γ-glutamyltransferase and pathogenesis of cardiovascular diseases

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Cardiovascular epidemiology has recently highlighted a clear link between serum γ-glutamyltransferase (GGT) and risk for stroke, infarction and cardiovascular death, associated with the evolution of atherosclerosis-related conditions, such as coronary artery and cerebrovascular disease. Thus, serum GGT is now recognized as a cardiovascular prognostic marker. However, the reasons for the association between serum GGT elevations and unfavorable prognosis remain to be defined. Histochemistry and biochemistry have provided intriguing clues, focusing new research approaches on the complex issue of monitoring and treatment of chronic cardiovascular conditions.

A major challenge in cardiovascular medicine is the identification of patients prone to acute, life-threatening events. Much attention has been given to factors participating in the atherosclerotic degeneration of the vessel wall, particularly to those responsible for 'instability' of atherosclerotic lesions, facilitating rupture and thrombosis. Over the last few decades, epidemiological studies have identified a large number of parameters that have the potential to become cardiovascular prognostic markers. Further work by clinical pathologists is required to determine their mechanism of action and elucidate their biological potential as new markers, thus enabling the development of preventative and/or therapeutic strategies.

γ-glutamyltransferase (GGT), whose activity level in serum has recently become a novel cardiovascular prognostic marker, is an example of this.

Serum γ-glutamyltransferase: epidemiological evidence of an independent relationship with cardiovascular diseases

Serum GGT has long been interpreted as an index of hepato–biliary dysfunction and alcohol abuse [1], and its association with morbidity has been reported [2]. Subsequent studies suggested that GGT can have a predictive value irrespective of hepatic disease or alcohol consumption [3,4]. With regards to cardiovascular diseases, it was observed that serum GGT levels correlated with an increased risk of myocardial infarction [5,6] and of stroke [7,8]. These associations were partially explained by the known correlations of GGT with recognized factors in cardiovascular pathogenesis, such as changes in blood lipids, body mass index, hypertension, glucose intolerance, insulin resistance and Type 2 diabetes [9]. GGT levels also correlated positively with novel risk factors such as C-reactive protein, fibrinogen and F2-isoprostanes [10]. It was suggested that a genetic predisposition may exist to abdominal obesity, insulin resistance and other signs of the so-called metabolic syndrome, and that it may also include elevation of GGT and other liver enzymes [11]. Such an association has been unambiguously documented [12]. However, the Framingham Heart Study has recently established that an increase in serum GGT *per se* predicts onset of the metabolic syndrome [13].

Most of these studies refer to 'normal' serum GGT, values within the laboratory reference range, which would otherwise raise no specific health concern. Wannamethee and colleagues first focused their attention on ischemic heart disease (IHD) [14]. In a large prospective study (7613 middle-aged men with an 11-year follow-up), GGT levels within the normal range were strongly associated with all-cause mortality, and the association was largely due to a significant increase in deaths from IHD in the top quintile of GGT distribution. After adjustment for numerous variables (history of myocardial ischemia, diabetes, hypertension, total and high-density lipoprotein [HDL] cholesterol, heart rate and blood glucose), higher GGT levels (≥24 U/l) were still associated with a significant increase in mortality from all causes and from IHD. The increased risk of cardiac mortality was more marked for patients with evidence of IHD at screening, particularly those with previous myocardial infarction. The increased mortality in men without heart disease was obviously dependent on other causes; however, the findings in ischemic patients

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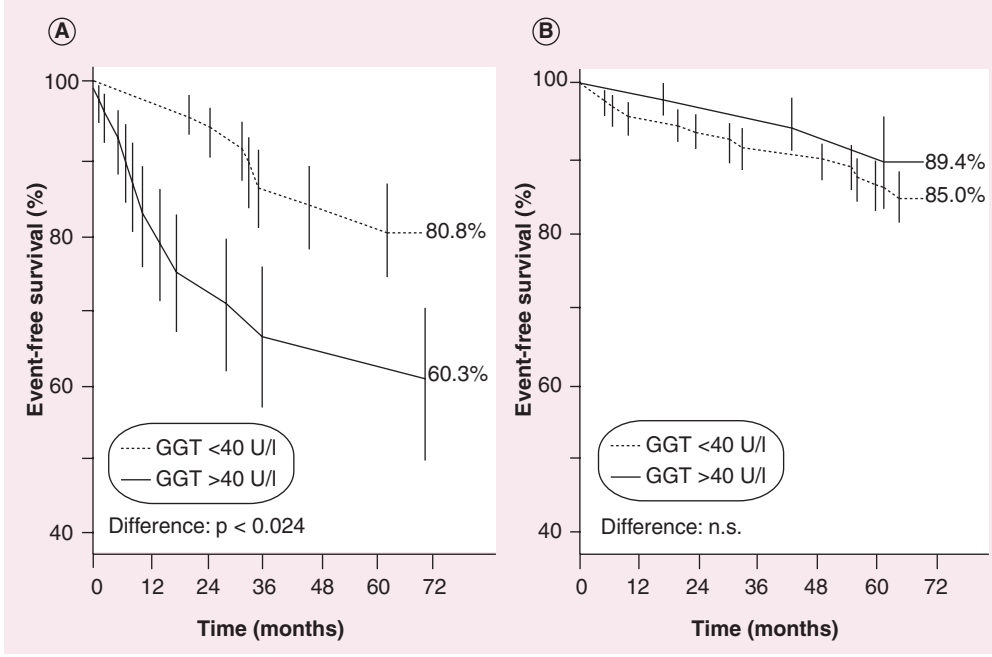
clearly pointed to a relation between GGT and underlying atherosclerotic coronary artery disease (CAD).

The link between GGT and atherosclerotic disease was assessed in a prospective study of 469 patients with ischemic syndrome and angiographically documented CAD performed by our group. After correction for other cardiovascular disease risk factors (e.g., age, smoking, serum cholesterol, left ventricular ejection fraction, body mass index and diabetes mellitus) or confounding factors (e.g., serum alanine aminotransferase and self-reported alcohol consumption), the prognostic value of serum GGT activity for cardiac death and nonfatal infarction was confirmed. In particular, the predictive power of serum GGT was higher in a subset of patients more prone to plaque complications, characterized by the association of diffuse atherosclerosis (multivessel disease) and a history of previous myocardial infarction (~36% of the whole population). The risk progressively increased at two different GGT cut-off values (25 or 40 U/l, both considered within the normal range), and the event excess was concentrated within the first 3-year period of the 6-year follow-up (**Figure 1**). The prognostic significance of serum GGT was found to disappear in patients undergoing revascularization procedures (**Figure 1B**) [15], for example angioplasty, which is regarded as a procedure capable of stabilizing plaque tissue [16]. These data suggest that the unfavorable prognosis associated with elevated serum GGT might specifically apply to patients with unstable plaques, and/or that GGT likely interacts with processes involved in plaque instabilization.

Alcohol consumption is a common confounding factor in studies involving serum GGT. The possible link between alcohol consumption, serum GGT and cardiovascular risk was analyzed by Jousilahti and colleagues [17]. In a random sample of 3666 men aged 25–74 years, the prevalence of IHD was correlated with serum GGT

Figure 1. Survival according to serum γ-glutamyltransferase activity in coronary artery disease patients.



(A) Event-free 6-year survival in a population of 168 patients with coronary artery disease, history of previous myocardial infarction and multiple vessel disease (119 with serum GGT <40 U/l and 49 with serum GGT >40 U/l). **(B)** Event-free 6-year survival in 262 patients with previous myocardial infarction, evaluated without considering revascularization (e.g., angioplasty and bypass grafting) as a cause of withdrawal from follow-up. Vertical lines represent confidence intervals.
GGT: γ-glutamyltransferase; n.s.: Nonsignificant; U/l: One unit is defined as the amount of enzyme activity that will catalyze the hydrolysis of 1 μmol of substrate/min.
Reproduced with permission from [9].

level, carbohydrate-deficient transferrin (CDT; a recognized marker of alcohol consumption) and self-reported alcohol consumption. CDT levels were inversely – and GGT levels positively – correlated with IHD risk. Furthermore, in a composite risk assessment, subjects with normal CDT (≤ 20 U/l) and elevated GGT levels (>80 U/l) had an approximate eightfold adjusted IHD risk compared with subjects with normal GGT and elevated CDT levels. Since CDT is actually a more reliable marker of alcohol consumption than GGT itself [18], these data appear to indicate that alcohol consumption may hold a protective effect on cardiovascular prognosis, as previously reported [19], and that the prognostic association of serum GGT must be independent of alcohol-related liver injury.

To date, a series of studies have confirmed in multivariable analyses the independent value of serum GGT in predicting all-cause mortality and, in particular, clinical evolution of cardiac and cerebrovascular diseases towards life-threatening events, such as myocardial infarction, stroke and cardiac death, irrespective of hepatic disease, alcohol consumption and other traditional risk factors. A recent large Austrian study, including data collected over 17 years (1985–2001) from 163,944 volunteers of the Vorarlberg Health Monitoring and Promotion Program, confirmed the prognostic value of serum GGT in identifying fatal events in chronic coronary heart disease, congestive heart failure and ischemic or hemorrhagic stroke [20]. The receiver operating characteristic analysis identified a GGT cut-off value of 15.5 U/l for men and 10.5 U/l for women, corresponding to 27.6 U/l and 18.7 U/l, respectively, for measurements made at 37°C, with a clear dose–response relationship. A stronger (1.5–2-fold higher) prognostic significance was observed in younger (<60 years of age) participants.

The relevance of GGT in subjects less than 60 years of age has been confirmed in a recent unselected cohort of 28,838 men and women [21]. This observation might indicate that a prolonged serum GGT elevation may be relevant in order to promote unfavorable effects, suggesting a direct involvement of GGT in the pathogenetic process. The study also reported a stronger association in subjects with Type 2 diabetes, indicating that the role of GGT may be additive to those specifically promoting the atherosclerotic process in diabetes (e.g., lipoprotein glycation). The same authors have observed that chronic exposure to persistent organic pollutants is a determinant of both serum GGT levels and incidence of Type 2 diabetes [22,23], thus envisaging a most intriguing relationship between serum GGT levels, diabetes and environmental pollution.

GGT activity inside atherosclerotic lesions: a reflection of the circulating enzyme?

Several of the epidemiological studies mentioned previously suggested a relationship between circulating GGT and the evolution of atherosclerosis, but how are these phenomena connected? Information was independently provided by studies that demonstrated the presence of GGT inside atherosclerotic lesions either from coronary or carotid arteries. Histochemical studies performed in our laboratories revealed intense GGT activity within the intimal layers of human atherosclerotic lesions, where it apparently locates in $CD68^+$ macrophage-derived foam cells [24,25]. GGT-positive cells were found to co-localize with immunoreactive oxidized low-density lipoprotein (LDL) [26], and catalytically active GGT was also detected in microthrombi adhering to the surface of atheromas [27]. Whether GGT activity found in plaque tissue is actually participating in the disease, or is rather an epiphenomenon, remains to be ascertained. However, two questions arise: is the serum level of GGT related to the enzyme detectable in plaques, and how could accumulated GGT affect the progression of lesions?

GGT enzyme circulating in serum is thought to be mainly released from the liver [1]. Constitutive high serum GGT has been reported in families as an inherited autosomic dominant characteristic; however, the importance of genetic factors is still a matter of debate, and the current view is that a mixture of genetic and environmental factors may influence serum GGT [28]. Another line of evidence suggests that the source of serum GGT may be identified in circulating platelets, as the two parameters are usually correlated. 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 [29]. A recent report, indicating a prognostic value of elevated serum GGT in stent restenosis, seems to support the potential role of platelets as a source of circulating GGT [30]. Significant levels of GGT are also carried by granulocytes [31]; the fact that serum GGT was found to correlate with white blood cell count [32] indicates that the latter could represent an additional source of circulating enzyme.

Interestingly, serum GGT is partially adsorbed onto circulating LDL [33,34]. Details of such an association were recently investigated in a population of patients with angiographically documented CAD [35]. As GGT activity in non-cholestatic patients is mainly associated with HDL and LDL [34], the amount of GGT activity associated with polycation-precipitated β-lipoproteins (β-LP) – which include LDL, intermediate-density lipoprotein and very low-density lipoprotein, but not HDL – was assumed as an index of LDL-associated GGT. It was observed that in both controls and CAD patients the amount of GGT associated with β-LP is directly proportional to total serum GGT levels; however, in CAD patients, the β-LP-associated fraction was significantly lower. Furthermore, serum GGT is known to increase with age, and such an increase was found in the control group; by contrast, significantly lower total serum GGT levels were detected in older CAD patients, although the ratio between β-LP-bound and total GGT remained constant [35]. It remains to be elucidated whether this phenomenon is caused by the absence in CAD patients of the factor(s) eliciting the increase of serum GGT with age, or by a selective loss of patients with higher GGT [15], owing to possibly GGT-related life-threatening complications of CAD.

The reasons for the association between GGT and LDL likely lie in the peculiar structure of GGT, a dimeric protein whose heavy chain contains the 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. 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 of an entire heavy chain, indicating that the amphiphilic form of the enzyme is involved [35]. It is known, however, that the incubation of amphiphilic GGT with isolated lipoprotein does not produce GGT–lipoprotein complexes [36], 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 [35].

As the β-LP-associated fraction of GGT is proportional to total serum activity, the binding of GGT with LDL might itself be related to the reported increased cardiovascular risk. What mechanisms could then account for the observed decrease of β-LP-bound GGT in CAD patients, and the decrease of total GGT levels in older patients? Conceivably, the reasons might be a less efficient association between GGT and LDL in these patients, enhanced release of GGT from LDL or 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 prompts a plausible interpretation of data: the enhanced uptake of LDL–GGT complexes, leading to the appearance of significant deposits of active GGT within the lesions, might explain the reported correlation between higher levels of serum GGT and the clinical consequences of atherosclerosis. The hypothesis implies a role of GGT activity in plaque instabilization: this may be related to the known ability of GGT to affect the oxidation/reduction (redox) equilibrium and the antioxidant status of cells and tissues.

Searching the link with pathogenesis: insights from GGT biochemistry

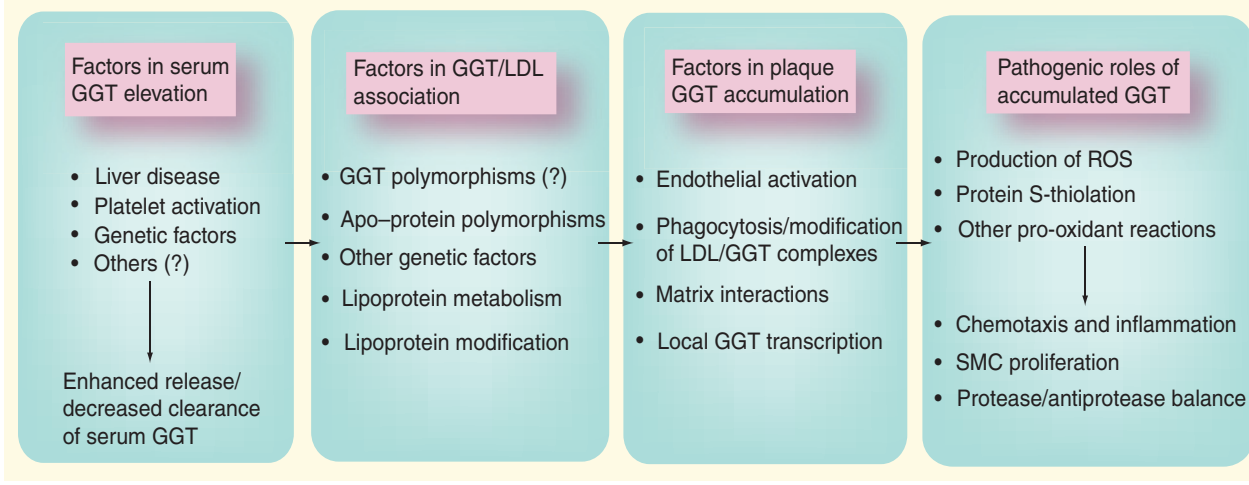
The main function of GGT is the degradation of glutathione (glutamate-cysteine-glycine; GSH), which is a major antioxidant involved in several important defence mechanisms, through hydrolysis of the γ-glutamyl bond between glutamate and cysteine. In this process, GGT releases the dipeptide cysteinyl-glycine, which is subsequently cleaved to cysteine and glycine by plasma membrane dipeptidase activities. Thus, GGT activity primarily provides cells with a means for the recovery of precursors needed to reconstitute intracellular GSH. Novel insights, however, demonstrated the ability of GGT to play a pro-oxidant role under selected conditions. Stark and colleagues were the first to demonstrate that the GGT-mediated cleavage of GSH can effect 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; e.g., superoxide anion) and the stimulation of oxidative reactions [37]. Subsequent studies from our own and other laboratories have repeatedly documented the production of ROS during the catabolism of GSH mediated by GGT. The biochemical processes involved have been characterized [38], and some of the redox effects occurring at the cellular level have been described, for example the effects on the activation status of transcription factors and apoptotic signaling pathways [39].

Oxidative reactions hold an important place among factors of plaque progression and instabilization [40]. One specific oxidative event – the oxidation of LDL – was highlighted by earlier studies as the single crucial event in pathogenesis of atherosclerosis, with special reference to the initiation of lesions. However, subsequent research has shown 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 demonstrated that oxidants and redox reactions are involved in the stimulation of smooth muscle cell proliferation, contributing to lesion thickness and expansion. The activities of matrix metalloproteinases and their inhibitors – major determinants of plaque stability – are modulated through redox changes in critical portions of their protein structure. Some aspects of endothelial dysfunction (e.g., impairment of nitric oxide [NO] production, expression patterns and concentration 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 function. Altogether, current experimental studies consistently indicate that redox processes may be frequently responsible for the changes capable of turning a mildly stenotic, silent plaque into a vulnerable, symptomatic and potentially fatal lesion. The activity of several enzymes, which are expressed by cellular elements present within the lesions (e.g., endothelium, phagocytes, smooth muscle cells and fibroblasts), have been proposed as sources of the oxidants involved; these include nicotinamide adenine dinucleotide phosphate-oxidases, NO-synthase, xanthine oxidase, myeloperoxidase, lipoxygenases and enzymes of mitochondrial respiration. GGT present in plaques – likely as a result of continued entry of LDL/GGT complexes into the vessel wall – can represent a further important pro-oxidant factor. Future research must establish the extent of its contribution.

Future objectives: refinement of marker specificity & therapeutic approaches

A considerable amount of data conclusively indicates that serum GGT is a critical factor in atherosclerotic disease and its complications. Research efforts should now be directed towards elucidating the details of the mechanisms involved in producing the scenarios outlined previously (**Figure 2**). First, reasons for mild elevations of serum GGT – apart from cholestasis and liver disease in general – are in need of clarification, and interesting insights might be provided by the genetic analysis of GGT protein expressed in patients, possibly identifying associations between GGT and specific polymorphisms. Second, it is well established that circulating GGT is a heterogeneous entity consisting of several molecular complexes, giving it distinct physico-chemical properties [33]. Some of these specific forms of serum GGT have been correlated with selected pathologies, for example, hepatocellular cancer [41,42]. Similarly, it is

Figure 2. Next steps and targets of future research concerning γ-glutamyltransferase and cardiovascular diseases.



Apo: Apolipoprotein; GGT: γ-glutamyltransferase; LDL: Low-density lipoprotein; ROS: Reactive oxygen species; SMC: Smooth muscle cell.

conceivable that specific forms of circulating GGT may have a critical role in cardiovascular disease, possibly presenting molecular characteristics that favor the process of their entry and/or accumulation in the vessel wall. The identification of such forms would clearly represent a diagnostic/preventive tool of great interest. In this respect, studies have been carried out in our laboratories aiming to establish molecular differences or similarities between GGT detectable in human atherosclerotic plaques and complexes circulating in serum [Franzini M, Corti A, Martinelli B *et al.*: Origins and potential role of γ-glutamyltransferase activity present in human atherosclerotic plaques (2007) (Submitted)]. As a confirmation of the hypotheses discussed above, biochemical 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 form plaque tissue present with some degree

Executive summary

Serum γ-glutamyltransferase is a prognostic marker for cardiovascular diseases

- A series of epidemiological studies has conclusively documented that a moderate elevation of serum γ-glutamyltransferase (GGT) activity is positively associated with cardiovascular mortality in patients affected by coronary artery disease, congestive heart failure and hemorrhagic or ischemic stroke.
- The prognostic significance is dose-dependent, and a stronger relationship between serum GGT and cardiovascular risk was observed in younger subjects (<60 years of age) and patients with diffuse atherosclerosis.
- Serum GGT actually correlates with several known cardiovascular risk factors, for example body mass index, hypertension, insulin resistance and Type 2 diabetes. However, the predictive value of GGT has been repeatedly proven to be independent from confounding factors.
- Several studies have documented that the prognostic association of serum GGT is independent of alcohol-related liver injury.

GGT activity inside atherosclerotic lesions: a reflection of the circulating enzyme?

- Histochemical studies independently revealed that enzymatically active GGT accumulates in human atherosclerotic lesions and co-localizes with macrophage-derived foam cells and immunoreactive oxidized low-density lipoprotein (LDL).
- GGT circulating in the blood is partially adsorbed onto high-density lipoprotein and LDL fractions.
- In coronary artery disease patients, the LDL-bound fraction of serum GGT is significantly lower. These data, and molecular analysis of GGT recovered from atherosclerotic plaques, strongly suggest that the entry and accumulation of GGT–lipoprotein complexes in the vessel wall may be facilitated in selected subjects.
- The factors possibly involved in such a process remain to be determined.

Searching the link with pathogenesis: insights from GGT biochemistry

- GGT enzyme activity specifically deals with the metabolism of the antioxidant glutathione.
- However, it has been shown that, in selected conditions, GGT activity can produce pro-oxidant reactions, resulting in the production of reactive oxygen species (e.g., superoxide anion and hydrogen peroxide).
- The plaque environment would conceivably allow such reactions to occur, since substrates and cofactors are present at suitable concentrations.
- One of the effects of GGT-dependent reactions is the formation of specific disulfide bonds on proteins (protein S-cysteylglycylation). Such a phenomenon has indeed been detected in atherosclerotic material.
- Pro-oxidant reactions are involved in several aspects of the progression of atherosclerosis, for example inflammation and chemotaxis, protease/antiprotease balance, smooth muscle cell proliferation and apoptosis.
- GGT-dependent pro-oxidant reactions might therefore participate in the progression and instabilization of plaques.

Future objectives: refinement of marker significance & therapeutic approaches

- Factors causing mild elevations of serum GGT – apart from cholestasis and liver disease in general – need to be clarified.
- Important insights might be provided by the genetic analysis of GGT proteins expressed in patients, possibly identifying associations between GGT and specific polymorphisms.
- Detailed analysis of lipoprotein complexes carrying GGT activity in serum is mandatory in order to verify the possibility that the pathogenetic relationship refers to selected molecular species. This would improve the ability of screening to identify patients at risk.
- It is conceivable that interventions aiming to remove these circulating GGT complexes – or to prevent their formation – might eventually lead to therapeutic applications.

Conclusion

- The interplay of epidemiology with basic investigation in histochemistry and biochemistry has provided important clues for understanding the roles possibly played by serum GGT in cardiovascular medicine.
- Current basic research studies are elucidating the details of the pathophysiological mechanisms involved, and will provide the basis for potentially important advances in diagnosis, prognosis and even treatment of chronic cardiovascular conditions.

of S-cysteyl-glycylation, the form of protein S-thiolation specifically catalyzed by GGT [43], thus indicating that the accumulated enzyme may be participating in pro-oxidant processes contributing to plaque instabilization.

Third, attention should be dedicated to factors possibly favoring accumulation of GGT in the intimal space, such as modification by phagocytes and/or interactions with matrix components. A local synthesis of GGT in the plaque environment has been detected [Franzini M *et al.* (2007) (Submitted)], suggesting the possibility that inflammatory cells may also participate in the process of GGT accumulation. Finally, studies to verify if, how and to what extent GGT-dependent redox reactions can affect progression and complication of atherosclerotic lesions are required.

As far as therapeutic perspectives are concerned, it is worth recalling that coronary revascularization procedures were demonstrated to suppress the prognostic value of elevated serum GGT for unfavorable prognosis (**Figure 1**) [15], suggesting that serum GGT elevation was specifically related to the instability of one or more culprit lesions in these patients. Accordingly, further studies have ascertained that low serum GGT identifies a subset of coronary ischemic disease patients with a very low cardiovascular risk [Emdin M, Passino C, Michelassi C *et al.*: γ-glutamyltransferase and mortality risk in coronary artery disease patients (2007) (Submitted)]: this seems to suggest that, besides identification of specific GGT molecular complexes, their removal from blood – or alternatively, strategies capable of preventing their formation – might produce beneficial effects.

Conclusions

The epidemiological observations that have indicated the value of serum GGT as a cardiovascular prognostic marker have been substantially supported by histochemical and biochemical data, suggesting that accumulation of enzyme activity in atherosclerotic lesions may play a pathogenetic role in progression/precipitation of cardiovascular diseases. A novel, exciting phase of research has begun, aimed at defining all factors potentially involved in the process(es) of serum GGT elevation, its association with circulating lipoproteins, and its entry and accumulation into the vessel wall (**Figure 2**). Available data suggest that the reported association between GGT and unfavorable cardiovascular prognosis may at least partly depend on a direct involvement of GGT enzyme activity in the pathogenesis and progression of atherosclerosis. Elucidation of these aspects will likely provide the basis for advances in diagnosis, prognostic stratification and possibly treatment of atherosclerosis-related cardiovascular diseases.

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