Non-Alcoholic Fatty Liver Disease Modifies Serum Gamma-Glutamyl Transferase in Cigarette Smokers

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Abstract

Background: Serum gamma-glutamyl transferase (GGT) is a marker of oxidative stress, associated with increased cardiovascular (CV) risk. The impact of smoking on oxidative stress may be aggravated in individuals with non-alcoholic fatty liver disease (NAFLD). We aimed to ascertain the association of smoking on GGT levels in the presence or absence of NAFLD.

Methods: We evaluated 6,354 healthy subjects (43 ± 10 years, 79% males) without clinical cardiovascular disease (CVD) undergoing an employer-sponsored physical between December 2008 and December 2010. NAFLD was diagnosed by ultrasound and participants were categorized as current or non-smokers by self report. A multivariate linear regression of the cross-sectional association between smoking and GGT was conducted based on NAFLD status.

Results: The prevalence of NAFLD was 36% (n = 2,299) and 564 (9%) were current smokers. Smokers had significantly higher GGT levels in the presence of NAFLD (P < 0.001). After multivariable adjustment, current smoking was associated with 4.65 IU/L higher GGT level, P < 0.001, compared to non-smokers. When stratified by NAFLD, the magnitude of this association was higher in subjects with NAFLD (β-coefficient: 11.12; 95% confidence interval (CI): 5.76 - 16.48; P < 0.001); however, no such relationship was observed in those without NAFLD (β: -0.02; 95% CI: -3.59, 3.56; P = 0.992). Overall the interaction of NAFLD and smoking with GGT levels as markers of oxidative stress was statistically significant.

Conclusions: Smoking is independently associated with significantly increased oxidative stress as measured by GGT level. This association demonstrates effect modification by NAFLD status, suggesting that smoking may intensify CV risk in individuals with NAFLD.

Keywords: Gamma-glutamyl transferase; Oxidative stress; Non-alcoholic fatty liver disease; Smoking; Cardiovascular risk

Introduction

Oxidative stress plays a crucial role in cardiovascular disease (CVD) mediating the pathway to atherosclerosis and inflammation [1, 2]. The oxidative stress theory is based on the assumption that the initial phase of atherosclerosis is dependent on low-density lipoprotein (LDL) oxidative modifications [3].

Gamma-glutamyl transferase (GGT) is a biomarker secreted mostly by the liver and found in epithelial cells of humans. It plays an important role in glutathione metabolism, an important component of the anti-oxidative process [4, 5]. This marker has been implicated in atherosclerosis, diabetes, metabolic syndrome and increased cardiovascular (CV) risk [6-16]. Elevated GGT levels have also been linked with hepatic steatosis (HS) and liver cancer [14, 17]. While non-alcoholic fatty liver disease (NAFLD) is the leading cause of chronic liver disease in the western world, evidence has also shown it as an independent risk factor for CVD [18, 19].

Similarly, it has been established that cigarette smoking is an important risk factor for atherosclerosis and associated with significant vascular disease [20-23]. Smoking enhances the production of reactive oxygen species which promotes oxidative stress and lipid peroxidation [24-27]. While smoking is associated with oxidative stress, its influence on GGT levels in individuals with NAFLD is not clearly defined. Our study examines the effect of the presence of NAFLD on the associa-

tion between smoking and oxidative stress by GGT levels in an asymptomatic population.

Materials and Methods

A total of 6,354 asymptomatic men and women, free of CVD, who had an obligatory clinical and laboratory health evaluation from December 2008 to December 2010 at the Preventive Medicine Center of the Albert Einstein Hospital in Sao Paulo, Brazil were evaluated. The examination protocol has been previously described in prior publications [28]. Individuals were provided with questionnaires for self-reported details of their demographics, medical history, quantitative alcohol consumption, smoking status and medication usage during their clinical visits. We excluded 110 individuals with missing information of either smoking status, GGT levels and presence of steatosis. Individuals with an established history of liver disease were also excluded. Individual smoking status was defined as either current smoker or non-smoker. While current smoker was defined as smoking at least one cigarette in the last 1 month, non-smoker was one who did not smoke in the last month. Diabetes mellitus was identified by a previous physician diagnosis or the use of glucose-lowering medication. Hypertension and dyslipidemia were ascertained by a previous history or the use of medications. Blood pressure measurements were made using an aneroid sphygmomanometer using the recommended method from the American Heart Association [29]. The waist circumference was the smallest diameter between the iliac crest and the costal margin using a plastic anthropometric tape.

All blood specimens were collected after an overnight fast. Plasma lipid, glucose, GGT and liver transaminase levels (alanine aminotransferase (ALT) and aspartate aminotransferase (AST)) levels were measured by standardized automated laboratory tests using a Vitros platform (Johnson & Johnson Clinical Diagnostics, New Brunswick, New Jersey). High-sensitivity C-reactive protein (hs-CRP) levels were determined by immunonephelometry (Dade-Behring GMbH, Mannhein, Germany). All tests were performed at the Central Laboratory of the Albert Einstein Hospital.

NAFLD was diagnosed after at least a 6-h fast using an ACUSON XP-10 device (Mountain View, CA) and was identified by the presence of an ultrasonographic pattern of a bright liver, with evident contrast between hepatic and renal parenchyma and using a standardized algorithm (indicating HS) [30, 31]. Obesity was defined as a body mass index (BMI) of greater than 30 kg/m^2. The metabolic syndrome (MS) was defined using criteria from the American Heart Association/National Heart, Lung, and Blood Institute published scientific statement on the metabolic syndrome [32]. Patients with $\geq$ 3 of the following risks were classified as having the MS: truncal obesity ($\geq$ 102 cm (40 inches) for men and $\geq$ 88 cm (36 inches) for women), high blood pressure (blood pressure $\geq$ 130/85 mm Hg or the use of antihypertensive medications), hyperglycemia (fasting blood glucose $\geq$ 100 mg/dL), low high-density lipoprotein cholesterol (HDL-C) ($\leq$ 40 mg/dL for men and $\leq$ 50 mg/dL for women) and hypertriglyceridemia ($\geq$ 150 mg/dL) [32]. This study did not involve any animal study and appropriate IRB approval was obtained prior to the administration of surveys to the participants.

All data were de-identified prior to analysis and determined not to meet criteria for protected health information (PHI) [28].

Baseline characteristics of individuals with a combination of the presence or absence of NAFLD and smoking status was compared using the analysis of variance test for continuous variables and the Pearson's χ^2 test for categorical variables. The non-parametric Kruskal-Wallis test was used to compare the median of variables with skewed distribution (ALT, AST, GGT and hs-CRP). A multivariate linear regression was used to evaluate associations of smoking and GGT levels. The regression analysis was done by stratifying the presence of NAFLD and obesity to ascertain an independent association. For all regression analyses, a hierarchical model approach was used, adjusting first for age and gender and then simultaneously adjusting for other confounding factors (waist circumference, triglycerides, HDL-C, systolic blood pressure (SBP), fasting glucose, LDL-C, CRP, lipid lowering and antihypertensive medications and alcohol use). Alcohol use was quantified by the alcohol use disorders identification test (AUDIT) score [33]. The AUDIT score was developed and validated by the World Health Organization among men and women in different countries. We categorized a total AUDIT score of $\geq$ 8 as high alcohol consumption for men and $\geq$ 4 for women [34]. A model including an interaction term for HS and smoking was fitted to assess whether smoking modified the association between HS and GGT. All statistical analyses were performed using STATA statistical software, release 12 (College Station, TX).

Results

The characteristics of the study population were stratified by the combination of smoking status and the presence of NAFLD (Table 1). There were 6,354 subjects analyzed: 3,731 (59%) non-smokers without NAFLD, 2,060 (32%) non-smokers who had NAFLD, 324 (5%) smokers without NAFLD and 239 (4%) smokers with NAFLD. There were statistically significant differences in demographics, anthropometric and biochemical profiles between the four groups. Individuals with NAFLD were more likely to be men, older and have a higher burden of risk factors (Table 1). However, smoking combined with the presence of NAFLD further worsened this association. Smokers with NAFLD had the highest prevalence of the MS.

Smokers with NAFLD were more likely to have the highest level of GGT levels (P < 0.001). Individuals who smoked and had NAFLD had the highest median levels compared to individuals who had NAFLD alone, smoked without NAFLD or were non-smokers without NAFLD. Figures 1 and 2 show the effect of smoking on the GGT levels in the presence and absence of NAFLD. Although the presence of NAFLD alone was associated with a high GGT level, it was even higher when the presence of NAFLD is combined with a positive smoking status (Fig. 1). Figure 2 shows similar effect of cigarette smoking on the quartiles of GGT levels when stratified by the presence of NAFLD. While non-smokers without NAFLD have predominantly GGT levels in the lowest quartiles, there is a steady redistribution to the higher quartiles in smokers without NAFLD, non-smokers with NAFLD and smokers with

Table 1. Baseline Characteristics of Study Population

Clinical, anthropometric and bio-chemical characteristics	Smokers (-) and NAFLD (-) (N = 3,731)	Smokers (-) and NAFLD (+) (N = 2,060)	Smokers (+) and NAFLD (-) (N = 324)	Smokers (+) and NAFLD (+) (N = 239)	P-value
Male (%)	71	94	70	95	< 0.001
Mean age, years (± SD)	42 (9)	46 (9)	42 (10)	47 (9)	< 0.001
Mean BMI, kg/m^2 (± SD)	25 (3)	29 (4)	25 (3)	29 (3)	< 0.001
Mean waist circumference, cm (± SD)	87 (10)	100 (10)	87 (11)	100 (9)	< 0.001
Obesity, BMI ≥ 30 kg/m^2 (%)	9	46	12	45	< 0.001
Mean SBP, mm Hg (± SD)	116 (12)	124 (12)	116 (12)	124 (12)	< 0.001
Mean DBP, mm Hg (± SD)	75 (8)	80 (7)	75 (8)	80 (8)	< 0.001
HTN present (%)	8	22	8	21	< 0.001
Median AST, IU/L (interquartile range)	27 (24 - 32)	32 (28 - 39)	26 (23 - 31)	30 (26 - 37)	< 0.001
Median ALT, IU/L (interquartile range)	29 (23 - 37)	43 (33 - 56)	28 (21 - 36)	39 (31 - 53)	< 0.001
Median GGT, IU/L (interquartile range)	26 (19 - 36)	40 (30 - 56)	28 (21 - 43)	42 (33 - 62)	< 0.001
Median TG, mg/dL (interquartile range)	99 (74 - 136)	149 (109 - 208)	113 (84 - 163)	179 (124 - 240)	< 0.001
Mean HDL, mg/dL (± SD)	52 (14)	43 (10)	49 (13)	42 (10)	< 0.001
Mean LDL, mg/dL (± SD)	128 (32)	136 (34)	129 (35)	135 (34)	< 0.001
Mean FBG, mg/dL (± SD)	87 (9)	93 (12)	87 (9)	93 (12)	< 0.001
Median hs-CRP, mg/L (interquartile range)	1 (0.5 - 0.9)	1.7 (0.9 - 3)	1.2 (0.5 - 2.6)	1.8 (1 - 3.5)	< 0.001
High hs-CRP (≥ 3 mg/L) (%)	28	36	32	41	< 0.001
Mean uric acid, mg/dL (± SD)	5.4 (4.4 - 6.3)	6.4 (5.7 - 7.2)	5.4 (4.6 - 6.4)	6.3 (5.6 - 7)	< 0.001
Antihypertensive (%)	8	21	7	21	< 0.001
Lipid lowering medication (%)	7	12	7	14	< 0.001
Subjects with metabolic syndrome (%)	8	41	11	46	< 0.001
Median TG/HDL-C (interquartile range)	1.9 (1.3 - 3)	3.5 (2.4 - 5.4)	2.4 (1.5 - 3.8)	4.4 (2.8 - 6.5)	< 0.001

NAFLD: non-alcoholic fatty liver disease; BMI: body mass index; SD: standard deviation; SBP: systolic blood pressure; DBP: diastolic blood pressure; HTN: hypertension; ALT: alanine aminotransferase; AST: aspartate aminotransferase; GGT: gamma-glutamyl transferase; HDL-C: high-density lipoprotein cholesterol; LDL: low-density lipoprotein; FBG: fasting blood glucose; hs-CRP: high-sensitivity C-reactive protein; TG: triglycerides.
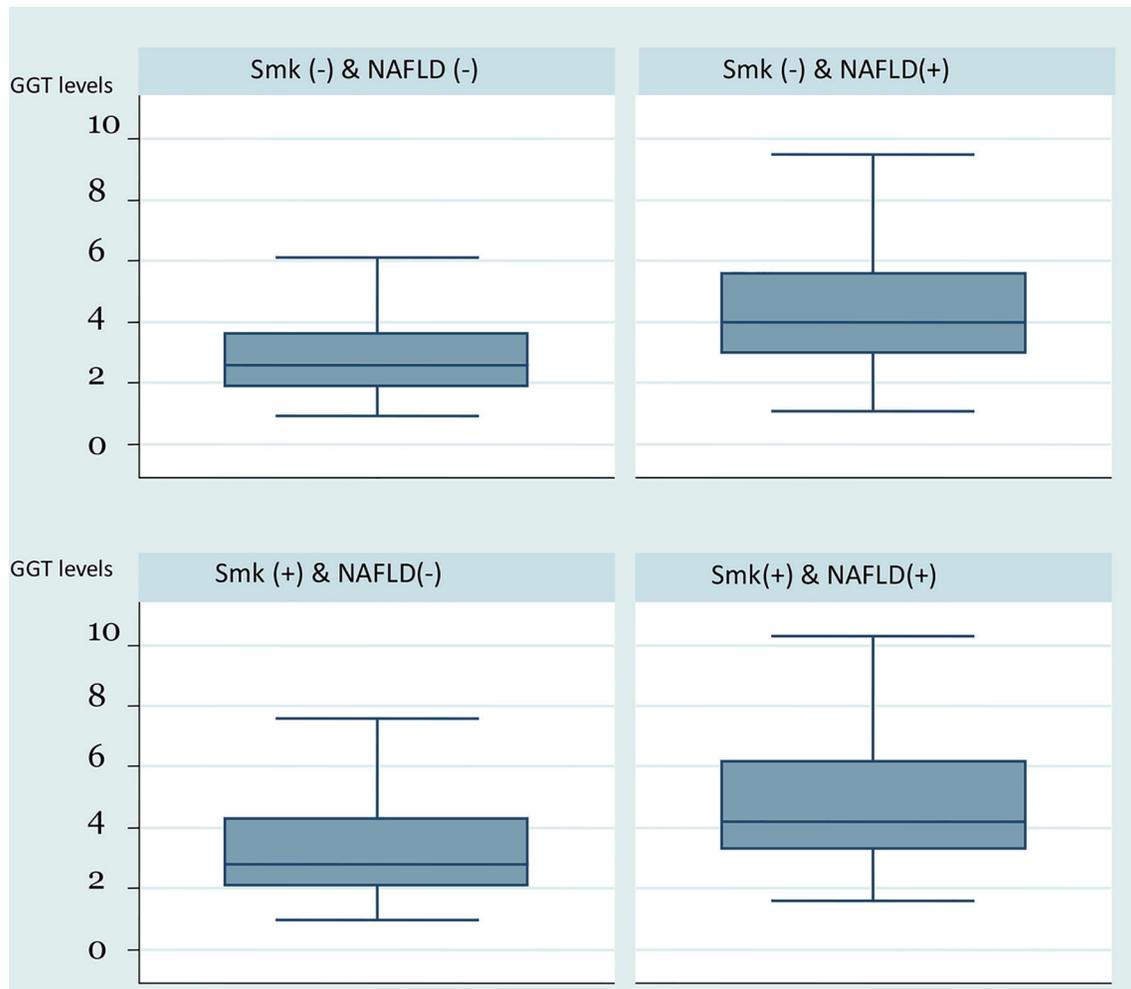
Figure 1. Box plot of GGT levels by the combination of smoking status and presence of HS. GGT: gamma-glutamyl transferase; HS: hepatic steatosis.

NAFLD. The GGT levels in smokers with NAFLD were predominately in the highest quartile. Figure 3 presents a similar relationship when stratified by MS.

Table 2 presents multivariate regression of the relationship between smoking and serum GGT levels. The results are presented from both the unadjusted and adjusted models for possible confounders. The analysis was also stratified by the presence of NAFLD. While there was a significant relationship between smoking and GGT in both the combined population and among individuals with NAFLD, the association was however not significant when NAFLD was absent. In the combined population, the GGT level of smokers was 4.65 IU/L higher. It was higher among individuals with NAFLD in the stratified analysis, as the GGT level of smokers in this group was 11.12 IU/L higher. The model testing for interaction demonstrated a statistically significant interaction of NAFLD and smoking in the association of smoking on GGT levels. We also found a similar association when stratified by the presence of obesity (Table 3). Obesity interacted with the association of smoking and the GGT levels and we noted the effect of smoking on GGT was independent of gender (Fig. 4).

Discussion

Our study of 6,354 asymptomatic individuals assessed the impact of smoking on oxidative stress in NAFLD. While 9% of the population were smokers, 36% had NAFLD. We demonstrated the presence of NAFLD modified the impact of cigarette smoking on GGT levels. Smokers who had NAFLD had higher GGT levels with evidence of interaction between smoking and NAFLD on the effect of smoking on GGT levels. This effect modification was also demonstrated with obesity and independent of gender.

This findings support prior studies that showed a relationship between smoking, NAFLD and GGT levels [24, 35, 36]. Smoking promotes oxidative stress and has a role in the development and progression of NAFLD [24]. While liver fat is a useful measure of visceral adiposity, it is also associated with increased risk of CVD [19-21].

Cigarette smoking has enormous consequences on health. It has been implicated in the development of NAFLD and known to cause inflammation and oxidative stress. It affects in-
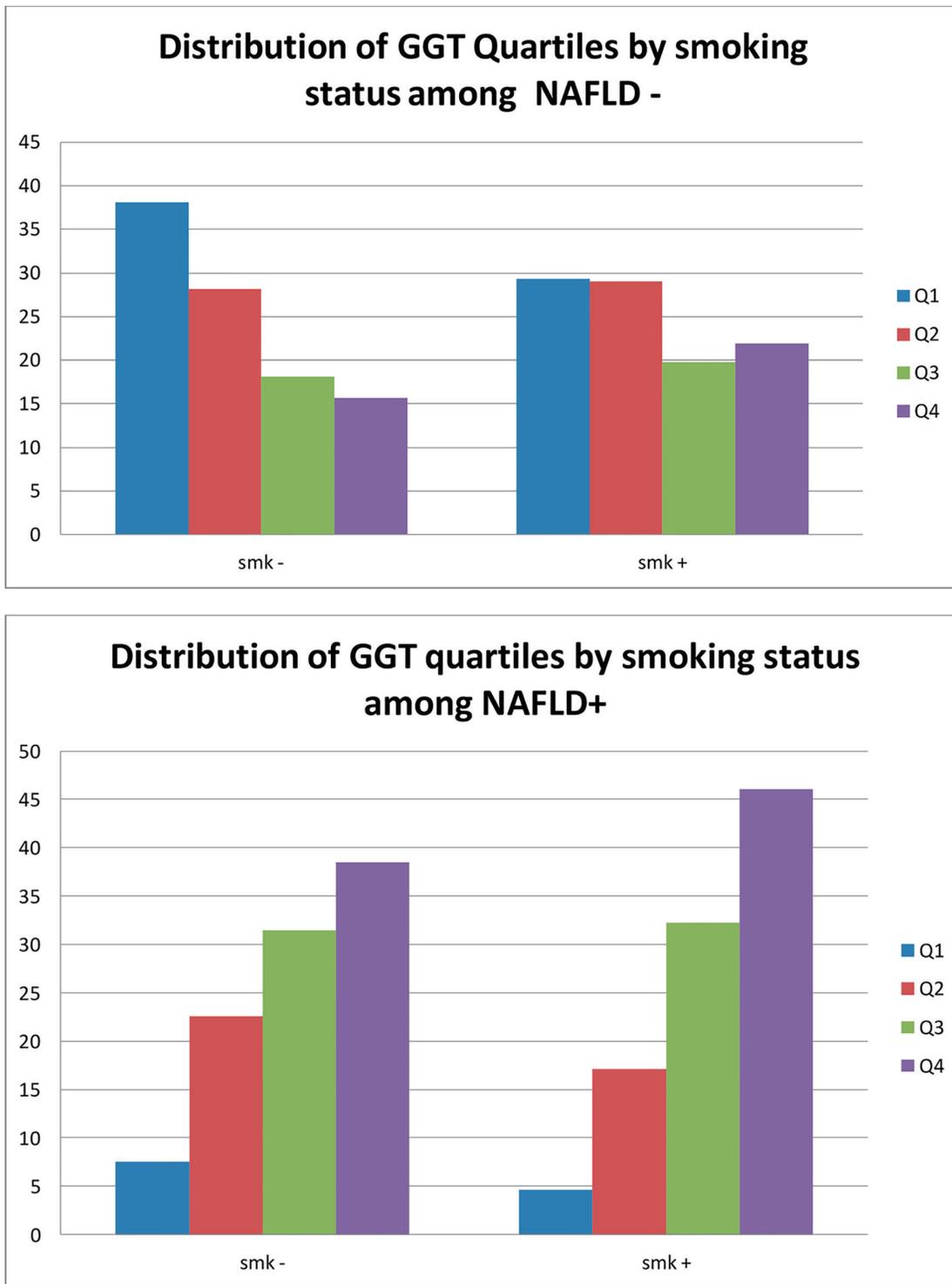
Figure 2. Distribution of GGT quartiles by smoking status and presence of NAFLD. GGT: gamma-glutamyl transferase; NAFLD: non-alcoholic fatty liver disease.

sulin resistance (IR) [35, 37, 38], which may play a role in the development/progression of HS, a component of NAFLD [24]. Importantly, it has been demonstrated that smoking is a risk factor for glucose intolerance and diabetes [39-41]. NAFLD

pathogenesis has been linked to IR and the promotion of steatogenesis by increasing the availability of free fatty acids and glucose from adipose tissue and excess dietary intake. This increases *de novo* hepatic lipogenesis, decrease fatty acid oxi-
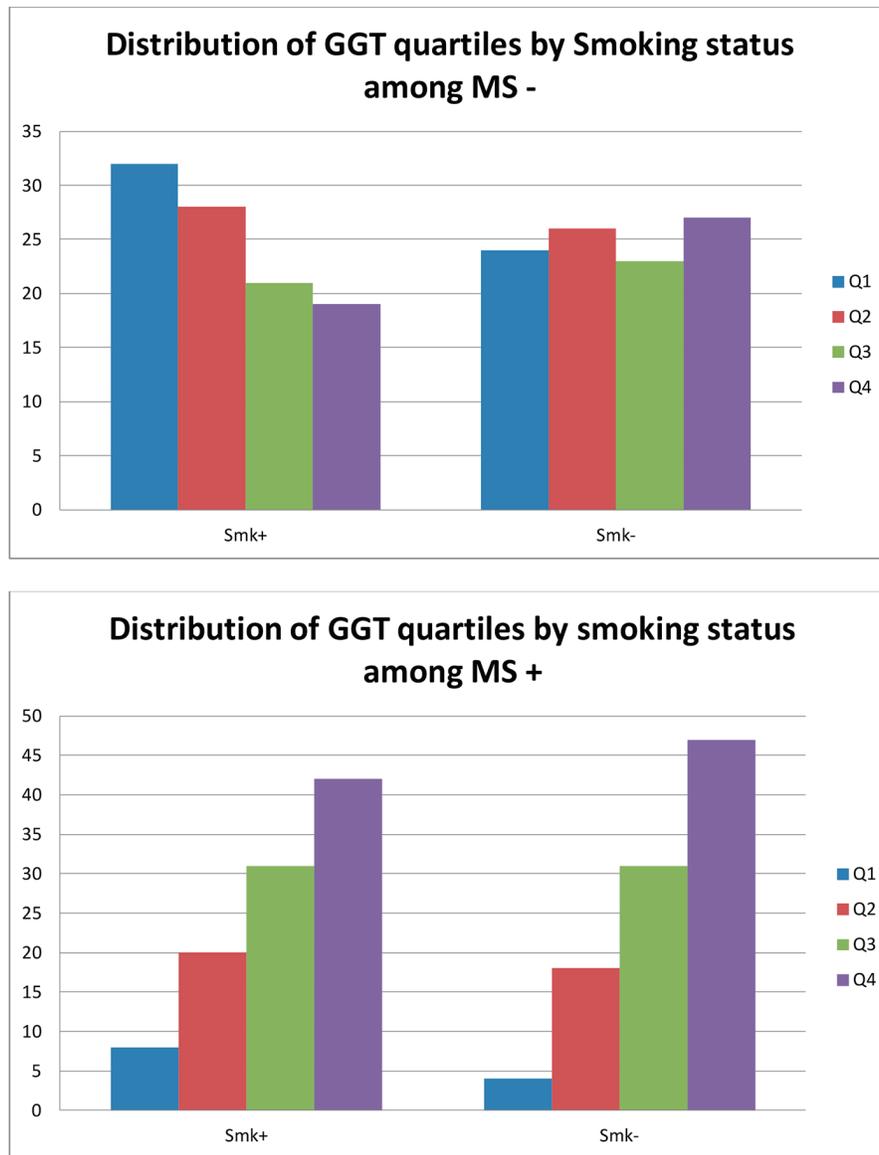
Figure 3. Distribution of GGT quartiles by smoking status and presence of metabolic syndrome. GGT: gamma-glutamyl transferase.

dation and decrease release of lipids from hepatocytes into circulation [18, 42]. Hamabe et al reported cigarette smoking was an independent risk factor for the onset of HS [24]. This study was supported by Lui et al, who reported that passive smoking and heavy active smoking were both associated with prevalent HS [36]. In a cross-sectional study subjects with NAFLD who smoked had a higher frequency of the MS compared to subjects with NAFLD who did not smoke [43]. Experimental data have also reported that smoking could worsen liver injury in NAFLD [44]. Smoking encourages the production of activated NADPH oxidase-induced reactive species, which enhances oxidative stress and lipid peroxidation [24]. Oxidative stress is a known mechanism of injury in HS [35, 45]. This study shows that smoking increased GGT levels in general and much more among persons with HS. As expected a similar relationship

was seen with MS and obesity. Our finding supports previous studies that have shown that smokers had higher GGT levels [46-48].

GGT is an enzyme responsible for the extracellular catabolism of the antioxidant glutathione [49, 50]. Although used as a diagnostic marker for hepato-bilary disease and alcohol abuse, strong epidemiological and clinical evidence suggests that serum GGT is a potential biomarker for CV morbidity and mortality [51]. Population studies reported significant independent associations between serum GGT concentrations and risk factors, like hypertension, type 2 diabetes and stroke independent of alcohol consumption [6, 8, 10, 15, 52]. Although the mechanism of this association is not clearly defined, it is likely related to oxidative stress [49, 53], which could be due to the GGT-linked pro-oxidant effect of glutathione catabolism

Table 2. Linear Regression of the Association Between Smoking and GGT Level Stratified by NAFLD

	Total (standardized coefficient)	HS+ (standardized coefficient)	HS- (standardized coefficient)	Interaction (HS-smoking)
Model 1	6.83 (3.69 - 9.96)***	10.31 (4.68 - 15.95)***	2.36 (-1.14 - 5.85)	P = 0.012
Model 2	6.51 (3.46 - 9.56)***	10.19 (4.57 - 15.81)***	2.57 (-0.83 - 5.9)	P = 0.015
Model 3	4.65 (1.61 - 7.69)**	11.12 (5.76 - 16.48)***	-0.02 (-3.59 - 3.56)	P < 0.001

*P < 0.05, **P < 0.005, ***P < 0.001. Model 1: unadjusted. Model 2: adjusted for age, gender. Model 3: model 2 + SBP, waist circumference, fasting glucose, lipid lowering medications, hypertension meds, LDL-C, AST, alcohol consumption and C-reactive protein. GGT: gamma-glutamyl transferase; NAFLD: non-alcoholic fatty liver disease; HS: hepatic steatosis; SBP: systolic blood pressure; LDL-C: low-density lipoprotein cholesterol; AST: aspartate aminotransferase.

Table 3. Linear Regression of the Association Between Smoking and GGT Level Stratified by Obesity

	Obesity+ (standardized coefficient)	Obesity- (standardized coefficient)	Interaction (obesity-smoking)
Model 1	11.14 (3.24 - 19.03)*	4.65 (1.43 - 7.87)*	P = 0.072
Model 2	10.46 (2.64 - 18.27)*	4.60 (1.48 - 7.73)**	P = 0.096
Model 3	12.73 (5.5 - 19.96)**	1.74 (-1.45 - 4.94)	P < 0.001

*P < 0.05, **P < 0.005, ***P < 0.001. Model 1: unadjusted. Model 2: adjusted for age, gender. Model 3: model 2 + SBP, fasting glucose, lipid lowering medications, hypertension meds, LDL-C, AST, alcohol consumption and C-reactive protein. GGT: gamma-glutamyl transferase; SBP: systolic blood pressure; LDL-C: low-density lipoprotein cholesterol; AST: aspartate aminotransferase.

[49]. GGT notably participates in the proliferation of atheromatous plaques [50] and it is believed that oxidative stress may be the mediating factor of the association between GGT levels and CVD through atherosclerosis [49, 53]. GGT plays a key role in maintaining intracellular anti-oxidant systems through its mediation of extracellular glutathione transport into cells. The association of elevated GGT with CVD is remarkable; Wannamethee et al described a significant relationship
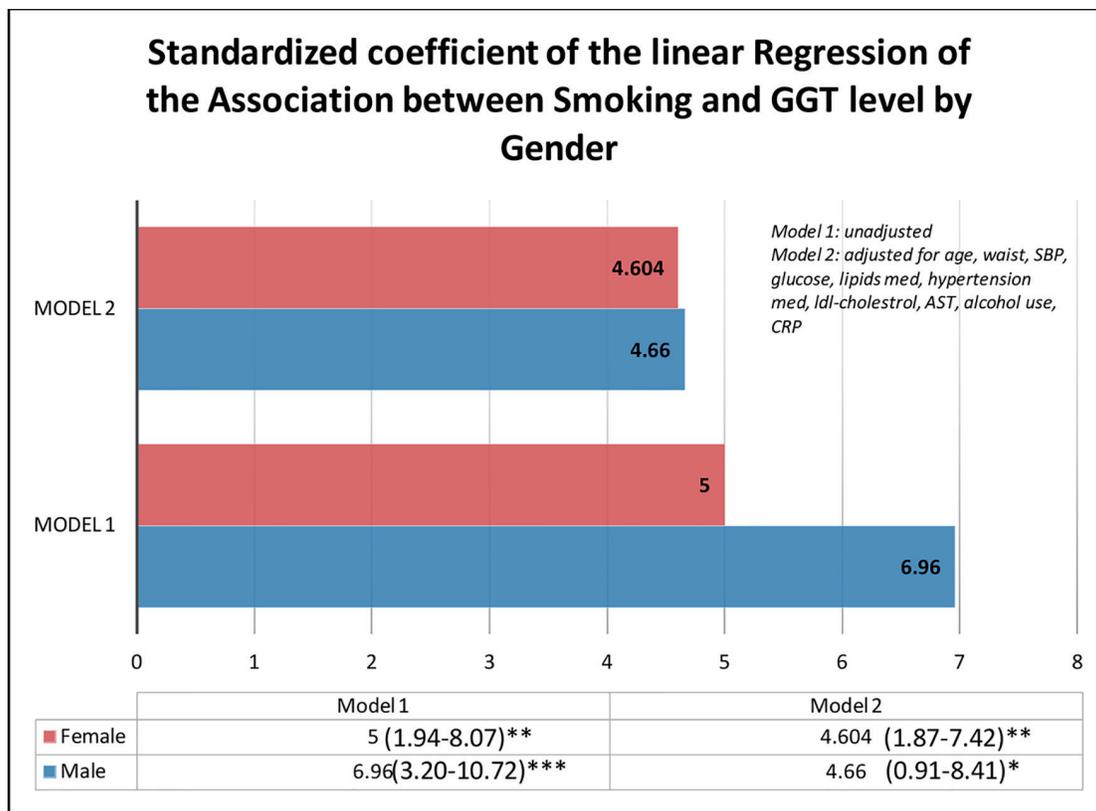

Figure 4. β-coefficient of the linear regression of smoking and GGT level stratified by gender. GGT: gamma-glutamyl transferase.

between GGT and CV events in a large prospective study with follow-up up to 24 years [54]. Furthermore, increased GGT levels have been demonstrated to be connected to coronary artery stenoses [1, 9]. GGT forms complexes with lipoprotein within atheromatous lesions and in plaques [9, 50].

However, there are questions whether the CV risk of increased GGT is modifiable [1]. Although there is no convincing evidence that risk for CV events is lessened by decreasing GGT, there are existing reports of the use of fibrates reducing GGT concentrations in individuals with hypertriglyceridemia, improving their lipid profile [55, 56]. This may be related to the role of fatty liver in generating increased serum GGT and its association with an increased CV risk [1, 57]. Since NAFLD is mostly treated by life style modifications, serum GGT levels could also be modifiable through these measures.

Implications

Our study provides evidence that GGT, an increasingly recognized biomarker for CV risk, may be worsened by cigarette smoking in persons with non-alcoholic liver fat. This further re-enforces the importance of life style modifications among persons with NAFLD. Although the presence of NAFLD is an established risk for future CV morbidity and mortality, smoking provides additional insult to the liver and ultimately aggravates the risk. Since oxidative stress has been implicated in many disease processes, including inflammation and atherosclerosis, this study provides further motivation for physicians to emphasize both screening for smoking and smoking cessation efforts in the routine care of individuals with HS and MS. Individuals with elevated GGT may be candidates for NAFLD screening.

Study limitations

There are a few limitations in this study. First, our study is cross-sectional and as such inferences are weakened as temporality cannot be ascertained. Secondly, the classification of smoking status as current smoking and non-smoker did not provide any information on history of smoking and as such presents the challenge of a residual bias. Also, there was no information on the quantity of cigarettes smoked, as such a dose response association could not be verified. Thirdly, the number of smokers with HS compared to the rest of the population may be a limitation to the interpretation of the results. Fourthly, our diagnosis of liver fat was based on ultrasound findings. Although ultrasound is a useful non-invasive and cheap tool for identifying NAFLD, its sensitivity for detecting fatty changes within the liver is reduced when less than moderate severity [28, 58]. Imaging using ultrasound is most effective when greater than a third of the liver is affected.

Strengths

Despite the limitations, this study has relevant strengths. An important strength for this study is the large number of par-
ticipants free of coronary heart disease. This has allowed for adjustment for multiple confounding risk factors to ascertain the relationship. The cohort size also allowed for evaluation of the relationship within demographic and clinical subgroups.

Conclusion

Oxidative stress plays a significant step in the development and progression of CVD. Serum GGT is an established and measurable biomarker of oxidative stress. In this study of asymptomatic individuals, smoking significantly raised oxidative stress as measured by GGT level among persons with NAFLD. While elevated GGT levels are associated with both NAFLD and CVD risk, smoking may further worsen this risk. Although future longitudinal studies to ascertain this association are imperative, this study provides further motivation for physicians to emphasize smoking screening and cessation in the routine care of individuals with NAFLD and MS.

Conflict of Interest

None to declare.

Informed Consent

This study did not involve any animal study and appropriate IRB approval was obtained prior to the administration of surveys to the participants.

Author Contributions

EO, EA, MB, EV and KN contributed to the conception, analysis and interpretation of the data. EO and KN performed the statistical analysis. EO, VF, MB, RC, JC, JM, RS and KN were involved in drafting the manuscript and revising it critically for important intellectual content. All authors read and approved the final manuscript.

Abbreviations

ALT: alanine aminotransferase; AST: aspartate aminotransferase; AUDIT: alcohol use disorders identification test; CVD:

cardiovascular disease; CV: cardiovascular; DM: diabetes mellitus; GGT: gamma-glutamyl transferase; HDL-C: high-density lipoprotein cholesterol; hs-CRP: high-sensitivity C-reactive protein; HS: hepatic steatosis; IR: insulin resistance; NADPH: nicotinamide adenine dinucleotide phosphate (reduced); NAFLD: non-alcoholic fatty liver disease; PHI: protected health information

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