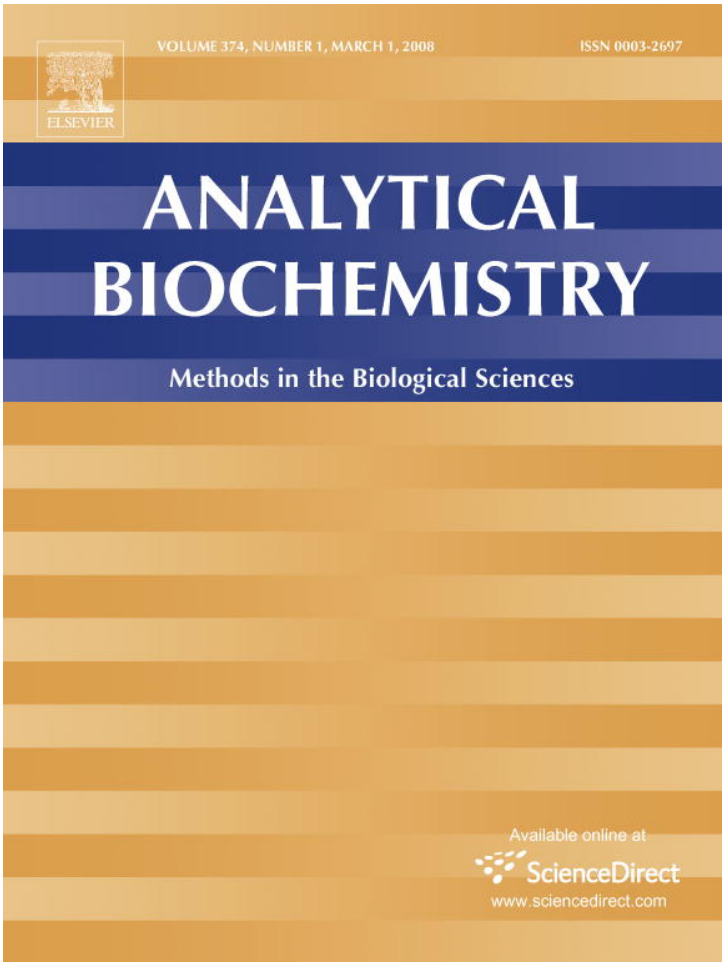

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A high performance gel filtration chromatography method for γ-glutamyltransferase fraction analysis

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Abstract

The clinical relevance of serum γ-glutamyltransferase (GGT) activity, in areas other than hepatic function, has recently been increased by several epidemiological associations. Still, GGT remains a nonspecific test because of the influence of various pathophysiological factors. We devised a procedure based on gel filtration chromatography, followed by postcolumn injection of fluorescent GGT substrate (γ-glutamyl-7-amido-4-methylcoumarin), permitting the quantification of GGT fractions in serum or plasma. Plasma GGT molecular weight distribution was analyzed in healthy volunteers (20 males; mean ± SD age 38 ± 10 years; 20 females; age 44 ± 13; total GGT 21 ± 11 for males vs 13 ± 7 for females; $P < 0.01$). The method is highly sensitive (determination limit: 0.5 U GGT/L), with a linear dynamic range between 0.5 and 150 U/L for each fraction. Four GGT fractions of different molecular weight were detected in all subjects of both genders: b-GGT, m-GGT, s-GGT (likely lipoprotein-bound, molecular masses >2000, 940, and 140 kDa, respectively), and a free fraction (f-GGT, 70 kDa). f-GGT and s-GGT were the main fractions in subjects with lower and higher total GGT activity, respectively. Higher total GGT activity in males is related mainly to f-GGT ($P < 0.01$). GGT fraction analysis may increase the sensitivity and specificity of the GGT activity test.

Keywords: γ-Glutamyltransferase activity; γ-Glutamyltransferase fractions; Gel filtration chromatography

Elevation of serum γ-glutamyltransferase (GGT)[1] activity is frequently interpreted as an index of hepatobiliary dysfunction and as a nonspecific marker of excessive alcohol use [1]. However, more recent studies have demonstrated that serum GGT, at values within the reference range, is associated with evolution of the atherosclerotic process [2,3], and predicts the onset and outcome of related diseases. In particular, type II diabetes [4,5] and metabolic syndrome (from the Framingham study) [6], coronary artery disease-related events, and stroke are predicted by serum GGT activity levels; this evidence has come from studies conducted either in unselected populations [7–9] or in patients known to have cardiovascular disease [10]. Furthermore, its prognostic value has been demonstrated in kidney disease [11], hepatobiliary disease, and neoplasms, even those without hepatic involvement [12]. This

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[1] *Abbreviations used:* GGT, γ-glutamyltransferase; γGluAMC, γ-glutamyl-7-amido-4-methylcoumarin; GlyGly, glycylglycine; γGluNA, γ-glutamyl-4-nitroanilide.

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confirms earlier epidemiological studies indicating the predictive value of serum GGT in overall and cardiac mortality [13–15].

At present, however, the nonspecificity of the test [1] makes risk stratification based on total serum GGT activity in patients with systemic, metabolic, and cardiovascular diseases unsuitable.

GGT is present in serum as part of several molecular complexes, with distinct physicochemical properties [16–18]; some have been associated with specific diseases, for example, intra- and extrahepatic jaundice [19], hepatocellular carcinoma [20], and cirrhosis and alcoholic liver disease [21]. Unfortunately, the low sensitivity and reproducibility of the different procedures adopted so far to separate and detect multiple forms of serum GGT have allowed the assessment of GGT fractions only in subjects with GGT values above the reference range, yielding conflicting results as far as the number and biological significance of GGT fractions are concerned.

The aim of the present study was therefore to develop a sensitive and reproducible procedure for the determination of distinct serum GGT fractions in subjects with serum GGT activity within the reference range of this enzyme. This would allow its clinical use in prognostic stratification for cardiovascular events by eventual definition of specific predictive GGT fraction profiles; moreover, it might permit us to describe specific GGT fraction profiles for each clinical condition with associated high GGT.

The new procedure is based on liquid chromatography for the separation of GGT fractions on the basis of their molecular weight and is coupled with a postcolumn enzymatic reaction in which a fluorescent substrate (γ-glutamyl-7-amido-4-methylcoumarin) is hydrolyzed to release fluorescent 7-amino-4-methylcoumarin [22–24]. We chose this substrate because it is more sensitive than the potentially carcinogenic 4-nitroanilide derivatives normally adopted for routine determination of serum GGT [25,26]. This method was applied to the analysis of plasma samples obtained from healthy volunteers.

Materials and methods

Chemicals and stock solutions

γ-Glutamyl-7-amido-4-methylcoumarin (γGluAMC, G7261), glycylglycine (GlyGly, G1002), bovine γ-glutamyltransferase (G8040), γ-glutamyl-4-nitroanilide (γGluNA, G1135), and a kit for molecular weight markers (MWGF1000; blue dextran, thyroglobulin, apoferritin, β-amylase, bovine serum albumin, carbonic anhydrase) were purchased from Sigma–Aldrich. Analytical reagent-grade chemicals were used without further purification. γGluAMC solution was prepared by adding 3.6 mmol/L in 0.005 N NaOH and immediately diluted 20-fold into 0.25 M Tris–HCl buffer, pH 8.5 (25 °C). This stock solution was prepared weekly and stored at +4 °C.

Subjects

Fasting blood samples were obtained between 8:00 and 9:00 from 40 healthy individuals (20 males, mean ± SD age 38 ± 10 years, range, 21–54; 20 females, age 44 ± 13 years, range 22–60), with serum GGT values between 6 and 47 U/L for men and between 6 and 34 U/L for women.

The presence of acute or chronic diseases was excluded by clinical examination and laboratory tests.

Laboratory analyses

Total plasma GGT values were determined by enzyme kinetic assay (Beckman Synchron CX 9-PRO analyzer). Within-run and between-run variation (coefficient of variation, %) was 3.5%; reference values by this procedure (5th and 95th percentile) were 10–54 U/L for males and 7–28 U/L for females (determination performed at 37 °C). Blood was collected into tubes containing EDTA as both anticoagulant and antioxidant. After centrifugation (1500*g*, 10 min) at room temperature, plasma samples were stored at −20 °C and analyzed within 1 month. The study was approved by the institutional ethics committee, and all subjects gave informed consent.

Chromatographic apparatus and conditions

A HPLC System Gold apparatus (Beckman 126) equipped with a 100-μL loop and a spectrofluorometric detector (821-FP, Jasco) was used. Separation was performed by gel filtration chromatography using a Superose 6 HR 10/300 GL column (GE Healthcare) by isocratic elution with 0.1 mol/L sodium phosphate buffer (pH 7.4) containing 0.2 mol/L NaCl, 0.1 mmol/L EDTA, and 5.4 mmol/L GlyGly to support GGT reaction [25]. Flow was set at 0.5 mL/min. The column was calibrated for molecular weight determination using a mixture of standard proteins of known molecular weight. Plasma samples were filtered with a 0.45-μm PVDF filter (Millipore) before injection. Total run time was 50 min.

Online GGT fraction determination

The flow eluting from HPLC column was mixed postcolumn in a low-dead-volume mixing tee with the γGluAMC fluorescent substrate delivered by a syringe pump (Braun) operating at a flow rate of 0.1 mL/min. Enzymatic reaction proceeded for 4.5 min [24] in a reaction coil (PFA, 2.6 mL) kept at the constant temperature of 37 °C in a water bath. The pH in the reaction coil after mixing was 8.2. AMC signal was specifically detected by a fluorescence detector operating at excitation wavelength $\lambda_{ex} = 380$ nm and emission wavelength $\lambda_{em} = 440$ nm [23]. Intensity of fluorescence signal was expressed in arbitrary fluorescence units (f.u.).

A pooled sample from 10 different subjects was analyzed through gel filtration chromatography, and GGT activity

fractions were collected, concentrated with a 3-kDa ultrafiltration membrane (Amicon, Millipore USA), and rechromatographed, as described above.

Offline GGT fraction determination

Spectrophotometric determination of GGT was performed using γGluNA as substrate and GlyGly as transpeptidation acceptor [26]. The amounts of *p*-nitroaniline formed were measured by reading the absorbance at 405 nm. GGT activity of chromatographic fractions ($V = 0.5$ mL) was measured in 96-well microtiter plates after incubation (4 h, 37 °C) of 100 µl of each fraction with 200 µl of reaction mixture. For spectrophotometric determinations, a multilabel plate reader was used (Wallac1420-Victor3, Perkin–Elmer).

Statistics

Correlations between various variables were presented as the squared Pearson correlation coefficient (r^2 value), and linear regression analyses were performed to assess associations. GGT fraction values were compared with the nonparametric Mann–Whitney test because of the skewness of the GGT distribution. $P < 0.05$ was considered significant.

Results

Optimization of online GGT reaction

The HPLC apparatus coupled to the postcolumn reaction system and spectrofluorometric detector was operated in flow injection mode, to optimize the online determination of GGT activity. Injections of commercial bovine GGT solution were used to optimize the concentration of the acceptor GlyGly (varying between 1 and 20 mmol/L) and γGluAMC (varying between 0.1 and 50 µmol/L). Maximum signal/noise ratio was obtained at 4.5 mmol/L GlyGly and 30 µmol/L γGluAMC. The calibration curve for GGT under these optimized conditions was linear in the range 0.5–150 U/L, with a sensitivity factor (±SD) of 3.81 ± 0.12 f.u. × min × L × U^{-1} ($r^2 = 0.95$, $P < 0.001$, $n = 7$) and a determination limit of 0.5 U/L (detection limit = 0.167 U/L).

Application: Plasma GGT elution profiles

The proposed method, under the operating conditions described above, was applied to the analysis of plasma samples obtained from healthy subjects. Fig. 1 shows the online GGT-specific elution profiles of a representative plasma sample obtained by using the liquid chromatographic system coupled to a postcolumn reaction with γGluAMC and fluorescence detection. The profile obtained with the online procedure was completely superimposable on that obtained by using γGluNA as substrate and determining offline GGT activity in the collected fractions. Under these conditions, reagent cost was approximately 1 Euro for each analysis.

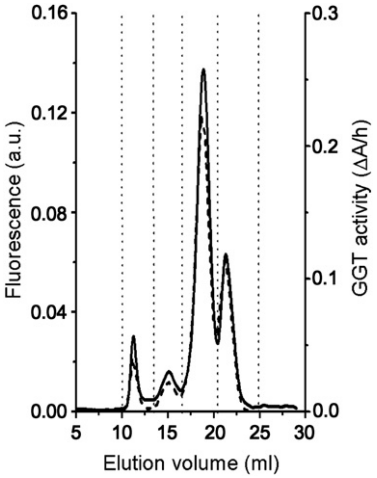

Fig. 1. Online GGT-specific elution profile of a representative plasma sample of a healthy subject with total plasma GGT activity 31 U/L (continuous line, left *y* axis). Offline GGT profile of the same plasma sample obtained by using γGluNA as substrate and determining enzyme activity in the collected chromatography fractions (dashed line, right *y* axis). Vertical lines represent GGT fraction elution limits.

All GGT activity elutes between 10 and 25 mL. On the basis of the molecular weight calibration curve of the column, four elution broad peaks can be identified for all samples and are defined as *big*-GGT (b-GGT, eluting between 10.0 and 13.5 mL, MW > 2000 kDa), *medium*-GGT (m-GGT, eluting between 13.5 and 16.6 mL, MW 940 kDa), *small*-GGT (s-GGT, eluting between 16.6 and 20.4 mL, MW 140 kDa), and *free*-GGT (f-GGT, eluting between 20.4 and 25 mL, MW 70 kDa).

Collection, concentration, and rechromatography of the f, b, m, and s fractions led to single peaks at the original elution volume.

Reproducibility

Reproducibility was determined by calculating both within-day and between-day coefficients of variation (CVs), by using a pool, obtained by mixing 10 distinct plasma samples. Within-day and between-day CVs for total area were 3.1% ($n = 3$) and 2.2% ($n = 5$), respectively.

Quantification of GGT fractions

Quantification of each GGT fraction was performed on the basis of a calibration curve obtained by plotting the total area under the four chromatogram peaks in the region 10 to 25 mL as function of known total GGT activity injected (Fig. 2). The calibration curve was linear between 5 and 47 U/L (sensitivity factor ± SD = $1.6 \times 10^{-5} \pm 0.6 \times 10^{-5}$ f.u × L^2 × U^{-1}; intercept ± SD = $3.4 \times 10^{-5} \pm 1.1 \times 10^{-5}$ f.u. × L; $r^2 = 0.952$, $P < 0.0001$,

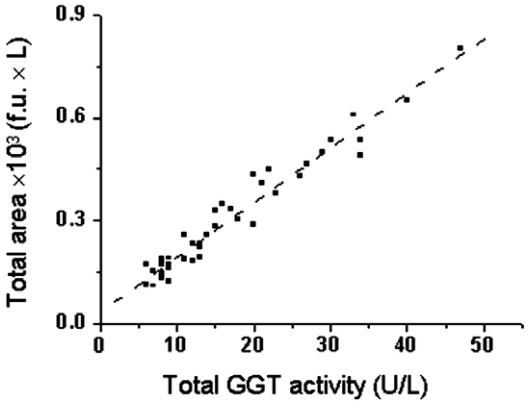

Fig. 2. Linear correlation between total area under the chromatographic curve (between 10 and 25 mL, elution volume) and total plasma GGT values. $y = 1.6 \times 10^{-5}x + 3.4 \times 10^{-5}$; $r^2 = 0.952$; $P < 0.0001$; $n = 40$.

$n = 40$). GGT activity associated with each GGT fraction was calculated by dividing the area of each single peak by the sensitivity factor; peak area was calculated using the x axis as baseline and the elution limits reported above. Table 1 and Fig. 3 summarize the results obtained with the 40 samples examined: higher values of total plasma GGT activity in males ($P < 0.01$) were reflected by a significant difference in f-GGT activity ($P < 0.01$).

Although a positive linear correlation was observed between total plasma GGT activity and activity of each of four GGT fractions (Table 2), their relative contributions to total GGT activity were different in both genders (Fig. 4). In fact, in subjects with lower GGT activity, f-GGT was the dominant fraction (up to 75%), whereas in subjects with higher GGT values, f-GGT contribution decreased to 20%, with a relative increase in s-GGT and b-GGT fractions.

Table 1
Total and fractional GGT activities (mean ± SD, U/L) in both genders

	Males ($n = 20$)	Females ($n = 20$)	P
GGT total	21.3 ± 11.1	13.1 ± 1.6	<0.01
b-GGT	2.6 ± 2.3	1.6 ± 1.1	NS
m-GGT	1.0 ± 0.7	0.7 ± 0.4	NS
s-GGT	8.1 ± 6.0	5.1 ± 4.4	NS
f-GGT	11.5 ± 4.1	8.2 ± 2.5	<0.01

Note. The nonparametric Student t test was applied. NS, not significant.

Discussion

Our method permits the sensitive and reproducible determination of four serum GGT fractions in all plasma samples examined (Fig. 1), with a considerably higher sensitivity; the determination limit, in fact, was 0.5 U/L for each fraction, as compared with 5 U/L—total plasma activity value—for the routine method.

The molecular weights determined for the b-GGT, m-GGT, and s-GGT fractions are compatible with lipoprotein-associated enzyme, that is, very low density lipoprotein, low-density lipoprotein and high-density lipoprotein, as previously proposed [16,17], while indicating a free enzyme form for f-GGT. Nevertheless, the gel filtration elution volumes of individual fractions (i.e., their molecular weights) did not change, independently of the amount of associated GGT activity (Fig. 1). This suggests a specific interaction of GGT enzyme with each lipoprotein particle, rather than aspecific absorption of lipophilic GGT to circulating lipoproteins; the latter would produce a shift in elution volume peaks, as a consequence of the varying extents of GGT protein absorption.

As expected [1], males had a higher total GGT value than females: this difference was due mainly to higher f-GGT levels (Table 1 and Fig. 3). In addition, we found that, within the reference values for the routine method, in healthy subjects of both genders (Fig. 4) f-GGT, the "free enzyme," was the predominant fraction in the presence of low serum GGT levels, whereas higher total

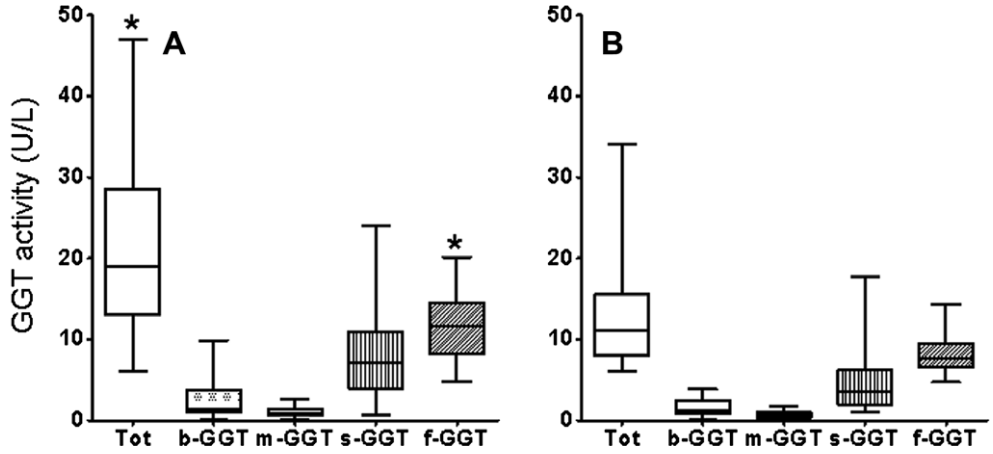

Fig. 3. Distribution of total plasma GGT activity and GGT activity associated with each plasma fraction in males (A) and females (B). The box extends from the 25th to the 75th percentile with a line at a median. The highest and lowest values are shown above and below the box, respectively. *Significantly different as compared with the corresponding values for females ($P < 0.01$).

Table 2
Linear correlation between GGT fractional and total activity (U/L) in both genders

	Males ($n = 20$)			Females ($n = 20$)		
	Sensitivity factor	Intercept	r^2	Sensitivity factor	Intercept	r^2
b-GGT	0.17 ± 0.03[a]	-1.05 ± 0.66	0.678	0.12 ± 0.02[a]	0.08 ± 0.33	0.607
m-GGT	0.03 ± 0.01[b]	0.28 ± 0.30	0.301	0.02 ± 0.01[b]	0.36 ± 0.18	0.192
s-GGT	0.51 ± 0.04[a]	-2.70 ± 1.01	0.889	0.56 ± 0.04[a]	-2.31 ± 0.54	0.927
f-GGT	0.32 ± 0.04[a]	4.64 ± 1.06	0.745	0.26 ± 0.04[a]	4.73 ± 0.65	0.666

Note. Values are expressed as means ± SD.
[a] $P < 0.0001$.
[b] $P < 0.01$.

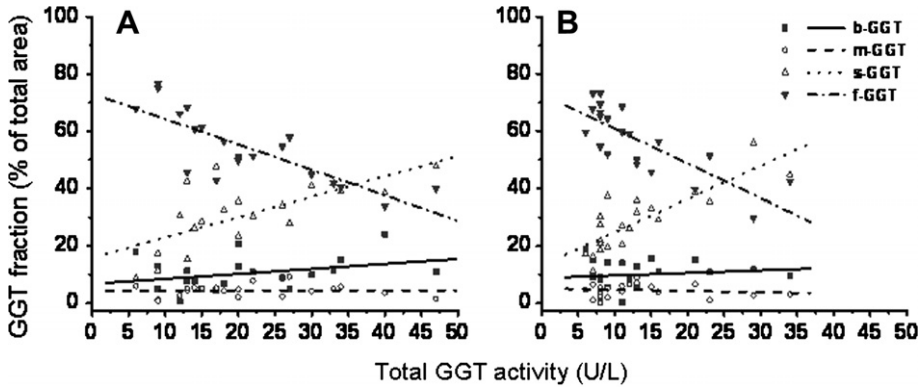

Fig. 4. Relative GGT fraction activity as a function of total GGT values for male (A, $n = 20$) and female (B, $n = 20$) subjects. Lines represent linear regressions. b-GGT (■, solid line), m-GGT (○, dashed line), s-GGT (□, dotted line), f-GGT (▼, dash-dot line). Males, linear regression: b-GGT $y = 0.17x + 6.7$ ($P = 0.15$, $r^2 = 0.11$), m-GGT $y = 0.01x + 4.3$ ($P = 0.93$, $r^2 = 0.01$), s-GGT $y = 0.71x + 15.9$ ($P < 0.001$, $r^2 = 0.47$), f-GGT $y = -0.89x + 73.1$ ($P < 0.0001$, $r^2 = 0.64$). Females, linear regression: b-GGT $y = 0.09x + 8.8$ ($P = 0.54$, $r^2 = 0.02$), m-GGT $y = -0.04x + 0.1$ ($P = 0.56$, $r^2 = 0.02$), s-GGT $y = 1.15x + 13.2$ ($P < 0.0001$, $r^2 = 0.68$), f-GGT $y = -1.21x + 72.9$ ($P < 0.0001$, $r^2 = 0.62$).

GGT values corresponded to increased b-GGT and s-GGT fractions, that is, higher-molecular-weight complexes.

The ability of our method to define the heterogeneous nature of total GGT activity of plasma and to describe a different fraction profile dependent on gender and changing total GGT levels make it suitable for the purpose of better characterizing the clinical value of plasma GGT activity in various disease conditions. Moreover, the increase in GGT fractions likely associated with lipoproteins, observed in subjects with plasma GGT values in the high-normal range, may explain the reported prognostic value of plasma GGT activity in cardiovascular diseases [6,7,10]. Conceivably, further studies might allow the association of variations in specific plasma GGT fractions with other disease conditions.

The potential effects of variation in very low density lipoprotein, low-density lipoprotein, and high-density lipoprotein and their ratios on GGT fractions may have important clinical correlates. Total serum GGT activity is known, from epidemiological studies, to be positively associated with total cholesterol and triglycerides, and large prospective studies are needed to clarify whether the prognostic relevance of lipid/lipoprotein concentration is associated with specific GGT fraction profiles. Furthermore, pharmacological studies could clarify if drug modulation of plasma lipids and its expected prognostic benefits are associated with possible peculiar modification of GGT fractions.

In conclusion, GGT fraction analysis is a reproducible and sensitive method, suitable for studying qualitative and quantitative distributions of GGT activity in plasma. Further studies on selected patient cohorts will establish precise connections between individual GGT fractions and their determinants, and their clinical value in various disorders.

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References

[1] J.B. Whitfield, Gamma glutamyl transferase, Crit. Rev. Clin. Lab. Sci. 38 (2001) 263–355.
[2] A. Paolicchi, M. Emdin, E. Ghliozeni, E. Ciancia, C. Passino, G. Popoff, A. Pompella, Human atherosclerotic plaques contain gamma-glutamyl transpeptidase enzyme activity, Circulation 109 (2004) 1440.
[3] M. Emdin, A. Pompella, A. Paolicchi, Gamma-glutamyltransferase, atherosclerosis, and cardiovascular disease: triggering oxidative stress within the plaque [editorial], Circulation 112 (2005) 2078–2080.

[4] D.H. Lee, K. Silventoinen, D.R. Jacobs Jr, P. Jousilahti, J. Tuomileto, Gamma-glutamyltransferase, obesity, and the risk of type 2 diabetes: observational cohort study among 20,158 middle-aged men and women, J. Clin. Endocrinol. Metab. 89 (2004) 5410–5414.

[5] J.S. Lim, D.H. Lee, J.Y. Park, S.H. Jin, D.R. Jacobs Jr., A strong interaction between serum gamma-glutamyltransferase and obesity on the risk of prevalent type 2 diabetes: results from the Third National Health and Nutrition Examination Survey, Clin. Chem. 53 (2007) 1092–1098.

[6] D.S. Lee, J.C. Evans, S.J. Robins, P.W. Wilson, I. Albano, C.S. Fox, T.J. Wang, E.J. Benjamin, R.B. D'Agostino, R.S. Vasan, Gamma glutamyl transferase and metabolic syndrome, cardiovascular disease, and mortality risk: the Framingham Heart Study, Arterioscler. Thromb. Vasc. Biol. 27 (2007) 127–133.

[7] E. Ruttmann, L.J. Brant, H. Concin, G. Diem, K. Rapp, H. Ulmer, γ-Glutamyltransferase as a risk factor for cardiovascular disease mortality: an investigation in a cohort of 163,944 Austrian adults, Circulation 112 (2005) 2130–2137.

[8] D.H. Lee, K. Silventoinen, G. Hu, D.R. Jacobs Jr., P. Jousilahti, J. Sundvall, J. Tuomilehto, Serum gamma-glutamyltransferase predicts non-fatal myocardial infarction and fatal coronary heart disease among 28,838 middle-aged men and women, Eur. Heart J. 27 (2006) 2170–2176.

[9] C. Meisinger, A. Doring, A. Schneider, H. LowelKORA Study Group, Serum gamma-glutamyltransferase is a predictor of incident coronary events in apparently healthy men from the general population, Atherosclerosis 189 (2006) 297–302.

[10] M. Emdin, C. Passino, C. Michelassi, F. Titta, A. L'Abbate, L. Donato, A. Pompella, A. Paolicchi, Prognostic value of serum gamma-glutamyl transferase activity after myocardial infarction, Eur. Heart J. 22 (2001) 1802–1807.

[11] S. Ryu, Y. Chang, D.I. Kim, W.S. Kim, B.S. Suh, Gamma-glutamyltransferase as a predictor of chronic kidney disease in nonhypertensive and nondiabetic Korean men, Clin. Chem. 53 (2007) 71–77.

[12] L. Kazemi-Shirazi, G. Endler, S. Winkler, T. Schickbauer, O. Wagner, C. Marsik, Gamma-glutamyltransferase and long-term survival: is it just the liver? Clin. Chem. 53 (2007) 940–946.

[13] K.M. Conigrave, J.B. Saunders, R.B. Reznik, J.B. Whitfield, Prediction of alcohol-related harm by laboratory test results, Clin. Chem. 39 (1993) 2266–2270.

[14] G. Wannamethee, S. Ebrahim, A.G. Shaper, Gamma-glutamyltransferase: determinants and association with mortality from ischaemic heart disease and all causes, Am. J. Epidemiol. 142 (1995) 699–708.

[15] H. Brenner, D. Rothenbacher, V. Arndt, S. Schuberth, E. Fraisse, T.M. Fliedner, Distribution, determinants, and prognostic value of gamma-glutamyltranspeptidase for all-cause mortality in a cohort of construction workers from south Germany, Prev. Med. 26 (1997) 305–310.

[16] N.E. Huseby, Multiple forms of serum gamma-glutamyltransferase: association of the enzyme with lipoproteins, Clin Chim Acta 124 (1982) 103–112.

[17] P.R. Wenham, D.B. Horn, A.F. Smith, Physical properties of γ-glutamyltransferase in human serum, Clin. Chim. Acta 141 (1984) 205–218.

[18] E. Nemesanszky, J.A. Lott, Gamma-glutamyltransferase and its isoenzymes: progress and problems, Clin. Chem. 31 (1985) 797–803.

[19] L. Sacchetti, G. Castaldo, F. Salvatore, The gamma-glutamyltransferase isoenzyme pattern in serum as a signal discriminating between hepatobiliary diseases, including neoplasias, Clin. Chem. 34 (1988) 352–355.

[20] M. Bellini, E. Tumino, R. Giordani, G. Fabrini, F. Costa, R. Galli, M. Rucco, C. Belcari, C. Michelassi, L. Murri, G. Maltinti, S. Marchi, Serum γ-glutamyl-transpeptidase isoforms in alcoholic liver disease, Alcohol Alcoholism 32 (1997) 259–266.

[21] M. Pompili, G. Addolorato, G. Pignataro, C. Rossi, C. Zuppi, M. Covino, A. Grieco, G. Gasbarrini, G.L. Rapaccini, Evaluation of the albumin-γ-glutamyltransferase isoenzyme as a diagnostic marker of hepatocellular carcinoma-complicating liver cirrhosis, J. Gastroenterol. Hepatol. 18 (2003) 288–295.

[22] G.D. Smith, J.L. Ding, T.J. Peters, A sensitive fluorimetric assay for gamma-glutamyl transferase, Anal. Biochem. 100 (1979) 136–139.

[23] E. Prusak, M. Siewinski, A. Szewczuk, A new fluorimetric method for the determination of gamma-glutamyltransferase activity in blood serum, Clin. Chim. Acta 107 (1980) 21–26.

[24] D.L. Blackmon, A.J. Watson, M.H. Montrose, Assay of apical membrane enzymes based on fluorogenic substrates, Anal Biochem 200 (1992) 352–358.

[25] F. Schiele, Y. Artur, D. Bagrel, C. Petitclerc, G. Siest, Measurement of plasma gamma-glutamyltransferase in clinical chemistry: kinetic basis and standardisation propositions, Clin. Chim. Acta 112 (1981) 187–195.

[26] N.E. Huseby, J.H. Stromme, Practical points regarding routine determination of γ-glutamyltransferase (γ-GT) in serum with a kinetic method at 37 °C, Scand J. Clin. Lab. Invest. 34 (1974) 357–363.