [15, 16]. Meta-inflammation develops due to obesity-related changes in adipose tissue morphology such as adipocyte hypertrophy, immune cell infiltrate and adipokine production [15]. This can disrupt metabolic pathways and drive insulin resistance and metabolic disease [16] and ultimately exacerbate NAFLD [15, 17]. Both visceral (VAT) [18–21] and subcutaneous adipose tissue (SAT) [21, 22] inflammation have been implicated in liver damage. Furthermore, some studies show that these inflammatory changes may be reflected in serum [23] and therefore could potentially identify those at risk of NAFLD.

Improved understanding of the epidemiology of NASH in obesity would be of great clinical utility. Current uncertainty of prevalence and associations of NASH in bariatric surgical patients is accompanied by a wide variation in clinical approach. This was demonstrated by the LABS study, where biopsy rates varied widely between the 38 participating surgeons, from 94.1% down to no intraoperative biopsies [9]. A better understanding of this cohort could improve guidelines on obesity-related liver disease, based on factors other than the presence or absence of obesity.

We aimed to study the prevalence of NAFLD and NASH in an obese population and describe obesity-associated factors that were associated with NAFLD and NASH. Specifically, we aimed to:

- 1) Prospectively measure the prevalence and severity of NAFLD in a group of obese bariatric surgical patients
- 2) Determine the effect of increasing levels of obesity and metabolic health status on severity of NAFLD
- 3) Determine whether adipose tissue or serum based inflammation and characteristics were associated with risk of more severe NAFLD

We hypothesized that in a well-characterized prospectively recruited cohort, the overall prevalence of NASH and steatofibrosis would be lower than previously reported but would increase with more severe obesity and metabolic disease. We further hypothesized that NASH and steatofibrosis would additionally be strongly correlated with the presence of adipose tissue inflammation and cellular hypertrophy.

## Methods

All participants provided informed consent. Ethics approval was obtained (Alfred (195/15), Avenue (190) and Cabrini (09-31-08-15) Human Research Ethics Committees). This study was registered with the Australian Clinical Trials Register (ACTRN12615000875505).

## Patients

We prospectively enrolled all eligible obese patients undergoing bariatric surgery in three metropolitan hospitals in Melbourne (Australia) between July 2015 and August 2017.

Inclusion criteria were (1) age  $\geq 18$  years, (2) BMI  $\geq 35$  kg/m<sup>2</sup> and (3) alanine aminotransferase (ALT) or aspartate aminotransferase (AST)  $> 0.5$  times upper limit normal (ULN) [24]. Patients were excluded if they had evidence of other liver disease, including viral hepatitis, medication-related, autoimmune, familial/genetic causes or a history of excessive alcohol use, as defined by the American Association for the Study of Liver Diseases [25].

## Outcomes

### Clinical and Biochemical Data

Medical history and examination were performed pre-operatively. Metabolic syndrome was defined as detailed below. Obstructive sleep apnoea was screened with the STOP-BANG questionnaire [26], and patients at risk were referred for polysomnography.

Fasting blood tests were taken prior to induction of anaesthesia. Serum was collected and frozen at  $-80\text{ }^{\circ}\text{C}$  for cytokine analysis.

Participants were analysed in groups according to body mass index (BMI) categories: *obese*—BMI  $< 40\text{ kg/m}^2$ ; *morbidly obese*—BMI  $40\text{--}50\text{ kg/m}^2$ ; and *super obese*—BMI  $> 50\text{ kg/m}^2$ .

### Intraoperative Biopsies

Intraoperative wedge liver biopsies,  $\geq 1\text{ cm}$  in depth, were taken for histology with a section frozen at  $-80\text{ }^{\circ}\text{C}$ . A single pathologist graded the biopsies in a blinded manner, according to the Clinical Research Network (CRN) NAFLD activity score (NAS) [27] and Kleiner classification of liver fibrosis [28]. For this study, we have classified NAFLD into the following groups: (a) *normal*: steatosis  $\leq 5\%$ , no other abnormality; (b) *non-NASH NAFLD*: steatosis  $> 5\%$  and does not reach criteria for NASH or steatofibrosis; (c) *NASH*: NASH by NAS criteria (NAS  $\geq 5$ ), with or without stage 1 fibrosis (F1); and (d) *steatofibrosis*: stage 2–4 fibrosis (F2–4).

Visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT) were taken intraoperatively for histological examination and frozen for cytokine analysis.

Full details on tissue processing are found in the [Supplementary Materials](#).

### Metabolic Disease

There are currently multiple published definitions for MHO (Supplementary Table 1) and metabolic syndrome [12]. We have used a modified version of the National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATPIII) criteria [29], used previously for defining MHO [30]. The criteria are (a) systolic blood pressure (SBP)  $> 130\text{ mmHg}$  and/or diastolic blood pressure (DBP)  $> 85\text{ mmHg}$ ; (b) triglycerides  $\geq 1.70\text{ mmol/L}$ ; (c) high-density lipoprotein (HDL)  $< 1.03\text{ mmol/L}$  for males and  $< 1.29\text{ mmol/L}$  for females; and (d) fasting blood glucose (FBG)  $\geq 5.6\text{ mmol/L}$ .

MHO is the absence of any of these criteria. *Borderline MHO* has been defined as fulfilment of only one of these criteria. *Metabolically abnormal obese* (MAO) is defined as fulfilling two or more of these criteria.

### Tissue and Serum Inflammation

Liver and adipose tissue were analysed by quantitative real-time polymerase chain reaction (qRT-PCR) to quantify mRNA expression of interleukin-6 (IL-6), interleukin-1 $\beta$  (IL-1 $\beta$ ) and C-C motif chemokine ligand 2 (CCL2), as objective markers of inflammation. Serum cytokines were measured via enzyme-linked immunosorbent assay (ELISA). Full laboratory methods are found in the [Supplementary Methods](#).

### Adipose Tissue Cell Size

Image analysis was used to objectively quantify adipose tissue cell size (Fiji, ImageJ, Madison, WI, USA) [31].

### Statistical Analysis

Continuous variables were expressed as mean  $\pm$  standard deviation (SD) for normally distributed data and median and interquartile range (IQR) for non-normally distributed data. Independent Student's *t* test and one-way ANOVA with post-hoc Bonferroni test and Mann Whitney *U* test and Kruskal-Wallis test were used where necessary. Categorical variables were expressed as numbers (with percentages). Pearson chi-squared or Fisher exact test were used for independent categorical variables. Logarithmic transformation ( $\log_{10}$ ) of qRT-PCR data was conducted prior to analysis as data was heavily skewed. Odds ratios were calculated as effect size, with 95% confidence intervals (CI).

Logistic and linear regression analyses were performed on binary categorical and continuous outcomes to determine associations with NASH/steatofibrosis and inflammation. Covariates used included age, gender, metabolic health status, obstructive sleep apnoea and smoking status. Variables were removed by backward elimination, using a criterion of  $p < 0.05$ .

A  $p$  value  $\leq 0.05$  was considered statistically significant. Data analysis was performed in SPSS v23 (SPSS Inc., Chicago, IL, USA) and Prism 7 (GraphPad Software Inc., La Jolla, CA, USA).

## Results

### Patients

There were 216 consecutive eligible bariatric surgical patients (Table 1). Average age was  $44.4 \pm 12.1$  years, with 163 females (75.5%). The average body mass index (BMI) was  $45.9 \pm 8.9\text{ kg/m}^2$ , corresponding to 58 (26.9%) with BMI  $< 40$ , 106 (49.1%) with BMI  $40\text{--}50$  and 52 (24.1%) with BMI  $> 50$ .

**Table 1** Baseline characteristics of cohort, showing clinical and biochemical characteristics of all patients, and according to non-alcoholic fatty liver disease (NAFLD) status

Variables	All patients	NAFLD status			p value
		Normal	Non-NASH NAFL	NASH/steatofibrosis	
n =	216	56	123	37	
Age (years)	44.4 ± 12.1	43.8 ± 13.7	44.5 ± 11.6	44.8 ± 11.1	0.899
Male gender	53 (24.5%)	10 (17.9%)	30 (24.4%)	13 (35.1%)	0.061
Waist circumference (cm)	125.3 ± 21.1	120.1 ± 16.7 <sup>^</sup>	126 ± 21.3	132 ± 25.2 <sup>^</sup>	0.044
Weight (kg)	129.0 ± 29.8	120.7 ± 23.2 <sup>^</sup>	127.8 ± 26.3 <sup>#</sup>	145.4 ± 41.9 <sup>^#</sup>	< 0.001
Body mass index	45.9 ± 8.9	43.6 ± 7.2 <sup>^</sup>	45.5 ± 7.8 <sup>#</sup>	50.5 ± 12.6 <sup>#</sup>	0.001
BMI < 40	58 (26.9%)	20 (35.7%)	32 (26%)	6 (16.2%)	0.076
BMI 40–50	106 (49.1%)	28 (50%)	62 (50.4%)	16 (43.2%)	
BMI > 50	52 (24.1%)	8 (14.3%)	29 (23.6%)	15 (40.5%)	
Metabolic disease status					
Type II diabetes mellitus	56 (26.0%)	8 (14.3%)	31 (25.2%)	17 (47.2%)	0.02
Hypertension	99 (46.0%)	26 (46.4%)	51 (41.5%)	22 (61.1%)	0.782
Pre-diagnosed dyslipidemia	43 (20.1%)	14 (25.5%)	17 (13.8%)	12 (33.3%)	0.164
Obstructive sleep apnoea	64 (29.6%)	14 (25%)	37 (30.1%)	13 (35.1%)	0.557
Metabolic syndrome parameters					
Waist circumference (male > 102 cm, female > 88 cm)	216 (100%)	56 (100%)	123 (100%)	37 (100%)	–
Triglyceride level (> 1.7 mmol/L)	86 (39.8%)	16 (28.6%)	47 (38.2%)	23 (62.2%)	0.063
High-density lipoprotein (male > 1.03, female > 1.29 mmol/L)	171 (79.2%)	44 (78.6%)	94 (76.4%)	33 (89.2%)	0.885
Fasting glucose (< 7 mmol/L)	73 (33.8%)	12 (21.4%)	39 (31.7%)	22 (59.5%)	0.015
Blood pressure (> 135/80 mmHg)	101 (46.8%)	27 (48.2%)	50 (40.7%)	24 (64.9%)	0.652
Metabolic health status					
MHO	18 (8.3%)	4 (7.1%)	13 (10.6%)	1 (2.7%)	0.553
Borderline MHO	73 (33.8%)	23 (41.1%)	44 (35.8%)	6 (16.2%)	
MAO	125 (57.9%)	29 (51.8%)	66 (53.7%)	30 (81.1%)	
Biochemical variables					
Fasting glucose (mmol/L)	6.1 ± 2.5	5.4 ± 1.1	6 ± 2.3	7.1 ± 4	0.007
Albumin (g/L)	36.4 ± 4.1	36 ± 5 <sup>^</sup>	36 ± 4	38 ± 4 <sup>^</sup>	0.027
Bilirubin (μmol/L)	9.5 ± 5.1	9 ± 5	9 ± 5	11 ± 6	0.098
ALT (IU/L)	33 (24–50)	25 (17–35)	33 (26–51)	46 (36–70)	< 0.001*
AST (IU/L)	27 (21–35)	22 (17–28)	27 (22–35)	34 (27–51)	< 0.001*
GGT (IU/L)	33 (20–45)	23 (18–37)	34 (21–42)	44 (34–88)	< 0.001*
ALP (IU/L)	73.9 ± 25.1	72 ± 21 <sup>^</sup>	71 ± 20 <sup>#</sup>	86 ± 39 <sup>^#</sup>	0.007
Total cholesterol (mmol/L)	4.2 ± 1	4.1 ± 1.1	4.1 ± 1	4.3 ± 1.1	0.639
HDL (mmol/L)	1.0 ± 0.3	1.1 ± 0.3	1 ± 0.3	0.9 ± 0.2	0.072
LDL (mmol/L)	2.5 ± 0.8	2.5 ± 0.9	2.4 ± 0.9	2.5 ± 0.8	0.751
Triglyceride (mmol/L)	1.5 ± 0.7	1.2 ± 0.5 <sup>^#</sup>	1.6 ± 0.7 <sup>^</sup>	1.9 ± 0.8 <sup>#</sup>	< 0.001
HbA1c (%)	5.7 (5.4–6.3)	5.6 (5.3–5.9)	5.7 (5.4–6.35)	6.35 (5.6–8.15)	< 0.001*
C-peptide (pmol/L)	795	646	810	966	< 0.001*
	(588–1119)	(481–911)	(588–1119)	(706–1850)	
Insulin (mIU/L)	7.6 (4.7–13.9)	5.5 (3.6–10.9)	7.2 (4.8–12.4)	12.3 (7.7–25.6)	< 0.001*
HOMA2 IR	1.0 (0.6–1.9)	0.76 (0.5–1.6)	0.95 (0.6–1.7)	1.91 (1.1–3.6)	< 0.001*
Haemoglobin (g/L)	132.7 ± 12.8	129.8 ± 12	133.3 ± 13	136 ± 13.2	0.086
White cell count (× 10 <sup>9</sup> )	7.5 ± 2.3	7.1 ± 2.3	7.5 ± 2.3	7.8 ± 2.5	0.315
Platelet (× 10 <sup>9</sup> )	239.8 ± 57.6	230 ± 64	245 ± 56	240 ± 50	0.31

Data shown as mean ± standard deviation, median (interquartile range) and number (percentage). Independent Student's *t* test and ANOVA used, unless specified

MHO metabolically healthy obese, MAO metabolically abnormal obese

\*Mann-Whitney *U* test or Kruskal-Wallis test

<sup>+</sup> Significant difference between all pairs

<sup>^</sup> or <sup>#</sup> Significant difference in pairs

Fifty-six participants had diabetes (26.0%), 99 had hypertension (46.0%) and 43 had pre-diagnosed dyslipidemia (20.1%). Many participants had abnormal fasting lipid levels, with low HDL in 171 (79.2%) and high triglycerides in 86 (39.8%). Therefore, there were 18 (8.3%) participants who were metabolically healthy and obese (MHO), 73 (33.8%) borderline MHO and 125 (57.9%) were MAO.

**Prevalence of NAFLD and NASH**

NAFLD (> 5% steatosis) was present in 160 participants (74.1%). More severe disease (i.e. NASH or steatofibrosis) was found in 37 participants (17.1%), with NASH in 26 (12.0%) and steatofibrosis in 11 participants (5.1%) (Supplementary Table 2).

**Factors Affecting Overall NAFLD Prevalence**

Any degree of NAFLD was present in similar proportions regardless of degree of obesity (Table 2; Fig. 1a). Increased rates of NAFLD were seen in those with type II diabetes mellitus (T2DM) (85.7% vs 69.8%, *p* = 0.020); however, overall NAFLD was not affected by hypertension, dyslipidemia or obstructive sleep apnoea (OSA). Neither number of

metabolic risk factors nor metabolic risk status had significant impact on presence of any NAFLD in obese patients.

**Factors Affecting NASH/Steatofibrosis Prevalence**

The presence of more significant disease, in the form of NASH/steatofibrosis, was significantly influenced by various obesity-related factors (Table 2). Increasing BMI category significantly affected rates of NASH/steatofibrosis (10.3% vs 15.1% vs 28.8%, *p* = 0.027). Additionally, T2DM, hypertension and dyslipidemia all affected NASH prevalence rates. This was reflected in increasing rates of NASH/steatofibrosis with increasing numbers of metabolic risk factors, as well as increasing rates with worsening metabolic health status (5.6% vs 8.2% vs 24.0%, *p* = 0.007).

**Effect of Obesity and Metabolic Disease on NASH/Steatofibrosis Risk**

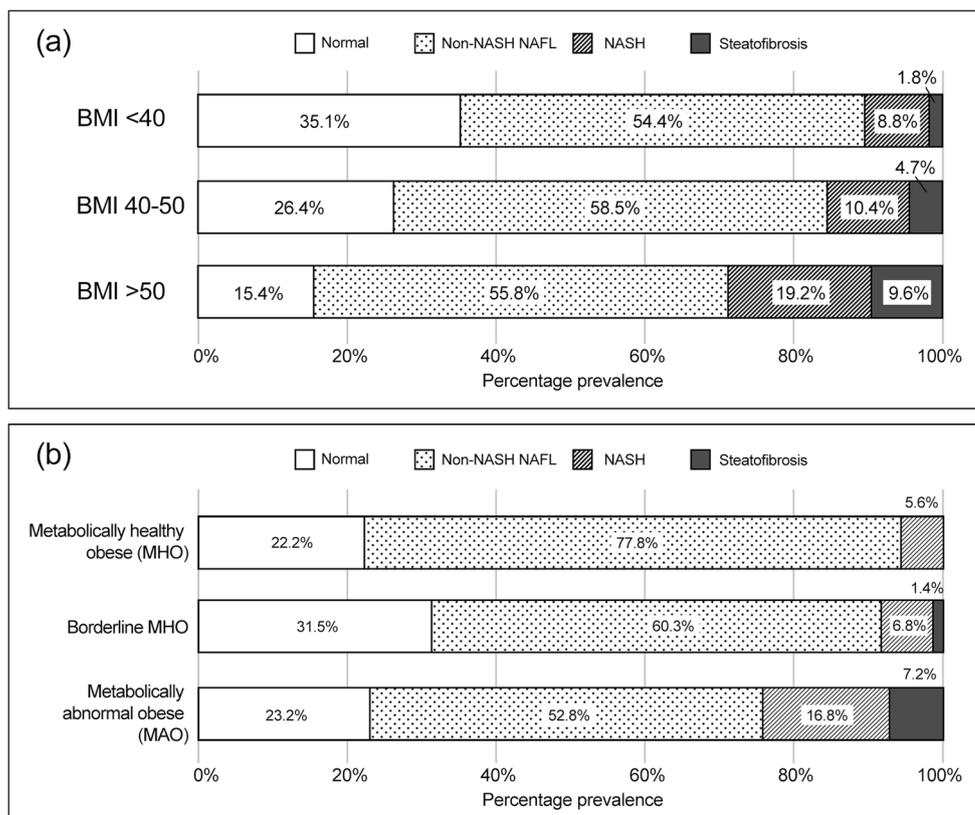
Table 3 shows the risk of severe progressive disease (NASH or steatofibrosis) according to BMI and metabolic health status groups. The BMI > 50 group had significantly higher risk of having severe progressive disease (NASH or steatofibrosis)

**Table 2** Factors significantly related to more significant disease (NASH and steatofibrosis)

Variable	Any NAFLD	<i>p</i> value	NASH/steatofibrosis	<i>p</i> value
<b>BMI category</b>				
< 40 kg/m <sup>2</sup>	38 (65.5%)	0.073	6 (10.3%)	0.027
40–50 kg/m <sup>2</sup>	78 (73.6%)		16 (15.1%)	
> 50 kg/m <sup>2</sup>	44 (84.6%)		15 (28.8%)	
<b>Individual metabolic risk factors</b>				
T2DM	No	111 (69.8%)	19 (11.9%)	0.002
	Yes	48 (85.7%)	17 (30.4%)	
Hypertension	No	86 (74.1%)	14 (12.1%)	0.047
	Yes	73 (73.7%)	22 (22.2%)	
Dyslipidemia	No	130 (76.0%)	24 (14.0%)	0.03
	Yes	29 (67.4%)	12 (27.9%)	
OSA	No	110 (72.4%)	24 (15.8%)	0.42
	Yes	50 (78.1%)	13 (20.3%)	
<b>Metabolic syndrome score (ATP III)</b>				
1	14 (77.8%)	0.171	1 (5.6%)	< 0.001
2	50 (68.5%)		6 (8.2%)	
3	31 (66.0%)		5 (10.6%)	
4	40 (83.3%)		14 (29.2%)	
5	25 (83.3%)		11 (36.7%)	
<b>Metabolic health status</b>				
MHO	14 (77.8%)	0.407	1 (5.6%)	0.007
Borderline MHO	50 (68.5%)		6 (8.2%)	
MAO	96 (76.8%)		30 (24.0%)	

Percentage prevalence with number of participants in brackets. Significance testing by chi-squared test  
*BMI* body mass index, *T2DM* type II diabetes mellitus, *OSA* obstructive sleep apnoea, *ATP III* Adult Treatment Panel III, *MHO* metabolically healthy obese, *MAO* metabolically abnormal obese

**Fig. 1** **a** Prevalence and severity of NAFLD with class I (BMI 30–40), class II (BMI 40–50) and class III obesity (BMI > 50). **b** Prevalence and severity of NAFLD with metabolic health status



compared to BMI 40–50 (odds ratio (OR) 2.28,  $p = 0.044$ ) and BMI < 40 (OR 3.46,  $p = 0.019$ ). MAO had significantly increased odds of NASH/steatofibrosis compared to *borderline MHO* (OR 3.52,  $p = 0.008$ ), as well as a combined *MHO/borderline MHO* group (OR 3.79,  $p = 0.003$ ).

Odds of disease were substantially increased with combined risk from metabolic disease and high BMI. Those with

*MAO* and BMI > 50 had a markedly increased OR of 9.71 for NASH/steatofibrosis ( $p < 0.001$ ), compared with healthier individuals (*MHO/borderline MHO* individuals with BMI  $\leq 50$ ).

Multivariate analysis confirmed that the presence of *NASH/steatofibrosis* was significantly associated with both metabolic health status ( $\beta = 1.44$ ,  $p = 0.002$ ) and body mass index ( $\beta =$

**Table 3** Odds ratio of having significant disease (NASH or steatofibrosis) in patients with differing body mass index, metabolic health status and a combination of both

Variables		Odds ratio (OR) (95% CI)	$p$ value
Body mass index (BMI)			
BMI < 40*	BMI 40–50	1.51 (0.56–4.10)	0.418
BMI < 40*	BMI > 50	3.46 (1.22–9.72)	0.019
BMI 40–50*	BMI > 50	2.28 (1.02–5.08)	0.044
Metabolic health status			
MHO*	Borderline MHO	1.52 (0.17–13.5)	0.706
MHO*	MAO	5.37 (0.69–42.0)	0.11
Borderline*	MAO	3.52 (1.39–8.94)	0.008
MHO/borderline*	MAO	3.79 (1.58–9.08)	0.003
Metabolic health status and body mass index (BMI)			
MHO/borderline + BMI $\leq 50$ *	MHO/borderline + BMI > 50	3.19 (0.65–15.7)	0.154
	MAO + BMI $\leq 50$	3.40 (0.89–12.9)	0.073
	MAO + BMI > 50	9.71 (2.83–33.3)	< 0.001

*NASH* non-alcoholic steatohepatitis, *BMI* body mass index, *MHO* metabolically healthy obese, *MAO* metabolically abnormal obese

\*Reference group

0.061,  $p=0.002$ ) (Supplementary Table 3). The presence of *any fibrosis* (F1–4) and *steatofibrosis* (F2–4) was associated with BMI ( $\beta=0.013$ ,  $p<0.001$  for *any fibrosis* and  $\beta=0.004$ ,  $p=0.002$  for *steatofibrosis*), but not metabolic health (Supplementary Table 3).

### Adipose Tissue and Serum Effects on NAFLD

#### Tissue Characteristics

Analysis of liver IL-6 levels confirmed significantly increased inflammation in non-NASH NAFLD ( $\log_{10}$  fold change 0.035 vs 0.534,  $p=0.024$ ) and NASH/steatofibrosis (0.035 vs 0.937,  $p=0.033$ ) compared to normal liver (Fig. 2a).

Neither visceral adipose tissue (VAT) nor subcutaneous adipose tissue (SAT) cytokine mRNA expression correlated with NAFLD severity (Fig. 2c, d; Supplementary Figs. 1 and 2). Similarly, SAT cell size did not change with NAFLD severity. There were, however, clear differences in VAT characteristics, with increased adipocyte size seen

in non-NASH NAFLD (cell diameter 85.6  $\mu\text{m}$  vs 90.0  $\mu\text{m}$ ,  $p=0.005$ ) and NASH/steatofibrosis (85.6  $\mu\text{m}$  vs 92.3  $\mu\text{m}$ ,  $p=0.003$ ) compared to normal (Fig. 2e, f).

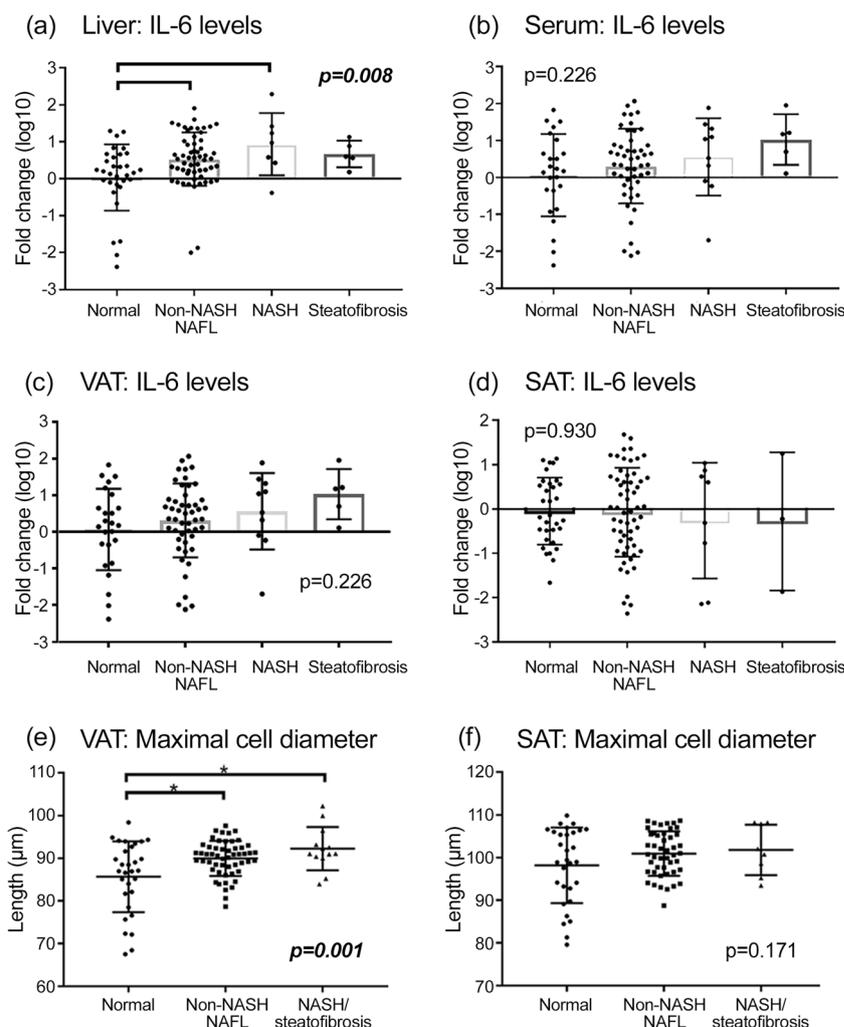
Some of the differences seen in liver and adipose tissues between normal and NASH are demonstrated in Fig. 3, showing increased liver steatosis and inflammation and adipose tissue cell characteristics associated with NASH.

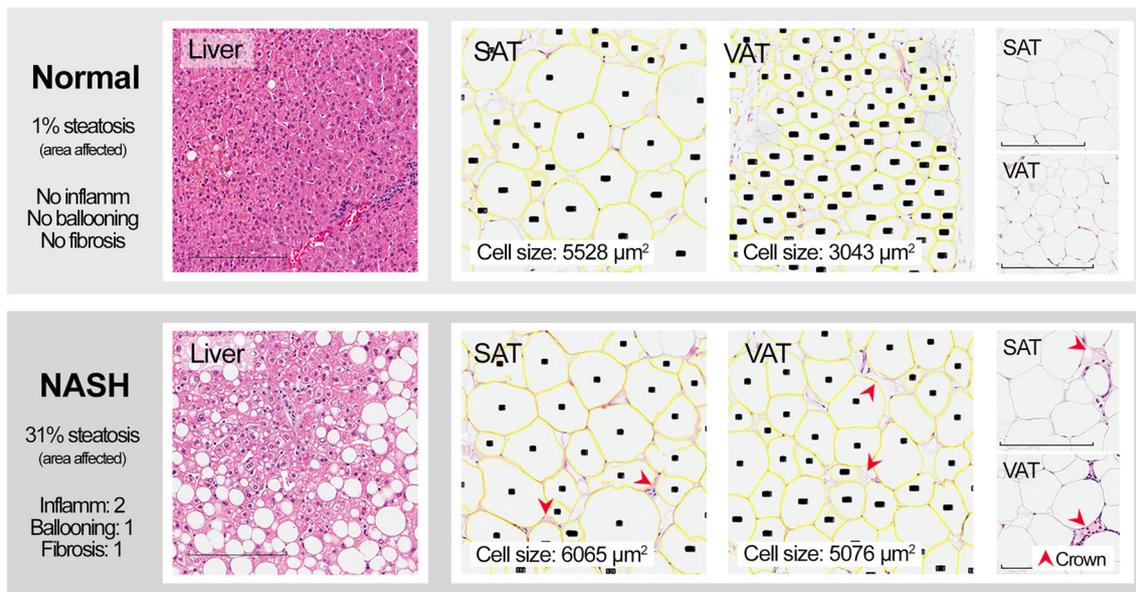
#### Serum Inflammation

Serum cytokines (interleukin-6 (IL-6), interleukin-1 $\beta$  (IL-1 $\beta$ ), interleukin-10 (IL-10) and tumour necrosis factor alpha (TNF- $\alpha$ )) were measured to compare differences in inflammation in NAFLD. There were no significant differences in serum IL-6 levels or other cytokines between NAFLD categories (Fig. 2b; Supplementary Fig. 2).

Full data for liver and fat mRNA expression and serum protein levels for IL-1 $\beta$ , CCL-2, IL-10 and TNF- $\alpha$  are provided in Supplementary Figs. 1 and 2.

**Fig. 2** Changes in interleukin 6 (IL-6) in the **a** liver, **b** serum, **c** visceral adipose tissue (VAT) and **d** subcutaneous adipose tissue (SAT) and changes of adipocyte characteristics in **e**, **f** visceral adipose tissue





**Fig. 3** Liver and adipose tissue characteristics in a patient with normal liver histology versus non-alcoholic steatohepatitis (NASH). This shows increased levels of steatosis and inflammation on liver histology, and

visceral adipose tissue cell hypertrophy, associated with signs of adipocyte death (“crown-like” structures)

## Discussion

In this prospective bariatric surgical cohort with histologically defined NAFLD, we have shown that liver steatosis is exceedingly common in severely obese patients, with around three quarters of individuals affected. However, despite significant metabolic disease burden and substantial obesity in this population, strikingly lower overall rates of NASH (12.0%) and steatofibrosis (5.1%) were observed, in comparison to previously published data. Both increasing body mass index (BMI) and metabolic health status independently affected the risk of having NASH/steatofibrosis (OR 2.3–3.8). The risk was markedly increased when both were present, with almost ten times the odds of NASH/steatofibrosis (OR 9.7,  $p < 0.001$ ). We were unable to demonstrate that adipose tissue inflammation mediated risk of NASH/steatofibrosis; however, differences in adipocyte size may indicate underlying contribution of VAT to liver disease.

One possible explanation for the surprisingly lower rates of NASH and steatofibrosis is the inherent differences in study setting, design and recruitment strategy. Although our results differed from some previous reports [5–7], our rates were similar to those reported by Lassailly et al. [8], with a NASH rate of 7.3% in a cohort of bariatric surgical patients who similarly had routine liver biopsies. Many other studies with histologically defined NAFLD have enrolled pre-selected patients via liver clinics or with substantially abnormal aminotransferases [5–7, 32]. Such selection bias would increase the prevalence of severe disease. By including consecutive bariatric surgical patients in a prospectively registered clinical trial, our population more likely reflects the

general obese population, and this may account for the lower, but potentially more representative, prevalence of severe disease.

Several observations can be made from this data regarding the influence of obesity and metabolic disease on NAFLD severity. Firstly, any degree of NAFLD (i.e.  $> 5\%$  steatosis) appears to be widespread in obesity and not significantly affected by degree of obesity or metabolic risk factors. The majority of these patients have simple steatosis, without inflammation or fibrosis. Evidence suggests that simple steatosis has very little hepatic sequelae, and the clinical importance of identifying simple steatosis in obesity is not clear [33, 34].

More significant disease, such as NASH and/or steatofibrosis, is substantially and independently influenced by both obesity severity and metabolic disease. The odds for NASH/steatofibrosis are more than threefold with greater BMI and nearly fourfold for metabolically abnormal individuals. Being both metabolically unhealthy as well as being super obese (BMI  $> 50$ ) compounds this risk, with a tenfold increased risk of having NASH/steatofibrosis. This is of significant importance, as NASH and fibrosis are associated with progression of liver disease and liver-related mortality [3, 35, 36].

The effects of obesity and obesity-related metabolic disease may contribute to the wide variation in reported prevalence of NASH and steatofibrosis within obese populations. Whilst these factors have been associated with NAFLD and NASH [37], the impact of degrees of obesity and metabolic disease severity has not previously been assessed in a prospective histologically defined cohort. This knowledge is vital for

stratifying NASH risk, particularly in exclusively obese and bariatric surgical cohorts.

We did not find a convincing correlation between adipose tissue or systemic inflammation and NAFLD. This is in contrast to other groups, who have shown that increased VAT cytokine expression and adipose tissue macrophages are associated with liver inflammation and damage [18, 20, 21]. We did find that VAT cellular hypertrophy was evident in states of NASH as well as non-NASH NAFLD, compared to normal. Cellular hypertrophy occurs when adipose tissue depots stored more lipids [15] and may also reflect a reduced capacity for adipogenesis, that is, the production of new adipocytes. These changes may lead to venous drainage of lipotoxic by-products and adipokines into the portal circulation, although direct support for this hypothesis is currently lacking. Overall, the lack of strong associations between adipose inflammation and NAFLD likely indicates the complexities that underlie NAFLD development and progression in obesity [38].

These data have substantial clinical application. It highlights the burden of NAFLD in the bariatric surgical population and reinforces the need to consider the possibility of undiagnosed liver disease and counsel patients appropriately. This study emphasizes the importance of obesity severity and obesity-related factors in determining likely risk of more significant disease. Factors such as increased BMI, particularly above 50 kg/m<sup>2</sup> and those with metabolic syndrome, independently contribute to a greater risk. A combination of both risk factors should alert clinicians to the greatly increased odds of disease and therefore the necessity for pre-operative work-up and consideration of intraoperative liver biopsy.

A significant strength of this study was the large number of well-documented patients and the prospective nature of the study. Additionally, we have used the gold standard, liver biopsy to accurately characterize NAFLD and correlated this histological assessment with clinical and inflammation data. Finally, we focused on the effects of increasing BMI and degrees of metabolic disease on NAFLD. Existing studies often comment on obesity in general, but few stratify by increasing obesity severity. These data are particularly important to bariatric surgeons and physicians, where the patient population is exclusively obese and vary by obesity class, rather than presence or absence of obesity.

This study has some drawbacks that warrant discussion. Firstly, the prevalence of NASH and steatofibrosis was lower than anticipated. This, in itself, is a noteworthy observation but has diminished our statistical power to find associations with severe disease. Secondly, there was a low prevalence of metabolically healthy obese individuals, compared to the metabolically abnormal and borderline patients. This is a potential cause for the lack of statistically significant differences between MHO cohort alone and borderline or MAO patients. A larger study that captures more MHO patients would

strengthen this analysis and could provide adequate power to demonstrate any significant differences in this group. Thirdly, we did not stratify for body composition. In particular, visceral fat deposition has been associated with increased metabolic disease, and specific measurement of this depot could be the focus of a future endeavour. Finally, we have targeted a few of the most prominent inflammatory markers associated with meta-inflammation. Future studies could analyse a wider range of cytokines.

In conclusion, this study provides significant insights into the impact of obesity, metabolic disease and inflammation on NAFLD in the obese. Whilst NAFLD was found to be highly prevalent in obesity, more severe NAFLD (i.e. NASH and steatofibrosis) is less common than previously reported. Increasing obesity and metabolic disease both independently increase the risk of NASH and steatofibrosis, with a substantially compounded effect when both are present. Whilst previous studies have shown significant correlation of inflammation with NAFLD, we failed to demonstrate this relationship, which could be due to underlying complexities in this interaction. Clinicians should be aware that increasing obesity independently increases the risk of more severe NAFLD, and the additional presence of metabolic disease should raise suspicion for NASH and fibrosis.

## Compliance with Ethical Standards

Ethics approval was obtained (Alfred (195/15), Avenue (190) and Cabrini (09-31-08-15) Human Research Ethics Committees).

**Conflict of Interest** GJO, PRB, CL and WAB report being affiliated with the Centre for Obesity Research and Education. The Centre has received funding for research purposes from Allergan and Apollo Endosurgery, the manufacturers of the LapBand™. The grant is not tied to any specific research project, and neither Allergan nor Apollo Endosurgery have control of the protocol, analysis and reporting of any studies. The Centre also receives a grant from Applied Medical towards educational programs.

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The remaining authors have no other disclosures or conflict of interest.

**Ethical Approval Statement** All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

**Informed Consent** Informed consent was obtained from all individual participants included in the study.

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