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WOMEN IN THE AGE GROUP OF 20 TO 50 YEARS ARE LESS PRONE TO DEVELOP NAFLD IN KERALA, SOUTH INDIA: A POPULATION BASED STUDY

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ABSTRACT

Aim: To investigate frequency, gender-specific differences and impact of polymorphisms in Non alcoholic Fatty Liver Disease (NAFLD) among the population of Kerala State, South India with a high incidence of type 2 Diabetes Mellitus (Type 2 DM).

Methods: In a Community based study, data were collected on anthropometry, blood pressure, morbidity patterns and Ultrasound was carried out in 484 subjects. 121 subjects were excluded due to other findings on ultrasonography, hepatitis B and C. 363 subjects, 126 with NAFLD (cases) and 237 with no NAFLD (controls) were evaluated. Blood samples collected after 12 hours fasting were analysed for blood glucose, Liver Function Tests, Lipid profile and Genetic studies for MTP polymorphisms.

Results: A low prevalence of NAFLD was noted in women below the age of 50 years ($P<0.05$). Prevalence of Type 2 DM was 14% and more common in men ($P=0.002$). Mean BMI was 25.5 for the NAFLD group and 23.2 for controls ($p< 0.001$). Women with NAFLD had higher BMI ($P=0.003$), systolic blood pressure (SBP) ($P=0.030$), LDL ($P=0.001$), HDL ($P=0.001$) and alkaline phosphatase ($P<0.001$) than men with NAFLD. Women with NAFLD had increased total cholesterol, higher AST/ALT ratio, ($P<0.001$) and diastolic BP ($P=0.061$) compared to women controls.

Microsomal triglyceride transfer protein (MTP) T-substitution at -493 was significantly more common in NAFLD than controls ($P=0.003$). High prevalence was noted among females as carriers ($P=0.002$). More than 80% of the T-allele carriers were females in the 20-50 years of age group, compared to 25% among males.

Conclusion: Women under 50 years of age had less NAFLD but more often had type 2 DM, metabolic syndrome, high BMI and the MTP -493 mutation. The specific age cut-off, for differences, indicates that estrogens may play an important role.

KEYWORDS : Non alcoholic fatty liver disease, diabetes, gender, Microsomal triglyceride transfer protein, India.

INTRODUCTION

Non alcoholic fatty liver disease (NAFLD) , with a prevalence of approximately 25 %, is the most common chronic liver disease in the world today [1] .This condition is primarily associated with obesity [2] non-insulin-dependent diabetes mellitus [3] , hyperlipidemia [4, 5] and insulin resistance, [6] i.e. the major characteristics of the metabolic syndrome, and is considered as a part of this syndrome [7].

Fatty liver, or simple steatosis, can progress to non alcoholic steatohepatitis or NASH [8, 9]. Further progression involves increasing cell damage and hepatic fibrosis, cirrhosis and, possibly, hepatocellular carcinoma, [9,3]. Currently, there is no effective drug to reduce the fibrosis, although suggestions regarding treatment with vitamin E[10], metadoxine [11] , saroglitazone [12] and obitocholic acid [13] are emerging.

The fatty infiltrated liver is insulin resistant [14] and it has been proposed that there exists a relationship between the hepatic fat content and the degree of insulin resistance. Furthermore, fatty livers overproduce glucose [14] and VLDL [15] and NAFLD has recently been reported to be an important emerging risk factor for cardiovascular disease [16,17]. In Kerala, a small state in southern India, NAFLD, insulin resistance, metabolic syndrome and incidence of Type 2 DM are high [18].

Several studies have reported genetic variations explaining NAFLD incidence [19-22], progression to NASH [20,23] and impact on alcoholic fatty liver disease [24]. In order to show if metabolic syndrome was the major factor or if genetic factors are involved, we chose to study polymorphisms in lipid regulating genes. There are contradictory reports on the importance of the microsomal triglyceride transfer protein (MTP) gene and especially the -493 polymorphism and its effect on cholesterol and triglyceride levels [25-28].

The MTP T-allele is described as a risk factor for severe hepatic steatosis grade in combination with hepatitis C infection [29,30] In order to study the impact of *obesity and* Type 2 DM, we evaluated the prevalence and gender difference of NAFLD in a population based study. In addition, we wanted to demonstrate whether polymorphisms in lipid regulating genes could be a modifying factor.

METHODS

Subjects:

This study was performed in the Trivandrum district of southern Indian state of Kerala. This state has the highest rate of literacy in India, contains groups of people with a variety of diets and ethnic and religious backgrounds in addition to exhibiting health indices more favourable than those in other regions of India. It also keeps good records and demographic documentation.

Our subjects were selected using random sampling of 5 of the 81 wards in accordance with a model described previously from the city of Trivandrum.[31,32] Each ward was subdivided into clusters of seven houses . 3–5 such clusters from each ward were then selected randomly for inclusion. We excluded subjects using medication such as phenytoin sodium, valproic acid, Isoniazid or sulfonamides; a history of alcohol or substance abuse (see also below). Written informed consent was received from each subject prior to enrolment in this investigation.

484 subjects (256 men and 228 women in the age range 20-70), selected from the population of approximately 200 000, answered questions regarding their medical history, nutritional profile, smoking, intake of alcohol and physical activity. Anthropometry ,blood pressures and ultrasonography of their livers were performed. Blood samples were taken after fasting. All subjects were examined for hepatitis B and C virus (HBV and HCV) by HBs antigen ELISA and anti HCV ELISA, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase, serum bilirubin, immunoglobulins, albumin, ferritin, lipid profile, blood glucose and fasting levels of serum insulin, total level of triglycerides, high-, low- and very low- density lipoprotein (HDL, LDL, VLDL) and total cholesterol.

Ultrasound was performed with a Logic 400 Ultrasound Scanner (Wipro G E) with a 3.5 MHz probe. After scanning, all subjects were evaluated according to the four level scale for ultrasound grading of fatty liver [33] Level 0 = a normal hepatic echo pattern, Level 1 = a slight increase in the echo pattern but normal appearance of vessels and the diaphragm. Level 2 = a moderate elevation in echogenicity along with

reduced visualization of portal veins and the diaphragm and Level 3 = a pronounced enhancement in the hepatic echo pattern with poor visualization of intrahepatic vessels and the posterior right lobe of the liver. 32 patients were excluded from the study (one upon finding a cystic region in the pancreas, eleven due to kidney cysts and three because of cystic areas in the liver, one because of calculi in the liver, two from calculi in in the kidney and nine from calculi in the gall bladder . One patient was excluded due to signs of liver cirrhosis, one because of a gall bladder polyp and three who had coarse parenchymal echotexture of the liver).

Cases of NAFLD (n=126) were identified on the basis of the ultrasonographic findings in combination with their daily intake of alcohol (< 20g for women, <30g for men). The individuals exhibiting normal liver ultrasonography and no other evidences of liver disease served as controls (n=237).

MTP -493 genotyping

DNA was purified from 180 persons (89 with NAFLD and 91 controls) from whole blood and buffy coat samples using the QIAamp® Mini Kit from Qiagen Ltd. (West Sussex UK). Kit was used according to manufacturer's instructions.

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) using specified primers (shown below) were used for evaluating each SNP. Generally, we used 30-35 cycles of PCR with 200µM dNTP, 0.4µM of primer, 0.025U/L of Taq Polymerase and 1.5mM $MgCl_2$ concentration. PCR was followed by restriction enzyme analysis to verify the presence or absence of cleavage-site due to polymorphism.

The MTP -493 G/T polymorphism was evaluated as previously described [29]. Briefly, we used nested PCR with first round being; 35 cycles at 94°C for 30 seconds, 55°C for 60 seconds, and 72°C for 3 minutes. We also increased the $MgCl_2$ concentration to 2.0 mM to enhance the specificity of the amplification. Primers were: Outer-Frw5': CCCTCTTAAT CTCTTTCCTAGAA. Outer-Rev5': AAGAATCATATTGACCAG CAATC. The second round of PCR the number of cycles was changed to 35 at 94°C for 30 seconds, 57°C for 60 seconds, and 72°C for 2 minutes. The -493 G/T SNP does not cause a restriction site with any common restriction enzyme. However, a mutation in the 5' primer used for PCR of a gene product including the -493 site gave rise to an *Hph*1 cutting site for the -493 G allele. The PCR (Inner Frw5'-GGA TTT AAA TTT AAA CTG TTA ATT CAT ATC AC and Inner Rev5'-AGT TTC ACA CAT AAG GAC AAT CAT CTA) gave rise to a 109-bp fragment, and the gene product was cleaved by *Hph*1. Analyzing the fragments on a 2,5% agarose gel (Agarose-1000) the -493 T allele gave rise to a full-length fragment (109 bp), whereas the -493 G allele gave rise to two fragments of 89 and 20 bp.

Definition Of Metabolic Syndrome

Metabolic syndrome was defined as per the International Diabetes Federation (IDF) 2005 [34]. This definition stipulates the mandatory presence of central obesity (with a waist circumference of >80 cm for women and>90 for men in Asian Indians) in combination with at least two of the following criteria: total serum level of triglycerides >1.7 mmol/L (150 mg/dl); HDL <1.3 or <1.0 mmol/L (<51 or <39 mg/dl) for women, and men respectively; blood pressure >130/85 or on-going anti-hypertensive treatment; a fasting level of plasma glucose >5.6 mmol/L (100.8 mg/dl); and/or Type 2 DM.

Insulin resistance according to HOMA2

Insulin resistance and insulin sensitivity were calculated in accordance with the updated version of the homeostasis model assessment (HOMA2) utilizing the HOMA calculator (version 2.2; Diabetes Trials Unit, University of Oxford) (see http://www.dtu.ox.ac.uk/index.php?maindoc=/homa/index.php)

Statistical Analysis

All of the data sets were first evaluated for deviation from a normal distribution. Thereafter, values not exhibiting normal distribution were evaluated for statistical significance employing the Kruskal-Wallis H and non-parametric median tests; normally distributed values were tested using the independent t-test, ANOVA (coupled to post-hoc Scheffe), multivariate linear regression or multinominal logarithmic regression. All computations and calculations were performed with the SPPS v25 for windows (Statistical Package for the Social Sciences, SPSS Inc, U.S.A) .

Ethical Permission

This study was approved by the Human Ethical Committee of the Medical College of Trivandrum and Sree Gokulam Medical College and Research Foundation. Written informed consent was received from each subject prior to enrolment in this investigation.

RESULTS

We had 126 subjects with fatty liver (67 men and 59 women) and 237 controls (103 men, 134 women). Age of the cases and controls were (mean ± sd) 48.5 ± 11.1 and 48.4 ± 12.2 respectively for the cases and controls (p *=0.971)*. In the NAFLD group , the average age was 48.5 years (48.5 for men and 48.4 for women) and 48.4 years (51.9 for men and 45.8 for women) for the controls. Both groups were similar with respect to their distribution of age and sex (Table 1).

Anthropometry , biochemical and morbidity pattern

This is represented in Table1. The mean BMI for the subjects with NAFLD was 25.5 kg/M^2 (24.4 for men and 26.8 for women) and 23.2 kg/M^2 (22.2 for men and 24.0 for women) for the control group. The subjects with NAFLD had larger waist and hip measurements, a higher HOMA2 IR in comparison to the controls. The AST/ALT ratio was lower than in controls.

Type 2 DM and the metabolic syndrome.

There were no differences between NAFLD and controls , 14% (n=18) and 14% (n=34) respectively (P= 0.569) nor for NAFLD to diabetes among women (P=0.431) or men (P=0.841) . Sub-group results are illustrated in figure 1 but groups were deemed too small to merit statistical analysis.

Table1. Gender-specific Differences Within And Between The NAFLD And Control Groups.

Factor	Men without NAFLD (n=103)	Men with NAFLD (n=67)	*P-value (Men without NAFLD vs Men with NAFLD)*	Women without NAFLD (n=134)	Women with NAFLD (n=59)	*P-value (Women without NAFLD vs Women with NAFLD)*	*P-value (Men vs women Without NAFLD)*	*P-value (Men vs women with NAFLD)*
Age (years)	51.9 ± 11.1	48.5 ± 11.0	*0.327*	45.8 ± 12.3	48.4 ± 11.4	*0.535*	*0.001*	*1.000*
BMI (kg/m^2)	22.2 ± 3.1	24.4 ± 3.3	*0.002*	24.0 ± 3.8	26.8 ± 4.5	*< 0.001†*	*0.006†*	*0.003†*
SYS (mm Hg)	128 ± 19	128 ± 13	*1.000*	125 ± 15	134 ± 18	*0.004*	*0.475*	*0.264*
DIA (mm Hg)	82 ± 10	83 ± 8	*0.841*	81 ± 8	85 ± 11	*0.061*	*0.940*	*0.742*
HOMA2 IR	0.74 ± 0.48	1.07 ± 0.73	*0.002†*	0.81 ± 0.63	1.14 ± 0.75	*< 0.001†*	*0.588†*	*0.549†*
Diabetes	21	12	*0.690*	13	6	*0.874*	*0.021*	*0.245*
Metabolic syndrome	26	23	*0.622*	39	28	*0.125*	*0.700*	*0.224*
AST (IU/L)	23.7 ± 12.8	30.0 ± 16.4	*0.013†*	18.0 ± 7.8	30.9 ± 15.3	*< 0.001†*	*<0.001†*	*0.467†*
ALT (IU/L)	17.6 ± 14.4	28.9 ± 22.6	*0.001†*	14.3 ± 10.8	31.0 ± 17.2	*< 0.001†*	*0.110†*	*0.140†*
AST/ALT	1.92 ± 1.48	1.42 ± 0.78	*0.221†*	1.62 ± 1.22	1.07 ± 0.33	*< 0.001†*	*0.102†*	*0.123†*
Ferritin (pmol/L)	99 ± 98	146 ± 115	*0.005†*	41 ± 39	73 ± 42	*< 0.001†*	*<0.001†*	*<0.001*
TG (mg/dL)	113 ± 70	159 ± 74	*< 0.001†*	103 ± 65	145 ± 57	*< 0.001†*	*0.141†*	*0.437†*
HDL (mg/dL)	38 ± 11	42 ± 12	*0.290*	41 ± 13	54 ± 14	*< 0.001†*	*0.085†*	*0.001†*
LDL (mg/dL)	127 ± 41	133 ± 50	*0.909*	131 ± 42	167 ± 52	*< 0.001†*	*0.968†*	*0.001†*
VLDL (mg/dL)	23 ± 14	34 ± 21	*<0.001†*	21 ± 14	29 ± 11	*< 0.001†*	*0.132†*	*0.425†*
Total Cholest. (mg/dL)	187 ± 50	209 ± 59	*0.100*	193 ± 52	249 ± 58	*< 0.001†*	*0.980†*	*< 0.001†*
Alb.	4.1 ± 0.70	4.2 ± 0.70	*0.462†*	3.89 ± 0.75	4.33 ± 0.61	*< 0.001†*	*0.0.14†*	*0.392†*

Statistically significant differences were examined for as described in the Materials and Methods †) Values not demonstrating a normal distribution and therefore analyzed using the Kruskal-Wallis H non-parametric test. Abbreviations: SYS= Systolic blood pressure, DIA= Diastolic blood pressure, HDL= high- density lipoprotein, LDL= low- density lipoprotein and VLDL=very low- density lipoprotein. TG= Triglycerides. AST= Aspartate aminotransferase. ALT=Alanine aminotransferase.

Type 2 DM was more common in men (P=0.002). (OR; and 95% confidence interval c.i) 2.31 (1.33-4.01). One hundred and sixteen (49 men and 67 women) had metabolic syndrome. Among those with NAFLD, 51 subjects had metabolic syndrome (23 men and 28 women, (40.0%)), in comparison to 65 (26 men and 39 women (27%)) of the controls (P=0.173). There was no significant correlation of NAFLD to metabolic syndrome among either women (P= 0.125) or men (P= 0.622). However, the HOMA2 IR values for the NAFLD group were 1.10 (1.07 for men and 1.14 for women), compared to 0.78 (0.74 for men and 0.81 for women) for the controls (P< 0.001).

Gender-specific findings

Calculated from the 180 genotyped persons, NAFLD was significantly more common among men than women up to the age of 50, (P=0.036) but not after, as shown in figure 1. However, including non-genotyped persons, male prevalence is still higher but not significantly so. Based on 363 persons, there was also a tendency towards increased fatty infiltration, as determined by ultrasound, for male diabetics (P=0.092) but not female (P=0.524). Among the cases with NAFLD women exhibited significantly higher levels of LDL and HDL cholesterol than men. No such gender difference was seen among the healthy controls. Metabolic syndrome was higher in NAFLD women younger than 50 years (table 2). Similarly, high BMI, high blood pressure and high serum lipids were seen higher in this group than men with NAFLD (not shown in table) (P<0.001). After the age of 50, there are no significant differences between the genders. Certain gender-specific differences, e.g a higher serum level of ferritin, were observed for both NAFLD cases and controls (Table 2).

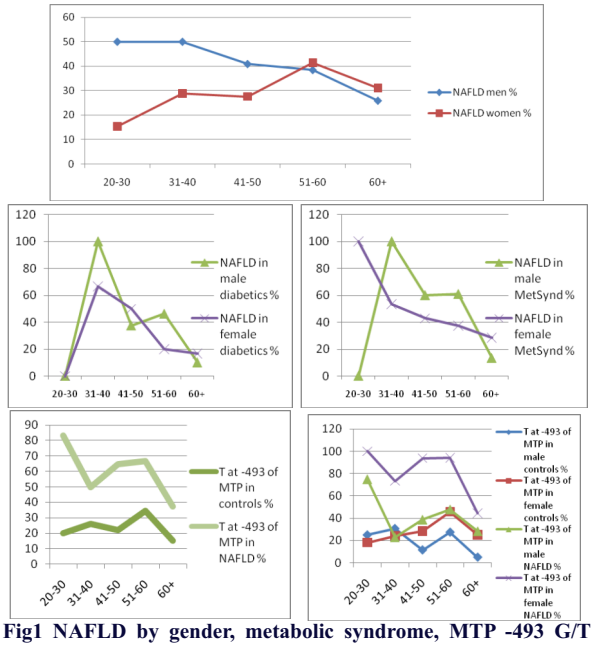

Fig1 NAFLD by gender, metabolic syndrome, MTP -493 G/T polymorphism

Table 2 Diabetes mellitus , Homa2 IR , metabolic syndrome and T at -493 0f MTP by age and gender in cases and controls

Variable	Non NAFLD Controls						NAFLD Cases					
Mean ± sd age* (range)	48.4 ± 12.2 ((24-66))						48.5 ± 11.1 (24-68)					
Age group	20-50			50+			20-50			50+		
Gender	M	F	Total	M	F	Total	M	F	Total	M	F	Total
Number	43	90	133	60	44	104	35	33	68	32	26	58
Diabetes mellitus	5	3	8	16	10	26	5	4	9	7	2	9
HOMA2 IR	0.74 ± 0.48			0.77 ± 0.67			1.08 ± 0.64			1.11 ± 0.73		
Metabolic syndrome**	4	19	13	22	20	42	7	18	25	16	10	25
T at -493 of MTP	8	23	31	11	11	22	13	28	41	14	20	34

** P-value age (*Non-NAFLD controls *vs NAFLD) 0.971*

** Metabolic syndrome was significantly more common in women up to the age of 50 (P=0.029).

MTP-493 G/T polymorphism

The allele frequencies of the MTP-493 G/T SNP followed the Hardy-Weinberg equilibrium and showed NAFLD: G: 0.42, T: 0.58; Controls: G: 0.59 and T: 0.41.

The T-substitution at -493 was significantly associated with an increased risk of NAFLD (P= 0.003) (figure 1). The odds ratio (OR; and 95% confidence interval c.i) for the T-substitution in the NAFLD cohort (as calculated for the T-dominant model) was 2.91 (1.42-5.94). T/T at position -493 was also associated with increased total cholesterol (P=0.033), LDL (P=0.038) and HDL (P=0.003) but not VLDL compared to G/G. Triglyceride (P=0.044) and VLDL (P=0.026) values were significantly lower for T homozygotes, compared to G/G and G/T in people with NAFLD, as were Alkaline Phosphatase values (P=0.032). As shown in figure 1, T at position -493 was also significantly overrepresented in women (P=0.002), with 45.1% of the women being carriers compared to 27.6% among the men. In some age-groups almost 100 % of the women with NAFLD were carriers.

DISCUSSION

In this study, there is a high incidence of NAFLD, with a gender-specific pattern, in a region with very high incidence of type 2 DM in a population. Insulin resistance but not type 2 DM is correlated to NAFLD and a weak tendency that metabolic syndrome and NAFLD may be correlated. Furthermore, we also find genetic differences between the NAFLD- and the control group. Major differences were found between men and women below the age of 50. To our knowledge, this is the first report to emphasize the significant differences between men and women with NAFLD in an epidemiologically selected population.

We used the sampling technique as described in earlier studies [31, 32]. It provides an unbiased sample of subjects from areas representative of the region as a whole. We used ultrasound in order to detect NAFLD which is an accepted method, although patients with less than 5-10% fat in the liver are not identified [33]. It is unehical to perform a liver biopsy on persons not seeking health care. 35% had NAFLD which is higher than reported from Europe and the United States[35-37] and the high incidence of type 2 Diabetes Mellitus may be an important factor. A high prevalence of NAFLD has been reported before in this region [38] and previous author have speculated that the high frequency of metabolic syndrome and DM type 2 is of importance. In our study we also found a high prevalence of MTP polymorphisms which indicates that genetic factors are important in combination with dietary factors.

Interestingly, although the total group with NAFLD did have higher BMI values than the healthy controls these values were still not very high and indicative of overweight rather than obesity. Only women with NAFLD had a mean BMI over 25. Moreover, we found that approximately 20% of the non-diabetic subjects with NAFLD were of normal weight, but nonetheless met the IDF criteria for presence of the metabolic syndrome as have been reported to similar extent before[8]. In addition we found no significant correlation between the metabolic syndrome and NAFLD except in specific age-groups and although our study population demonstrated a high incidence (>14%) of Type 2 DM, the presence of NAFLD did not significantly correlate with that of diabetes. This is not unheard of and it has been shown that although NAFLD is usually associated with obesity and diabetes the latter conditions are not in any way essential for the development of NAFLD [16].

The fact that patients with and without NAFLD had the same prevalence of Diabetes is interesting and Diabetes may be something that increase the risk for NAFLD but other factors, which we have described, are necessary in order to increase the amount of triglycerides in the liver and cause NAFLD. Much more research, maybe on a genetic level, is needed to respond to this question. However, there was a correlation between NAFLD and HOMA2 insulin resistance. According to recent investigations, this correlation may indicate impaired insulin clearance and secretion, rather than altered insulin sensitivity [15].

Blood tests showed that serum levels of Alkaline phosphates were higher in NAFLD patients, something which has been shown for NASH patients before [5] . However that AST/ALT ratio were lower in NAFLD patients than controls which is an unexpected finding since the reversed would be expected, also previously shown in NASH patients. HDL levels were higher in women, which is known since before and is caused by estrogen. It is however not clear why HDL was higher in the female NAFLD group compared to female controls and we have no explanation for this. This was not found in men.

We found that T at position -493 is significantly associated with NAFLD. This is in agreement with recent reports describing correlation between the T-allele and advanced hepatic steatosis in subjects with hepatitis C viral infection [29,30] , Interestingly, the T allele frequency was much higher in this cohort compared to the Framingham offspring study which analyzed 2510, mostly Caucasian, subjects [27] . Furthermore, we found that women are overrepresented by almost a 2 to 1 ratio as carriers of the T-dominance. Some investigators have reported NAFLD prevalence to vary between men and women [35-37.] and other interesting genetic variations possibly explaining NAFLD incidence [19-22] , progression to NASH [20, 23] and impact on alcoholic fatty liver disease [24] have also been described. We found no significant difference when analyzing the total cohort. NAFLD was more common among males, in our cohort, in younger ages but the difference disappears for older subjects. A reason for this might be estrogen, suggested as a protective factor in liver disease including NAFLD/NASH/ASH in human subjects as well as animal models [40-43] .

Thus, after menopause, the protective effect is diminished and, women over the age of 50 have equal (or even more based on the genotyped persons) NAFLD than men of the same age. A fact that may be explained by the overrepresentation of MTP -493 T-substitution in women. We suggest that men in this cohort develop NAFLD partially due to "classical" reasons like type 2 diabetes, with an increased risk stemming from the MTP polymorphism, and that women who are overrepresented for the T-substitution, would have increased prevalence if not protected by estrogen. Thus, we suggest that these findings are indicative of gender-specific profiles for NAFLD.

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