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Lean NAFLD: A distinct entity shaped by differential metabolic adaptation

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Abstract (Word count = 177)

Non-alcoholic fatty liver disease (NAFLD) affects a quarter of the population. A significant subset of patients are lean, but the underlying pathophysiology in this group is poorly understood. We investigated the role of bile acids, FGF-19 levels (a surrogate for intestinal FXR activity) and gut microbiota profiles in the pathogenesis of lean NAFLD in a cohort of Caucasian

patients with biopsy proven disease (n=582), and in experimental murine models. Lean NAFLD patients had a more favourable metabolic and histological profile ($p < 0.05$ for all comparisons). Patients with lean NAFLD had higher serum bile acid levels, particularly secondary bile acids, and higher FGF19 levels ($P < 0.05$ for both). These differences were more profound in the early stages of lean NAFLD with fibrosis. Lean patients also demonstrated an altered gut microbiota profile. Consistent differences were observed in murine models of NAFLD. The pathophysiology of lean NAFLD differs from those with non-lean NAFLD disease with respect to gut microbiota composition and bile acid profile. Differences in the metabolic milieu between lean and non-lean NAFLD, at least in part, likely explains the pathophysiology.

Introduction

Non-alcoholic fatty liver disease (NAFLD) affects about 20-30% of the world's population and is a leading cause for end-stage liver disease, cancer and transplantation ¹. Despite this, the existence and clinical course of the entity known as “lean NAFLD” has been the subject of intense debate and controversy. To many, lean NAFLD refers to individuals having the disease in the context of a normal body mass index (BMI), but having excess visceral adiposity and insulin resistance, the so called metabolically obese normal-weight (MONW) individual. The latter refers to individuals considered “normal-weight” or “lean” by conventional measures, with metabolic dysfunction that is typically observed in people with obesity². The prevalence of lean NAFLD varies widely according to the criteria used for its definition, but ranges from 5 to 45% ³. By this interpretation, lean NAFLD is similar if not identical to NAFLD associated with overweight and obesity, with insulin resistance at its core, but detected at an earlier stage.

Accumulating evidence however suggests that lean NAFLD might be a distinct pathophysiological entity with about half (47-65%) having NASH ⁴. While “lean NAFLD” was first described in Asia, it has since been recognised globally¹. Most aspects of lean NAFLD including its operational classification have not been systematically characterised. The most frequently used definition is that of hepatic steatosis with a BMI < 25 kg/m² (or less than 23 kg/m² in Asians) ⁵. The natural history of lean NAFLD is even less well characterised; some data suggests that they have worse mortality and accelerated disease progression, despite a more favourable metabolic risk profile^{6,7}. Lastly, the pathogenesis and mechanisms for the favourable metabolic profile are puzzling and poorly understood.

For metabolic homeostasis, in addition to the neuroendocrine axis, caloric intake and physical activity, the enterohepatic circulation, including bile acids (BA) and their metabolites, and gut microbiota are intimately involved. Bile acids are the principal route for cholesterol catabolism, and recent evidence demonstrates that a high intake of dietary cholesterol⁸, elevated levels of hepatic cholesterol⁹⁻¹² and disrupted hepatic cholesterol homeostasis are pivotal drivers of NAFLD⁹⁻¹². However, individual responses to changes in dietary cholesterol vary widely, suggesting a modifying role for other environmental or genetic factors. Of particular interest, it has been suggested that cholesterol intake is higher in lean compared to obese NAFLD¹³⁻¹⁵. BA also regulate glucose and lipid metabolism, and energy expenditure¹⁶ and in turn, their production, transport and metabolism are regulated by a specific nuclear BA receptor, the Farnesoid X receptor (FXR) and circulating Fibroblast Growth Factor 19 (FGF-19) likely via dependent and independent mechanisms¹⁶⁻¹⁸. The gut microbiome is also intimately involved in the pathogenesis of a number of metabolic diseases including body weight regulation, NAFLD, and liver cancer, in part through direct interactions with BAs¹⁹⁻²¹.

We hypothesized that the pathogenesis of lean and obese NAFLD and their distinct metabolic and histological profiles is caused by more than just differences in body weight and body mass index. We considered that the clinical phenotype of lean NAFLD might reflect differences in the integration of signals from the diet and the systemic metabolic milieu as also the enterohepatic axis comprising both bile acids and the gut microbiota. We tested this hypothesis in a large well-phenotyped biopsy proven cohort of 582 Caucasian patients with NAFLD.

Results

Clinical and histological characteristics of patients with lean NAFLD

A total of five hundred and eighty two patients with biopsy proven NAFLD were included. One hundred and eleven (19%) were lean. The clinical and biochemical characteristics of lean NAFLD compared to their counterpart non-lean patients are presented in **Table 1**. In addition to lower BMI, lean patients had a better metabolic profile, including a significantly lower frequency of diabetes and dyslipidaemia, lower serum triglycerides, fasting blood glucose and HOMA-IR values compared to their non-lean counterparts. Histologically, lean patients had lower fibrosis and NAS scores ($p < 0.001$ for both), as well as lower serum ALT. In total, lean patients have favourable metabolic and histological features compared to non-lean NAFLD.

Serum bile acid profile is associated with NAFLD severity, but not steatosis

Although there is increasing evidence to suggest a critical role for BA in metabolic diseases including NAFLD, their correlation with disease severity is conflicting, likely due to the limited sample sizes of previous studies. We explored the association between the BA profile and liver histology.

No differences in total BA, total primary or total secondary BA levels was noted between patients with none or mild steatosis (S0-S2) compared to those with severe steatosis (S3) (**Supplementary Fig 1**). Next, the association with steatohepatitis activity incorporating the severity of inflammation and hepatocyte ballooning was tested. In this analysis, significantly higher total BA ($p = 0.004$), primary BA ($p = 0.025$) and secondary BA ($p < 0.001$) was found in patients with hepatocyte ballooning compared to those without ballooning (**Supplementary Fig 2A**).

When comparing degrees of inflammation, higher grades of lobular inflammation, were associated with higher total and secondary BA ($p = 0.02$, for both), while there was no significant difference in primary BA levels (**Supplementary Figures 2B and C**). Lastly, the association of BA with NAFLD fibrosis stage was tested. The distribution of all individual BA between patients with none/mild fibrosis and those with significant fibrosis is depicted in **Figure 1A**. Patients with significant fibrosis ($\geq F2$) had higher total ($p = 0.008$), primary ($p = 0.008$) and secondary BA levels ($p = 0.030$) compared to those with none/mild fibrosis (F0-1) (**Figure 1B**). At the level of individual BAs, the level of cholic acid was significantly higher in patients with significant fibrosis compared to those with none/mild fibrosis ($p = 0.044$) (**Figure 1C**). Similarly, the levels of glycine and taurine conjugated BAs were significantly higher in patients with significant fibrosis compared to those with none/mild fibrosis ($p = 0.001$, for both) (**Figures 1D and 1E**).

Higher levels of total BA ($p = 0.001$), primary BA ($p = 0.001$) and secondary BA ($p = 0.001$) were seen in patients with higher NAS score, defined as ≥ 4 compared to patients with lower scores (**Supplementary Figure 3**). Similarly, patients with NASH had higher levels of total, primary and secondary BA compared to patients with steatosis, but only the secondary BA level was significantly different between the two groups ($p = 0.049$).

Bile acid profile in lean NAFLD patients

Next, we explored the differential bile acid profile between patients with lean and non-lean NAFLD. Interestingly, patients with lean NAFLD had higher total, primary and secondary BA levels compared to those with non-lean NAFLD, though this was only significant for the secondary bile acids ($p=0.01$) (**Figure 2B**). The composition of individual BA also differed

between lean and non-lean NAFLD patients, wherein lean patients had lower DCA, GCDCA and CDCA, but more GCA compared to the non-lean patients (**Figure 2A**).

Given the strong correlation between BA profiles and fibrosis, we examined the relationship between bile acids and lean NAFLD stratified by fibrosis stage. When stratified in this way, in those with mild fibrosis (F0-1), higher total and total secondary BA levels were observed in lean compared to non-lean NAFLD patients ($p = 0.037$ for total BA, $p = 0.002$ for total secondary BA). No significant difference between lean and non-lean patients was observed in those with more severe fibrosis (**Figures 2C and D**). The predominant secondary BA contributing to this difference were deoxycholate (DCA) and ursodeoxycholic acid (UDCA) ($P < 0.05$ for both) (**Figures 2E and F**). Glycocholic acid (GCA) was also higher in lean NAFLD patients; however, the difference was not significant (**Supplementary Figure 4B**). The secondary to primary BA ratio was also significantly higher in patients with lean NAFLD compared to non-lean NAFLD ($P = 0.01$) (**Supplementary Figure 4A**).

For further confirmation, in a subsequent analysis, we determined the relevant clinical factors associated with secondary BA levels. Consistently, on univariate analysis, BMI, fibrosis and ballooning were associated with secondary bile acid levels. On multivariate analysis, only BMI and fibrosis stage remained independently negatively and positively associated with secondary BA levels, respectively (**Supplementary Table 1**).

Serum FGF-19 concentration

FXR dysregulation has been implicated in the pathogenesis of NAFLD so we were interested to determine if differential effects are observed in lean versus non-lean NAFLD ^{22,23}. To examine for this, serum FGF-19, a surrogate marker of FXR activity was measured. In this analysis, reduced levels of FGF-19 were observed with the advancement of fibrosis stage ($p =$

0.005) (**Figure 3A**). Patients with lean NAFLD had significantly higher FGF-19 levels compared to non-lean NAFLD patients ($p = 0.047$) (**Figure 3B**). Interestingly when stratified according to fibrosis severity, the differences were more profound in those with mild fibrosis (F0-F1) ($p = 0.008$), with the reverse being true as fibrosis severity increased; this was however not significant ($p = 0.3$) (**Figure 3C**).

Microbiome analysis

The composition of the gut microbiome and their interaction with BA affects FXR-mediated signalling in both the liver and intestine, and is implicated in NAFLD pathogenesis^{22,24}. Hence, we determined gut microbiome composition in an exploratory subset of patients with available stool samples by 16S rRNA amplicon sequencing (N = 29, 5 lean and 24 non-lean). At the phylum level, no differences in taxonomic composition of the gut microbiome were observed according to lean versus obese BMI status. At the genus level, Erysipelotrichaceae UCG-003, as well as several bacterial genera within the Clostridiales order including *Ruminococcus*, *Clostridium sensu stricto 1*, *Romboutsia* and Ruminococcaceae UCG-008 were enriched in lean patients, while *Ruminiclostridium* and *Streptococcus* was enriched in obese NAFLD patients (Mann-Whitney test, $p < 0.05$) (**Figure 4**). These changes remained significant for Ruminococcaceae UCG-008 when corrected for multiple comparison testing (FDR $p = 0.010$).

A murine lean NAFLD model has increased bile acids and altered gut microbiota

To further test our hypothesis, we investigated the alteration in BA levels and the gut microbiome in a murine model of lean versus non-lean NAFLD. Mice were fed either a cholesterol rich or a high sucrose diet for 16 weeks. Mice fed the cholesterol rich diet remained lean, despite the development of NAFLD, consistent with previous studies^{25,26}, while those fed

the high sucrose diet demonstrated significant weight gain. Similar to our human results, mice fed the cholesterol rich diet had significantly higher total BA ($p = 0.01$), primary ($p = 0.02$) and secondary bile acids ($p = 0.06$) (**Figure 5A**). Analysis of their microbiota demonstrated a change in composition (PERMANOVA $P = 0.009$, pseudo- $F = 18.58$, 126 permutations), as reflected broadly by significant changes in the relative abundances of the two major phyla, Firmicutes and Bacteroidetes, as well as Actinobacteria, Cyanobacteria and Proteobacteria. Mice fed with the cholesterol rich diet were observed to have an increased relative abundance of Bacteroidetes and a decrease in Firmicutes, compared to those fed with high sucrose (**Figure 5B**). As, we observed in humans, similar trends were observed for the abundance of members of the Ruminococcaceae bacterial family in the high cholesterol diet fed (lean NAFLD) mice. These changes were also observed for several phylotypes within the Erysipelotrichaceae.

Discussion

Lean NAFLD constitutes a significant proportion of NAFLD patients, though its pathogenesis is not well understood. Herein we provide a testable hypothesis for the pathophysiological distinction between lean and non-lean NAFLD that can be examined in other cohorts. Using biopsy proven Caucasians patients in whom the lean NAFLD entity is less frequent than in cohorts from Asia, we demonstrate that lean patients have distinct metabolic, histologic and bile acid profiles, as well as differences in FXR activity and gut microbiota compared to their non-lean counterparts.

Consistent with other reports¹, around 1 in 5 Caucasian NAFLD patients are lean and have a favourable metabolic and pathological profile at biopsy, with less insulin resistance and dyslipidaemia, and milder liver histology. A reciprocal and intimate interaction between bile acids and gut microbiota is associated with, and thought to regulate, metabolic and hepatic traits^{16,20}. Although myriad factors could explain the differences we observed, our results *in toto* suggest that the balance and interaction between the systemic metabolic milieu and changes in the intestinal microbiome and bile acid physiology governs the expression of hepatic disease and the onset of NAFLD in patients with a normal BMI.

To elaborate, increased bile acid levels as we observed in lean NAFLD, are reported to mediate resistance to diet-induced obesity, a phenomenon called “obesity-resistance”²⁷⁻²⁹. Obesity-resistant rodents are able to burn more dietary fat by increasing energy expenditure. Of relevance, bile acids (including major bile acid species such as CA, TCA, DCA and CDCA) increase energy expenditure²⁸ and CDCA increases human brown adipose tissue activity³⁰. FGF19, which was also increased in lean NAFLD is reported to be a key regulator

of energy expenditure and improves glucose and lipid homeostasis ³¹, while gut-restricted FXR agonism promotes metabolic improvements and enhances thermogenesis and browning of white adipose tissue (WAT) in mice ³². At microbiota level, patients with lean NAFLD had distinct gut microbiota compared to those who were non-lean. Lean NAFLD had an increased abundance of members belonging to the *Clostridium* genus, and as well *Ruminococcaceae* that are involved in the formation of bile acids ^{33,34}. Consistently, in an experimental model that involved feeding mice a cholesterol rich diet ^{25,26}, we recapitulated several features of the phenotype including lean body weight, steatohepatitis and less insulin resistance, compared to mice receiving a high sucrose diet, with similar changes in bile acid profiles with higher total bile acid levels and similar trends observed in gut microbiota. Thus, we surmise that patients with lean NAFLD have an obesity-resistant phenotype in part mediated by greater levels of bile acids and FGF19 and microbiota changes.

Notably, we did not observe any association between bile acid levels and hepatic steatosis, indicating a potential lack of a protective effect of bile acids on the development of steatosis, as opposed to changes in peripheral tissues. Alternatively, changes in microbiota might explain the development of steatosis ³⁵. Lean patients had an increased relative abundance of several phylotypes within the Erysipelotrichaceae family in both patient and murine models, that have been repeatedly linked to host lipid and cholesterol phenotypes in different species (humans, mice, and hamsters) and positively associates with changes in liver fat in humans ³⁶. Use of plant sterol esters (PSE) to reduce cholesterol in hamsters likewise reduced Erysipelotrichaceae abundance ³⁶. Ruminococcaceae UCG-008, *Clostridium sensu stricto* 1 and *Romboutsia*, which were also enriched in lean NAFLD are reported to be strongly correlated with hepatic triglycerides ³⁷. Thus, the microbiota profile we observed

might be an additional contributor to steatosis development in response to an obesogenic environment (and perhaps a diet enriched in animal protein and cholesterol). Thus, lean NAFLD might be consistent with an “obesity-resistance” phenotype where patients are still prone to develop steatosis in response to an obesogenic environment, likely by gut-driven mechanisms.

The milder disease and favourable metabolic profile of patients with lean NAFLD could also be explained by the current findings. There is strong evidence that activation of bile acid signalling induces improvements in metabolic (glucose and lipid) phenotype in murine models ³⁸. Furthermore, in humans and murine models, elevated bile acids play a role in the metabolic improvements after bariatric surgery, including in type 2 diabetes, dyslipidemia and NASH resolution, even before significant weight loss ³⁹⁻⁴². Thus, we suggest that lean patients are able to adapt metabolically and excrete greater amounts of bile acids while their obese counterparts are those less able to excrete adequate amounts of bile acids to rid themselves of excess cholesterol, even if they are able to maintain a plasma cholesterol level comparable to that of lean NAFLD. Consistently, in humans, lean and obese patients have differential defence mechanisms to maintain stable serum cholesterol levels, wherein dietary cholesterol appears to preferentially induce bile acid synthesis in lean compared to obese patients ⁴³.

We observed that differences between lean and non-lean patients were more profound in those with early stages of liver fibrosis. This suggests that with disease progression, these homeostatic responses might possibly no longer be able to limit inflammation and fibrosis, leading ultimately to long-term adverse outcomes, despite a favourable baseline metabolic and histological profile^{6,7}. This hypothesis is supported by the higher serum bile acid levels and

lower FGF19 levels in patients with significant fibrosis. Longitudinal studies would be needed to confirm the findings.

A number of investigational drugs targeting the BA pathway, such as the FXR agonist obeticholic acid and the FGF-19 analogue NGM282 are currently in phase 3 and 2 clinical trials in patients with NAFLD ⁴⁴⁻⁴⁶. Hence, a better understanding of the role of BA physiology in NAFLD is crucial. Notably, clinical trials have focused on obese NAFLD and our data would suggest that stratification in trial design of lean versus obese NAFLD might be important.

The strengths of our report includes the study of a large well defined, biopsy-proven Caucasian cohort, and as detailed an investigation as is possible, from cross-sectional data. However, our study also has limitations. First, patients were seen in tertiary referral centres, and may suffer from selection bias. In addition, dietary histories were not available, while the cross-sectional design did not allow for interventions or longitudinal outcomes and thus, a causal relationship cannot be demonstrated.

In conclusion, in contrast to non-lean NAFLD, lean patients are likely to have a distinct pathophysiology. We suggest that the onset of disease occurs at a lower BMI set point (with lower measures of insulin resistance and dyslipidaemia), triggered or influenced by early alterations in the bile acid and gut microbiota profile. These changes might reflect altered dietary composition (perhaps with an excess of dietary cholesterol, as previously reported in patients with lean NAFLD¹³⁻¹⁵, altered cholesterol metabolism due to genetic or environmental factors, or limitations in adipocyte numbers in childhood). Secondary or concomitant alterations in gut microbiota composition also drives the phenotype to a greater extent than in patients with non-lean NAFLD. This hypothesis does not negate the possibility

that there are overweight/obese NAFLD patients with a similar pattern of compensatory mechanisms in bile acids and microbiota profile, but suggests that lean patients have a preponderance of a gut-mediated phenotype. Further studies are needed to investigate the contribution of early-stage adaptive mechanisms on the long-term hepatic and extrahepatic outcomes of this disease. Our hypothesis would suggest that these individuals will have more severe and progressive liver disease, but this hypothesis needs to be proven.

Figure legends

Figure 1: Serum bile acid levels and liver fibrosis. A) Bile acid composition as a percentage according to hepatic fibrosis. The x axis shows patients with absent/mild (METAVIR stage F0-F1, left, n = 329) and moderate/severe fibrosis (METAVIR stage F2-F4, right, n = 232), and the y axis shows the percentage composition of each individual bile acid in %. B) Mean concentration of total bile acids, total primary bile acids and total secondary bile acids according to hepatic fibrosis. The x axis shows hepatic fibrosis dichotomized as absent/mild (METAVIR stage F0-F1, n = 329) or moderate/severe (METAVIR stage F2-F4, n = 232), and the y axis shows the mean concentration of bile acid levels in $\mu\text{mol/L}$. C) Mean concentration of cholic acid (CA) according to hepatic fibrosis. The x axis shows hepatic fibrosis dichotomized as absent/mild (METAVIR stage F0-F1, n = 329) or moderate/severe (METAVIR stage F2-F4, n = 232), and the y axis shows the mean concentration of bile acid levels in $\mu\text{mol/L}$. D) Mean concentration of glycine conjugated bile acids according to hepatic fibrosis. The x axis shows hepatic fibrosis dichotomized as absent/mild (METAVIR stage F0-F1, n = 329) or moderate/severe (METAVIR stage F2-F4, n = 232), and the y axis shows the mean concentration of bile acid levels in $\mu\text{mol/L}$. E) Mean concentration of taurine conjugated bile acids according to hepatic fibrosis. The x axis shows hepatic fibrosis dichotomized as absent/mild (METAVIR stage F0-F1, n = 329) or moderate/severe (METAVIR stage F2-F4, n = 232), and the y axis shows the mean concentration of bile acid levels in $\mu\text{mol/L}$. Results are expressed as mean \pm SEM and P value was calculated using the Mann-Whitney non-parametric t-test. $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$.

Figure 2: Serum bile acid levels in lean and non-lean NAFLD patients. A) Mean concentration of total bile acids, total primary bile acids and total secondary bile acids in lean and non-lean NAFLD patients. The x axis shows lean (n = 111) and non-lean NAFLD patients (n = 471), and the y axis shows the mean concentration of bile acid levels in $\mu\text{mol/L}$. B) Bile acid composition in percentage between lean and non-lean NAFLD patients. The x axis shows lean (left, n = 111) and non-lean NAFLD patients (right, n = 471), and the y axis shows the

percentage composition of each individual bile acid in %. C) Serum bile acid levels in lean and non-lean patients with absent/mild fibrosis. Mean concentration of total bile acids, total primary bile acids and total secondary bile acids according to BMI and hepatic fibrosis. The x axis shows lean (n = 78) and non-lean (n = 251) patients with absent/mild (METAVIR stage F0–F1) hepatic fibrosis and the y axis shows the mean concentration of bile acid levels in $\mu\text{mol/L}$. D) Serum bile acid levels in lean and non-lean NAFLD patients with moderate/severe fibrosis. Mean concentration of total bile acids, total primary bile acids and total secondary bile acids according to BMI and hepatic fibrosis. The x axis shows lean (n = 27) and non-lean (n = 205) patients with moderate/severe (METAVIR stage F2–F4) hepatic fibrosis and the y axis shows the mean concentration of bile acid levels in $\mu\text{mol/L}$. E) Mean concentration of deoxycholic acid (DCA). The x axis shows lean (n = 111) and non-lean patients (n = 471), and the y axis shows the mean concentration of bile acid levels in $\mu\text{mol/L}$. F) Mean concentration of Ursodeoxycholic acid (UDCA). The x axis shows lean (n = 111) and non-lean NAFLD patients (n = 471), and the y axis shows the mean concentration of bile acid levels in $\mu\text{mol/L}$. Results are expressed as mean \pm SEM and P value was calculated using the Mann-Whitney non-parametric t-test. $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$.

Figure 3. Serum FGF-19 levels and liver fibrosis in lean NAFLD. A) Mean concentration of FGF-19 according to hepatic fibrosis. The x axis shows hepatic fibrosis dichotomized as absent/mild (METAVIR stage F0–F1, n = 329) or moderate/severe (METAVIR stage F2–F4, n = 232), and the y axis shows the mean concentration of FGF-19 in pg/mL. B) Serum FGF-19 levels in lean and non-lean NAFLD patients. Mean concentration of FGF-19 in lean and non-lean NAFLD patients. The x axis shows lean (n = 111) and non-lean (n = 471) NAFLD patients, and the y axis shows the mean concentrations of FGF-19 levels in pg/mL. C) Serum FGF-19 levels in lean and non-lean NAFLD patients according to stage of fibrosis. Mean concentration of FGF-19 levels according to BMI and hepatic fibrosis. The x axis shows lean and non-lean NAFLD patients with absent/mild (METAVIR stage F0–F1, left panel, n = 78 for lean and n = 251 for non-lean NAFLD) and moderate/severe (METAVIR stage F2–4, right panel, n = 27 for lean and n = 205 for non-lean NAFLD) hepatic fibrosis, and the y axis shows the mean concentration of FGF-19 levels in pg/mL. Results are expressed as mean \pm SEM and P value was calculated using the Mann-Whitney non-parametric t-test. $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$.

Figure 4. Gut microbiota associated with lean NAFLD. Abundance of bacterial genera and species that differ between patients with lean (n=5) and obese NAFLD (n=24). Results are expressed as mean \pm SEM and P value was calculated using the Mann-Whitney non-parametric t-test. P < 0.05, ** P<0.001, *** P<0.0001.

Figure 5. Serum bile acid levels and microbiota profile in a murine experimental model of NAFLD. A) Mean concentrations of total bile acids, total primary bile acids, and total secondary bile acids in a mouse model of lean NAFLD. The x axis shows mice fed a diet high in cholesterol (black bar, n = 9) and mice fed a diet high in sucrose (grey bar, n =5); the y axis shows the mean concentrations of bile acid levels in $\mu\text{mol/L}$. B) Relative abundance of the phyla Firmicutes and Bacteroidetes in caeca of mice fed the high cholesterol or the high sucrose diet, as determined by 16S rRNA sequencing. Results are expressed as mean \pm SEM; P value was calculated using the Mann-Whitney non-parametric t-test. P < 0.05, ** P<0.001, *** P<0.0001.

Figure 6: Proposed model for the differential pathophysiology between lean and obese patients with NAFLD. Lean patients had better metabolic and liver histology profiles. Consistent with the notion that lean patients have appropriate metabolic adaptation to an obesogenic environment, they are obesity resistant. The compensatory mechanisms include increases in bile acids and FXR activity and distinct gut microbiota profiles that explain the favourable profile, despite steatosis development. Similar features were observed in murine models of lean and obese NAFLD. We suggest that the relative contribution of the systemic milieu versus that of the gut governs the lean versus non-lean phenotype.

Methods

Patient selection

Five hundred and eighty two consecutive Caucasian patients with histologically characterized NAFLD were included. Patients were recruited from hepatology clinics at four centres: Australia (Storr Liver Centre, Westmead Hospital, Sydney) and Italy (Unit of Metabolic Diseases and Clinical Dietetics, University of Bologna; Gastroenterology unit, University of Palermo, and Division of Gastroenterology and Hepatology, University of Turin).

Inclusion criteria were liver biopsy for suspected NAFLD with available serum samples and clinical data. Individuals with alternative diagnoses were excluded including excess alcohol intake (>20 g per day for women; and >30 g per day for men), chronic viral hepatitis (hepatitis B and hepatitis C), autoimmune liver diseases, hereditary hemochromatosis, α 1-antitrypsin deficiency, Wilson's disease and drug-induced liver injury. Patients with any secondary cause of steatohepatitis including previous gastrointestinal surgery or ingestion of medications known to impact hepatic steatosis or bile acid metabolism were excluded as previously described in another study ⁴⁷. Ethics approval was obtained from the Human Ethics committee of the Western Sydney Local Health District and the University of Sydney. All other sites had ethics approval from their respective ethics committees. Written informed consent was obtained from all participants.

Clinical and laboratory assessments

A complete physical examination was performed on all patients on the day of the liver biopsy including measurement of body mass index. Weight (in kilograms) and height (in centimetres) were measured by staff at the time of biopsy and used to calculate BMI, expressed

as kg/m². Following WHO criteria for Western populations, patients with BMI of less than 25kg/m² were defined as lean, and ≥ 25 kg/m² as non-lean ⁴⁸. Hypertension was defined as a registered diagnosis in patient medical records, a resting blood pressure of $\geq 130/85$ mm Hg, or having any antihypertensive medication prescribed. Type 2 diabetes mellitus (T2DM) was defined as a registered diagnosis in patient medical records, a fasting plasma glucose value ≥ 5.6 mmol/L (or 100 mg/dL) or having any antidiabetic medication prescribed.

Venous bloods were collected on the morning of liver biopsy after an overnight 12-hour fast for serum transaminases, bilirubin, albumin, lipid profile, glucose and insulin. Serum insulin was determined by a radioimmunoassay technique (Phadaseph Insulin RIA; Pharmacia and Upjohn Diagnostics, Uppsala, Sweden). All other biochemical tests were performed using conventional automated analyzers within each local department. Insulin resistance was calculated using the homeostasis model (HOMA-IR) using the formula: $\text{HOMA-IR} = \text{fasting insulin (mU/L)} \times \text{plasma glucose (mmol/L)} / 22.5$ ^{49,50}.

Histopathology

Biopsies were interpreted by a single expert liver pathologist at each center who was blinded to patient clinical characteristics and serum measurements. All biopsies had a minimum of 11 portal tracts, and inadequate biopsies were excluded. Disease activity was assessed according to the NAFLD Activity Score; fibrosis was staged according to the NAFLD clinical research network ⁵¹. Some of these patients have been the subject of previous publications ⁵²⁻⁵⁴. The concordance between pathologists within this cohort was very good for steatosis and good for fibrosis, with coefficients for inter-observer agreement for fibrosis and steatosis grade of 0.78, 0.85, respectively ⁵⁵. The histology scoring was available for 562 (96.39%) of the cohort.

High throughput bile acid profiling

Bile acids were extracted from serum samples as per previously described^{47,56}. Briefly, 80µL of acetonitrile containing internal standard (cholic2,2,4,4-d4acid, Quebec, Canada) was added to 20µL of the serum sample. After centrifugation, the supernatant was evaporated to dryness and stored at -20⁰ C until time of analysis. The dried bile acid residue was then reconstituted in mobile phase containing 50:50 water and acetonitrile and analysed on a Ultra Performance Liquid Chromatography (UPLC, Shimadzu, Kyoto, Japan) system using an ACQUITY (WATERS, Milford, MA) column in combination with a Q-TRAP 5500 Mass Spectrometer (AB SCIEX, Toronto, Canada) to quantitate concentrations of 19 bile acids. The mass spectrometer was operated in negative ion mode. The calibration solution containing all 19 analytes prepared at a series of concentrations in pooled naïve plasma depleted of bile acids using activated charcoal to generate the calibration curve. The detection limit for individual bile acids was 0.01 – 0.05 umol/L.

FGF-19 analysis

FGF-19 level was measured using the Human FGF-19 Elisa kit (EHFGF19, Thermo Scientific) on the serum of subjects according to the manufacturer's instructions.

Microbiome analysis

DNA extraction was performed using a common protocol for both human stool and mouse caecum samples. Briefly, a single stool sample was collected from 29 patients with biopsy-proven NAFLD (5 lean NAFLD and 24 non-lean NAFLD). Mouse caeca were harvested following 16 weeks of cholesterol rich or high sucrose diet were used for microbiota composition

analysis. Genomic DNA isolation from these material were performed using the QIAGEN DNeasy Powerlyzer Powersoil kit (QIAGEN, Germany) according to the manufacturer's instructions. The DNA extracts were used for sequencing of the V4 hypervariable region of the 16S ribosomal RNA (rRNA) gene, as previously described⁵⁷. Briefly, amplicons were generated and indexed using the Illumina Miseq 16S Metagenomic Sequencing Library Preparation protocol

(http://support.illumina.com/downloads/16s_metagenomic_sequencing_library_preparation.html

) with modifications. Paired-end 16S rRNA sequencing (2 x 300 bp sequence reads) was performed on an Illumina Miseq platform at the David R Gunn Genomics Facility (South Australian Health and Medical Research Institute). Bioinformatics processing of the 16S rRNA sequence reads were performed using the Quantitative Insights into Microbial Ecology (QIIME) software (version 2-2018.2). Denoising was performed using the DADA2 pipeline, and chimera filtering and operational taxonomic unit (OTU) assignment was performed against the SILVA 16S rRNA reference database (release v132) clustered at 97% similarity. A minimum subsampling depth of 8,335 reads and 10,698 reads was selected for microbiota composition analysis of the human stool and mice caecum samples, respectively. Taxa present in ≥ 3 samples and in > 5 sequence reads were used to analyse genera that are differentially abundant between groups. The Benjamini-Hochberg method was used to control the false discovery rate for multiple testing correction.

NAFLD mice models

Male C57BL/6 mice were obtained from Animal Resources Centre (Perth, Australia) and used for diet studies commencing at week 8. Mice were exposed to a 12 hr light/dark cycle with free access to food and water. Mice were given either a 33% sucrose diet (SF09-079, Specialty

Feed Service, Glen Forest, Australia) or a diet containing 33% Sucrose, 2% cholesterol and 0.5% cholate (SF09-080, Specialty Feed Service, Glen Forest, Australia) starting at 8 weeks of age for 16 weeks. A separate group of male mice were fed normal chow (NC) (meat free rat and mouse chow; Specialty Feed Service, Glen Forest, Australia). At the time of harvest, mice were anesthetized with i.p. ketamine (100 mg/kg)/xylazine (10 mg/kg) injection after a 4 hr fasting period. Blood was collected by cardiac puncture. Liver and cecum samples were harvested, rapidly snap frozen in liquid nitrogen and stored at -80 °C. A thin slice of liver tissue was formalin fixed for histology. All procedures were approved by the Western Sydney Local Health District Animal Ethics Committee and conducted in accordance with Animal Experimentation guidelines of the National Health and Medical Research Council (NHMRC) of Australia.

Statistical analysis

Data was analysed using SPSS version 24.0 (IBM, Armonk, NY). Values are expressed as mean \pm standard deviation, median and interquartile range or frequency (percentage) as appropriate. P-values for comparisons of distributions between groups were assessed using Fisher's exact test. The Mann-Whitney non-parametric test was used to obtain significance between two means of continuous variables. The strength of associations between continuous variables was reported using Spearman's rank correlations. Univariate analysis of variance (ANOVA) was used to examine factors associated with increasing total secondary bile acid levels as continuous variables. Multiple regression analysis was then undertaken to determine which factors significant on ANOVA, remained independent predictors for total secondary bile acid levels when adjusted for other clinically relevant variables including age, gender, BMI, hypertension, diabetes, dyslipidaemia, total cholesterol, HOMA and histological profile (fibrosis, steatosis, ballooning, portal inflammation, lobular inflammation and NAS). Hepatic steatosis was

graded from 0 to 3 and was dichotomized into mild steatosis (NASH CRN grades 0-2) and more severe steatosis (grade 3) for the purposes of statistical analysis. Hepatocyte ballooning was dichotomized into no ballooning and any ballooning for analysis purposes. Lobular and portal inflammation was dichotomized to mild (grade 0-1) and severe (grade 2 or more) ⁵⁸. Fibrosis stage was dichotomized to mild fibrosis (F0-1) and significant fibrosis (F2-4). This was based on a recent systematic review, which showed that the risk of liver-related mortality increases exponentially from stage 2 fibrosis onwards ⁵³. Statistical significance was considered as $p < 0.05$ throughout.

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Table 1. Clinical and histological characteristics of lean and non-lean NAFLD patients

	Lean NAFLD (n = 111)	Non-lean NAFLD (n = 471)	p-value
Age (years)	46 ± 11.7	47 ± 13	0.435
Male (%)	76 (68.5)	308 (52.9)	0.579
BMI (kg/m ²)	23.2 ± 1.5	30.8 ± 4.7	<0.001
ALT (IU/ml)	56.6 ± 35.7	72.4 ± 46.6	<0.001
Waist/ Hip ratio*	0.917 ± 0.061	0.972 ± 0.079	<0.001
Diabetes (%)	14 (12.6)	129 (27.4)	<0.001
Hypertension (%)	28 (25.2)	164 (34.8)	0.057
Dyslipidaemia (%)	47 (42.3)	256 (54.4)	0.026
Total Cholesterol (mmol/L)	5.1 ± 1.2	5.2 ± 1.2	0.594
HDL-C (mmol/L)	1.4 ± 0.6	1.2 ± 0.3	<0.001
LDL-C (mmol/L)	3.6 ± 1.6	3.5 ± 1.6	0.519
Triglyceride (mmol/L)	1.6 ± 1.4	1.9 ± 1.1	<0.001
Fasting blood sugar (mmol/L)	5.3 ± 1.8	5.9 ± 1.8	<0.001
HOMA-IR	2.73 ± 1.88	5.39 ± 5.88	<0.001
Fibrosis (%)			
F0-F1 (%)	78 (70.3)	251 (53.3)	<0.001
F2-F4 (%)	27 (24.3)	205 (43.5)	
Ballooning (%)			
No ballooning (%)	40 (36)	137 (29.1)	0.1318
Any ballooning (%)	65 (58.6)	314 (66.7)	
Steatosis (%)			
Grade 0-2 (%)	90 (81.1)	371 (78.8)	0.2550
Grade 3 (%)	14 (12.6)	84 (17.8)	
Lobular inflammation			
No inflammation (%)	28 (25.2)	93 (19.7)	0.1092
Any inflammation (%)	75 (67.6)	363 (77.1)	
NAS score	3 ± 2	4 ± 2	<0.001

Values are mean ± SD, or number (%), p-value was calculated using Fisher's exact test and student's t-test. *Value based on 61 lean NAFLD and 250 non-lean NAFLD samples. The histology scoring was available for 562 (96.39%) of the cohort.