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ORIGINAL ARTICLE

Epidemiology/Genetics

Combining diabetes, sex, and menopause as meaningful clinical features associated with NASH and liver fibrosis in individuals with class II and III obesity: A retrospective cohort study

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Abstract

Objective: Steatotic liver disease (SLD) is frequent in individuals with obesity. In this study, type 2 diabetes (T2D), sex, and menopausal status were combined to refine the stratification of obesity regarding the risk of advanced SLD and gain further insight into disease physiopathology.

Methods: This study enrolled 1446 participants with obesity from the ABOS cohort (NCT01129297), who underwent extensive phenotyping, including liver histology and transcriptome profiling. Hierarchical clustering was applied to classify participants. The prevalence of metabolic disorders associated with steatohepatitis (NASH) and liver fibrosis (F ≥ 2) was determined within each identified subgroup and aligned to clinical and biological characteristics.

Results: The prevalence of NASH and F ≥ 2 was, respectively, 9.5% ($N = 138/1446$) and 11.7% ($N = 159/1365$) in the overall population, 20.3% ($N = 107/726$) and 21.1% ($N = 106/502$) in T2D patients, and 3.4% ($N = 31/920$) and 6.1% ($N = 53/863$) in non-T2D patients. NASH and F ≥ 2 prevalence was 15.4% (33/215) and 15.5% (32/206) among premenopausal women with T2D vs. 29.5% (33/112) and 30.3% ($N = 36/119$) in postmenopausal women with T2D ($p < 0.01$); and 21.0% (21/100) / 27.0% (24/89) in men with T2D ≥ age 50 years and 17.9% (17/95) / 18.5% (17/92) in men with T2D < age 50 years (NS). The distinct contribution of menopause was confirmed by the interaction between sex and age with respect to NASH among T2D patients ($p = 0.048$). Finally, several NASH-associated biological

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traits (lower platelet count; higher serum uric acid; gamma-glutamyl transferase; aspartate aminotransferase) and liver expressed genes *AKR1B10* and *CCL20* were significantly associated with menopause in women with T2D but not with age in men with T2D.

Conclusions: This study unveiled a remarkably high prevalence of advanced SLD after menopause in women with T2D, associated with a dysfunctional biological liver profile.

INTRODUCTION

Steatotic liver disease (SLD) [1] is a chronic disease characterized by excessive accumulation of triglycerides in hepatocytes and subsequent liver steatosis. If inflammation and liver injury are also present, patients may develop nonalcoholic steatohepatitis (NASH) that may progress to liver fibrosis, cirrhosis, and hepatocellular carcinoma (HCC). In parallel with obesity, the number of NASH cases is expected to increase significantly, with an estimated prevalence of SLD of nearly 30% by 2030 [2, 3]. Patients with a higher risk of NASH may benefit from current and future efforts to prevent, diagnose, and treat the disease. Until clinically validated and useful biomarkers are developed, the diagnosis of NASH requires a liver biopsy. It is therefore key to determine simple features that will help primary care providers identify subgroups at a higher risk of NASH among individuals with obesity. Among the clinical characteristics associated with the risk of SLD, three in particular deserve special attention: type 2 diabetes (T2D), sex, and menopausal status. First, T2D appears to be the most important predictor of NASH and significant liver fibrosis in individuals with obesity [4]. A recent meta-analysis estimated that NASH is present in 37.3% (95% confidence interval [CI]: 24.7-50.0) of patients with T2D while fibrosis is in 17% (95% CI: 7.3-34.9) [5]. This bidirectional relationship between T2D and NASH suggests common pathogenic mechanisms [6, 7]. Second, evidence also suggests that NASH is a sex-dimorphic disease, being more frequent in males [8]. Moreover, insulin resistance, hepatic lipid influx, and hepatic adaptation to lipids (e.g., regional fat distribution, visceral adiposity, low-grade systemic inflammation, sarcopenia, hepatic beta-oxidation, and triglyceride synthesis in the liver) are all affected by sex and sex hormones [8–10]. In line with this, PNPLA3 genetic variants have been found to have sex-specific effects on disease progression in primary sclerosing cholangitis [10]. Genome-wide analysis of transcriptomic liver profile further supports the sexually dimorphic nature of NASH and its link with fibrosis [11]. Sex appears therefore as a significant determinant of the progression of NASH and should be included in future practice guidelines [8]. Third, epidemiological studies have consistently reported that the risk of SLD increases after menopause in women [11–13], thus identifying natural age-related hormone fluctuations as a disease-modifying factor [8]. Postmenopausal women also have a higher risk of NASH and fibrosis than premenopausal women [14, 15] probably due to the loss of the inhibitory effects of estrogen on stellate cell activation and fibrogenesis [8]. Premature menopause and a longer duration of estrogen deficiency further increase the risk of fibrosis [16].

Additionally, it was shown in T2D that stratifying the disease according to clinical phenotype differs in disease mechanism and progression, risk of complications, and response to treatment [17–21]. Here, we hypothesized that patients could be usefully grouped into qualitative clusters based on simple clinical features associated with increased prevalence of NASH, such as T2D, sex, and menopausal status, which could share an underlying biological level dysfunction. We assessed whether combining these three features could improve SLD stratification in an adult population with obesity with histologically proven NASH and/or significant liver fibrosis. We also tested whether this stratification was related to the clinical, biological, and molecular profiles of the defined subgroups.

METHODS

Study design and patients

This retrospective study analyzed data from the ABOS (Atlas Biologique de l'Obésite Sévère) cohort (NCT01129297), a prospective study aiming to identify determinants for the outcome of bariatric surgery. All ABOS participants who underwent bariatric surgery between 2006 and 2020 at Lille University Hospital were enrolled in the present study (Figure S1). Informed consent was obtained from all participants. Demographic characteristics, anthropomorphic measurements, medical history, medication use, including hormone replacement therapy (HRT), and clinical laboratory tests were prospectively collected before surgery, as previously described [22]. Diabetes status was defined at baseline, based on American Diabetes Association guidelines (Supplementary Material and Methods). Menopausal status was classified according to self-reported reproductive information and a history of bilateral oophorectomy.

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Liver histology

The SLD/NASH diagnosis and the classification of the SLD cases among participants from the ABOS cohort were performed in the Department of Pathology at Lille University Hospital, France. Liver biopsies were systematically planned during the surgical procedure. Needle biopsies were performed during the first part of the surgical procedure after trocar insertion and abdominal exploration, within 10 min after pneumoperitoneum installation. The Hepafix needle biopsy system was used until 2010, and the MONOPTY needle biopsy system (16G, ref: 121620; C. R. Bard, Tempe AZ, USA) was used thereafter. The same slides were evaluated simultaneously by two different expert liver pathologists, unaware of the clinical information, who gave a consensual central diagnosis as previously published [11, 23–25]. As previously described [23], the diagnosis of NASH was defined by the presence and pattern of specific histological abnormalities on liver biopsy using the Brunt scoring system for NASH [26]. Accordingly, NASH diagnosis required the association of liver steatosis, hepatocyte ballooning, and inflammation. Brunt criteria included the following parameters: amount of fat: graded 1–3 according to the percentage of fatty droplets (1, 0%–33%; 2, 34%–66%; 3, 67%–100%) and necroinflammation: graded 0 (absent) to 3 (1, occasional ballooned hepatocytes) and no or very mild inflammation; 2, ballooning of hepatocytes and mild to moderate portal inflammation; 3, intra-acinar inflammation and portal inflammation). Liver fibrosis was assessed semiquantitatively using the Kleiner score (defined as follows: F0, normal; F1, stages are divided into three subclasses: 1a, mild pericellular fibrosis in zone 3, 1b, moderate pericellular fibrosis in zone 3, and 1c, portal fibrosis; F2, perivenular and pericellular fibrosis confined to zone 2 and 3, with or without portal or periportal fibrosis; F3, bridging or extensive fibrosis with architectural distortion and no clear-cut cirrhosis; and F4, cirrhosis) [27]. Patients with excessive daily alcohol consumption (>30 g/day for male patients, >20 g/day for female patients) and/or nonmetabolic acute, chronic liver disease (alcoholic, drug-induced, or viral hepatitis) were excluded. Biopsies were obtained from individuals who were not in a drug treatment trial and were not receiving any specific drug therapy (Figure S1).

Outcomes

The primary outcome was the diagnosis of NASH defined by the joint presence of steatosis, lobular inflammation, and hepatocellular ballooning [26] independently of total SLD activity score. The secondary outcome was the diagnosis of moderate-to-advanced liver fibrosis (F ≥ 2) defined by a grade ≥2 according to Kleiner scoring (F ≥ 2) [27]. Additionally, we evaluated clinico-biological traits available in the ABOS cohort associated with NASH as well as the expression of liver genes previously related to NASH progression.

Microarray data

Transcriptomic data were available in a subset of 869 patients enrolled from 2006 to 2016 [25]. Microarray data generation, quality control, and preprocessing have been previously described and are detailed in the Supplementary Material and Methods.

Study Importance

What is already known?

- Although type 2 diabetes (T2D), male sex, and menopause have been previously associated with a higher risk of nonalcoholic steatohepatitis (NASH) in individuals with obesity, they have always been analyzed separately.
- T2D appears to be the most important predictor of NASH and significant liver fibrosis in individuals with obesity.
- Sex appears as a significant determinant of the progression of NASH.
- Postmenopausal women have a higher risk of NASH and fibrosis than premenopausal women.

What does this study add?

- We showed that combining these simple clinical features improves the stratification of individuals with class II and class III obesity, according to the prevalence of NASH.
- We also identified liver-specific clinico-biological and molecular profiles associated with menopause in women with obesity and T2D, in parallel with the higher prevalence of significant liver disease observed in this subgroup.

How might these results change the direction of research or the focus of clinical practice?

- This study from a large histologically characterized steatotic liver disease (SLD) cohort may provide new insight into disease heterogeneity and suggest new hypotheses to advance precision medicine. Our results highlight the importance of considering sex/menopause in investigating risk factors for the progression of SLD in individuals with T2D and obesity. It is likely that various populations will benefit from pharmacotherapies specific to the metabolic and molecular abnormalities responsible for their SLD.

Statistical analysis

A hierarchical clustering was first applied to stratify patients according to binary variables of diabetes status, sex, and menopause for women, without any supervision of outcomes. This clustering was computed with the R function hclust with *gower* distance calculated using the R package cluster [28]. We then calculated the rates of patients with NASH and fibrosis in each subgroup. In parallel, to disentangle the

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effect of menopause from the effect of age, we run logistic regressions to test the interaction between age and sex with respect to the prevalence of NASH. For that purpose, among T2D patients, we compared the two models with and without interaction through a likelihood ratio test. The interaction was also analyzed after adjustment for other potential confounding factors (body mass index [BMI] and arterial hypertension). In all statistical analyses, age was included as a binary input variable using an empirical cutoff value. This cutoff value was defined as the one that best separates premenopausal and postmenopausal women in our cohort, according to a receiver operating characteristic (ROC) curve analysis (Figure S2). Additionally, we performed sex-specific comparisons between pre- and postmenopausal women on one hand and between men younger and older than the cutoff value on the other hand.

Clinico-biological data were reported as the mean with standard deviation (SD) for continuous variables normally distributed, median with interquartile range (IQR) for other continuous variables and frequencies (in percentages) for categorical variables. These clinic-biological variables were compared among the defined subgroups using ANOVA for continuous variables normally distributed, Kruskall–Wallis tests for other continuous variables, and χ^2 tests for categorical variables. *P* values were corrected for multiple comparisons using the Bonferroni method to control the family-wise error rate. Proportions of NASH or fibrosis (F ≥ 2) in each subgroup were compared with the average proportion of all subgroups using a binomial test. The clinico-biological variables between NASH and non-NASH individuals were compared using Welch *t* tests for continuous variables normally distributed, Wilcoxon tests for other continuous variables, and χ^2 tests for categorical variables. A cutoff of 0.05 was chosen on raw *p* values to select clinical characteristics related to NASH. The same strategy was used to compare these selected variables between pre- and postmenopausal women with T2D and between men with T2D younger and older than the cutoff value. In these analyses, the *p* values were corrected using Bonferroni method. A cutoff of 0.05 was chosen on adjusted *p* values for statistical significance. Clinico-biological traits were represented by ratios between two subgroups (post- vs. premenopausal status for women, old vs. young for men, NASH vs. non-NASH). The 95% CIs of ratios were computed using the R package DescTools. For comparing NASH and fibrosis (F ≥ 2) in pre- and postmenopausal women with T2D, the statistical power of the khi-2 test was determined. For this, we calculated the effect size as the square of the ratio of the Khi-2 statistic to the number of observations N, which corresponds in our case to Cramer's V. The statistical power was then calculated using the pwr.chisq.test function from the pwr R package (version 1.3–0).

For transcriptomic data, differential analysis between NASH and non-NASH was performed using moderated *t* tests from the R Bioconductor package limma with a Benjamini–Hochberg correction for multiple testing to control the false discovery rate genes were called differentially expressed if their adjusted *p* values were below 0.05 and their fold changes (FC) were higher than 1.5 (decrease or increase). Therefore, we focused our analysis on the expression of a subgroup of 25 genes that have been previously associated with the progression of NAFL to severe liver disease (NASH and/or fibrosis [F ≥ 2]) [29] including *AKR1B10*, *ANKRD29*, *CCL20*, *CFAP221*, *CLIC6*, *COL1A1*, *COL1A2*, *DRNA*, *DUSP8*, *EPB41L4A*, *FERMT1*, *GDF15*, *HECW1*, *IL32*, *ITGBL1*, *LTBP2*, *PDGFA*, *PPAPDC1A*, *RGS4*, *SCTR*, *STMN2*, *THY1*, *TNFRSF12A*, *TYMS*, and *HSD17B14*. Then, the expression of these genes was compared between pre- and postmenopause for women with T2D and between men with T2D younger and older than the cutoff value with Welch *t* tests. No adjustment was performed on raw *p* values due to the small number of tests. The differences were considered significant when raw *p* values were below 0.05. All statistical analyses were performed using R statistical software version 3.6.3.

RESULTS

Patient characteristics

Among 1545 ABOS participants, we enrolled in the present study 1446 patients with available liver histology who had no exclusion criteria including a history of excessive alcohol consumption and/or having a nonmetabolic acute, chronic liver disease (alcoholic, drug-induced, or viral hepatitis) (Figure S1). The mean age and BMI were 42.0 (±11.7) years and 46.5 (±8.7) kg/m^2, respectively. The prevalence of NASH and liver fibrosis (F ≥ 2) was, respectively, 9.5% ($n = 138$, 95% CI: 8.0-11.1) and 11.7% ($n = 159$, 95% CI: 10.0-13.4), in the overall population and 20.3% ($n = 107$, 95% CI: 16.9-23.8) and 21.1% ($n = 106$, 95% CI: 17.6-24.7) in T2D patients and 3.4% ($n = 31$, 95% CI: 2.2-4.5) and 6.1% ($n = 53$, 95% CI: 4.5-7.7) in non-T2D patients (Figure 1).

Stratification of patients

Hierarchical clustering indicated the presence of T2D as the first node separating patients, as shown in Figure 1A,B. Proportions of NASH and fibrosis in the six subgroups of the clustering revealed differences in the T2D patients. Across the T2D subgroups, the proportion of NASH was higher in premenopausal women ($n = 33/215$, 15.4%; 1.6-fold increased change above the average, 95% CI: 1.1-2.3, $p = 0.004$), postmenopausal women ($n = 36/119$, 30.3%, 3.2-fold increased change above average; 95% CI: 2.3-4.3, $p < 0.001$), and men ($n = 38/192$, 19.8%, 2.1-fold increased change above the average, 95% CI: 1.5-2.9, $p < 0.001$). Of note, the proportion of NASH in postmenopausal women with T2D was twice as high as in premenopausal women with T2D (2-fold increase, 95% CI: 1.3-3.0, $p = 0.002$), and 1.5 times higher than in men with T2D (1.5-fold increase, 95% CI: 1.0-2.3, $p = 0.049$). In contrast, NASH prevalence was similar in men with T2D below 50 years (21.0%, $N = 21/100$; 95% CI: 13.0-29.0) versus above 50 years (17.9%, $N = 17/95$, 95% CI: 10.6-26.4) of age ($p = 0.80$).

In nondiabetic participants, the proportion of NASH was similar in pre- and postmenopausal women ($n = 20/613$, 3.3%, 95% CI: 1.9-4.7

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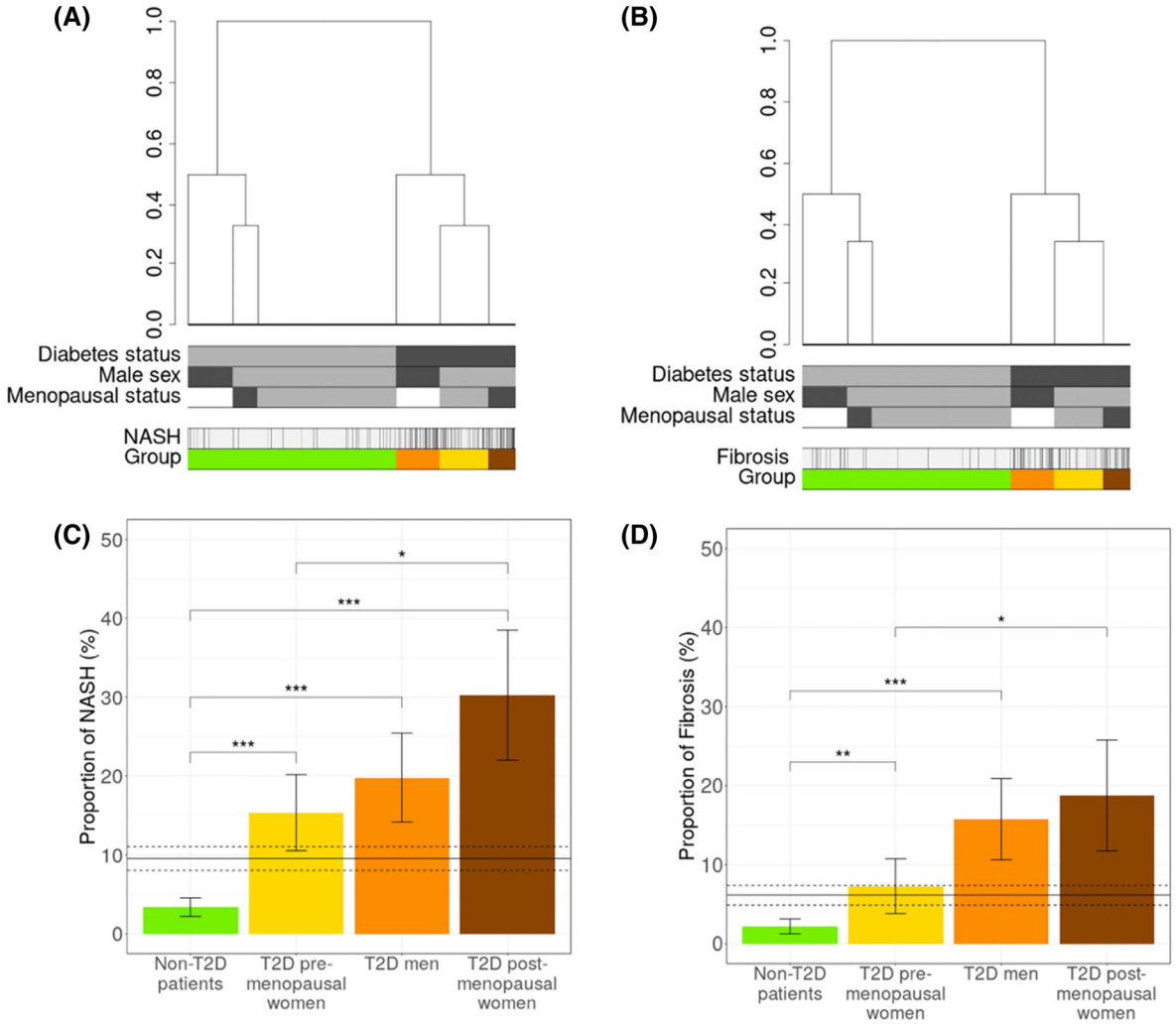

FIGURE 1 Risk stratification of (A) NASH and (B) fibrosis in the ABOS cohort based on T2D status, male sex, and menopausal status. The cohort was stratified by hierarchical clustering based on T2D status, male sex, and menopausal status in four subgroups displaying distinct prevalence of NASH and/or advanced fibrosis. Diabetes status, male sex, and menopausal status are illustrated in the first three bars under the dendrogram and colored in dark gray for modality TRUE, light gray for modality FALSE, and white for missing values. Each individual presenting with NASH and/or advanced fibrosis is figured as a gray line on the fourth bar under the dendrogram. The last-colored bar indicates the group allocation: Group 1 (green) corresponds to non-T2D patients, group 2 (yellow) for premenopausal women with T2D, group 3 (orange) to men with T2D, and group 4 (brown) to postmenopausal women with T2D. Prevalence of (C) NASH and/or (D) advanced fibrosis (Kleiner fibrosis score ≥2) in the different subgroups. The χ2 test was used for group differences in percentages. Adj. *p* values with Bonferroni ***<0.001; **<0.01; *<0.05). The black line indicates the average prevalence in the entire cohort and the dashed line indicates 95% CI. NASH, nonalcoholic steatohepatitis; T2D, type 2 diabetes. [Color figure can be viewed at wileyonlinelibrary.com]

and $n = 3/109$, 2.75%, 95% CI: 0.0-5.8, respectively) and in men ($n = 8/198$, 4.0%, 95% CI: 1.3-6.8). Thus, all patients without T2D were combined in a single subgroup of individuals who had a statistically significantly lower than average prevalence of NASH (2.8-fold decrease; 95% CI: 1.9-4.1, $p < 0.001$). For that reason, we suggest a final stratification focusing on four subgroups: patients without T2D, premenopausal women with T2D, postmenopausal women with T2D, and men with T2D.

Similar results among subgroups were observed regarding the proportion of fibrosis (F ≥ 2), the second studied outcome. Among the T2D subgroup, the proportion of patients with fibrosis (F ≥ 2) was 15.5% in premenopausal ($n = 32/206$, 95% CI: 10.6-20.5), 29.5% in postmenopausal women ($n = 33/112$, 95% CI: 21.0-37.9, 22.3%), and 22.3% in men ($n = 41/184$, 95% CI: 16.3-28.3). The proportion of F ≥ 2 in postmenopausal women with T2D and in men with T2D was statistically significantly different from the cohort average proportion (2.9-fold increase, 95% CI: 1.8-4.5, $p < 0.001$ and 2.5-fold increase, 95% CI: 1.7-3.6, $p < 0.001$). Among the non-T2D patients, the proportion of fibrosis (F ≥ 2) was 3.8% in premenopausal ($n = 22/577$; 95% CI: 2.3-5.4), 5.9% in postmenopausal women ($n = 6/102$, 95% CI: 1.3-10.5), and 13.6% in men ($n = 25/184$, 95% CI: 8.6-18.5). In contrast, F ≥ 2 prevalence was similar in men with T2D below 50 years (18.5%, $N = 17/92$; 95% CI: 10.2-25.6.0) versus above 50 years of age (27.0%, $N = 24/89$, 95% CI: 17.8-36.2) ($p = 0.19$).

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TABLE 1 Patient characteristics based on group allocation in ABOS cohort

	N	Non-T2D	T2D			
			Premenopausal women	Postmenopausal women	Men	Adj. *p*
Women/men [%]	1446	722/198 (78/22)	215/0 (100/0)	119/0 (100/0)	0/192 (0/100)	$<10^{-5}$
Age [years]	1446	38.62 (11.46)	41.78 (7.92)	56.26 (4.56)	49.28 (9.29)	$<10^{-5}$
Age of menopause [years]	188	50 (6.25)	/	50 (5)	/	1
Menopause duration [years]	188	5 (5.25)	/	6 (7)	/	1
BMI [kg/m^2]	1446	45.9 (9.02)	48.21 (8.63)	47.36 (6.57)	46.98 (7.73)	0.08815
Diabetes duration [years]	482	/	3 (6)	6 (10)	4 (9.75)	0.00352
Antidiabetic drugs [%]	1312					$<10^{-5}$
0 [%]		920 (100)	54 (31)	18 (23)	30 (22)	
1 [%]*		0 (0)	68 (39)	34 (43)	69 (50)	
≥ 2 [%]*		0 (0)	52 (30)	27 (34)	40 (29)	
Patients on insulin treatment [%]	1445	0 (0)	40 (19)	40 (34)	52 (27)	$<10^{-5}$
Fasting glucose [mmol/L]	1404	5.21 (0.73)	7.12 (2.87)	7.78 (3.51)	7.93 (3.51)	$<10^{-5}$
120 min glucose postload [mmol/L]	1364	6.38 (2.33)	12.65 (6.24)	14.84 (8.45)	13.37 (6.74)	$<10^{-5}$
Fasting insulin [mUI/L]*	1271	13.35 (9.72)	16 (14.75)	13.4 (12.2)	19.25 (14.95)	$< 10^{-5}$
120 min insulin postload [mUI/L]*	1236	58.4 (67.05)	57.7 (82.9)	53.8 (67.6)	59.35 (59.12)	1
Fasting plasma C-peptide [ng/mL]	1103	3.4 (1.5)	4. (1.9)	4.13 (2.65)	4.4 (2.41)	$<10^{-5}$
120 min C-peptide [ng/mL]	998	10.42 (4.02)	10.44 (4.9)	10.59 (4.27)	9.37 (4.53)	1
HbA1c [mmol/mol]	1426	5.6 (0.5)	6.8 (1.8)	7. (1.8)	7.3 (2.1)	$<10^{-5}$
Leucocytes [$\times 10^9$/L]	1445	8.34 (2.25)	8.76 (2)	7.94 (2.19)	8.3 (2.27)	0.39442
Thrombocytes [$\times 10^9$/L]	1443	283.97 (66.09)	299.19 (67)	262 (55.93)	247.79 (62.68)	$<10^{-5}$
Serum alpha2 macroglobulin [g/L]	1422	1.71 (0.47)	1.75 (0.51)	1.93 (0.63)	1.83 (0.82)	0.00328
Serum uric acid [mg/L]	1282	58.43 (13.25)	55.1 (13.71)	62.08 (16.93)	64.36 (16.55)	$<10^{-5}$
Serum protein [g/L]	1422	75.72 (4.08)	75.68 (4.51)	75.95 (3.82)	75.43 (4.35)	1
Serum C-protein reactive [mg/L]	1170	7.14 (6.18)	8.62 (5.17)	6.89 (6.37)	5.57 (6.21)	0.00436
Total cholesterol [mmol/L]	1428	5 (0.94)	4.91 (0.94)	4.89 (0.97)	4.75 (1.18)	0.51988
High-density lipoprotein cholesterol [mmol/L]	1428	1.18 (0.27)	1.11 (0.25)	1.2 (0.29)	0.98 (0.21)	$<10^{-5}$
Low-density lipoprotein cholesterol [mmol/L]	1417	3.17 (0.82)	3 (0.85)	2.84 (0.9)	2.67 (0.88)	$<10^{-5}$
Triglycerides [mmol/L]	1429	1.29 (0.75)	1.59 (0.98)	1.66 (0.98)	1.99 (1.61)	$<10^{-5}$
Alanine aminotransferase (ALAT) [UI/L]	1442	25 (18)	26 (16.75)	29 (26)	37 (26.75)	$<10^{-5}$
Aspartate aminotransferase (ASAT) [UI/L]	1435	22.5 (11)	22 (13)	25.5 (18.5)	28 (17)	$<10^{-5}$
Gamma-glutamyltransferase (Gamma-GT) [UI/L]	1436	27 (23)	32 (28.75)	45.5 (51)	51 (45)	$<10^{-5}$
Hypolipidemic treatment [%]	1446	77 (8)	61 (28)	67 (56)	104 (54)	$<10^{-5}$
Systolic blood pressure [mmHg]	1439	135.34 (18.92)	135.32 (17.22)	140.97 (16.62)	141.23 (18.27)	0.00082

(Continues)

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TABLE 1 (Continued)

	N	Non-T2D	T2D			Adj. *p*
			Premenopausal women	Postmenopausal women	Men	
Diastolic blood pressure [mmHg]	1439	76.84 (14.96)	76.37 (14.91)	76.29 (13.94)	79.67 (14.85)	1
Hypotensive treatment [%]	1446	249 (27)	101 (47)	102 (86)	143 (74)	$<10^{-5}$
Treatment with HRT	1022	316 (45)	78 (37)	5 (4)	0 (0)	$<10^{-5}$
Histological features						
NASH [Brunt], N (%)	1446	31 (3)	33 (15)	36 (30)	38 (20)	$<10^{-5}$
Fibrosis [Kleiner] Grade ≥2, N (%)	159/1365	53 (6)	32 (15)	33 (30)	41 (23)	$<10^{-5}$

Note: Groups were compared using the $\chi 2$ test for categorical variables, ANOVA *F*-tests for continuous variables having normal distribution, and Kruskall–Wallis test for continuous having no normal distribution and Bonferroni correction was applied. Values are mean ± SD, median ± IQR, or *n* (%).

Abbreviations: HRT, hormone replacement therapy; NASH, nonalcoholic steatohepatitis; T2D, type 2 diabetes.

*Insulin-treated individuals were not included.

Age and sex interaction with respect to the prevalence of NASH in T2D patients

To further explore the distinct role of menopause in women with T2D, we then analyzed the interaction between sex and age with respect to NASH prevalence among all patients with T2D. The likelihood ratio test between the two logistic regression models with and without interaction term showed a statistically significant interaction ($p = 0.048$), which remained significant after adjusting for other potential confounding factors (BMI and arterial hypertension), ($p = 0.049$). The ROC curve analysis (area under the curve 96.3%, 95% CI: 95.0-97.6) showed that the age cutoff that best separates pre- and postmenopausal women was 50 years (Figure S2). In line with the interaction previously identified, we found that the prevalence of NASH in women with T2D was significantly different between younger and older women ($p = 0.002$). In contrast, we observed no significant difference in the proportion of NASH between younger ($n = 89$) and older men ($n = 103$) with T2D, respectively (1.2-fold-increase, 95% CI: 0.7-2.0, $p = 0.75$). Likewise, we observed no significant difference in the proportion of fibrosis (F ≥ 2) between younger and older men with T2D (1.4-fold decrease, 95% CI: 0.7-2.7, $p = 0.48$).

Distinct clinico-biological profiles related to NASH and menopausal status in women with T2D

Clinical, biological, and liver histology of the subgroups are presented in Table 1. We examined 35 clinico-biological features available in the ABOS cohort and identified 26 of them that were significantly associated with NASH in our cohort (Figure S3). Noteworthy, most features related to impaired glucose control were positively associated with NASH. In contrast, exposure to HRT was negatively associated with an increased prevalence of NASH in women ($p = 0.001$).

We then focused on identifying among these 26 clinico-biological traits, those that were specifically associated with NASH induced by menopause in women with T2D. For this, we conducted two parallel analyses, comparing pre- and postmenopausal women with T2D and men with T2D younger and older than the age of 50 years (Figure 2).

The first analysis identified six traits associated with menopause in women with T2D: lower platelet count (Adj. $p < 0.001$), higher level of serum uric acid (Adj. $p = 0.008$), gamma-glutamyltransferase (Gamma-GT; Adj. $p = 0.02$), and serum glutamate oxaloacetate transaminase (SGOT; Adj. $p = 0.029$), and higher frequency of hypolipidemic (Adj. $p < 0.001$) and hypotensive treatments (Adj. $p < 0.001$) (Figure 2A). In the second analysis, which was conducted in men with T2D, the variables associated with age under and over 50 years were higher low-density lipoprotein level and macroglobulin levels as well as a higher frequency of hypolipidemic and hypotensive treatments (Adj. $p < 0.001$, Adj. $p = 0.04$, Adj. $p < 0.001$, and Adj. $p < 0.001$, respectively) (Figure 2B).

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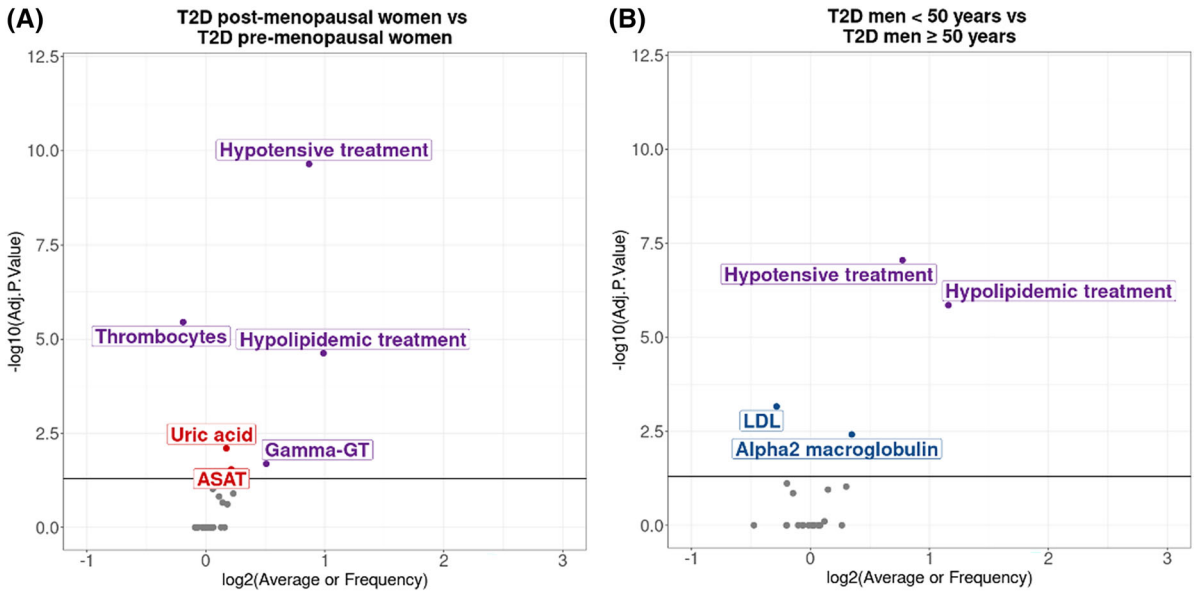

FIGURE 2 Comparison of the mean, ranks, or frequency of nonalcoholic steatohepatitis-related clinico-biological characteristics (A) between pre- and postmenopausal women with T2D and (B) between men with T2D before and after 50 years. For each analysis, the differences between the mean, rank, or frequency ratio of the two groups were compared using χ2 tests for categorical variables and *t* tests or Wilcoxon tests for continuous variables. *P* values were corrected with the Bonferroni method. Continuous variables are represented by means or medians ratio between postmenopausal status and premenopausal status for women and means or medians ratio between men with age ≥50 years and <50 years status. Categorical variables were represented as the percentage ratio between postmenopausal status and premenopausal status for women and means ratio between men with age ≥50 years and <50 years status. In red are variables statistically significantly different only in premenopausal versus postmenopausal women, violet represents statistically significant differences in premenopausal versus postmenopausal women and in men with age <50 years and ≥50 years, blue is statistically significantly differences only in men with age <50 years and ≥50 years, and in gray are the variables not statistically significantly different in each of the comparisons. The *y*-axis corresponds to the −log10 (Adj. *p* value), and the *x*-axis displays the log2 of average or frequency. T2D, type 2 diabetes. [Color figure can be viewed at wileyonlinelibrary.com]

Hepatic gene expression profiles related to NASH and menopausal status in women with T2D

Our objective was to investigate whether the specific link between severe liver disease and menopause in women with T2D was associated with specific changes in the liver transcriptome profile. To achieve this, in our cohort, we analyzed the liver expression profile of a subgroup of 25 genes (see Methods) that have previously been linked to the progression of nonalcoholic fatty liver (NAFL) to severe liver diseases, such as NASH and advanced fibrosis [29]. Out of these 25 genes, 23 were available for analysis in the ABOS cohort. Among these 23 genes, we identified two that exhibited significant differential expression with clinically relevant FC (≥1.5) in postmenopausal compared with premenopausal women with T2D. *AKR1B10* showed an FC of 1.6 with a *p* value of 0.011, indicating a significant difference in expression between these two groups. *CCL20* demonstrated an FC of 1.7 with a *p*-value of 0.019 (Table S1, Figure S4). Of note, we did not find any differential expression with clinically relevant FCs among these 23 NASH-related genes, when comparing younger and older men with T2D (Table S1). The distinct expression of these two NASH-related genes observed after menopause in women with T2D further supports the specific impact of menopause on the development of NASH.

DISCUSSION

In this cross-sectional study, we stratified a large cohort of individuals with class II and class III obesity by combining three simple clinical features: diabetes, sex, and menopausal status. We identified four distinct subgroups with various prevalences of NASH: postmenopausal women with T2D, men with T2D, premenopausal women with T2D, and patients without T2D. Although diabetes, male sex, and menopause have been previously associated with a higher prevalence of NASH in individuals with obesity, they have always been analyzed separately. In the present study, we identified a new stratification based on the combination of these three simple features, which are all readily available in the primary care setting. Our main finding was the distinct association observed between menopause and the prevalence of severe SLD (NASH and/or liver fibrosis [F ≥ 2]) in women with T2D. In contrast, older age had no significant impact on the prevalence of NASH in men with T2D. In addition, we observed that in the absence of T2D, male sex and postmenopausal status in women had no significant influence on the prevalence of NASH, which remained inferior to 5% in all cases.

One major strength of this study is the large number of unselected patients with severe obesity and a detailed phenotype and liver histology available at enrollment. This enabled us to analyze the

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clinical and biological characteristics associated with NASH across the entire cohort and look for a distinct profile of the disease across the various subgroups. We identified four clinico-biological variables including lower platelet count, higher level of serum uric acid, Gamma-GT, and SGOT that paralleled the higher prevalence of NASH observed in postmenopausal women in the presence of T2D. In contrast, these features were not associated with older age in men with T2D. Hyperuricemia is a known risk factor for SLD, and it has been shown to be higher in women [30–32]. In postmenopausal—but not premenopausal—women, serum uric acid levels were found to be associated with SLD, even after adjusting for factors associated with metabolic syndrome. Noteworthy, estrogen replacement therapy was negatively related to NASH in our study, and previous research has linked estrogen deficiency with hyperuricemia [33–35]. Finally, in postmenopausal women, uric acid has been associated with inflammation and major adverse cardiovascular events [36]. Adding to the available evidence, our findings suggest that higher uric acid levels may play a distinct role in NASH development, especially among women with T2D. The mechanisms underlying this are still unknown. Lower platelet count found in postmenopausal women with T2D is in line with menopause-related differences in blood count, the low estrogen level in postmenopausal women being considered to be responsible for the decreased platelet count [37, 38] and with age-related changes in platelet function being found more profound in women than in men [38]. Additionally, it was shown in a randomized clinical trial that combined HRT significantly decreased aminotransferase levels in postmenopausal women with T2D and presumed SLD compared with placebo controls [39]. Collectively, current evidence suggests that estrogen may protect against SLD.

Another strength of our study was the analysis of liver transcriptomic data from the vast majority of the study participants, which allowed us to show that among genes previously linked to NASH and/or liver fibrosis, *AKR1B10* and *CCL20* were differentially expressed depending on the women's menopausal status. The gene *AKR1B10* has been associated with lipid metabolism and oxidative stress [40, 41]. Its increased expression in postmenopausal women with T2D compared with premenopausal women with T2D might indicate a higher level of oxidative stress or lipid accumulation in the liver after menopause in women with T2D. Similarly, the *CCL20* gene is involved in the recruitment of immune cells to the site of inflammation [42], and its upregulation in postmenopausal women with T2D compared with premenopausal women with T2D might indicate an enhanced inflammatory response in the liver of postmenopausal women with T2D. These two genes are linked to liver inflammation and may also be involved in menopause-related mechanisms that lead to NASH. These two genes may also play a role in the reproductive system and may be involved in menopause-related mechanisms that lead to NASH. Estradiol, for example, is an important regulator of *CCL20* by uterine epithelial cells [43]. Additionally, postmenopausal women had significantly higher levels of *AKR1B10* than premenopausal women [44]. This merits further exploration and provides new insight into disease heterogeneity and suggests new hypotheses to advance precision medicine.

One of the limitations of the study is that participants from the ABOS cohort do not reflect a general patient cohort, and our results may not be generalizable to individuals with lower levels of obesity, where the pathophysiology of SLD may be different. In our study, liver biopsy specimens were indeed obtained during bariatric surgery procedures in all participants. Importantly, these needle biopsies were performed in the first 10 min of the intervention, with the same material used for percutaneous biopsies. This systematic approach allowed us to evaluate accurately and consistently the presence of NASH and liver fibrosis (F ≥ 2) in our study population. This rigorous methodological approach strengthens the reliability of our findings regarding the prevalence rates of NASH and liver fibrosis in this specific patient population. We acknowledge also the lower prevalence rates of histologically proven NASH and liver fibrosis (F ≥ 2) in our cohort of patients with obesity submitted to bariatric surgery compared to previous studies. However, our study benefits from a prospective design that involves systematic histologic evaluation of the liver at the time of surgery for all participants. This approach allows for a more accurate assessment of NASH prevalence by avoiding the potential bias of overestimation. It is also important to consider the potential bias of other studies introduced by including only SLD patients specifically referred for liver biopsy. This selective referral for biopsy based on clinical indications, such as elevated liver enzymes, can overestimate the prevalence of NASH [45, 46]. We also acknowledge that the relative homogeneity of this single-center cohort, primarily of Caucasian ethnicity, and the retrospective design of our study may limit the applicability of the findings to other populations. Moreover, the prevalence of NASH and liver fibrosis (F ≥ 2) was rather low in our cohort, yet the number of patients enrolled in the various subgroups was sufficient to show significant differences in NASH prevalence in these subgroups. Like any study using liver biopsy as a standard, sampling errors can result in misdiagnosis of disease activity and fibrosis stage on biopsy. Finally, although exposure to HRT was negatively associated with an increased risk of NASH in the whole cohort, there were not enough postmenopausal women to draw any conclusions on the potential beneficial association between HRT and the risk of NASH in this population. Finally, future studies on more heterogeneous populations will be needed in order to determine whether our findings are generalizable.

CONCLUSION

The main and novel point addressed by our study is the specific contribution of menopause (as opposed to age) in the higher prevalence of NASH observed in postmenopausal women with T2D as compared with men younger and older than 50 years. We also identified liver-specific clinico-biological and molecular profiles associated with menopause in women with obesity and T2D, in parallel with the higher prevalence of significant liver disease observed in this subgroup. We showed that combining these simple clinical features improves the stratification of individuals with class II and class III obesity, according to the prevalence of NASH.

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Moreover, we also identified specific clinico-biological and molecular profiles associated with the high prevalence of NASH after menopause in women with obesity and T2D. This study of transcriptomic data from a large histologically characterized SLD cohort may provide new insight into disease heterogeneity and suggest new hypotheses to advance precision medicine. Overall, the study results suggest that menopausal women with T2D may share dysfunction of an underlying biological profile. Our results also highlight the importance of considering sex/menopause in investigating risk factors for progression in SLD, given the robust evidence to suggest multifaceted sexual dimorphism in NASH. Studying these patients could provide a more direct path into NASH physiopathology, helping us unravel the intricated physiopathology of NASH. Incorporating sex and menopause status may elucidate sex-specific mechanisms that allow further risk stratification of patients with SLD and promote individualized disease management. Indeed, as more is understood about the heterogeneity of SLD and precision medicine becomes a reality, it is likely that various populations will benefit from pharmacotherapies specific to the metabolic and molecular abnormalities responsible for their SLD.

Prospective studies are needed to determine whether it is possible to treat NASH and its progression as a group of distinct qualitative subtypes.O

AUTHOR CONTRIBUTIONS

Francois Pattou, Violeta Raverdy, Estelle Chatelain, Guillaume Lasailly, Robert Caiazzo, Jimmy Vandel, Helene Verkindt, Camille Marciniak, Benjamin Legendre, Viviane Gnemmi, Pierre Bauvin, Naima Oukhouya-Daoud, Gregory Baud, Mikael Chetboun, Emmanuelle Leteurtre, Bart Staels, Marie-Christine Vantyghem, Philippe Lefebvre, Philippe Mathurin, Guillemette Marot, and Francois Pattou contributed substantially to the conception and design of the study, the acquisition of data, or the analysis and interpretation. Estelle Chatelain performed the statistical analysis. Violeta Raverdy, Estelle Chatelain, and Francois Pattou drafted the article. Philippe Lefebvre, Guillemette Marot, Guillaume Lasailly, and Jimmy Vandel reviewed/edited the manuscript. All authors contributed to the interpretation of data and critical revision of the article. Francois Pattou is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors read and approved the final manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declared no conflict of interest.

DATA AVAILABILITY STATEMENT

The study protocol and methods as well as the ABOS cohort profile have been published (ClinicalTrials.gov identifier NCT01129297) and are unrestrictedly available. Clinical data sets generated during and/or analyzed during the current study are subject to national data protection laws and restrictions imposed by the ethics committee to ensure the data privacy of the study participants. They are not publicly available; however, they can be applied for through an individual project agreement with the principal investigator of the University Hospital of Lille, France. Affymetrix raw files are available at Gene Expression Omnibus (GEO) under the accession number GSE130991.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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