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CIRRHOSIS AND LIVER FAILURE

Circulating gamma-glutamyltransferase fractions in cirrhosis

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Keywords
b-GGT/s-GGT ratio – cirrhosis – end-stage liver disease – fractions – gamma-glutamyltransferase

Abbreviations
ALB, albumin; ALP, alkaline phosphatases; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUC, area under the curve; b-GGT, b-GGT fraction; BIL, bilirubin; CHOL, total cholesterol; CI, confidence interval; CrCl, creatinine clearance; EDTA, ethylenediamine-tetra-acetic-acid; FBG, fibrinogen; f-GGT, f-GGT fraction; FPLC, fast protein liquid chromatography; GGT, gamma-gultamyltransferases; HCC, hepatocellular carcinoma; INR, international normalized ratio; kDa, kiloDalton; LDH, lactate dehydrogenases; LDL, low density lipoprotein; LFT, liver function tests; MELD, model for end stage liver disease; MET, metabolic cirrhosis; m-GGT, m-GGT fraction; NAFLD, non-alcoholic fatty liver disease; ROC, receiver operator characteristic curve; s-GGT, s-GGT fraction; TRG, triglycerides; VIR, viral cirrhosis; VLDL, very low density lipoprotein.

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Abstract

Background & Aims: Four gamma-gultamyltransferases (GGT) fractions (b-, m-, s-, and f-GGT) have been identified in human plasma and their concentrations and ratios vary in different pathological conditions. To assess the behaviour of fractional GGT in cirrhotic patients evaluated for liver transplantation. *Methods:* This was a single-centre, cross-sectional study; GGT fractions were determined by gel-filtration chromatography. *Results:* 264 cirrhotic patients (215 males; median age 54.5 years) were included and compared against a group of 200 healthy individuals (100 males; median age 41.5). Median (25th–75th percentile) total and fractional GGT were higher in cirrhotics, with s-GGT showing the greatest increase [36.6 U/L (21.0–81.4) vs. 5.6 U/L (3.2–10.2), $P < 0.0001$], while the median b-GGT/s-GGT ratio was lower in cirrhotics than in healthy controls [0.06 (0.04–0.10)] vs. 0.28 (0.20–0.40), $P < 0.0001$]. The ratio showed higher diagnostic accuracy (ROC-AUC, 95% CI: 0.951, 0.927–0.969) then either s-GGT (0.924, 0.897–0.947; $P < 0.05$) or total GGT (0.900, 0.869–0.925; $P < 0.001$). The diagnostic accuracy of the ratio was maintained (0.940, 0.907–0.963) in cirrhotic patients ($n = 113$) with total GGT values within the reference range. The s-GGT fraction consisted of two components, with one (s2-GGT) showing a significant positive correlation with serum aspartate aminotransferases, alanine aminotransferase, lactate dehydrogenases (LDH), alkaline phosphatases and bilirubin, and negative with albumin. The b-GGT fraction showed a positive correlation with albumin, fibrinogen, and platelet counts, and negative with international normalized ratio, bilirubin and LDH. *Conclusions:* The ratio performs as a sensitive biomarker of the liver parenchymal rearrangement, irrespective of aetiology of cirrhosis and presence of hepatocellular carcinoma, even in patients with total GGT values within the reference range.

In the liver, the enzyme gamma-glutamyltransferase (GGT) is expressed in the biliary canaliculi and on the luminal side of the bile ducts (1, 2). Liver diseases are the main causes of plasma GGT increase (1), making GGT a biomarker of hepato-biliary disease and alcohol abuse (3, 4). During the last decade, a series of

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large-scale, prospective studies have shown that plasma GGT values above the median, albeit within the reference range, are independent predictors of the risk of cardiovascular mortality (5), stroke (6, 7) and metabolic syndrome (8–10), including its individual components, such as hyperglycaemia, hypertension and dyslipidaemia, and irrespective of alcohol consumption and liver diseases (11–14).

By use of a high-sensitivity molecular size-exclusion procedure we previously reported that four GGT fractions (i.e. b-GGT, m-GGT, s-GGT, and f-GGT) with molecular weights ranging from 2000 to 70 kDa are present in the human plasma (15, 16), and that different patterns of fractional GGT are associated with distinct pathological conditions (17, 18). The evidence collected to date suggests that the higher concentrations of the high-molecular-weight b-GGT are associated with cardiovascular and metabolic disease (17, 19, 20), while the s-GGT is a biomarker of liver injury and architectural rearrangement (17, 18). The s-GGT is a supramolecular complex with a molecular weight similar to high density lipoprotein (HDL) cholesterol, and its increase has been reported in alcohol abuse (18) and chronic hepatitis (17). On the opposite, the b-GGT has a molecular size similar to very low density lipoprotein (VLDL), and increased levels have been reported in non-alcoholic fatty liver disease (NAFLD) concurrent with heightened s-GGT expression (17). In turn, the f-GGT fraction, which corresponds to the hydrophilic form of the enzyme, is the most frequent one expressed in healthy individuals (16). Studies in humans, rodents and large laboratory animals have shown that the four-GGT-fraction pattern is shared by all adult mammalians, and that during embryonic development the b-GGT is the first to appear and the f-GGT is the last, suggesting that a fully functional liver architecture is required to deploy and maintain this pattern (21).

Scanty information is still available on the fractional GGT pattern expressed in cirrhosis. To that regard, we designed a cross-sectional study to explore the expression of total and fractional GGT in cirrhotic patients referred for liver transplantation (LT), and to assess their correlation with routine biomarkers of liver function.

Materials and methods

Study design and patient selection

This was a single-centre, cross-sectional study on patients with cirrhosis referred for evaluation of eligibility to LT and compared against a historical cohort of healthy individuals enrolled in previous research on total and fractional GGT (16). This study was initiated in February 2008 at the Hepatobiliary Surgery and Liver Transplantation Unit of the University of Pisa Medical School Hospital in collaboration with the Department of Translational Research and Novel Technologies, the Department of Cardiovascular Medicine, Gabriele Monasterio Foundation, National Research Council, and the Sant'Anna Institute of High Education, Pisa, Italy.

The inclusion criteria called for adult (≥18 years), consenting patients affected with cirrhosis of viral, metabolic, toxic and autoimmune aetiology, and undergoing evaluation for deceased donor LT. Patients were excluded if unable to provide an informed consent; on active alcohol use ≤6 months prior to evaluation; affected with hepatocellular carcinoma (HCC) with macrovascular infiltration and/or extra-hepatic disease; affected with non-HCC malignancies; on active steroid and/or anti-hepatitis C virus (HCV) treatment; with renal failure with a creatinine clearance (CrCl) ≤30 ml/min according to the Cockroft Gault's formula; affected with systemic infection of extrahepatic origin; affected with fulminant liver failure; and if on dialysis or mechanical ventilation.

Blood samples were obtained after an overnight fasting on the first day of the outpatient and/or inpatient pretransplant workup and included liver function tests (LFT), serology, virology and the autoimmune panel. A diagnosis of cirrhosis was based as the composite of medical history, physical examination, liver function tests, serologic studies, radiology and histology, as clinically appropriate. HCC was diagnosed according to the American Association for the Study of Liver Disease 2005 guidelines, eventually updated in 2010 (22). The severity of liver disease was graded according to the model for end-stage liver disease (MELD) scoring system, which is based on 3 variables (serum bilirubin, serum creatinine and international normalized ratio (INR) and is able to estimate 3-month mortality in patients with chronic end-stage liver disease more accurately than the Child-Turcotte-Pugh score (23, 24). MELD is the score of choice for deceased donor allocation since 2002 in the United States and 2006 in the euro-transplant countries (25, 26).

This study was approved by the internal review board and carried out in compliance with the principles set forth in the Declaration of Helsinki.

Laboratory analyses and score calculations

Standard assay of all blood tests were simultaneously performed according to the clinical laboratory procedures by automated analysers at the Clinical Laboratories of the Pisa University Hospital, and included: red blood count; white blood count; platelet count; INR; fibrinogen (FBG); aspartate aminotransferases (AST); alanine aminotransferases (ALT); alkaline phosphatases (ALP); bilirubin (BIL); serum electrolytes; total protein; serum albumin (ALB); total triglycerides (TRG); total cholesterol (CHOL); low density lipoprotein (LDL); HDL; VLDL; viral serology for hepatitis B (HBV), HCV, hepatitis delta, and human immunodeficiency virus as appropriate. The CrCl was calculated with the Cockcroft-Gault formula [CrCl ml/min = [(140 − age)

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× kg/(creatinine mg/dl × 72)] × 0.85 for women)] (27). The LDL cholesterol was calculated using the Friedewald formula [LDL-C = total cholesterol − HDL cholesterol − (triglycerides/5)]. The MELD score was derived according to the formula: MELD = 9.57 × ln (creatinine) (mg/dl) + 3.78 × ln(total bilirubin) (mg/dl) + 11.20 × ln(INR) + 6.43 (23).

Total and fractional GGT determination

Analysis of total and fractional GGT was performed, as previously described (15, 16) (pat. pend. WO2009/001290-A3, The University of Pisa), on plasma-EDTA (ethylenediamine-tetra-acetic acid) samples 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). Separation of fractional GGT was obtained by gel filtration chromatography and the enzymatic activity was quantified by post-column injection of the fluorescent substrate for GGT, gamma-glutamyl-7-amido-4-methylcoumarin (gGluAMC). Enzymatic reaction, in the presence of gGluAMC 0.030 mmol/L and glycylglycine 4.5 mmol/L, proceeded for 4.5 min in a reaction coil (PFA, 2.6 ml) kept at the 37°C in a water bath. The fluorescence detector operating at excitation/emission wavelengths of 380/440 nm detected the AMC signal; the intensity of the fluorescence signal was expressed in arbitrary fluorescence units (f.u.), and the area under curve is proportional to GGT activity.

The total GGT and fractional GGT areas were calculated by a MatLab program (Version 7 MathWorks, Inc., Natick, MA, USA) 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 analysing 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 (16).

A 4.5 mmol/L stock solution of gGluAMC was prepared in ethanol 30% w/w containing 0.005 N NaOH and stored at –20°C. This solution was daily diluted 25-fold into 0.25 M Tris-HCl buffer pH 8.5 (25°C).

Statistical analysis

Data are presented as means ± standard deviation, medians, percentiles, frequencies and ranges as appropriate. According to the and level of distribution of the variables, the statistical analysis was carried out with the Student's *t*-test or 1-way ANOVA followed by the Bonferroni's multiple comparison test for continuously distributed values. Total GGT, b-, m- and s-GGT fractions, as well as b-GGT/s-GGT ratio, triglycerides and body mass index values were ln-transformed to reduce the distribution skewness. f-GGT fraction data were not ln-transformed because they were normally distributed (16). Bivariate linear correlations between biological variables and fractional GGT activity were evaluated with the Spearman's correlation coefficient. A Receiver Operating Characteristics curve analysis (ROC) was performed to assess the diagnostic accuracy of total and fractional GGT values and of the b-GGT/s-GGT ratio. The ROC analysis and the comparison between ROC curves were performed with MedCalc 11.5 analysis software on the basis of the De Long method described elsewhere (28). Positive and negative predictive value have been calculated as if the ratio of healthy and diseases subjects considered in this study reflects the prevalence of disease.

Results

Patients

Between February 2008 and April 2011, we enrolled a total of 264 patients (215 males (81.4%)) of a median age (25th–75th percentile) of 54.5 (50–60). Based on previous research on total and fractional GGT (17), patients were divided according to aetiology of liver disease and/or presence of HCC: 17 (6.4%) were affected with metabolic cirrhosis (MET); 22 (8.3%) with alcoholic cirrhosis (ALC); 96 (36.4%) with viral cirrhosis because of HCV and/or HBV infection (VIR); and 129 (48.9%) with HCC in the setting of cirrhosis of viral origin (HCC). Two hundred healthy individuals included in previous research on total and fractional GGT reference values were used as the historical control cohort [100 males (50%); median age (25th–75th percentile) 41.5 (35.0–50.0) years] (16). Table 1 illustrates the demographic, clinical and biochemical characteristic of the study population of cirrhotics and of the 200 healthy controls. The two populations were different for gender and age, with cirrhotics being predominantly males and older than healthy individuals (Table 1).

Laboratory tests

The study group of cirrhotics showed higher AST, ALT, ALP, total bilirubin, lactate dehydrogenases (LDH), INR and triglycerides, while serum albumin, total cholesterol and platelet counts were lower vs. healthy controls (Table 1). Total and fractional GGT were higher in cirrhotics (Table 1). The s-GGT showed a higher increase, and was the most abundant fraction in cirrhosis (Table 1). The b-GGT/s-GGT ratio was significantly lower in cirrhosis vs. the healthy controls (Table 1). In both groups the total GGT level, all of its fractions and the b-GGT/s-GGT ratio showed a right-skewed distribution ($P < 0.0001$). Of the 264 cirrhotic patients, 113

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Table 1. Comparison of the characteristics of the healthy subjects (#200) and study patients (#264)

	Healthy Subjects (n = 200)		Cirrhosis (n = 264)		P
Males, n (%)	100 (50%)		215 (81%)		<0.0001
Age, years	41.5	35.0–50.0	54.5	50.0–60.0	<0.0001
MELD			11.9	9.1–14.9	
BMI*, kg/m^2	24.3	22.2–26.6	24.8	23.1–26.8	n.s.
SBP, mmHg	120.0	110.0–120.0	120.0	110.0–130.0	n.s.
DBP, mmHg	80.0	70.0–80.0	70.0	70.0–80.0	0.0003
Heart rate, bpm	70.0	64.0–75.5	68.0	60.0–75.0	n.s.
Glucose, mg/dl	93.0	86.0–100.0	101.0	93.0–116.0	<0.0001
Creatinine, mg/dl	0.9	0.8–1.0	0.8	0.7–0.9	n.s.
CrCl, ml/min	104.5	91.5–119.2	107.5	89.5–130.7	n.s.
CHOL, mg/dl	187.5	160.8–209.3	139.5	118.8–166.0	<0.0001
HDL cholesterol, mg/dl	51.0	43.0–62.0	49.0	37.0–61.0	0.0306
LDL cholesterol, mg/dl	116.5	97.0–135.0	75.0	56.8–96.0	<0.0001
TRG*, mg/dl	73.0	50.0–106.5	83.5	69.5–112.0	0.0008
AST, U/L	17.0	14.0–21.0	70.0	43.8–106.3	<0.0001
ALT, U/L	17.0	12.0–24.5	50.0	33.0–81.0	<0.0001
BIL, mg/dl	0.7	0.6–0.9	1.6	0.9–2.9	<0.0001
ALP, U/L			129.5	94.8–182.0	
LDH, U/L			198.5	166.0–244.8	
ALB, g/dl			3.9	3.6–4.3	
Haemoglobin, g/dl	14.7	13.6–15.8	12.9	11.9–14.5	<0.0001
WBC, $\times 10^9$ L	6.2	5.4–7.6	4.3	3.6–5.7	<0.0001
PLT, 10^9/L	249.0	212.0–283.0	75.5	58.0–111.5	<0.0001
INR			1.4	1.2–1.5	
FBG, mg/dl			217.0	183.8–270.3	
Total GGT*, U/L	18.8	13.8–28.7	61.1	40.3–113.5	<0.0001
b-GGT*, U/L	1.6	0.9–3.0	2.6	1.4–5.3	<0.0001
m-GGT*, U/L	0.6	0.4–1.1	3.1	1.5–6.7	<0.0001
s-GGT*, U/L	5.6	3.2–10.2	36.6	21.0–81.4	<0.0001
f-GGT, U/L	10.4	8.6–13.4	16.2	13.6–20.4	<0.0001
b/s ratio*	0.28	0.20–0.40	0.06	0.04–0.10	<0.0001

Data are reported as median, 25th–75th percentile. CrCl was estimated with the Cockcroft–Gault formula; LDL cholesterol was estimated with the Friedewald formula. ALP, LDH, albumin, INR and fibrinogen were not assessed in healthy subjects (blood donors).

*Student's t-test performed on ln-transformed data.

ALB, albumin; ALP, Alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BIL, total bilirubin; BMI, body mass index; CHOL, total cholesterol; CrCl, creatinine clearance; DBP, diastolic blood pressure; FBG, fibrinogen; INR, international normalized ratio; GGT, gamma-glutamyltransferase; LDH, lactate dehydrogenase; MELD, model for end-stage liver disease (MELD) scoring system; SBP, systolic blood pressure; TRG, triglycerides; WBC, white blood cells. n.s., not significant.

Table 2. Diagnostic power of total, fractional GGT and of the b-GGT/s-GGT ratio in patients with cirrhosis and healthy subjects (HS)

	HS (n = 200) vs. Cirrhosis (n = 264)		P-value vs. b/s ratio	Tot-GGT within ref. range: HS (n = 200) vs. Cirrhosis (n = 113)		P-value vs. b/s ratio
Total GGT	0.900	0.869–0.925	0.010	0.789	0.740–0.833	<0.0001
b-GGT	0.616	0.570–0.661	<0.0001	0.628	0.672–0.773	<0.0001
m-GGT	0.868	0.833–0.897	<0.0001	0.724	0.671–0.773	<0.0001
s-GGT	0.924	0.897–0.947	0.0460	0.843	0.789–0.881	<0.0001
f-GGT	0.805	0.766–0.840	0.0001	0.672	0.617–0.724	<0.0001
b/s ratio	0.951	0.927–0.969		0.940	0.907–0.963	

Data are presented as ROC-AUC (95% CI). ROC-AUC, area under the receiver operating characteristic curve.

[42.8%; 104 males (92.0%)] showed median (25th–75th percentile) total GGT values within the reference range (16), i.e. 35.6 U/L (25.8–45.7) (Supplementary Table S1) (males: total GGT <60.5 U/L, females: <30.9 U/L).

ROC analysis

When comparing healthy subjects with cirrhotics, the s-GGT showed higher specificity and sensitivity for cirrhosis [area under the curve (AUC), 95% CI: 0.924,

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0.897–0.947], but the b-GGT/s-GGT ratio showed a significantly higher AUC (0.951, 0.927–0.969) vs. either the s-GGT ($P = 0.046$) or total GGT (0.900, 0.869–0.925; $P = 0.010$) (Table 2; Figure 1A). The best cut-off value for b-GGT/s-GGT ratio was 0.13 [sensitivity (95% CI): 87.1% (82.5–90.0); specificity: 91.0% (86.1–94.6); posi-

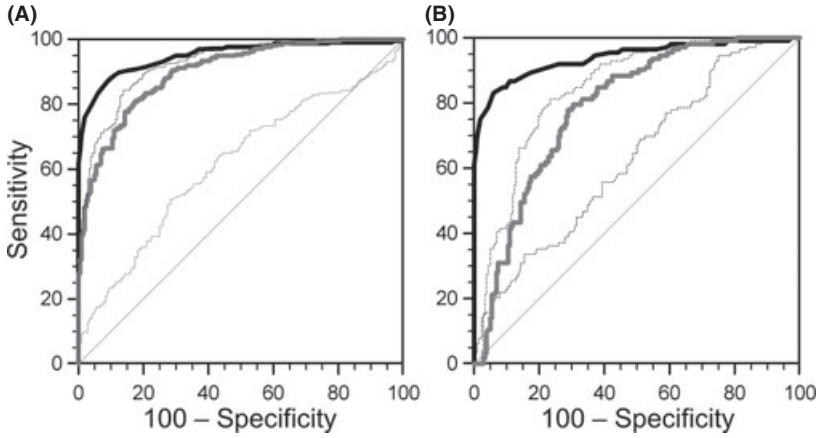

Fig. 1. ROC analysis of total and fractional GGT in patients affected with cirrhosis vs. healthy subjects: (A) whole population; (B) total GGT within reference range. b-GGT/s-GGT, black solid line; b-GGT, dotted line; s-GGT, dashed line; total GGT, gray solid line.

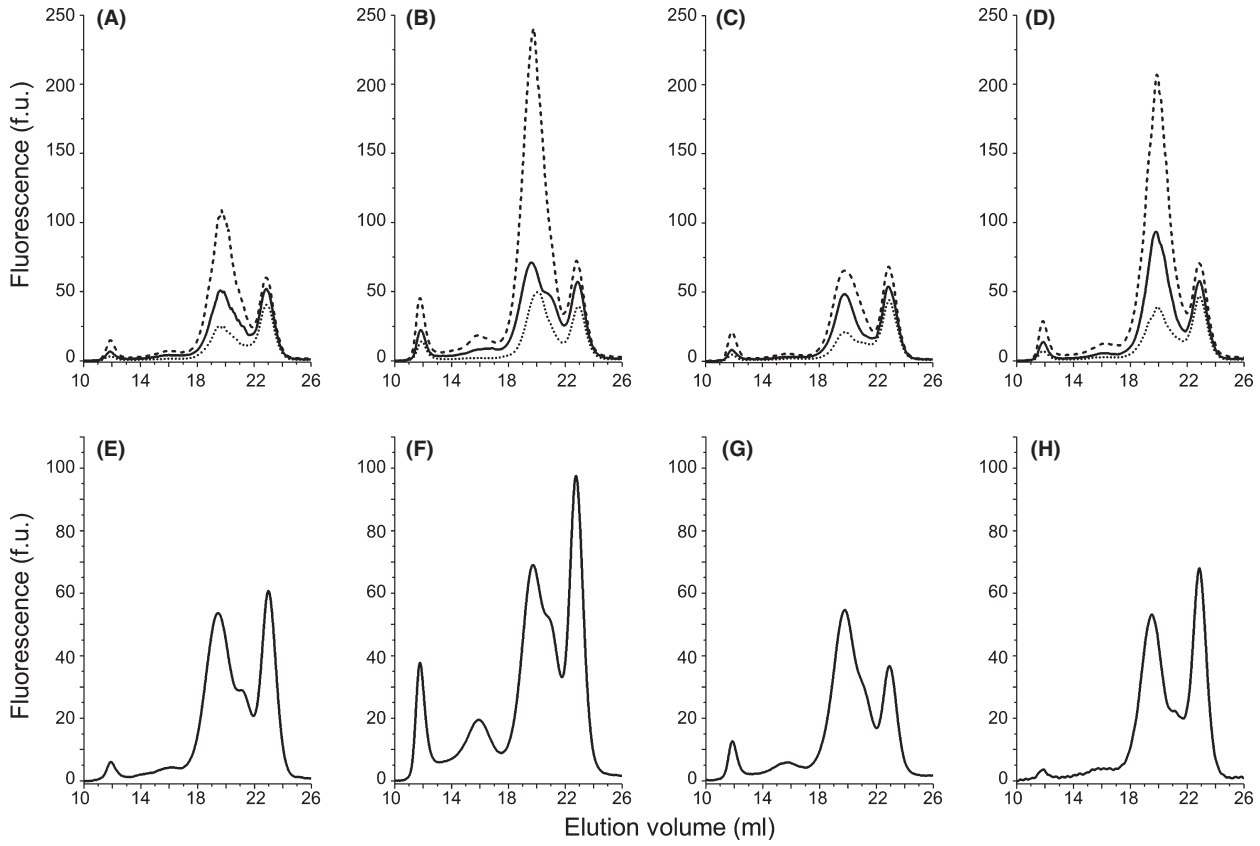

Fig. 2. Calculated elution profile (A–D) of fractional GGT activity corresponding to the 25th (dotted line), 50th (solid line) and 75th (dashed line) percentile and a representative chromatogram (E–H) of patients affected by: viral cirrhosis (A, E), metabolic cirrhosis (B, F), alcoholic cirrhosis (C, G) or cirrhosis and hepatocellular carcinoma (D, H). Fractional GGT analysis was performed on plasma-EDTA samples by high performance gel-filtration chromatography; GGT activity was specifically detected by an online post-column reaction with a fluorescent substrate.

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tive likelihood ratio: 9.68; negative likelihood ratio: 0.14; positive predictive value: 92.7; negative predictive value: 84.3].

In patients with total GGT within the reference range (n = 113) the diagnostic accuracy of the b-GGT/s-GGT ratio was maintained (AUC, 95% CI: 0.940, 0.907–0.963), while that of all other fractions dropped to lower values (m-, s-GGT amd f-GGT) or showed a moderate (b-GGT) (Table 2, Figure 1B). In this case the best cut-off value for b-GGT/s-GGT ratio was 0.12 [sensitivity (95% CI): 83.2% (75.0–89.6); specificity: 94.0% (89.8–96.9); positive likelihood ratio: 13.8; negative likelihood ratio: 0.18; positive predictive value: 88.7; negative predictive value: 90.8].

Fractional GGT activity

To better assess the behaviour of the GGT fractions in cirrhosis and HCC, the four subcohorts of patients (HCC, VIR, MET, ALC) were compared. Figure 2 shows the calculated elution profile of each of the three subgroups, corresponding to the 25th, 50th and 75th percentile of the distribution, and the actual chromatographic profile of a representative individual patient of each group. In all subcohorts, the s-GGT elution profile was broader than in healthy individuals and consisted of two Gaussian components that were eventually mathematically defined and named s1-GGT and s2-GGT. The s-GGT double profile was not observed in healthy controls. The differences observed across groups regarding total and fractional GGT (Table 3) were that in VIR patients both the b-GGT and the b-GGT/s-GGT ratio were lower vs. MET, while HCC patients showed higher values of total, b-, m- and s1-GGT compared with the VIR group, but not to the MET or ALC groups. In all of the four groups (Table 3), the b-GGT/s-GGT ratio was lower than in controls (Table 1) and was not correlated with total GGT, aetiology of cirrhosis or the presence of HCC.

The results of the correlation analysis between fractional GGT and laboratory tests showed that TRG and CHOL were positively correlated with all of the GGT fractions, as well as the b-GGT/s-GGT ratio. However, some specific patterns of associations emerged between fractional GGT and LFTs (Table 4). Namely, the b-GGT showed a positive correlation with ALB and FBG, and a negative correlation with INR, but no or negative correlation with AST, and ALT, ALP, BIL and LDH. As a result, the b-GGT showed a negative correlation with the MELD score. The s2-GGT fraction showed a statistically significant positive correlation with AST, ALT, LDH, ALP and BIL, but a negative association with ALB, and no association with INR and FBG. The two s-GGT fractions – s1-GGT and s2-GGT showed independent behaviour for some variables (e.g. ALB, INR and FBG were significantly correlated with one only), and sometimes opposite behaviour for others (BIL and LDH showed opposite correlations with s1 and s2-GGT). The

Table 3. Total and fractional GGT activity (U/L) in patients with hepatocellular carcinoma and cirrhosis (HCC) or affected by viral (VIR), metabolic (MET) or alcoholic (ALC) cirrhosis

	HCC (n = 129)		VIR (n = 96)		MET (n = 17)		ALC (n = 22)		P HCC vs. VIR	P HCC vs. MET	P HCC vs. ALC	P VIR vs. MET	P VIR vs. ALC	P MET vs. ALC
Total GGT*	72.0	41.8–133.5	53.0	34.4–91.2	61.6	43.2–159.2	48.3	32.9–114.8	<0.05	n.s.	n.s.	n.s.	n.s.	n.s.
b-GGT*	2.8	1.6–6.0	1.8	0.9–3.6	4.6	3.0–11.4	2.3	1.1–5.2	<0.01	n.s.	n.s.	<0.05	n.s.	n.s.
m-GGT*	3.4	1.5–8.0	2.9	1.0–4.7	5.5	1.7–11.6	2.4	1.3–7.8	<0.05	n.s.	n.s.	n.s.	n.s.	n.s.
s1-GGT*	40.6	18.7–90.3	24.2	13.6–59.5	33.2	20.8–112.1	25.1	9.1–66.7	<0.05	n.s.	n.s.	n.s.	n.s.	n.s.
s2-GGT*	5.2	2.8–7.6	4.7	3.0–7.1	5.8	3.0–10.5	4.0	3.2–8.9	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
f-GGT	16.9	13.6–20.6	15.2	11.6–18.5	25.6	12.1–22.2	18.8	13.5–24.1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
b/s*	0.06	0.04–0.11	0.05	0.04–0.08	0.09	0.07–0.17	0.07	0.04–0.13	n.s.	n.s.	n.s.	<0.05	n.s.	n.s.

Data are reported as median, 25th–75th percentile. Statistical analysis: 1-way ANOVA followed by Bonferroni's multiple comparison test.
*Statistical analysis performed on ln-transformed data.
HCC, hepatocellular carcinoma; n.s., not significant.

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Table 4. Linear correlation analysis between biological variables and fractional GGT activity

Variables	TOT GGT	b-GGT	m-GGT	s1-GGT	s2-GGT	f-GGT	b-GGT/s-GGT
MELD score	−0.216†	−0.283‡	n.s.	−0.300‡	0.166§	n.s.	n.s.
CHOL, mg/dl	0.456‡	0.508‡	0.480‡	0.473‡	0.166§	0.277‡	0.214†
TRG, mg/dl	0.337‡	0.351‡	0.420‡	0.301‡	0.214†	0.289‡	0.142*
PLT, 10^9/L	n.s.	0.180§	n.s.	n.s.	n.s.	n.s.	0.190§
BIL, mg/dl	−0.181‡	−0.268‡	n.s.	−0.264‡	0.175§	n.s.	n.s.
ALP, U/L	0.232†	n.s.	0.315‡	0.155*	0.415§	0.359‡	n.s.
AST, U/L	0.157*	n.s.	0.199§	n.s.	0.413‡	0.283‡	−0.344‡
ALT, U/L	0.276‡	n.s.	0.255‡	0.249‡	0.409‡	0.320‡	−0.306‡
LDH, U/L	n.s.	−0.181§	n.s.	−0.148*	0.200‡	0.183§	n.s.
ALB, g/dl	n.s.	0.260‡	n.s.	n.s.	−0.240†	n.s.	0.300‡
INR	−0.269‡	−0.361‡	−0.172§	−0.327‡	n.s.	n.s.	−0.161§
FBG, mg/dl	0.264‡	0.438‡	0.228†	0.286‡	n.s.	0.163§	0.355‡

Data are reported as Spearman correlation coefficients.

Statistical significance levels: *$P < 0.05$; §$P < 0.01$; †$P < 0.001$; ‡$P < 0.0001$.

ALB, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CHOL, cholesterol; FBG, fibrinogen; INR, international normalized ratio; LDH, lactate dehydrogenase; TRG, triglycerides. n.s., not significant.

m-GGT and f-GGT also displayed different association with the LFTs, with the f-GGT mostly corresponding to s2-GGT. The b-GGT/s-GGT ratio showed negative correlations mostly with indexes of hepatocellular damage (i.e. AST, ALT), or positive with marker of hepatocyte function (i.e. albumin, FBG), accordingly to an increase of s-GGT or b-GGT fraction respectively. Representative scatter plot for correlations are provided in Supplementary Figure S1.

Discussion

The current cross-sectional study shows that the b-GGT/s-GGT ratio has a high sensitivity and specificity for cirrhosis, irrespective of the levels of total and fractional GGT. The area under the ROC curve as well as the best cut-off were comparable in the whole cohort of cirrhotics vs. the subset of patients with total GGT within the reference range, and the b-GGT/s-GGT ratio was lower than in controls across all aetiologies or the presence of HCC. These data suggest that the b-GGT/s-GGT ratio behaves as a specific biomarker for the liver structural rearrangement, and that the mechanisms responsible for a decreased b-GGT/s-GGT ratio are different than those leading to the increase of total GGT values observed in liver disease. Consistently with this, in a previous study we already reported a decreased b-GGT/s-GGT ratio in chronic HCV-related hepatitis vs. NAFLD (17). Furthermore, the b-GGT/s-GGT ratio observed in the cirrhotic patients enrolled in this study (0.06, 0.04–0.1) was lower than in previous chronic HCV-related hepatitis patients (0.10, 0.07–015) (17), suggesting that the b-GGT/s-GGT ratio may act as an indicator of progression of liver fibrosis.

With regard to the individual GGT fractions, our study underlines that the b-GGT is correlated with liver function, as suggested by the positive association with serum albumin, fibrinogen, platelet counts, and the negative association with INR. On the opposite, the s-GGT – and its component s2-GGT in particular – behaves as a marker of cell injury and cholestasis, showing a positive association with AST, ALT, LDH, ALP and bilirubin, and a negative association with serum albumin. On the other hand the correlations found are quite low, suggesting that we have not identified yet the main role of GGT in liver metabolism. Elucidation of the mechanisms leading to production and release of the GGT fractions is pivotal to better understanding of the link between GGT, its fractions and liver physiopathology, thus explaining also the correlations found in this study.

Despite their heterogeneity – with molecular weights ranging from 2000 (b-GGT) to 70 kDa (f-GGT) – all of the GGT fractions share an identical GGT peptide encoded by the *GGT1* gene. However, the GGT fractions differ in that this peptide is linked to different carriers whose nature has not yet been entirely described (b, m-, s-GGTs), or it originates from hydrolysis of an otherwise strongly hydrophobic GGT peptide by a yet unknown protease (f-GGT). Describing in detail these mechanisms will likely improve our understanding of liver function and of pathogenesis of liver disease. The evidence collected to date confirms that the biogenesis of the GGT fractions replicates liver function and structure. Studies in rodents have shown that the b-GGT represents the fetal form of the circulating GGT, while s-GGT appears at birth, and f-GGT becomes the main fraction at weaning (21). In healthy controls, the b-GGT showed a strong association with the traditional risk factors for cardiovascular disease and metabolic syndrome, such as serum lipids and arterial blood pressure (19).

In conclusion, the b-GGT/s-GGT ratio showed to be a highly sensitive biomarker of liver structural rearrangement, even in patients with total GGT values within the reference range. The diagnosis of cirrhosis in patients evaluated for liver transplantation does not require any further biochemical analysis by a clinical point of view, anyway this study together with the previous ones (17, 18) represent the base for planning future

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studies aimed to verify if the b-GGT/s-GGT ratio may act as an indicator of progression of liver fibrosis and to compare its specificity and sensitivity with established test, such as Fibroscan (29). Recently, several algorithms, based on the dosage of serum biomarkers, have been validated as surrogate markers of liver fibrosis; some of these include also total GGT values, i.e.: FibroTest-ActiTest (30), Hepatoscore (31), the model developed by Forns and colleagues (32). Thus, it will be of interest to verify if GGT fractions and the b-GGT/s-GGT ratio may increase the predicting power of these tests, bettering the stratification of patients.

Further research on the nature and biological role of the GGT fractions is strongly favoured and will help improve the use of this biomarker for diagnosis and prognosis of liver disease and monitoring of progression of fibrosis.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Total and fractional GGT activity (U/L) in patients affected by cirrhosis and with total GGT values within the reference range (n = 113; 104 males).

Figure S1. Representative scatter plots for correlations between fractional GGT, the b-GGT/s-GGT ratio and a representative marker of hepatocellular damage (ALT), liver synthetic capability (albumin) and liver metabolism (total cholesterol).

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