CAUCASIAN LEAN SUBJECTS WITH NON-ALCOHOLIC FATTY LIVER DISEASE SHARE LONG-TERM PROGNOSIS OF NON-LEAN: TIME FOR REAPPRAISAL OF BMI-DRIVEN APPROACH?

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Abbreviations:

NAFLD non-alcoholic fatty liver disease, NASH non-alcoholic steatohepatitis, T2DM type 2 diabetes mellitus, BMI body mass index, ALT alanine aminotransferase, AST aspartate aminotransferase, GGT gamma-glutamyl-transpeptidase, ALP alkaline phosphatase

Keywords

NAFLD, NASH, Lean-NASH, Lean-Outcomes, PNPLA3

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ABSTRACT

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Background & aims: The full phenotypic expression of NAFLD in lean subjectsis incompletely characterized. We aimed to investigate prevalence, characteristics and long-term prognosis of Caucasian lean subjects with NAFLD.

Methods: the study cohort comprises 1,352 biopsy-proven NAFLD subjects from four countries (Italy, United Kingdom, Spain and Australia), stratified into lean and non-lean (BMI</≥25 kg/m^2). Liver/non-liver-related events and survival free of transplantation were recorded during follow up, compared by by log-rank testing and reported by adjusted hazard ratios (aHR). **Results:** Lean patients represented 14.4% of the cohort and were predominantly of Italian origin (89%). They had less severe histological disease (lean vs non-lean: NASH 54.1% vs 71.2% p<0.001; advanced fibrosis 10.1% vs 25.2% p<0.001), lower prevalence of diabetes (9.2% vs 31.4%, p<0.001), but no significant differences in the prevalence of the *PNPLA3* I148M variant (p=0.57). During a median follow up of 94 months (>10,483 person-years), 4.7% of lean vs 7.7% of non-lean patients reported liver-related events (p=0.37). No difference in survival was observed compared to non-lean NAFLD (p=0.069).

Conclusions: Caucasian lean subjects with NAFLD may progress to advanced liver disease, develop metabolic comorbidities and experience CVD as well as liver-related mortality, independent of longitudinal progression to obesity and *PNPLA3* genotype. These patients represent one end of a wide spectrum of phenotypic expression of NAFLD where the disease manifests at lower overall BMI thresholds.

Lay Summary

Non-Alcoholic Fatty Liver may affect and progress in both obese and lean individuals. Lean subjects are predominantly males, have a younger age at diagnosis and are more prevalent in some geographic areas. During follow up, lean subjects can develop hepatic and extra-hepatic disease, including metabolic comorbidities, in the absence of weight gain. These patients represent one end of a wide spectrum of phenotypic expression of NAFLD.

Summary Box

What is already known about this subject?

- NAFLD may occur in lean patients with a BMI < 25 kg/m^2 in both Asian and Caucasian ethnicities.
- Although some longitudinal studies have been carried out in the Asian population, the natural history of NAFLD in lean Caucasian patients is still only partially explored.

What are the new findings?

- NAFLD may progress in Caucasian patients with a normal BMI in the absence of longitudinal progression to obesity and independent of their PNPLA3 profile.
- Despite a more favourable metabolic profile at baseline, lean NAFLD patients experienced both hepatic and extrahepatic complications, including HCC and CVD events
- Caucasian lean subjects with NAFLD have a predominant geographical localization

How might it impact on clinical practice in the foreseeable future?

- Lean subjects with NAFLD should not be overlooked in clinical practice, as they develop all disease outcomes in the long-term
- Well-defined phenotyping strategies should be applied in clinical trials to separate the outcome in lean and non-lean NAFLD subjects.

INTRODUCTION

The worldwide burden of Non-Alcoholic Fatty Liver Disease (NAFLD) has been usually associated with the global widespread of obesity(1). However, there is growing interest towards understanding the development of NAFLD in specific subgroups of individuals. "Lean" NAFLD patients have been identified as subjects with a Body Mass Index (BMI) below the ethnic-specific cut-off of 25 kg/m^2 in Caucasian and 23 kg/m^2 in Asian subjects(2). Studies involving this subpopulation has been mainly carried out in Asia, where epidemiological studies consolidated a stable prevalence of this phenotype in many Asian countries and most, but not all, cross-sectional and longitudinal studies suggested less severe disease(3-10)

On the other hand, prevalence and outcomes of lean subjects with NAFLD among Caucasian patients are controversial. In the US NHANES III cohort, Younossi et al. reported a NAFLD prevalence of 7.4% in lean subjects. The phenotype was associated with younger age, greater insulin resistance and hypercholesterolemia. However, NAFLD diagnosis was not based on histological criteria(11). Characteristics and outcomes of lean Caucasian patients with an histological diagnosis of NAFLD had been reported by Francanzani(12) and Hagstrom(13). In both cases, single-country experiences were described, in Italy(12) and Sweden(13) respectively. Regretfully, a longitudinal study describing features and long-term outcomes of a multi-ethnic and internationally recruited cohort of biopsy-proven lean NAFLD was only published in its abstract form(14).

In this study we aimed to describe baseline characteristics and long-term outcomes of Caucasian lean subjects with biopsy-proven NAFLD, prospectively recruited in tertiary centres in Europe and Australia.

PATIENTS AND METHODS

This is an observational, multicentre cohort study of well-characterized Caucasian patients with biopsy-confirmed NAFLD who had been prospectively enrolled and followed up in tertiary centres in Europe and Australia. The study has two components: the first, a cross-sectional analysis to characterize the clinical presentation of lean patients with NAFLD compared with non-lean, and the second, a longitudinal, follow-up analysis to determine the long-term morbidity and mortality of lean subjects with NAFLD compared with non-lean ones. Patients included were consecutively biopsied and were managed in academic medical centres in four different countries (Italy, UK, Spain and Australia). Patients had been prospectively enrolled between 1990 and 2016 and their data included in secured, local databases(15). Inclusion criteria were age ≥18 years and diagnosis of NAFLD confirmed by liver biopsy. Patients with clinically overt cirrhosis (significant thrombocytopenia, prolonged prothrombin time and/or US and CT imaging showing cirrhosis and/or splenomegaly and/or varices on endoscopy in the absence of decompensation) were excluded because liver biopsy is usually not performed as it is deemed not required for diagnosis confirmation. Exclusion criteria included a liver disease of other aetiology such as alcohol-induced or drug-induced liver disease, autoimmune or viral hepatitis, and cholestatic or metabolic/genetic liver disease including Wilson's, hemochromatosis and alpha 1 antitrypsin deficiency. All the aforementioned liver diseases had been ruled out by specific clinical, laboratory or radiographic criteria and by a histological diagnosis other than NAFLD at liver biopsy. All patients had a negative history of alcohol abuse as indicated by a weekly ethanol consumption of <140 g in women, and <210 g in men. The history of alcohol consumption was investigated by interviewing the patients and in many cases by also interviewing close relatives during both the first and subsequent visits. In more than 90% of cases, the liver biopsy was performed because of persistent elevation of liver enzymes in patients with fatty infiltration of the liver detected on repeated imaging studies. In the remaining cases, biopsy was performed for repeatedly imaging evidence of severe hepatic steatosis. For the purpose of this study, an extensive review of all the data from clinic visits, laboratory and imaging data and liver biopsy reports was performed and only patients of Caucasian

ancestry with an unequivocal diagnosis of NAFLD were included. Patients were excluded when data on alcohol consumption was not detailed in the medical records.

Extensive clinical and laboratory data were collected at the time of the liver biopsy. Laboratory evaluation included routine liver biochemistry; complete blood count; lipid profile; fasting glucose; iron parameters; viral serology for hepatitis B and C infection done either at the time of liver biopsy or during the follow-up visits; autoantibodies; alpha 1 antitrypsin levels and phenotype; and ceruloplasmin levels. Patients with advanced fibrosis or newly diagnosed cirrhosis at liver biopsy underwent endoscopic screening for gastroesophageal varices and screening for HCC at regular intervals following standard of care recommendations or guidelines in place at specific times as proposed by liver societies.

The study was approved by appropriate regulatory bodies at all participating centres, and all patients had given written informed consent for participation in medical research.

Body mass index (BMI)

The BMI (body weight in kilograms divided by the height in meters squared [kg/m^2]) was calculated in every patient at the time of liver biopsy. Patients were categorized into those with normal BMI (<25 kg/m^2), overweight (BMI between 25 and 29.9 kg/m^2) and obese (BMI ≥30 kg/m^2). Lean NAFLD in this Caucasian cohort was defined by BMI <25 kg/m^2.

Other metabolic conditions were recorded, including 1) hyperglycaemia (fasting glucose ≥100 mg/dl) or previously diagnosed type 2 diabetes mellitus (fasting glucose ≥126 mg/dL or treatment with antidiabetic drugs), 2) hypertriglyceridemia (fasting triglycerides ≥150 mg/dl), 3) low HDL-cholesterol (<40 mg/dl in men, <50 mg/dl in women), and 4) central adiposity (waist circumference ≥88 cm in women, and ≥102 cm in men). When waist circumference was not recorded in the medical records, we used a BMI ≥30 kg/m^2 to define obesity.

Liver Histology

Liver biopsies were routinely stained with haematoxylin and eosin and Masson's trichrome. Liver biopsies were scored by experienced liver pathologists in each centre using the NASH Clinical Research Network scoring system to grade NAFLD diagnostic histological features and stage fibrosis(16) . Histological features analysed included steatosis grade (0-3), lobular inflammation grade (0-3), ballooning grade (0-2), and fibrosis stage (0-4) as recommended (16), based on a historical reading at the time of biopsy. There was no re-reading in batch by the pathologist, which could have accounted for changes in reading of the biopsies over time. A total of eight experienced liver pathologists scored the liver biopsy features (A.B. and D.T. in the Newcastle centre, E.D, F.M.V., M.M. and D.C. in the Italian centres, M.J.P. in the Seville centre, and D.M.L. in the Sydney centre). The majority of the hepato-pathologists are members of the FLIP/EPoS Histopathology group, where inter-observer agreement was previously reported to be overall ~77% (k = 0.54) using the NASH-CRN histology criteria(17). This high level of inter-observer agreement compares favourably to that reported by the NASH-CRN(16, 18). The presence of NASH was recorded and categorized as NASH or non-NASH based exclusively on the pathologists' opinion whether or not NASH was present (based on the pattern of injury and the combined presence of steatosis, ballooning and lobular inflammation)(19). All liver biopsy samples were of appropriate quality and length, and had an appropriate number of portal tracts for a confident grading and staging of the histological features as judged by the pathologist. A threshold of 5% of hepatocytes showing steatosis was required for the diagnosis of NAFLD. An exception was made for patients who underwent liver biopsy for NAFLD suspicion and revealed cirrhosis without steatosis (n=8 patients) as it is well demonstrated that steatosis may disappear at advanced fibrosis stages.

Genotyping

Genomic DNA was isolated from peripheral blood using the EZ1 DNA Blood 350 µl kit (Qiagen) according to the manufacturer's instructions. Genotyping of the *PNPLA3* rs738409 C>G variant (I148M) was performed by the TaqMan Single Nucleotide Polymorphism allelic discrimination assay (Applied Biosystems).

Follow-up

Patients were followed by GI specialists or hepatologists at 6-12 month intervals after the diagnosis of NAFLD. At each visit, a complete medical history and physical examination was performed along with a routine laboratory work-up to follow their liver disease and other medical conditions. In the long-term follow-up analysis, we included those patients who underwent the diagnostic liver biopsy before 2016; this date was chosen to have at least three years of follow-up for the last patient recruited. During follow-up visits staff researchers recorded liver events (end-stage cirrhosis by MELD score above 15, cirrhosis decompensation including ascites, hepatic encephalopathy and portal hypertensive bleeding), hepatocellular carcinoma occurrence (defined by imaging/histology criteria following current clinical guidelines)(20), cardiovascular events [acute coronary syndrome (myocardial infarction, unstable angina, need for coronary revascularization), peripheral arterial ischaemia, acute cerebrovascular event (transient ischemic attack, acute ischaemic or haemorrhagic stroke)], autoimmune disease occurrence, non-liver related cancers (including breast, colorectal, lung, prostatic, hematologic, melanoma, pancreatic and urinary tract cancers) and patient deaths. Giving the lack of approved therapies for NAFLD, treatment recommendations during the study period were similar in all centres and consisted of the standard recommendations to achieve and maintain appropriate body weight with increased physical activity and dietary changes. No specific dietary intervention or specific type of physical activity was used but subjects were encouraged to lifestyle change(21). Hypoglycaemic and lipid lowering therapy was prescribed according to international guidelines. Individuals who did not experience an outcome event and whose health status was unknown for more than 12 months from review in their medical records were considered lost to follow-up. No patients underwent bariatric surgery.

Statistical analysis

Patients were grouped into lean subjects with NAFLD (BMI <25 kg/m^2) and non-lean with NAFLD (BMI ≥25 kg/m^2). Data are presented as median (interquartile range [IQ]) or otherwise specified, and number (percentage) of patients with a condition. Baseline characteristics were compared between lean and non-

lean using standard non-parametric tests for continuous variables or the Chi-squared test for categorical variables. Cumulative overall mortality during follow-up was calculated using Kaplan–Meier analysis and compared by log-rank testing. Multivariate (adjusted) hazard rate ratio (aHR) estimates (relative risk) for the outcome was calculated by Cox proportional hazard regression analysis to control for the effect of potential risk factors (confounders), while taking into consideration varying lengths of follow-up. The multivariate model included the variables 'lean NAFLD', plus variables that may potentially bias the outcome i.e. age, gender, diabetes, advanced fibrosis, BMI and site. Variables independently associated with the outcome analysed were identified by a stepwise forward selection procedure using a threshold of $p<0.1$ for variable selection. Time at risk (T0) was from the date of liver biopsy to the date of outcome or last follow-up. Analyses were performed using IBM SPSS Statistics version 25.0 (SPSS Inc, Chicago, Illinois) software

RESULTS

A total of 1,704 Caucasian patients were initially identified, but after revision 365 were excluded because the diagnosis of NAFLD was uncertain/associated with another liver disease or their health status was unknown for more than 12 months from review of their medical records. As per protocol, we only recruited patients with a baseline liver biopsy (excluding historical biopsies), thus cirrhosis was a histological finding in the absence of clinical signs/symptoms of liver failure or complications. We included 1,339 patients in the present analysis; 195 (14.6%) of them were lean. Lean patients were significantly younger (median age 45 yrs. vs 49 yrs., p=0.03) and with a higher prevalence of male gender (147/195 [75.4%] vs 717/1144 [62.7%] respectively, p=0.001). **Table 1** describes the distribution of NAFLD patients (both total and lean) from each centre. Noteworthy, the majority (89.7%) of lean patients with NAFLD were derived from Italian sites; more specifically, sites in Torino, Milano and Roma had a higher prevalence of lean subjects (n=552, lean=150) compared with sites in the UK, Spain, Sydney and Palermo (n=787, lean=45).

Cross-sectional analysis

Table 2 shows the baseline characteristics of the total patient population and the comparison between lean and non-lean NAFLD subjects. Waist circumference was measured in 986 patients, and the proportion of patients with central obesity was significantly lower in the lean NAFLD group as compared to the non-lean group (12.2% [20/164] vs. 69.7% [572/822], respectively; p<0.001). On the metabolic side, lean patients with NAFLD had a significantly lower prevalence of diabetes and lower values of glucose and triglycerides. However, total cholesterol and HDL-cholesterol values did not significantly differ between the two groups and LDL-cholesterol was slightly higher in the lean NAFLD group (**table 2**). In order to avoid treatment bias for dyslipidaemia, we undertook a sub-analysis focused on diabetic patients who typically receive statins; the results confirmed higher values of LDL in the lean group (median 116; IQR 101, 159) vs the non-lean (median 108; IQR 81, 147; p=0.04). Overall, *PNPLA3* genotype was available for 799 patients; the two groups did not show significant differences when comparing the distribution of the three genotypes (CC, CG, GG).

Table 3 shows the histological features of the patient population and the comparison between lean and non-lean groups. Lean subjects with NAFLD had significantly less steatosis, less lobular inflammation, less ballooning and less advanced liver fibrosis as compared to the non-lean group. Accordingly, lean patients showed a significant lower prevalence of NASH (54.1% vs 71.2% in lean vs non-lean patients respectively, p<0.001). Nonetheless, half of the lean patients displayed a mild/moderate fibrosis (F1-2) and 10.2 % of them had advanced fibrosis or cirrhosis (F3-4). In lean patients, the prevalence of F3-4 fibrosis at liver biopsy was associated with the presence (p<0.001) but not with the number (p=0.614) of metabolic abnormalities. Conversely, lean NAFLD without metabolic abnormalities (26%) had a higher prevalence of F0-2 (p<0.001).

Long-term follow-up analysis

The long-term outcome analyses were performed only in patients for whom the occurrence/non-occurrence of the specific event was documented. Patients were followed up for a median period of 92

months (interquartile range 70 mo., 132 mo.) and the cohort had a total of 10,483.20 person-years of follow-up.

Long-term hepatic, extrahepatic events and mortality in lean versus non-lean patients with NAFLD

Figure 1 illustrates the frequency of liver and non-liver events recorded by physicians during nearly 8 years of median follow up, comparing lean and non-lean patients with NAFLD; data are also reported as Kaplan-Meier curves in **supplementary Figure 1**. In particular, the most common complication was new onset diabetes, which occurred in 90/785 of non-lean vs 11/177 of lean patients (aHR 1.55 [CI 0.83-2.9], p= 0.171). It was followed by cardiovascular events, occurring in 122/1083 of non-lean vs 14/192 of lean (aHR 1.3 [CI 0.73-2.2], p=0.39), and extra-hepatic cancers, which developed in 93/1076 subjects in the non-lean group vs 17/191 in the lean one (aHR 0.42 [CI 0.39-1.4], p=0.44). As expected, diabetes was an independent predictor of cardiovascular events [aHR 1.95 (CI 95% 1.36-2.81) p<0.001] and extrahepatic cancers [aHR 1.53 (CI 95% 1.01-2.31) p=0.042] in the entire cohort. Extrahepatic events did not show significant differences in terms of adjusted hazard ratios in the two groups, despite a slightly higher incidence of diabetes and CVD complications in non-lean NAFLD. Liver related events occurred in 88/1137 of non-lean patients compared to 9/193 in the lean group (aHR 1.4 [CI 0.7-2.7] p=0.39) and HCC developed in 29/1136 of non-lean subjects compared with 2/192 of lean patients (aHR 1.9 [CI 0.46-8.1], p=0.37).

During follow up, 53 patients died (5 of them were lean). Causes of death were available for 45% of the cases (total n=24; 20 non-lean and 4 lean). In the non-lean NAFLD cohort, 10 patients died of liver-related conditions (specifically: 7 patients died of liver failure, 3 because of HCC); cardiovascular events were the cause of death for 2 patients, while 8 patients died of extra-hepatic cancers. In the lean cohort, causes of death were available for 4/5 patients, and showed that 2 patients died of liver-related events (not-HCC), 1 patient of ischemic stroke and 1 patient died of septic shock complications. Although the survival curve appeared to be more favourable in the lean NAFLD group, overall survival analysis did not show a significant difference in terms of mortality when comparing lean subjects to overweight and obese

(log-rank 5.34, p=0.069) (**Figure 2A**). On the contrary, comparing survival in non-obese and obese patients, the latter had a significantly worse prognosis (Log-rank 5.34, p=0.021) (**Figure 2B**).

Of note, the age of NAFLD patients with BMI <25 was significantly lower than those with BMI ≥30 (P=0.003); the same holds true also for patients with BMI <30 compared with ≥30 (P<0.0001). This can lead to underestimation of the risk of events at follow up in low-BMI classes. In fact, there is a significant correlation between age and BMI, but age is also strongly related to all events at follow up and to death (**Supplementary Table 1**). Since BMI is not a dichotomous variable, and the definition of lean/non-lean patients can be somewhat arbitrary, we tested the impact of the exact BMI value treated as a continuous variable. When the values of BMI were used for prediction of the clinical events, apparent correlations appeared with diabetes, HCC and cardiovascular events at follow up. However, when we adjusted for age, the link between BMI and clinical events became negligible, indicating that the correlation only arises because age influences both BMI and the clinical events (**Supplementary Table 2**). This suggests that a BMI-driven approach to risk stratification and detection of patients with NAFLD may not take into account important variables and it should be reconsidered.

Long-term change in BMI in lean patients with NAFLD

A crucial point is whether lean patients who developed long-term events remained lean during follow-up or changed their BMI category. We retrieved the last available BMI of lean patients who had completed a follow up visit in the last 12 months or the last available BMI before death. The analysis included 98% of lean patients and results are summarised in **Figure 3**. In the 191 lean subjects with NAFLD, mean and median BMI were 23.3 and 23.6 kg/m^2 respectively at baseline and 23.7 and 23.8 kg/m^2 (p=ns) at the end of follow up. The vast majority (77.5%) of patients remained lean, with the following distribution: 40/57 (70.2%) in Turin, 39/48 (81.3%) in Milan, 20/24 (83.3%) in Palermo, 37/45 (82.2%) in Rome, 8/9 (88.9%) in Seville and 4/7 in Sydney (57.1%) (**Figure 3A**). Of the 43 patients who progressed, only 10 had a significant increase in BMI, defined as BMI ≥ 27. Among these subjects only 3 patients increased their BMI falling in the obesity category (≥ 30). The frequency of long-term events did not differ significantly when

analysed according to change in BMI category, confirming data reported on a larger scale in the entire cohort (**Figure 3B**). Similarly, no differences were noted in adjusted hazard ratios for hepatic and extra-hepatic events and mortality (**Supplementary Table 3**).

Clinical and histological predictors of outcome

In the entire cohort, univariate analysis identified baseline diabetes, age and advanced fibrosis as predictors of mortality. Supporting previous reports that both NAFL and NASH have the capacity to progress(22), baseline NASH status was not significantly predictive of liver events (HR 1.24, CI 0.8-1.9), HCC (HR 2.1, CI: 0.8-4.9), cardiovascular events (HR 1.4, CI: 0.9-1.9), or death (HR 0.9, CI: 0.5-1.6). At multivariate Cox regression analysis only advanced fibrosis (F3/F4) was independently associated with a higher risk of mortality in the whole cohort. In keeping with BMI not being an independent predictor of mortality, lean status did not significantly influence survival (**Table 4**).

Longitudinal Analysis in the Italian Population

Given the higher prevalence of lean patients in the Italian centres and to avoid the impact this heterogeneity may have on outcomes, we conducted a longitudinal sub-analysis focused on the Italian cohort including centres from Turin, Milan, Rome and Palermo. All analyses were adjusted for the principal confounders of outcomes (age, gender, BMI, diabetes, fibrosis status) and centre. The Italian cohort included 751 patients (lean=175, 23.3%) with a median follow up of 84 months (IQR 70, 115). At baseline, diabetes was diagnosed in 127/576 (22%) non-lean vs 14/175 (8%) lean patients ($p<0.001$). During follow up 33/449 non-lean vs 10/161 lean developed diabetes, with no significant difference between groups (aHR 1.47, CI: 0.71-3.1, p=0.31). Cardiovascular events were reported by 46/569 (8.1%) non lean vs 11/173 (6.4%) lean patients (aHR 1.4 CI: 0.7-2.7, p=0.35); while the number of patients who developed extra-hepatic cancers were 52/562 (9.3%) and 16/172 (9.3%) in the non-lean and lean population respectively (aHR 1.1, CI: 0.6-1.9, p=0.83). Liver events were reported by 37/569 (6.5%) non-lean patients vs 8/173 (4.6%) lean (aHR 1.5, CI: 0.7-3.3, p=0.3); while HCC was diagnosed in 11/568 (1.9%) non-lean subjects vs

1/172 (0.6%) lean (aHR 2.9, CI: 0.4-23.6, p=0.31). Overall survival was not significantly different comparing lean and non-lean subjects (log-rank 0.46, p= 0.51).

DISCUSSION

The major finding from this study is that NAFLD may develop and progress in Caucasian subjects with a normal BMI, predominantly males, in the absence of longitudinal progression to obesity and independent of their *PNPLA3* genotype. Our findings highlight that these subjects are not healthy but are best considered "lean metabolically diseased", being one end in the wide spectrum of phenotypic expression of NAFLD that share with the non-lean counterpart similar morbidity and mortality.

The histological features of Caucasian patients with NAFLD having a normal BMI confirmed that initially lean patients have milder liver damage (in term of steatosis, lobular inflammation, ballooning, NASH and advanced fibrosis) as compared to overweight and obese; nevertheless, they are not spared from the risk of progressive liver disease, as more than half of them had NASH and 1 in 10 had severe fibrosis at the time of diagnosis. The above mentioned findings are consistent with what has been previously reported by Hagstrom et al.(13) and McPherson et al (22). The lower prevalence of advanced fibrosis and NASH at baseline in lean subjects does not invariably translate into better prognosis, as patients with NAFL who progress have a higher prevalence of baseline inflammation or ballooning and disease activity has been identified as an important determinant of progression.

An important confounding factor is the younger age of lean subjects with NAFLD at liver biopsy, extensively reported in previously published studies, leading to an underestimation of the risk of progression as older age is a main predictor of morbidity and mortality. Indeed, when BMI was used as a continuous variable and corrected for age, the link between BMI and clinical event became negligible. These observations, supported by the results of a recent and comprehensive meta-analysis(23), suggest that a BMI-driven approach to detecting patients with NAFLD can be misleading and should be reconsidered as a) metabolic derangements can arise independent of obesity and b) in the absence of intervention patients' liver-related prognosis ultimately depends on the degree of liver damage.

Indeed, despite a more favourable metabolic profile at baseline, during a median follow of nearly 8-years lean NAFLD patients suffered both hepatic and extrahepatic complications, including onset of diabetes and CVD events, which was not explained by a concomitant significant weight gain. Lean subjects can be insulin resistant, although mechanisms may be different from those acting in obese patients (24). A study among lean Caucasians showed that NAFLD in lean individuals might have a distinct metabolomic profile, with lysophosphatidylcholine, phosphatidylcholine, tyrosine and valine levels being different from those in the obese NAFLD group (25). The skeletal muscle compartment is another important determinant of metabolic homeostasis. Sarcopenia is commonly present along with increased adipose tissue mass in patients with NAFLD (26) and peripheral insulin resistance might have a prominent role.

Another important finding of this study is the identification of a phenotype of Caucasian NAFLD patients with a predominant geographical localization. Lean NAFLD subjects mostly occurred in the Italian centres, where they represented 22% of the patients, but long-term outcomes of lean *versus* non-lean NAFLD did not differ significantly within the cohorts "enriched" of lean patients. In turn, this suggests real heterogeneity in Europe, as the frequency of lean patients in Italy was similar to that reported by Hagstrom et al. in Sweden (~ 19%)(13). This finding partially mirrors variation in the prevalence of obesity across Europe, ranging from around 20% of the general population in Italy and Sweden to approximately 24% in Spain, and 29% in UK (with peaks of 37% in NE England) and Australia(27). However, it might also suggest the presence of as-yet unidentified genetically determined "sub-clusters" within NAFLD patients of Caucasian ethnicity that are most evident in countries with a lower prevalence of obesity where these effects are not overwhelmed by environmental factors. Although *PNPLA3* genotype distribution did not significantly differ when comparing lean and non-lean patients, other variants, such those as *TM6SF2, GCKR* and *MBOAT7* have not been investigated (28-30). However, data from public available databases show there are no great differences in the minor allele frequencies of the common *PNPLA3* and *TM6SF2* variants across European countries (31, 32). A possible explanation is that NAFLD arises on a predisposed genetic background but liver damage is boosted by obesity; the latter however can blur the importance of genetic predisposition as well as of other cofactors encountered in a lifetime.

In addition, multiple local environmental factors may contribute to the development of this phenotype, influencing amount and distribution of body fat. Nutrition habits are quite different between Italy and Sweden. The Swedish traditional pattern is towards meat and meat products, sauce and potatoes and often bread and margarine(33), while specific aspects of the Italian food consumption pattern are a very large contribution from olive oil to fats and a large contribution from bread, pasta and pizza to cereals(34). In our cohort, cholesterol levels were no better, and LDL even worse, in lean compared to non-lean NAFLD even among diabetics, who commonly would be receiving statin treatment. This finding has also been observed in the NANHES III and in the Swedish cohorts(13) and it might reflect dietary patterns enriched of cholesterol from high level consumption of red meat, previously reported in lean NAFLD(35-37)and in the Italian population(34). It might also suggest an altered cholesterol metabolism; a different metabolic adaptation to the environment mediated by differences in FXR activity, bile acids composition and gut microbiota has recently been suggested in lean NAFLD patients(38). In particular, increased bile acids (BAs) levels observed in lean NAFLD are reported to mediate resistance to diet-induced obesity, a phenomenon called “obesity resistance”(39, 40); further, at the microbiota level, lean NAFLD had an increased abundance of members belonging to the Clostridium genus as well as Ruminococcaceae, which are involved in the formation of BAs(41, 42). Finally, discrepancies in lean patient prevalence across countries should take into account the different national health services and access modalities to tertiary care centres.

The main strengths of our study are the large number of patients included; the long-term follow up averaging nearly a decade per patient; complete follow-up in the vast majority of patients; having a liver biopsy confirming the diagnosis of NAFLD in every case; and having experienced liver pathologists grading and staging the biopsy features.

Our study has some limitations, such as enrolment bias at tertiary centres or absence of specific treatment protocol with uniform diet and exercise across all centres. Results are mainly driven by the Italian population, but this may simply mean that this population is not so overwhelmed by obesity and a lean phenotype can be discerned, yet. Although the overall group results have been confirmed in a

separate analysis of the Italian group, this potentially limits the generalisability of the results. Further, with about three decades of overall study duration, we were not able to adjust for specific treatment modalities. We acknowledge there was not an a-priori established protocol for follow-up data collection as this is a retrospective and real-life study of a prospectively recruited cohort. The lack of a central pathologist scoring the liver biopsies is another limitation of the study. However, all recruiting centres are well-known for their interest in NAFLD and involved in several scientific collaborations with homogeneous protocols for patient enrolment and NAFLD histological scoring through the years. The relative small sample of lean NAFLD and low event rate in the whole cohort can partially account for statistical differences, but this is expected in a population where cirrhosis was a histological finding and all patients were free of events at baseline. Further, PNPLA3 genotyping was performed in 799 patients and genotype distribution subgroups are small. On the other hand, this is the largest cohort of Caucasian lean NAFLD subjects with liver biopsy and longitudinal follow up available so far.

In conclusion, lean subjects with NAFLD can develop the full spectrum of metabolic comorbidities and liver damage that occurs in non-lean patients, in the absence of longitudinal progression to obesity and independent of their *PNPLA3* genotype. These individuals thus represent one end of the full phenotypic expression of NAFLD where the disease manifests at lower overall BMI thresholds and at a younger age. It is important to characterize this population by further “omics” studies and to make a reappraisal of a biased BMI-driven approach to NAFLD. These findings may have implications for clinical management and for drug development: lean subjects should not be overlooked in clinical practice and well-defined phenotyping strategies should be applied in clinical trials to separate the outcome in lean and non-lean NAFLD patients.

Figure legends

Fig.1: Incidence of clinical events recorded during follow up in lean and non-lean patients with NAFLD.

Fig.2: **Comparison of overall survival**. (A): lean patients with NALFD vs obese and overweight patients. (B): non-obese NAFLD vs obese NAFLD patients

Fig.3: **BMI category change in lean patients**. (A) Lean patients whose BMI changed vs non-change in the analysed centres. (C) Frequency of long-term events in patients whose BMI changed vs non-change

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Table 1. NAFLD patients distribution among centres and countries.

Centre	Country	Centre-specific NAFLD patients (% in the total cohort)	Centre-specific Lean NAFLD patients (% in the total Lean cohort)	% of Lean NAFLD patient in the Centre
Turin	Italy	17.9 (240/1339)	29.2 (57/195)	23.8
Milan	Italy	8.6 (115/1339)	24.6 (48/195)	41.7
Rome	Italy	14.7 (197/1339)	23.1 (45/195)	22.8
Palermo	Italy	14.9 (199/1339)	12.8 (25/195)	12.6
Seville	Spain	21.4 (286/1339)	4.6 (9/195)	3.2
Newcastle	UK	18.7 (250/1339)	2.1 (4/195)	1.6
Sydney	Australia	3.9 (52/1339)	3.6 (7/195)	13.5

Table 2. Clinical and demographic characteristics of the total patient population

Variable	[n]	Total (n = 1,339)	Lean (n = 195)	Non-Lean (n = 1,144)	P value
Age (years)	[1339]	48 (38, 57)	45 (36, 55)	49 (38, 58)	0.03
Gender					
Female		475	48 (24.6%)	427 (37.3%)	0.001
Male		864	147 (75.4%)	717 (62.7%)	
Body mass index (kg/m^2)	[1339]	29.8 (26.5, 34.5)	23.6 (22.8, 24.4)	31.1 (28, 35.9)	< 0.001
Waist circumference (cm)	[986]	101 (93,110)	89 (84, 92)	103 (97, 112)	< 0.001
Diabetes (yes)	[1339]	377/1339 (28.2%)	18/195 (9.2%)	359/1144 (31.4%)	< 0.001
ALT (UI)	[1330]	59 (41, 88)	56 (38, 80)	59 (42, 89)	0.145
AST (UI)	[1326]	38 (28, 54)	37 (27, 46)	37 (28, 54)	0.073
Total bilirubin (mg/dL)	[1194]	0.64 (0.47, 0.89)	0.8 (0.5, 1)	0.62 (0.47, 0.85)	< 0.001
Albumin (g/dL)	[1124]	4.5 (4.3, 4.8)	4.6 (4.4, 4.8)	4.5 (4.2, 4.8)	0.053
Alkaline phosphatase (IU/L)	[1181]	81 (63, 112)	83 (62, 127)	81 (63, 110)	0.495
Platelet (x10^9)	[1227]	227 (188, 272)	225 (195, 273)	227 (186, 271)	0.796
Glucose (mg/dL)	[1165]	95 (86, 112)	88 (83, 97)	97 (86, 115)	< 0.001
Triglycerides (mg/dL)	[1226]	133 (97, 195)	115 (80, 168)	142 (97, 195)	< 0.001
Total Cholesterol (mg/dL)	[1237]	194 (166, 228)	197 (174, 224)	197 (174, 224)	0.298
HDL-cholesterol (mg/dL)	[1148]	48 (41, 58)	46 (43, 58)	46 (40, 58)	0.562
LDL-cholesterol (mg/dL)	[921]	124 (97, 155)	128 (106, 159)	120 (95, 155)	0.014
Ferritin (ng/mL)	[1068]	174 (89, 309)	175 (105, 328)	174 (87, 308)	0.291
PNPLA3	[799]				0.569
CC		253	30 (27.5%)	223 (32.4%)	
CG		344	52 (46.8%)	292 (42.4%)	
GG		202	28 (25.7%)	174 (25.2%)	

FOOTNOTE: Data are presented as median (interquartile range), or number (proportion) of patients with a condition. Number in brackets after each variable indicates the number of patients who had that particular variable measured.

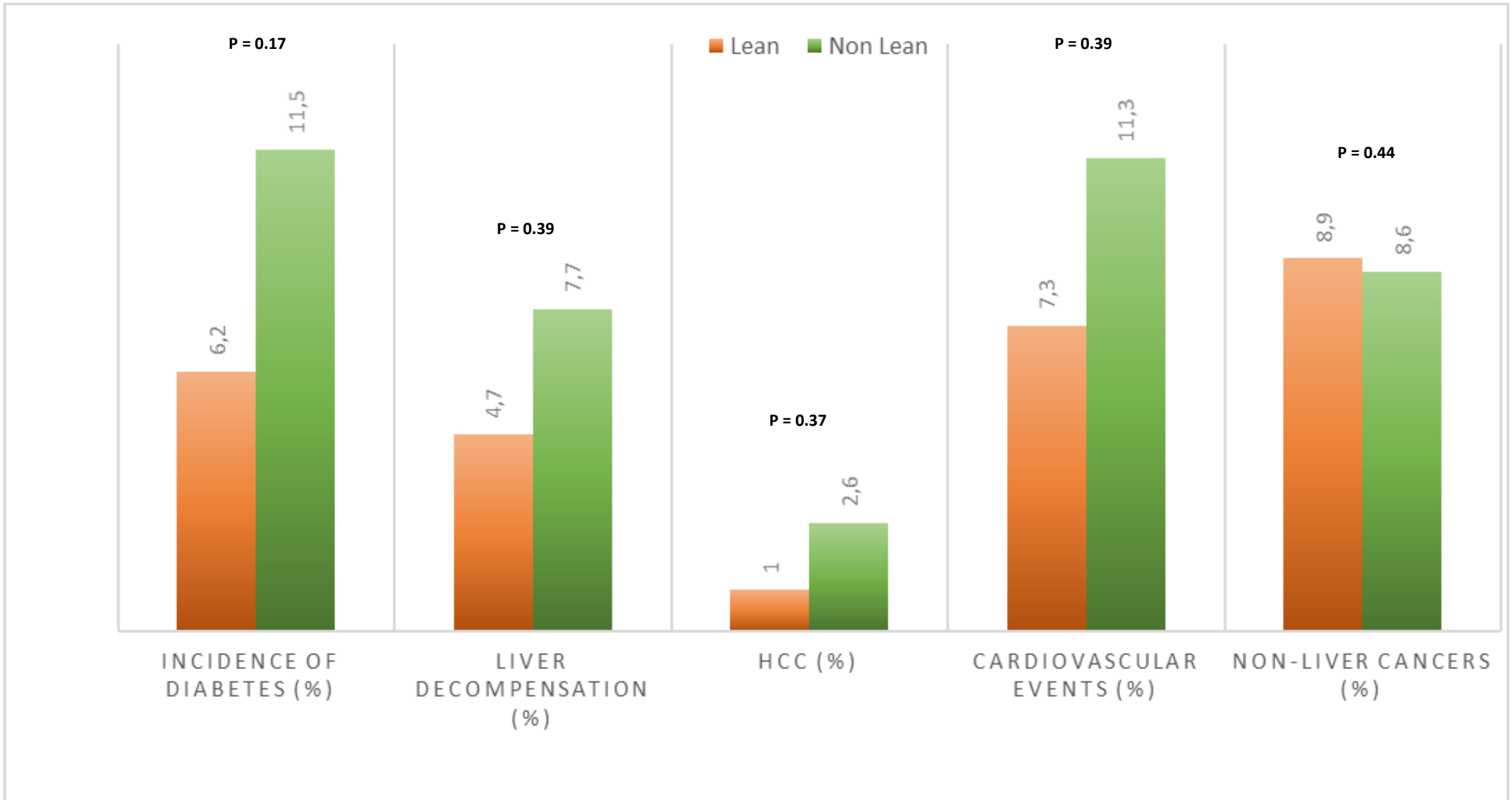
Table 3. Liver biopsy features of the total patient population

Variable	Total (n = 1,339)	Lean (n = 195)	Non-Lean (n = 1,144)	P value
Steatosis, grade				< 0.001
0*	8	3 (1.4%)	5 (0.4%)	
1	528	115 (59.9%)	413 (36.1%)	
2	511	57 (29.7%)	454 (39.7%)	
3	292	20 (10.4%)	272 (23.8%)	
Lobular inflammation, score				0.001
0	216	45 (23.1%)	171 (14.9%)	
1	764	118 (60.5%)	646 (56.5%)	
2	331	32 (16.4%)	299 (26.1%)	
3	25	0 (0.0%)	25 (2.2%)	
Ballooning, score				< 0.001
0	398	90 (46.4%)	308 (27%)	
1	656	74 (38.1%)	582 (51%)	
2	280	30 (15.5%)	250 (22%)	
NASH category				< 0.001
Non-NASH	417	89 (45.9%)	328 (28.8%)	
NASH	916	105 (54.1%)	811 (71.2%)	
Fibrosis stage				< 0.001
0	359	75 (38.5%)	284 (24.8%)	
1 - 2	674	100 (51.3%)	574 (50.2%)	
3 – 4	306	20 (10.2%)	286 (25%)	

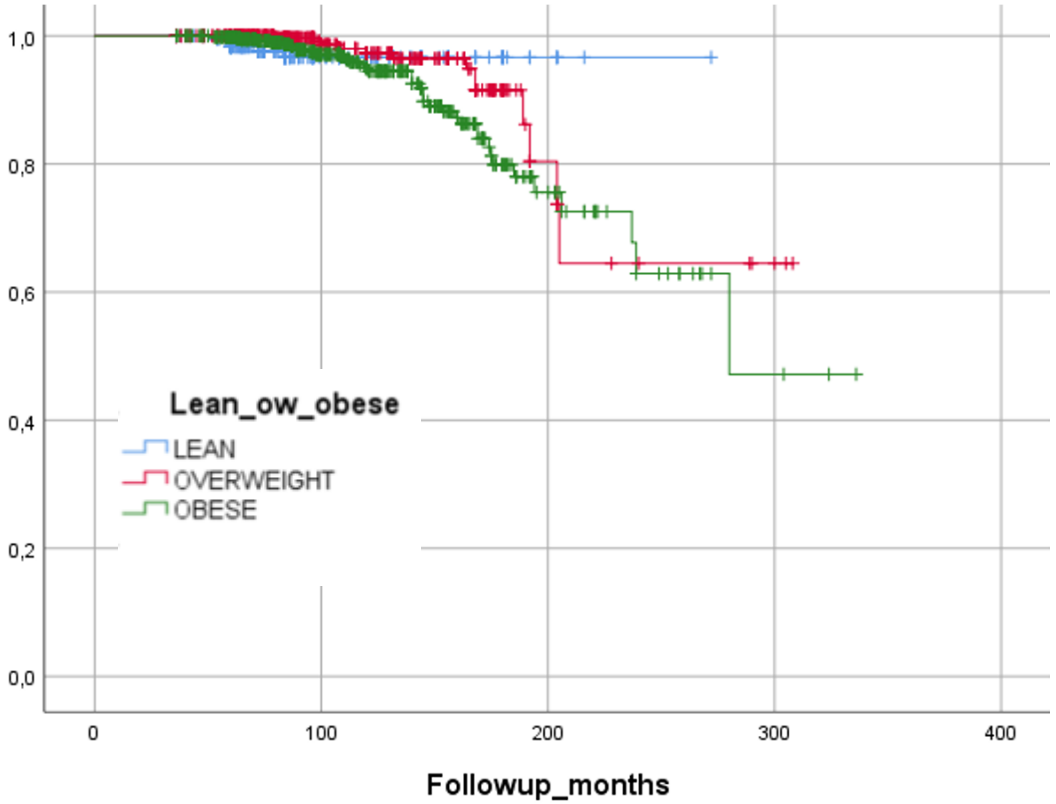
*Patients who underwent liver biopsy for suspicion of NASH and showed F4 fibrosis at histology, with steatosis less than 5%.

Table 4. Multivariate-adjusted hazard ratios and 95% CI's of outcome mortality

	Hazard Ratio	95% CI of HR	P value
Lean NAFLD	2.81	0.3, 30.1	0.4
Age	1.01	0.99, 1.16	0.071
Fibrosis, stage 3-4	7.4	1.3, 41.3	0.022
Diabetes at baseline	1.8	0.32, 10.3	0.49
Male gender	1.48	0.48, 8.2	0.48
PNPLA3 GG	1.4	0.26, 7.54	0.69


Lean
Non Lean
P = 0.17
6,2
11,5
INCIDENCE OF DIABETES (%)
P = 0.39
4,7
7,7
LIVER DECOMPENSATION (%)
P = 0.37
1
2,6
HCC (%)
P = 0.39
7,3
11,3
CARDIOVASCULAR EVENTS (%)
P = 0.44
8,9
8,6
NON-LIVER CANCERS (%)

(A)

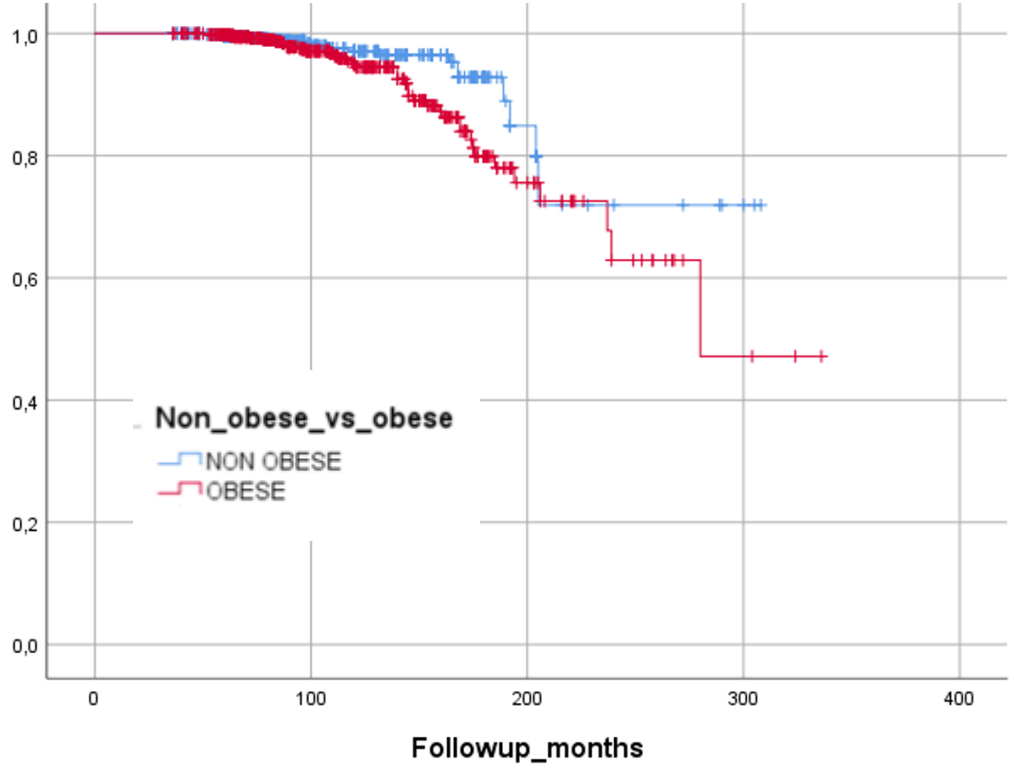


Number at risk

	0	50	100	150	200	250	300	350
(1)	195	184	73	29	6	1	0	0
(2)	496	472	192	84	12	5	2	0
(3)	661	647	321	126	31	12	3	0

(1) Lean
(2) Overweight
(3) Obese

(B)

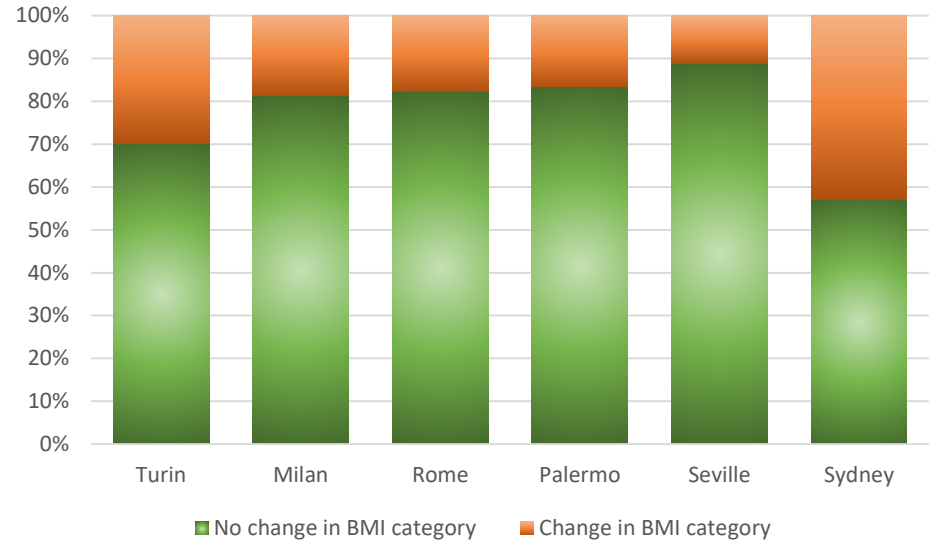


Number at risk

	0	50	100	150	200	250	300	350
(1)	691	656	265	113	18	6	2	0
(2)	661	647	321	126	31	12	3	0

(1) Non-obese
(2) Obese

A



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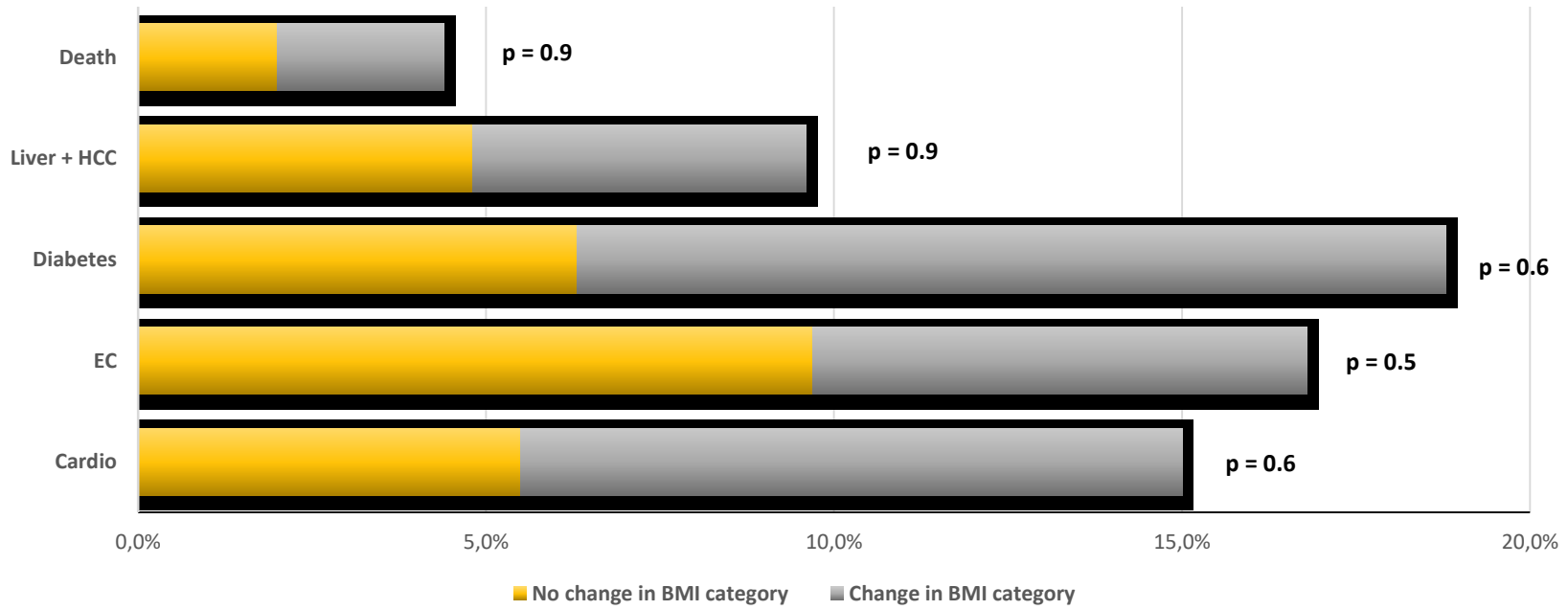

EC: Extrahepatic Cancers
HCC: Hepatocellular Carcinoma