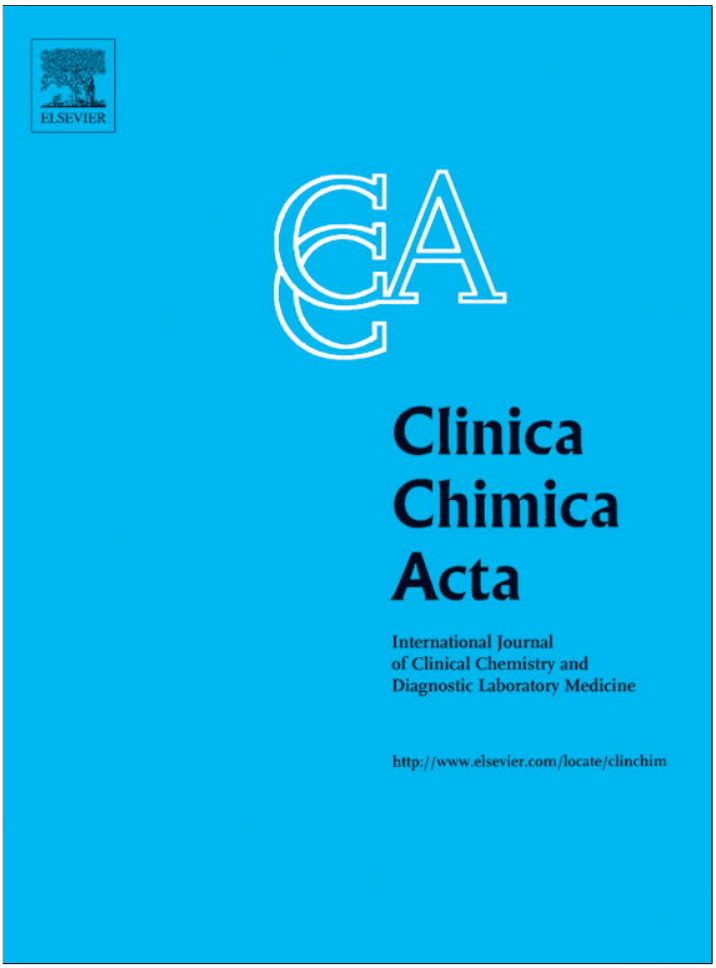

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Correlates and reference limits of plasma gamma-glutamyltransferase fractions from the Framingham Heart Study

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ABSTRACT

Background: We assessed GGT fractions correlates and their reference values in the Offspring Cohort of the Framingham Heart Study.
Methods: Correlates of GGT fractions were assessed by multivariable regression analysis in 3203 individuals [47% men, mean age (SD): 59 (10) years]. GGT fractions reference values were established by empirical quantile analysis in a reference group of 432 healthy subjects [45% men, 57 (10) years].
Results: Fractional GGT levels were higher in men than in women ($P<0.0001$). In both sexes, fractions were associated with: triglycerides were associated with b-GGT, alcohol consumption with m-, s- and f-GGT. C-reactive protein with m- and s-GGT, while plasminogen activator inhibitor-1 with b- and f-GGT. Body mass index, blood pressure, glucose and triglycerides correlated with b- and f-GGT. In comparison with the reference group [b-GGT/s-GGT median (Q1–Q3): 0.51 (0.35–0.79) U/L], subjects affected by cardiovascular disease or diabetes showed no change of *b/s* ratio [0.52 (0.34–0.79) U/L, 0.57 (0.40–0.83) U/L, respectively]. The *b/s* ratio was higher in presence of metabolic syndrome [0.61 (0.42–0.87) U/L, $P<0.0001$], while lower in heavy alcohol consumers [0.41 (0.28–0.64) U/L, $P<0.0001$].
Conclusions: Metabolic and cardiovascular risk markers are important correlates of GGT fractions, in particular of b-GGT.

1. Introduction

Total serum gamma-glutamyltransferase (GGT) activity is currently considered a sensitive but non-specific diagnostic marker of hepato-biliary disorders and of alcohol abuse [1].

Serum GGT activity is affected by genetic factors, with heritability estimated between 0.3 and 0.5 [2,3] but it has many other correlates within its normal range. GGT shows a positive association with alcohol consumption and smoking habit, heart rate (HR), systolic (SBP) and diastolic (DBP) blood pressure, obesity indexes, such as waist circumference and body mass index (BMI), and with serum level of glucose, triglycerides, total and LDL cholesterol and uric acid [1]. Also preexisting ischemic heart disease, diabetes mellitus, menopause and use of antihypertensive medication, lipid lowering drugs and oral contraceptives show a positive association with GGT level, while an inverse association has been observed with coffee consumption, physical activity, and lung function (FEV1) [1].

Several large epidemiological studies conducted in unselected populations have demonstrated that serum GGT elevation is, an independent predictor of all-cause mortality [4], and mortality due to either hepatic or neoplastic diseases [5]. Circulating total GGT activity has been also associated with an increased risk for arterial hypertension, diabetes, and metabolic syndrome [6–8]. Serum GGT levels within the upper normal range (25–40 U/L) were found to be associated with increased risk of cardiovascular events, independently of established cardiovascular risk factors, both in unselected populations (including the community-based Framingham Heart Study) [8–12] and in patients with prior coronary artery disease [13]. Accordingly, elevation of

Abbreviations: BMI, body mass index; *b/s*, b-GGT/s-GGT ratio; BNP, B-type natriuretic peptide; CRP, C-reactive protein; DBP, diastolic blood pressure; FEV1, lung function; GGT, gamma-glutamyltransferase; HR, heart rate; NAFLD, non-alcoholic fatty liver disease; PAI-1, plasminogen activator inhibitor 1; SBP, systolic blood pressure; WC, waist circumference; UACR, urine albumin to creatinine ratio.

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serum GGT concentrations was associated with an increase in the SCORE risk function [14], and GGT was also found to incrementally add to Framingham Risk Score function [15].

Recently, our group has set up a reproducible chromatographic method [16], disclosing that total plasma GGT activity corresponds, in healthy subjects, to four distinct fractions showing distinct physicochemical properties [17,18]. These fractions consist in three GGT-containing molecular complexes b-, m-, s-, with molecular weight >2000, 940, 140 kDa, respectively, and the free enzyme, f-GGT (70 kDa). f-GGT is the most abundant fraction [19], while b-GGT correlates with the level of serum triglycerides, LDL-cholesterol, C-reactive protein (CRP), and DBP [20]. Interestingly, the active enzyme found inside the atherosclerotic plaque [21–23] was shown to correspond to the b-GGT fraction [24].

While f-GGT is the major circulating GGT fraction in healthy subjects in both sexes, in the pathological conditions examined so far, b-GGT and s-GGT accounted for most of total GGT increase [25,26]. Chronic viral hepatitis C [25] and alcoholic-liver disease [26] are characterized by the increase in s-GGT and the decrease in the b-GGT/s-GGT (*b*/*s*) ratio. Conversely, non-alcoholic fatty liver disease (NAFLD) is associated with the increase in both b-GGT and s-GGT fractions, without change of the *b*/*s* ratio, in comparison with healthy controls [25].

The aims of the present investigation were to establish the reference values of GGT fractions, to assess their correlates in a large reference sample of healthy subjects from the Offspring Cohort of the Framingham Heart Study, and to study the clinical correlates in the larger community sample.

2. Materials and methods

2.1. Study participants

The Framingham Offspring Study began in 1971 with enrolment of 5124 offspring (and offspring spouses) of original cohort participants [27]. Participants from the Framingham Offspring Study who attended the sixth examination cycle (1995 to 1998), for which plasma-EDTA sample was available, were eligible for this investigation. The final sample was 3203 individuals [1497 men, mean age (SD): 58.9 (9.9) years; 1706 women, 58.5 (9.7) years].

At each Framingham Heart Study visit, attendees undergo a physical examination and a medical history (by a Heart Study physician), anthropometric measures, and laboratory assessment of vascular risk factors. All participants provided written informed consent, and the Institutional Review Board of the Boston Medical Center approved the study protocol.

2.2. Measurements and definitions

For the present investigations, hypertension was defined as a systolic blood pressure of 140 mm Hg or higher, a diastolic blood pressure of 90 mm Hg or higher, or the use of antihypertensive medications [28] Participants who smoked cigarettes regularly during the year preceding the Heart Study visit were considered "current" smokers. Alcohol intake was assessed by averaging the self-reported weekly consumption of alcoholic drinks; USA dietary guidelines recommend the consumption of less than 14 drinks/week for men and 7 drinks/week for women; one drink contains 0.6 US fluid ounces of alcohol (14 g of ethanol). Body mass index (BMI) was calculated as the weight in kilograms divided by the square of the height in meters (kg/m^2); obesity was defined as BMI ≥30 kg/m^2. Pre-diabetes condition was defined as fasting blood glucose levels greater or equal to 100 mg/dL and less than 126 mg/dL; diabetes was defined as a fasting blood glucose level of 126 mg/dL or greater, or the use of any hypoglycemic agent [29]. Metabolic syndrome was defined using modified National Cholesterol Education Program (NCEP) criteria, which required at least three of the following: (1) elevated serum triglycerides ≥150 mg/dL; (2) HDL cholesterol <40 mg/dL [men] or <50 mg/dL [women]; (3) BP ≥130 mm Hg systolic, ≥85 mm Hg diastolic, or use of antihypertensive therapy; (4) fasting blood glucose ≥100 mg/dL; and (5) BMI ≥30 kg/m2 [8]. Dyslipidemia was defined as total serum cholesterol ≥240 mg/dL or HDL cholesterol <40 mg/dL (men) or <50 mg/dL (women) or lipid lowering treatment. Cardiovascular disease (CVD) was defined as the presence of coronary heart disease, heart failure, cerebrovascular disease, and peripheral vascular disease. Renal disease was defined empirically as serum creatinine level ≥2 mg/dL.

Usual levels of physical activity were assessed by a questionnaire. Subjects were asked how many hours per day were spent engaging in sleep, sedentary activity, and slight, moderate, and heavy physical activity. Based on the answers to these questions a physical activity index was estimated, expressing how many times per week intense physical activity (enough to sweat) was performed [30].

2.3. Reference sample

A reference sample constituted by healthy subjects was obtained according to the following inclusion criteria: no smoking, no heavy alcohol intake, absence of CVD, hypertension, diabetes, obesity, renal diseases, metabolic syndrome and dyslipidemia. The final reference sample comprised 432 subjects (194 men and 238 women).

2.4. Laboratory analysis

Blood samples were obtained from fasting participants between 8 and 9 AM at the Heart Study visit. Analyses were performed using standard clinical laboratory procedures with automated analyzers available at the Framingham Heart Study. In particular, B-type natriuretic peptide (BNP) was measured using the Shionogi Assay, high sensitivity C-reactive protein (CRP) was measured through high sensitivity Dade Behring BN100 nephelometer, plasminogen activator inhibitor 1 (PAI-1) was determined by ELISA kit (Biopool International, Ventura CA); LDL cholesterol was calculated using the Friedewald formula.

2.5. Fractional GGT analysis

Archived plasma-EDTA (ethylenediaminetetraacetic acid) samples, frozen at −80 °C and thawed once, were used. Fractional GGT analysis was performed as described previously [16,19; pat. pend. WO2009/001290-A3, The University of Pisa] using an FPLC (fast protein liquid chromatography) system (AKTA purifier, GE Healthcare Europe, Milan, Italy) equipped with a gel-filtration column (Superose 6 HR 10/300 GL, GE Healthcare Europe) and fluorescence detector (Jasco FP-2020, Jasco Europe, Lecco, Italy). GGT activity was measured using gamma-glutamyl-7-amido-4-methylcoumarin (Nova Chimica, Milan, Italy) as substrate (0.03 mmol/L, final concentration) and glycylglycine (5.4 mmol/L, final concentration) as acceptor of the transpeptidation reaction. The fluorescence detector operating at excitation/emission wavelengths of 380/440 nm detected the amino-4-methylcoumarin signal; the intensity of the fluorescence signal was expressed in arbitrary fluorescence units.

Under this reaction conditions, area under curve is proportional to GGT activity. Total area, between 10 and 25 mL elution volume, and fractional GGT area was calculated by a MatLab program (Version 7 MathWorks, Inc.) to resolve overlapping peaks; the curve fitting was conducted with a nonlinear least-squares minimization algorithm using four exponentially modified Gaussian (EMG) curves. The reaction was calibrated analyzing plasma samples with known total GGT activity (standards). The slope of the calibration curve was used to convert total and fractional GGT area to U/L [19]. The sum of fractional GGT activity represents on average the 99% of total GGT activity.

A 4.5 mmol/L stock solution of gamma-glutamyl-7-amido-4-methylcoumarin was prepared in ethanol 40% v/v containing 0.01 N NaOH and stored at −20 °C. This solution was diluted 25-fold into 0.25 M Tris–HCl buffer pH 8.5 (25 °C) daily.

2.6. Statistical analysis

Total and fractional GGT values between males and females, healthy subjects and heavy alcohol consumers, or subjects with CVD, or with metabolic syndrome were compared using Wilconxon–Mann–Whitney test.

To perform correlation analysis, several variables (total and fractional GGT values, triglyceride, CRP, fibrinogen and PAI-1 levels) were natural-logarithmically transformed.

Bivariate linear (Pearson) correlations were estimated between anthropometric data (age, BMI, waist circumference), lifestyle (alcohol and coffee consumption, physical activity index), biological variables (glucose, HDL and LDL cholesterol, triglycerides, CRP, fibrinogen, PAI-1, HCys, BNP, etc.) and fractional GGT activity.

Multiple linear regressions, with total and fractional GGT activities as dependent variables, were performed to quantify the relations of several factors (age, waist circumference, alcohol consumption, systolic blood pressure, HDL and LDL cholesterol, triglycerides, CRP, fibrinogen PAI-1, HCys). Variables not significantly associated with GGT were removed from final models. Results were presented as a standardized regression coefficient. Considering the high number of hypothesis tests carried out, $P<0.01$ has been chosen as significance threshold to reduce the type I error (i.e. false positive).

In the healthy sample, fractional GGT activity reference limits were estimated by empirical quantile (quantiles 0.025, 0.50 and 0.975). Statistical analysis was performed with SAS analysis software (SAS/STAT version 9.2).

3. Results

3.1. Distribution of fractional GGT in the offspring cohort

The sample considered in the present study was of 3203 participants to the VI cycle of examination of the offspring cohort. The sample comprised 1497 men [mean (SD); age 58.9 (9.9) years] and 1706 women [58.5 (9.7) years]. Clinical and biochemical characteristics of the sample are presented in Table 1.

In both sexes, total plasma GGT, as well as all fractions and the *b*/*s* ratio, showed a skewed distribution and f-GGT was the prominent fraction. Total GGT activity was higher in men [median (Q1–Q3), 26.1 (19.6–38.4) *vs.* 18.8 (14.2–27.5), ($P<0.001$)], as it was for all four GGT fractions ($P<0.0001$). The *b*/*s* ratio was lower in men ($P<0.0001$; Table 1).

Table 1
Characteristics of the offspring cohort (examination 6).

	Men (1497)	Women (1706)
Age, years	59 (10)	59 (10)
BMI, kg/m^2	28.5 (4.3)	27.3 (5.7)
Waist circumference, cm	101 (11)	94 (15)
Smoking, *n* (%)	215 (14)	271 (16)
Alcohol consumption, drinks/week	7.3 (9.5)	3.2 (5.2)
Coffee consumption, cups/day	2.3 (2.4)	1.7 (1.8)
Physical activity index, times/week	2.7 (2.5)	2.1 (2.1)
Heart rate, bpm	62 (11)	65 (10)
SBP, mm Hg	130 (17)	127 (20)
DBP, mm Hg	77 (9)	74 (9)
Hypertension, *n* (%)	673 (45)	647 (38)
Obesity, *n* (%)	458 (31)	429 (25)
Pre-diabetes, *n* (%)	640 (43)	436 (26)
Diabetes mellitus, *n* (%)	184 (12)	137 (8)
Metabolic syndrome, *n* (%)	659 (44)	623 (37)
Renal diseases, *n* (%)	16.0 (1.1)	5.0 (0.3)
Cardiovascular diseases, *n* (%)	232 (15)	127 (7)
Coronary heart disease	177 (12)	77 (5)
Heart failure	43 (3)	31 (2)
Cerebrovascular disease	52 (3)	37 (2)
Peripheral vascular disease	22 (1.5)	13 (0.8)
Glucose, mg/dL	100 (94–110)	94 (88–102)
Creatinine, mg/dL	1.2 (1.1–1.3)	1.1 (1.0–1.2)
UARC, mg/g	4.8 (2.1–11.0)	8.5 (3.5–17.7)
Total cholesterol, mg/dL	197 (175–122)	209 (185–236)
HDL cholesterol, mg/dL	42 (35–50)	56 (46–67)
LDL cholesterol, mg/dL	126 (104–148)	125 (104–148)
Triglycerides, mg/dL	122 (85–175)	112 (78–165)
CRP, mg/dL	1.8 (0.9–3.8)	2.3 (1.0–5.7)
Fibrinongen, mg/dL	325 (288–376)	334 (294–381)
PAI-1, ng/mL	25.6 (16.9–36.1)	20.2 (12.2–31.8)
HCys, μmol/L	9.9 (8.4–12.0)	8.4 (7.0–10.3)
BNP, ng/L	6.7 (4.0–16.8)	10.0 (4.1–20.2)
Aldosterone, ng/dL	9.0 (7.0–13.0)	11.0 (7.0–15.0)
Renin, mUI/L	14.0 (8.0–24.5)	11.0 (6.0–19.0)
Aldosterone/renin	0.7 (0.4–1.2)	1.0 (0.6–1.7)
D-dimer μg/mL	299 (184–472)	335 (230–483)
Total GGT, U/L	26.1 (19.6–38.4)	18.8 (14.2–27.5)‡
b-GGT, U/L	3.8 (2.3–6.7)	2.7 (1.7–4.9)‡
m-GGT, U/L	0.6 (0.3–1.1)	0.3 (0.2–0.7)‡
s-GGT, U/L	7.9 (4.7–13.8)	4.9 (3.0–9.3)‡
f-GGT, U/L	13.4 (11.0–16.6)	10.1 (8.2–12.7)‡
b/*s* ratio	0.47 (0.33–0.68)	0.53 (0.36–0.80)‡

Data are presented as mean (SD) or median (Q1–Q3), unless otherwise indicated. BMI: body mass index; BNP: brain natriuretic peptide; CRP: C-reactive protein; DBP: diastolic blood pressure; HCys: homocysteine; PAI-1: plasminogen activator inhibitor 1; Q: quartile; SBP: systolic blood pressure; UACR: urine-albumin to creatinine ratio. Wilconxon–Mann–Whitney test, ‡$P<0.0001$.

3.2. Correlates of fractional GGT activity in men and women: linear correlation analysis

To study the biological correlations of each GGT fraction, we first performed a bivariate linear correlation analysis with known correlates of total GGT activity available for the sixth examination cycle of the offspring cohort (Table 2). Results for total GGT are reported in the Supplemental Data Table 1.

In both sexes, all plasma GGT fractions were associated with body mass index (BMI), waist circumference (WC), alcohol consumption (apart from b-GGT in women), heart rate, systolic and diastolic blood pressure, blood glucose, total cholesterol, triglycerides, C-reactive protein (CRP), and with plasminogen activator inhibitor 1 (PAI-1). Among these variables, triglycerides and PAI-1 levels showed the highest Pearson correlation coefficients with all the fractions. Age was correlated with b-, m- and f-GGT fractions in women and with the *b*/*s* ratio in both genders. Coffee consumption showed an inverse correlation only with b-GGT in women. Physical activity inversely correlated with b-GGT, both in men and women, and with f-GGT in men. Alcohol consumption was positively correlated with the fractions m-, s- and f-GGT in both sexes, the magnitude of the correlation being stronger in men.

Comparing correlation coefficients for each biological variables among GGT fractions, emerged that b-GGT and f-GGT, in both genders, showed the highest correlation coefficient with all the considered cardiovascular risk factors: high levels of waist circumference (WC), diastolic blood pressure (DBP), blood glucose, triglycerides and low levels of HDL cholesterol. Plasma b-GGT concentration showed the highest degree of correlation also with markers of inflammation (CRP and fibrinogen), while PAI-1 showed the highest correlation coefficients with f-GGT in both genders.

Compared to the individual fractions, the *b*/*s* ratio showed higher correlation with WC, fibrinogen and LDL cholesterol.

3.3. Clinical correlates of fractional GGT activity in men and women

Variables associated with total and GGT fractions were reported separately for men and women in Table 3. Results for total GGT are reported in the Supplemental Data Table 2.

Table 2
Linear correlation analysis, by sex, between biological variables and fractional GGT activity.

	Men					Women				
Variables	b-GGT	m-GGT	s-GGT	f-GGT	*b/s* ratio	b-GGT	m-GGT	s-GGT	f-GGT	*b/s* ratio
Age	−0.01	−0.06	−0.06	−0.06	0.09†	0.17‡	0.07§	0.04	0.12‡	0.19‡
BMI	0.17‡	0.15‡	0.09†	0.18‡	0.11‡	0.27‡	0.14‡	0.12‡	0.25‡	0.21‡
Waist circumference	0.20‡	0.15‡	0.10‡	0.20‡	0.14‡	0.32‡	0.17‡	0.15‡	0.30‡	0.24‡
Alcohol	0.15‡	0.21‡	0.28‡	0.24‡	−0.24‡	0.03	0.08§	0.13‡	0.09†	−0.16‡
Coffee	−0.06	−0.00	−0.04	0.01	−0.03	−0.07§	−0.01	−0.04	0.03	−0.05
Physical activity	−0.08§	−0.03	−0.05	−0.06	−0.04	−0.06§	−0.04	−0.04	−0.02	−0.03
Heart rate	0.20‡	0.18‡	0.16‡	0.17‡	0.05	0.22‡	0.14‡	0.13‡	0.21‡	0.11‡
SBP	0.07§	0.09†	0.07§	0.09†	−0.01	0.19‡	0.10‡	0.10‡	0.19‡	0.12‡
DBP	0.13‡	0.14‡	0.11‡	0.15‡	0.01	0.14‡	0.06	0.07§	0.15‡	0.07†
Creatinine#	−0.12‡	−0.08	−0.07†	−0.12‡	−0.07	−0.08†	−0.06	−0.06	−0.6	−0.03
Blood glucose	0.15‡	0.09†	0.08§	0.17‡	0.09†	0.27‡	0.17‡	0.15‡	0.31‡	0.15‡
Total cholesterol	0.26‡	0.27‡	0.16‡	0.30‡	0.14‡	0.30‡	0.24‡	0.13‡	0.19‡	0.23‡
HDL cholesterol	−0.10†	−0.05	0.11‡	−0.03	−0.34‡	−0.22‡	−0.13‡	−0.01	−0.21‡	−0.30‡
LDL cholesterol	0.07§	0.15‡	−0.01	0.04	0.13‡	0.25‡	0.24‡	0.07§	0.18‡	0.25‡
Triglycerides#	0.40‡	0.26‡	0.18‡	0.32‡	0.33‡	0.45‡	0.20‡	0.19‡	0.29‡	0.36‡
CRP#	0.20‡	0.20‡	0.13‡	0.17‡	0.10‡	0.30‡	0.24‡	0.19‡	0.20‡	0.14‡
Fibrinogen#	0.06	0.04	−0.05	−0.02	0.18‡	0.16‡	0.11‡	0.01	0.09†	0.22‡
PAI-1#	0.40‡	0.30‡	0.26‡	0.41‡	0.19‡	0.45‡	0.29‡	0.25‡	0.53‡	0.27‡
HCys#	−0.01	0.07§	0.01	0.02	−0.03	0.07§	0.07§	0.07§	0.13‡	0.00
UACR,#	0.04	0.04	0.01	0.04	0.03	0.03	0.04	0.04	0.09†	−0.00
BNP#	−0.08§	0.05	−0.04	−0.12‡	−0.07§	−0.08§	−0.05	−0.03	−0.07§	−0.07§
Aldosterone#	0.08§	0.06	0.06	0.11‡	0.03	0.09†	0.09†	0.08†	0.10‡	0.01
Renin#	0.06	0.06	0.04	0.04	0.04	0.08†	0.07§	0.07§	0.14‡	0.01
Aldosterone/Renin#	−0.01	−0.01	−0.01	0.02	−0.00	−0.03	−0.02	−0.02	−0.08†	−0.01
D-dimer#	−0.02	−0.01	−0.06	−0.06§	0.07§	0.12‡	0.08§	0.04	0.07§	0.12‡

Data are Pearson correlation coefficients. BMI: body mass index; BNP: brain natriuretic peptide; CRP: C-reactive protein; DBP: diastolic blood pressure; HCys: homocysteine; PAI-1: plasminogen activator inhibitor 1; SBP: systolic blood pressure; UACR: urine-albumin to creatinine ratio. #Linear correlation analysis have been performed on ln-transformed data, also fractional GGT data were ln-transformed. Statistical significance level: §$P<0.01$; †$P<0.001$; ‡$P<0.0001$, otherwise $P\geq 0.01$.

Table 3
Clinical correlates of fractional GGT, by sex, as determined by multivariable linear regression analysis.

	b-GGT			m-GGT			s-GGT			f-GGT			*b/s* ratio		
Variable	Std. estimate	95% CI		Std. estimate	95% CI		Std. estimate	95% CI		Std. estimate	95% CI		Std. estimate	95% CI	
Men (n = 1422)															
Age	−0.03	−0.08	0.01	−0.08†	−0.13	−0.03	−0.06	−0.11	−0.01	−0.06	−0.10	−0.01	0.09†	0.04	0.15
Waist circumference													0.03	−0.02	0.09
Alcohol	0.07§	0.03	0.12	0.16‡	0.12	0.21	0.18‡	0.13	0.23	0.19‡	0.14	0.23	−0.18‡	−0.23	−0.13
Heart rate	0.08§	0.03	0.12	0.08†	0.03	0.13	0.08†	0.03	0.13	0.06§	0.02	0.11			
SBP													−0.06	−0.11	−0.01
Blood glucose	0.03	−0.02	0.08	0.02	−0.03	0.07	0.02	−0.02	0.07	0.07§	0.02	0.12			
HDL cholesterol	0.12‡	0.07	0.18				0.20‡	0.14	0.26				−0.15‡	−0.21	−0.09
LDL cholesterol	0.05	0.01	0.10	0.14‡	0.09	0.19				0.05	−0.00	0.09	0.15‡	0.11	0.20
Triglycerides#	0.35‡	0.29	0.41	0.15‡	0.10	0.20	0.18‡	0.12	0.24	0.19‡	0.14	0.24	0.24‡	0.19	0.30
CRP#	0.11‡	0.06	0.15	0.18‡	0.12	0.24	0.16‡	0.10	0.22	0.15‡	0.09	0.20	−0.06	−0.12	0.00
Fibrinogen,#				−0.08†	−0.14	−0.02	−0.12‡	−0.18	−0.06	−0.14‡	−0.20	−0.08	0.12‡	0.06	0.18
PAI-1#	0.23‡	0.17	0.28	0.16‡	0.10	0.21	0.17‡	0.11	0.22	0.25‡	0.20	0.30	0.06	0.00	0.12
HCys#				0.04	−0.00	0.09	0.00	−0.05	0.05	0.01	−0.04	0.06	−0.05	−0.10	−0.00
Women (n = 1616)															
Age	0.02	−0.02	0.07	−0.02	−0.07	0.03	−0.04	−0.09	0.01	−0.01	−0.06	0.03	0.11‡	0.06	0.16
Waist circumference													0.07	0.01	0.13
Alcohol	0.05	0.01	0.095	0.08†	0.04	0.13	0.09†	0.04	0.13	0.07†	0.03	0.12	−0.06†	−0.11	−0.02
Heart rate	0.04	−0.00	0.08	0.02	−0.03	0.06	0.03	−0.02	0.07	0.05	0.01	0.09			
SBP													−0.03	−0.08	0.02
Blood glucose	0.04	−0.00	0.09	0.07§	0.02	0.12	0.05	−0.00	0.10	0.11‡	0.06	0.16			
HDL cholesterol	0.04	−0.01	0.09				0.11†	0.06	0.17				−0.11‡	−0.16	−0.05
LDL cholesterol	0.12‡	0.08	0.17	0.20‡	0.16	0.25				0.09†	0.04	0.13	0.14‡	0.10	0.19
Triglycerides#	0.28‡	0.23	0.33	0.04	−0.01	0.09	0.14‡	0.08	0.20	0.07§	0.03	0.12	0.20‡	0.15	0.26
CRP#	0.08†	0.04	0.13	0.18‡	0.12	0.23	0.14‡	0.08	0.19	0.02	−0.03	0.07	−0.09§	−0.14	−0.03
Fibrinogen,#				−0.06	−0.11	−0.00	−0.10†	−0.15	−0.04	−0.08§	−0.12	−0.03	0.11‡	0.06	0.16
PAI-1#	0.26‡	0.21	0.31	0.16‡	0.11	0.21	0.19‡	0.13	0.25	0.42‡	0.38	0.47	0.08§	0.02	0.13
HCys#				0.02	−0.02	0.07	0.06	0.01	0.11	0.07§	0.03	0.11	−0.09†	−0.13	−0.04

Standardized regression coefficients (95% CI) are shown. CRP: C-reactive protein; DBP: diastolic blood pressure; PAI-1: plasminogen activator inhibitor 1. #Linear regression analysis have been performed on ln-transformed data, also fractional GGT data were ln-transformed. Statistical significance level: §$P<0.01$; †$P<0.001$; ‡$P<0.0001$, otherwise $P\geq 0.01$.

Multivariable linear regression analysis showed that, in both sexes, the four fractions were mainly influenced by the same variables; triglycerides level was the main predictor of b-GGT, whereas alcohol consumption was specifically associated with m-, s- and f-GGT. CRP and PAI-1 levels also showed to be important correlates of all GGT fractions.

Serum lipid levels showed different associations with the three high molecular weight GGT fractions in men and women: triglycerides levels were associated with b-GGT and s-GGT in both sexes but with m-GGT only in men; LDL-cholesterol was associated with b-GGT and m-GGT both in men and women; while HDL-cholesterol was associated with s-GGT in both sexes and with b-GGT only in men. LDL-cholesterol showed higher standardized regression coefficient values in women in comparison with men; the opposite was true for HDL-cholesterol. Fibrinogen showed a negative association with m-, s- and f-GGT, both in men and women, in particular s- and f-GGT in men showed the highest standardized regression coefficients.

In both sexes, the main positive correlates of *b*/*s* ratio were LDL-cholesterol, triglycerides levels and fibrinogen while alcohol consumption and HDL-cholesterol showed an inverse association.

3.4. Total fractional GGT activity in the healthy subjects: reference values

To estimate the reference values for fractional GGT activity, among participants at the sixth examination cycle of the Offspring cohort, we selected a Reference sample consisting of 432 healthy subjects (13.2% of participants), 194 men [mean (SD), age 55.6 (9.8) years] and 238 women [age 57.6 (9.6) years]. We excluded all major medical conditions potentially associated with alteration of circulating GGT activity, as detailed in the Material and Methods section. The clinical and biochemical characteristics of these subjects are presented in the Supplemental Data Table 3; UACR, BNP, aldosterone, renin, D-dimer and homocysteine values were within normal range (data not shown).

Also in the reference sample, total plasma GGT values were higher in men than in women ($P<0.0001$), analogously all GGT fractions (b-GGT, $P<0.05$; m-GGT, $P<0.01$; s- and f-GGT, $P<0.0001$). Levels of *b*/*s* ratio, instead, were lower in men than in women ($P<0.001$; Supplemental Data Table 2).

Lower (2.5th quintile), median (50th quintile) and upper (97.5th quintile) reference limits are presented for total and each GGT fractions separately for men and women in Table 4.

3.5. Total and fractional GGT activity in subsets

We compared total and fractional GGT activities in the reference group of healthy subjects with corresponding levels in heavy alcohol consumers or subjects affected by cardiovascular disease (CVD), metabolic syndrome, or diabetes (Table 5), according to the definitions noted in Materials and methods section.

In both genders, all the aforementioned conditions were associated with a mild, but statistically significant, increase of total and fractional GGT values, except for s-GGT in cardio vascular disease (CVD) subset and b-GGT in women CVD-subset. Alcohol consumption was associated with the prevalent elevation of s-GGT (men: 2.0 fold; women: 1.8 fold) over the other fractions, thus the *b*/*s* ratio resulted significantly lower than in healthy subjects (men: $P<0.001$; women: $P<0.0001$). In both genders, subjects affect by CVD or diabetes experienced a proportional elevation of GGT fractions, in fact the *b*/*s* ratio did not change in comparison with healthy subjects. The latter, instead, was increased in presence of metabolic syndrome (men: $P<0.001$; women: $P<0.01$).

Table 4
Fractional GGT reference limits (U/L).

	Men ($n=194$)			Women ($n=238$)		
	2.5th	50th	97.5th	2.5th	50th	97.5th
Total GGT	11.4	23.1	108.8	8.8	16.8	87.3
b-GGT	0.8	3.1	18.1	0.8	2.5	30.1
m-GGT	0.01	0.48	4.19	0.01	0.29	3.51
s-GGT	2.0	6.3	71.6	1.4	4.1	46.4
f-GGT	6.9	11.7	24.2	5.6	9.4	20.9
b/*s* ratio	0.16	0.46	1.25	0.18	0.59	1.61

Data are from empirical quantile estimation.

4. Discussion

The analysis of Framingham Offspring cohort shows that the correlates of plasma activity vary for each GGT fraction: the b-GGT fraction is mostly associated with serum triglyceride levels in both sexes, while m- and s-GGT are mostly correlated with either alcohol consumption and HDL or LDL cholesterol level. Prominent correlates of f-GGT in both sexes were PAI-1 level and triglyceride level and alcohol consumption in men, and blood glucose in women.

We also established the reference values for each of the four GGT fractions in a subgroup of healthy subjects ($n=432$), values that correspond to median values previously reported in 200 blood donors [19]. Fractional GGT analysis showed significant differences in activity of all fractions between men and women.

Analysis of the clinical correlates in the whole community sample confirmed the results of bivariate correlations. Plasma total GGT activity is positively associated with already described factors such as alcohol consumption, triglycerides, LDL cholesterol, blood pressure, body mass index, waist circumference, serum glucose, fibrinogen and CRP [1], and negatively with HDL cholesterol, physical activity. The multivariable analysis showed that alcohol consumption, triglycerides, HDL and LDL cholesterol and CRP were the only independent correlates, together with PAI-1 hereby described for the first time.

As expected, bivariate correlations and multivariable linear regression analyses conducted separately for each GGT fraction showed that biological and clinical correlations described for total GGT actually depended on the diverse association of the above mentioned factors with specific fractions.

Alcohol consumption showed a prominent association with the m- and s-GGT fractions: in fact, we have previously reported that fractional GGT profile of alcohol addicts is characterized by a greatest increase in m- and s-GGT levels *vs.* other fractions [26]. We have also previously observed the elevation of s-GGT fraction in patients with chronic hepatitis C, this suggesting the s-GGT as a marker of hepatocellular damage. The negative correlation found between s-GGT and plasma fibrinogen in the Framingham cohort might correspond to underlying liver dysfunction.

Markers of metabolic syndrome (BMI, DBP, glucose, triglycerides) showed the highest positive correlation with the b- and f-GGT fractions. These results confirm and support the recent finding that b-GGT fraction holds the best specificity and sensitivity for the diagnosis of NAFLD [25]. b- and f-GGT showed a negative correlation with physical activity as it had been previously showed for total GGT activity. It is well known that regular exercise improves many cardiovascular and metabolic risks factors, including the intrahepatic triglyceride content [31].

The existence of a correlation between PAI-1 and total GGT levels has been previously reported only in small selected cohorts of hypertriglyceridemic and insulin-resistant patients [32,33]; in the present investigation we observed that plasma PAI-1 is among the strongest independent predictors of especially b-GGT and f-GGT. Enhanced expression of both PAI-1 and GGT has been shown in a variety of liver injury models, including bile duct ligation and alcohol-induced liver injury [34,35], affecting hepatic protein synthesis. Furthermore, plasma PAI-1 levels were strongly correlated with the cluster of variables defining the metabolic syndrome (i.e.: insulin-resistance, obesity, glucose and lipid metabolic imbalance) [36].

Table 5
Total and fractional GGT values in subgroups.

	Tot GGT	b-GGT	m-GGT	s-GGT	f-GGT	*b/s* ratio
Men						
Healthy ($n=194$)	23.1 (16.7–35.2)	3.1 (2.1–6.1)	0.48 (0.25–0.89)	6.3 (4.0–12.7)	11.7 (9.8–14.9)	0.46 (0.33–0.68)
Alcohol ($n=237$)	33.9 (23.7–60.1)‡	4.8 (2.7–9.6)‡	0.85 (0.49–2.1)‡	12.3 (6.7–28.8)‡	15.1 (12.3–19.5)‡	0.38 (0.25–0.58)†
CVD ($n=232$)	27.5 (20.0–38.9)†	4.2 (2.6–6.8)§	0.67 (0.35–1.2)§	8.4 (4.6–15.4)	13.6 (11.6–16.7)‡	0.49 (0.33–0.73)
MS ($n=575$)	30.1 (22.4–43.8)‡	4.9 (3.1–8.4)‡	0.74 (0.41–1.6)‡	8.6 (5.4–16.1)†	14.6 (12.0–18.0)‡	0.55 (0.40–0.77)†
Diabetes ($n=184$)	31.0 (21.0–46.7)‡	4.9 (2.9–8.4)‡	0.74 (0.41–1.5)‡	8.7 (4.6–18.4)§	14.8 (12.2–18.6)‡	0.51 (0.38–0.75)
Women						
Healthy ($n=238$)	16.8 (13.0–23.8)	2.5 (1.7–4.3)	0.29 (0.15–0.58)	4.1 (2.7–7.8)	9.4 (7.7–11.3)	0.59 (0.38–0.85)
Alcohol ($n=225$)	21.3 (16.3–31.5)‡	3.2 (1.9–5.5)§	0.49 (0.20–0.83)†	7.1 (3.9–13.0)‡	10.8 (8.8–13.0)‡	0.44 (0.31–0.70)‡
CVD ($n=127$)	20.0 (15.5–29.7)§	3.1 (2.0–5.6)	0.41 (0.21–0.77)§	5.1 (3.1–9.4)	10.6 (8.5–13.7)†	0.59 (0.36–0.93)
MS ($n=468$)	24.5 (17.9–37.8)‡	4.4 (2.8–7.6)‡	0.56 (0.23–1.1)‡	6.5 (3.6–13.3)‡	12.3 (10.1–15.3)‡	0.69 (0.46–0.97)§
Diabetes ($n=137$)	27.3 (19.9–47.3)‡	5.5 (3.1–9.1)‡	0.74 (0.41–1.53)‡	7.6 (4.0–15.7)‡	13.9 (11.0–16.7)‡	0.62 (0.42–0.97)

Data are presented as median (Q1–Q3). ALCOHOL: heavy alcohol consumers; CVD: cardiovascular diseases; MS: metabolic syndrome; Q: quartile. Statistical significance *vs.* Healthy subjects: $^{\S}P<0.01$; $^{\dagger}P<0.001$ $^{\ddagger}P<0.0001$, otherwise $P\geq 0.01$.

Comparison of total GGT values between the subsets of healthy subjects and of subjects affected by CVD, metabolic syndrome, diabetes, or characterized by heavy alcohol consumption confirmed that total plasma GGT activity is a sensitive but nonspecific marker. On the other hand, each subset was characterized by a specific fractional GGT pattern, better described by the *b/s* ratio. Based to our correlation analysis, heavy alcohol intake was characterized by the highest values of s-GGT and the lowest *b/s* ratio, while individuals with metabolic syndrome and diabetes had the highest values of both b-GGT and *b/s* ratio. As a perspective, the estimation of *b/s* ratio could improve the interpretation of total GGT elevation, as already observed in small selected cohorts of patients affected by liver steatosis or chronic viral hepatitis C, where an increase has been associated with a metabolic liver dysfunction or a decrease with hepatocellular damage [25].

In conclusion, the present study indicates that known cardiovascular and metabolic risk markers are important correlates of GGT fractions, in particular of b-GGT. The study of GGT fractions could permit a better understanding of the pathogenesis of diseases associated with GGT increase, thus allowing a better clinical use of the GGT test. Prospective studies are needed to establish the risk for metabolic disease and cardiovascular events associated with each GGT fractions.

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Appendix A. Supplementary data

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