



## A NOVEL MARKER OF PROGRESSION FOR ATHEROSCLEROSIS AND CARDIOVASCULAR DISEASES: SERUM GAMMA-GLUTAMYLTRANSFERASE ENZYME ACTIVITY

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### Abstract

Oxidative events – the oxidation of low-density lipoproteins (LDL) in the first place – have been shown to participate in the progression of atherosclerosis and precipitation of cardiovascular events. This occurs through modulation of important components of lesions of the vessel wall (smooth muscle cell proliferation, protease/anti-protease balance, endothelial functions). Our recent studies have provided evidence that the enzyme gamma-glutamyltransferase (GGT), normally found in serum, is often accumulated within the plaque environment in substantial amounts, and that this activity is a potential source of a variety of prooxidant species. Concurrently, epidemiological research has conclusively documented that the serum levels of GGT are an independent factor in prognosis of myocardial infarction and stroke in atherosclerotic patients. Several signs suggest that the GGT appearing in plaque tissue may originate from the serum enzyme, which in fact associates with the circulating lipoprotein fractions. Thus, data seem to point out that pathogenesis of atherosclerosis and in particular of the events leading to progression of the disease and acute cardiovascular events – might include an as yet unexplored pathway, based on the prooxidant effects of gamma-glutamyltransferase accumulating as a result of LDL entry in the vessel wall.

**Keywords:** serum gamma-glutamyltransferase; serum beta-lipoproteins; atherosclerosis; atherosclerotic plaque; oxidative stress; glutathione; protein oxidation.

### Introduction

Understanding factors participating in the progression of atherosclerotic lesions would allow the identification of high-risk lesions, in which the pathological changes produced by the disease process are more likely to result in plaque rupture, thrombosis and the associated ischemic/necrotic sequelae. Establishing timely treatment for such lesions would clearly represent a major advance in secondary prevention and therapy of cardiovascular patients.

Oxidative reactions occupy an important position among factors of plaque progression and “destabilization” [Stocker R., Keaney J.F. Jr., 2004].

One specific oxidative event – the oxidation of low density lipoprotein (LDL) – had been highlighted by earlier studies as the single crucial event in pathogenesis of atherosclerosis, with special reference to the initiation of lesions. Subsequent research has shown however that the implication of oxidative processes may be considerably wider, extending to include effects on several other important pathological changes occurring in the diseased vessel wall. Thus, it has been shown that oxidants and redox reactions are involved in stimulation of smooth muscle cell proliferation, contributing to lesion thickness and expansion. The activities of matrix metalloproteinases and of their inhibitors – major determinants of plaque stability – are modulated through redox changes in critical portions of

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their protein structure. Some aspects of endothelial dysfunction [e.g. impairment of nitric oxide (NO) production, expression patterns, and levels of adhesion molecules] are also affected by oxidative events, and several other redox-sensitive steps are present in signal transduction, apoptotic cell death and platelet functions. Current experimental studies consistently indicate that oxidation/reduction processes may be frequently involved in the production of changes capable of turning a mildly stenotic, silent plaque into a vulnerable, symptomatic, and potentially fatal lesion. A series of enzyme activities expressed by cellular elements present in the lesions (endothelium, phagocytes, smooth muscle cells, fibroblasts) have been proposed as sources of the oxidants involved; these include NADPH-oxidases, NO-synthase, xantine oxidase, myeloperoxidase, lipoxygenases and enzymes of mitochondrial respiration. Non-enzymic prooxidants also participate in the reactions, such as transition metals (iron, copper) and lipid peroxides. However, the relative roles and importance of each of the mentioned factors in the “oxidative progression” of lesions have not been elucidated yet, and are likely to depend on which aspect of the process is being considered; the whole matter is in fact the subject for extensive experimental research.

**Involvement of gamma-glutamyltransferase (GGT) activity in atherosclerosis.** Serum GGT levels are best known as a reliable index of hepatobiliary dysfunction and alcohol abuse [Whitfield J.B., 2001], but recent studies have repeatedly proposed an independent role of GGT in clinical evolution of cardiovascular diseases related with atherosclerosis [Pompella A. et al., 2004]. Several studies had documented that serum GGT is associated with overall mortality and cardiovascular events in both unselected populations [Conigrave K.M. et al., 1993; Wannamethee G. et al., 1995; Brenner H. et al., 1997; Jousilahti P. et al., 2000] and patients with ascertained coronary artery disease, independent of all confounders including liver function and alcohol consumption [Karlson B.W. et al., 2000]. A recent prospective study carried out in as many as

163,944 adult subjects [Ruttman E. et al., 2005] has now rather conclusively shown that serum GGT levels are an independent prognostic factor for fatal events of chronic forms of coronary artery disease, congestive heart failure and ischemic or hemorrhagic stroke. The finding was true for both sexes, with a clear dose-response relationship and a stronger significance as far as younger subjects were concerned.

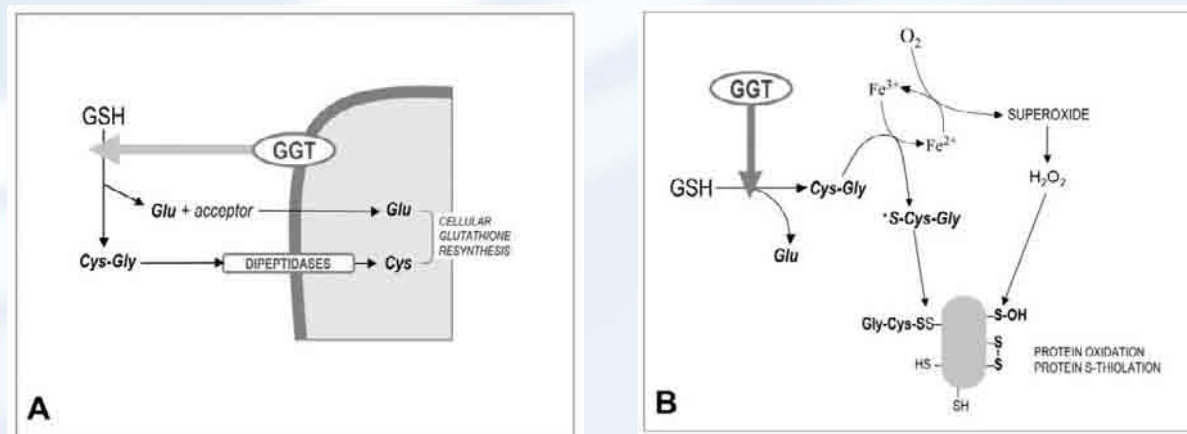
The prognostic value of serum GGT activity for cardiac death and non-fatal infarction had been previously documented in our prospective study, including a six-year follow-up of 469 patients with ischemic syndrome and angiographically documented coronary artery disease [Emdin M. et al., 2001]. In this study, the significance of serum GGT was more evident in a subset of patients prone to plaque complications, i.e. characterized by the association of diffuse atherosclerosis (multivessel disease) and a history of previous myocardial infarction (approx. 36% of the whole population). The risk was increasing using two different GGT cut-off values (25 or 40 U/l, both generally considered within the normal range), the event excess being concentrated within the first three-year period. The prognostic significance of serum GGT was thus correlated with the diffusion of coronary artery disease, and interestingly, the significance of serum GGT appeared to depend on the instability of plaques, as indicated by the fact that the prognostic value of GGT disappeared after revascularization by angioplasty, a procedure thought to effect plaque stabilization. The unfavourable prognosis signaled by elevated serum GGT appeared thus to apply specifically to patients with vulnerable plaques, suggesting that connections of some kind may exist between GGT and the processes involved in plaque stability [Emdin M. et al., 2001].

Importantly, all of the studies mentioned above refer to “normal” serum GGT, i.e. values within the laboratory reference range, such as in the large prospective study by Wannamethee et al. (7613 middle-aged men, with a 11-years follow-up), showing that GGT levels in the normal range

(top quintile of distribution) were strongly associated with a significant increase in deaths from ischemic heart disease [Wannamethee G. et al., 1995]. Serum GGT was positively correlated with preexisting ischemic heart disease (IHD), diabetes mellitus, antihypertensive medication, systolic and diastolic blood pressure, total and high-density lipoprotein (HDL) cholesterol, heart rate and blood glucose. After adjustment for these variables, elevated GGT (highest quintile,  $\geq 24$  U/l, vs. the rest) was still associated with a significant increase in mortality from all causes and from IHD. The increased risk of IHD mortality was more marked for patients with evidence of IHD at screening, particularly those with previous myocardial infarction [Wannamethee G. et al., 1995]. The increased mortality in men without heart disease was obviously dependent on other causes; on the other hand, the findings in ischemic patients again pointed to a connection of serum GGT with unknown aspects of the underlying atherosclerotic coronary artery disease.

An indirect confirmation for the implication of circulating GGT in the evolution of atherosclerosis derives from evidence obtained by histochemistry. Histochemical studies performed in our laboratories showed that intense GGT activity is often present in the intimal layers of human atherosclerotic lesions, where it is apparently expressed in CD68+ macrophage-derived foam cells [Paolicchi A. et al., 1999; Paolicchi A. et al., 2004]. GGT-positive foam cells were found to co-localize with immunoreactive oxidized LDL [Emdin M. et al., 2002], and catalytically active GGT could be also detected in microthrombi adhering to the surface of atheromas [Dominici S. et al., 2003]. Whether GGT activity found in plaque tissue is actually participating in the disease – or is rather an epiphenomenon lacking specific significance – remains to be ascertained. At least two questions arise: i) how could GGT activity affect the progression of the disease, and ii) how are serum levels of GGT related with the enzyme detectable in plaques?

**Functions of gamma-glutamyltransferase – unexplored aspects.** GGT enzyme activity has complex biochemical functions, and its overall physiological significance has not been entirely appreciated yet. Several aspects of GGT activity are indeed capable to promote effects of oxidative nature, which in principle might contribute to progression of the atherosclerotic process. Most of the knowledge about GGT functions is concerned with the roles played by this activity at the cellular level. High GGT levels are expressed in kidney tubules, biliary epithelium, and brain capillaries; however, lower or trace levels are detectable in many more cell types, e.g. blood cells, endothelium, and notably secretory and absorptive cells [Hanigan M.H., Frierson H.F. Jr., 1996]. Being located at the plasma membrane level, GGT catalyzes the first step in the degradation of extracellular glutathione (GSH), i.e. the hydrolysis of the gamma-glutamyl bond between glutamate and cysteine. In this process, GGT releases the dipeptide cysteinyl-glycine, which is subsequently cleaved to cysteine and glycine by plasma membrane dipeptidase activities. Thus, GGT activity provides cells first of all with a means for the recovery of precursors needed to reconstitute the intracellular levels of glutathione, the main cellular antioxidant (Figure 1A). Novel insights however have been prompted by studies showing the ability of GGT to play a prooxidant role under selected conditions. The key issue in this alternative function of GGT is related to the reducing properties of GSH and the thiol metabolites originating from GGT activity. Thiols – especially when dissociated to their thiolate anion forms (R-S<sup>-</sup>) – can effect the reduction of metal cations, e.g. iron and copper. Electrons can then be transferred in turn from metal ions to molecular oxygen, thus generating the reactive oxygen species superoxide anion and hydrogen peroxide, both capable of stimulating prooxidant reactions. The sequence – a true “redox cycling” of metals [Kappus H., Sies H., 1981] – can proceed with even minimal concentrations of metal ions as long as the electron donors (thiols) and electron acceptor (molecular



**Figure 1.** Functions of gamma-glutamyltransferase (GGT) activity: (GSH), glutathione; (Cys-Gly), cysteinyl-glycine.

(A) cellular supply of precursors for the resynthesis of intracellular stores of the antioxidant glutathione.

(B) promotion of metal-catalyzed redox reactions, leading to the appearance of reactive oxygen species, free radicals and oxidative modifications on proteins.

oxygen) are available. In this way, and paradoxically, the ‘reducing’ abilities of thiols are eventually turned into overall ‘oxidizing’ effects. Stark et al. [Stark A.-A. et al., 1993] were the first to propose that the catabolism of GSH can play a prooxidant role under selected conditions. These authors suggested that the GGT-mediated cleavage of GSH – allegedly through the generation of the more reactive thiol cysteinyl-glycine – could cause the reduction of ferric iron Fe(III) to ferrous iron Fe(II), thus starting an iron-dependent redox-cycling process liable to result in the production of reactive oxygen species (ROS) and the stimulation of oxidative reactions (Figure 1B). Subsequent studies from our and other laboratories have repeatedly documented the production of ROS during the catabolism of GSH mediated by cellular GGT. The biochemical processes involved have been characterized (reviewed in [Dominici S. et al., 2003]), and some of the redox effects occurring at the cellular level have been described, e.g. on the activation status of transcription factors and apoptotic signalling pathways [Del Bello B. et al., 1999; Paolicchi A. et al., 2002; Dominici S. et al., 2003].

The observation of such prooxidant reactions has refined our understanding of GGT functions, the significance of which appears to be dual. At a cellular level, it is conceivable that the prooxi-

dant effects of plasma membrane GGT are normally balanced by its established role in favouring the cellular uptake of precursors for GSH resynthesis, thus allowing the maintenance of cellular antioxidant defenses. On the other hand, the functions of soluble GGT activity in serum are less directly related to the cellular supply of GSH, and conditions likely exist in which its prooxidant effects may be prevailing. The free iron required for such processes can originate from iron storage proteins; it was in fact shown that GGT prooxidant effects are sustained by both transferrin and ferritin [Dominici S. et al., 2003; Corti A. et al., 2005]. The emerging prooxidant potential of serum GGT can help explain the recent observation that baseline serum GGT can predict future elevations of serum F2-isoprostanes, a sensitive marker of oxidative stress [Lee D.H. et al., 2003; Lee D.H. et al., 2004]. As far as the plaque environment, free iron is present [Pang J.H. et al., 1996] at levels sufficient to sustain the GGT-mediated prooxidant effects [Paolicchi A. et al., 1999].

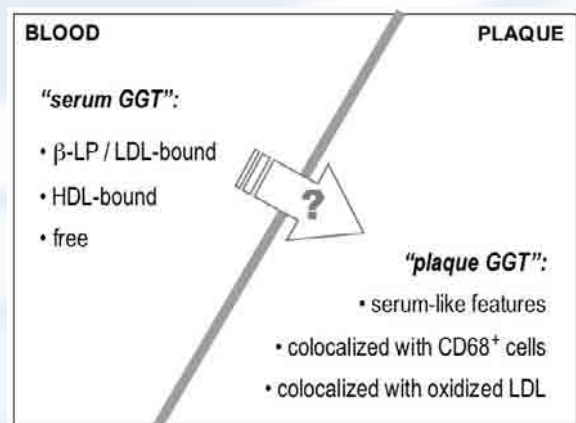
**Serum GGT vs. enzyme activity present in atherosclerotic plaques.** The liver being the apparent main source for circulating GGT activity [Stocker R., Keaney J.F. Jr., 2004], constitutive high serum levels have been reported in families as an inherited autosomic dominant character, and

in the apparent absence of any disease (see e.g. [Bibas M. et al., 1994]). Genetic effects could in principle involve either the release of GGT from liver cells or its clearance from circulation. Other liver enzymes partly share the same effects, and this seems to indicate that genetic factors may be operative at the level of GGT release in blood [Whitfield J.B. et al., 2002]. The importance of genetic factors is still a matter for discussion. The current view is that a mixture of genetic and environmental factors can influence serum GGT [Bathum L. et al., 2001; Whitfield J.B. et al., 2002]. Another line of evidence points to the possibility that the source of serum GGT may be identified in circulating platelets, as the two parameters are usually correlated [Bolodeoku J.A. et al., 1997]. Thus, it has been proposed that the increased GGT levels observed in atherosclerotic patients may originate from increased platelet consumption at the site of plaques [Krijgsman B. et al., 2003].

It has long been appreciated that serum GGT is partially adsorbed onto circulating LDL [Huseby N.E., 1982; Watanabe M. et al., 1984]. Details of such association were recently investigated by us in a population of patients with angiographically documented coronary artery disease (CAD) [Paolicchi A. et al., 2006]. As GGT activity in non-cholestatic patients is mainly associated with HDL and LDL [Watanabe M. et al., 1984], the amount of GGT activity associated with polycation-precipitated beta-lipoproteins (beta-LP) – which include LDL, intermediate density lipoprotein (IDL) and very low density lipoprotein (VLDL), but not HDL – was assumed as an index of LDL-associated GGT. It was thus observed that both in controls and in CAD patients the amount of GGT associated with beta-LP is directly proportional to total serum GGT levels; in CAD patients however, the beta-LP-associated fraction was significantly lower. Furthermore, serum GGT is known to increase with age, and such increase was indeed found in the control group. In contrast, significantly lower total serum GGT levels were detected in older CAD patients, though the ratio between beta-LP-bound and total GGT remained constant

[Paolicchi A. et al., 2006]. It remains to be elucidated whether this phenomenon depends on the absence in CAD patients of the factor(s) eliciting the increase of serum GGT with age, or rather to a selective loss of patients with higher GGT [Emdin M. et al., 2001], due to (GGT-related?) complications of CAD, i.e. cardiac death and stroke. The reasons for the association of GGT with LDL likely lie in the peculiar structure of GGT, a dimeric protein the heavy chain of which contains a lipophilic domain responsible for the plasma membrane insertion of the cellular enzyme. Several proteases are capable of digesting the lipophilic domain, thus producing a hydrophilic form of the protein [Meister A., 1983]. The specific association of GGT with LDL and the nature of the protein were further confirmed by ultracentrifugation. We found that the LDL-associated enzyme consists in an entire heavy chain, indicating that the amphiphilic form of the enzyme is involved [Paolicchi A. et al., 2006]. It is known however that the incubation of amphiphilic GGT with isolated lipoprotein does not produce GGT-lipoprotein complexes [Artur Y. et al., 1984], suggesting that the interaction of GGT with lipoprotein may be different from the one with plasma membrane lipids. Indeed, affinity-adsorbed LDL released most of its GGT activity following extensive washing, indicating a rather labile LDL/GGT association [Paolicchi A. et al., 2006].

The beta-LP-associated fraction of GGT is proportional to total serum GGT; thus, binding of GGT with LDL might itself be related to the reported increase of cardiovascular risk. What mechanisms could then account for the observed decrease of beta-LP-bound GGT in CAD patients and the decrease of total GGT levels in older ones? Conceivably, the reasons might be (i) a less efficient association of GGT with LDL in these patients, (ii) an enhanced release of GGT from LDL, or (iii) an enhanced uptake of LDL-GGT complexes within atherosclerotic lesions, possibly of those presenting with higher GGT contents. The latter hypothesis is particularly attractive, as it would allow the integration of an overall picture with the histochemical findings



**Figure 2.** Possible relationships between GGT activity circulating in serum in association with distinct lipoprotein fractions, and GGT activity detectable in atherosclerotic lesions. Available data suggest that the latter might result from the uptake of GGT/LDL complexes within the arterial wall.

(Figure 2): the enhanced uptake of LDL–GGT complexes, leading to the appearance of significant deposits of active GGT within the lesions, might be a discrete step in atherogenesis, potentially explaining the reported correlation between higher levels of serum GGT and the clinical consequences of atherosclerosis.

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## Conclusions

Current studies in our laboratory are aimed to a further biochemical characterization of GGT present in human atherosclerotic plaques. As a confirmation of the hypothesis discussed above, preliminary data indicate that the enzyme protein accumulating in lesions has molecular weight and chromatographic properties compatible with those of serum GGT. In addition, proteins recovered from plaque tissue present with some degree of S-cysteyl-glycylation, i.e. the form of protein S-thiolation specifically catalysed by GGT [Corti A. et al., 2005], thus witnessing the occurrence of prooxidant reactions promoted by the accumulated enzyme [Franzini M. et al., 2008]. It seems therefore reasonable to speculate that the prooxidant reactions promoted by GGT activity might contribute to the (oxidative) processes involved in several aspects of plaque destabilization. Further studies are necessary however in order to identify the factors that favour the uptake of GGT within plaques, and possibly accelerate it in selected subjects.

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