Brief Review: Circulating Markers of Liver Function and Cardiovascular Disease Risk

Giovanni Targher[1], Christopher D. Byrne[2,3]

[1] Division of Endocrinology, Diabetes and Metabolism, Department of Medicine, University and Azienda Ospedaliera Universitaria Integrata of Verona, Verona, Italy
[2] Nutrition and Metabolism, Faculty of Medicine, University of Southampton, Southampton, UK
[3] Southampton National Institute for Health Research Biomedical Research Centre, University Hospital Southampton, UK

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Address for correspondence:
Prof. Giovanni Targher, MD
Section of Endocrinology, Diabetes and Metabolism
Department of Medicine
University and Azienda Ospedaliera Universitaria Integrata
Piazzale A. Stefani, 1
37126 Verona, Italy
Fax: +39-045-8027314
E-mail: giovanni.targher@univr.it

Abstract

Measurement of serum concentrations of various liver enzymes and other non-enzymatic proteins and metabolites of heme metabolism (*e.g.*, bilirubin) is often undertaken in clinical practice. Measurement of these 'liver function tests' (LFTs) is simple, quick and relatively inexpensive. However, interpreting the LFT results in patients without evidence of liver disease is often challenging. Concentrations of some of liver enzymes, such as gamma-glutamyltransferase or alkaline phosphatase, and concentrations of liver-derived metabolites, such as bilirubin, may be influenced by metabolic processes beyond the liver, sometimes making interpretation of the test results very difficult. This scenario frequently occurs both in individuals at risk of cardiovascular disease (CVD) and in patients with known CVD, often resulting in the clinicians ignoring the test results. In this brief review, we discuss the evidence for associations between key serum LFTs and CVD risk and where associations are robust, we provide an interpretation for possible mechanistic links between the LFT and CVD.

Introduction

Serum measurements of liver-derived enzymes, non-enzymatic proteins and metabolites of liver metabolism (colloquially known as 'liver function tests' or 'LFTs') are frequently measured in clinical practice. However, more often than not, in patients without liver disease, interpreting the results can be difficult. The panel of LFTs usually reflect the standardised batch of tests measured by the laboratory auto-analyser and 'ticking' the box to request measurement of this panel of tests is all too easy. The standardised batch of LFTs usually comprises alanine aminotransferase (ALT) [and sometimes, aspartate aminotransferase (AST)], alkaline phosphatase (ALP), gamma-glutamyltransferase (GGT), other non-enzymatic proteins (*e.g.*, albumin) and metabolites of heme metabolites such as bilirubin. Subtle abnormalities of LFTs are very common in patients at risk of, and with, cardiovascular disease (CVD).

In this brief review, we discuss the evidence for associations and potential mechanistic links between altered serum concentrations of frequently measured LFTs and the risk of CVD. (For abnormalities of serum albumin concentration, since this most often involves serious renal disease and CVD, and because of the imposed word limit, we have omitted discussion of albumin and CVD).

Gamma-glutamyltransferase (GGT)

What is GGT?

Serum GGT is a glycoprotein consisting of two proteins, the larger chain with a molecular weight of 46,000 Da and the smaller one with a molecular weight of 22,000 Da.[1] The exact protein structure and pattern and regulation of gene expression are not well understood, but expression of GGT occurs in many tissues besides the liver, including placenta, lung and pancreas.[2] GGT is an important enzyme that hydrolyses glutathione into glutamate and a cysteinyl-glycine dipeptide, and inside the cell, the amino acids are subsequently reused, producing additional reduced glutathione, and as such elevated serum GGT concentrations are often considered an indirect measure of increased oxidative stress.[3]

Associations between GGT and CVD risk

Increased serum GGT levels were first shown to be associated with all-cause and CVD mortality in the British Regional Heart Study in 1995. This study evaluated 7,613 older men over 11.5 years in England, Wales and Scotland. GGT levels were strongly associated with all-cause mortality, largely due to a significant increase in deaths from coronary heart disease (CHD) and other non-cardiovascular disease causes, *i.e.,* non-cancer deaths, in the top quintile of the GGT distribution.[4] A recent systematic review and meta-analysis of published prospective cohort studies evaluating the associations of baseline GGT levels with all-cause mortality in 19 cohort studies with aggregate data on over 9.24 million participants and 242,953 all-cause mortality outcomes showed that there was a 60% increase in relative risk in the highest tertile of GGT levels.[5] The pooled relative risk showed a 7% increase in risk per 5 U/L increment in

GGT levels. In middle-aged and older people (≥55 years) from the population-based Rotterdam Study, participants with GGT in the top 5% had a 55% increase in risk for all-cause mortality.[6] Another recent meta-analysis of seven studies with 273,141 participants showed a 56% increase in all-cause mortality for the highest *vs.* lowest GGT quartile. Although a similar overall association was observed for CVD mortality, in sub-group analyses the association was not significant in the Asian subgroups.[7] To date, there are also fewer data in different ethnic groups and there are some conflicting data in Asian populations.[8-10] Thus, there may be ethnic differences affecting the relationships between GGT and organ-specific mortality outcomes.

The above-mentioned meta-analysis also showed a pooled relative risk for the highest *vs.* lowest GGT quartile of 1.52 (95% confidence interval 1.36-1.70) for CVD mortality and, importantly, there was considerable heterogeneity in the thresholds of GGT concentration that defined the highest GGT quartile and these ranged from >22 IU/L to >56 IU/L.[7] Results involving 10 prospective studies showed that a change of 1 IU/L of GGT was associated with a fully adjusted hazard ratio (HR) of 1.20 (1.02-1.40) for CHD and a HR of 1.54 (1.20-2.00) for stroke, respectively.[11] However, once again heterogeneity was noted between studies. The relationship between GGT and CVD risk has been also discussed by Ghouri *et al.*[12] These authors commented that the association between elevated GGT and CVD mortality was stronger in younger individuals.

Although recent evidence suggests that mildly elevated levels of GGT were an independent predictor of CVD mortality, and this association was independent of alcohol intake, there is limited data to date to indicate this effect is independent of non-alcoholic fatty liver disease (NAFLD). A recent Cochrane review has assessed the current evidence for the efficacy of statin therapy in NAFLD.[13] Based on the findings of this review the effect of statins on GGT levels remains uncertain. Recent data suggest that addition of GGT concentration to conventional risk factors does not substantially improve CVD risk prediction.[14,15]

Emerging evidence indicates that four different sub-fractions of GGT (named 'big', 'medium', 'small' and 'free') exist in human blood.[16] Interesting recent work from the same group of investigators shows that levels of the various sub-fractions correlate differentially with individual CVD risk factors.[17] For example, in the Framingham Heart Study cohort,[17] 'big' GGT levels correlated positively with plasma triglycerides, whereas body mass index, blood pressure, glucose and triglyceride levels correlated positively with both 'big' and 'free' GGT concentrations. However, to date the precise pathophysiological role of the different GGT sub-fractions remains uncertain.

Potential mechanisms linking GGT and CVD risk

On the cellular membrane and in the extracellular space, the cysteinylglycine moiety can act as a strong reducing agent of iron, with the stepwise development of the super-oxide ion and hydrogen peroxide.[1] GGT located in arterial atheromatous plaques has been shown to promote the LDL oxidation through a redox reaction and lead to the further development of atherosclerotic plaques.[18] It has been proposed that the hydrolysis of glutathione produces cysteinylglycine, which is a powerful reductant of Fe^{3+} which is present within the atherosclerotic plaque. This leads to the production

of Fe^{2+}, and a free thiyl radical. Thereafter, oxygen reactive species, produced from the same reaction, contribute to a pro-oxidant effect, leading to LDL oxidation and likely contributing to other processes, such as metalloproteinase activation, cell proliferation, and apoptosis.[18] Again, recent experimental data indicate the presence in human carotid plaques of a serum-like GGT protein.[19] These data suggest that a direct contribution of serum GGT to enzyme activity is possible within atherosclerotic lesions. Furthermore, the data confirm the occurrence of GGT-mediated redox reactions within the plaque environment, and the data emphasize that is plausible that these reactions could influence changes in the atherosclerotic plaque.[19] Thus, there are mechanistic data suggesting that GGT may have a direct role in promoting atherosclerotic plaque development. It has been also been hypothesized that GGT can mark exposure to various environmental pollutants that are capable of causing oxidative stress.[20,21]

Bilirubin

What is bilirubin?

Degraded red blood cells release heme, which is broken down by heme oxygenase to biliverdin, which in turn is reduced by biliverdin reductase into the hydrophobic compound bilirubin. Free, or unconjugated bilirubin, is a lipid-soluble molecule that must be made water-soluble to be excreted. Unconjugated bilirubin is carried by albumin to the liver, where it is conjugated into a water-soluble form by hepatic glucuronyl-transferase. The hepatic enzyme UDP-glucuronosyltransferase 1 (UGT1A1) converts bilirubin to a soluble (conjugated) form suitable for renal and biliary elimination.[22] UGT1A1 is also responsible for glucuronidation of many other vasoactive small lipophilic molecules, hormones and drugs that may affect the vasculature.[23]

Associations between bilirubin and CVD risk

Increased levels of total bilirubin (TB) may confer protection against CVD. A meta-analysis of eleven studies published over 10 years ago showed an unambiguous inverse relationship between TB levels and atherosclerosis.[24] More recently, in a meta-analysis of 12 population-based prospective studies, involving a total of 173,360 participants with 9,385 incident CVD cases, the pooled multivariate-adjusted relative risk for CVD was 0.93 (0.90-0.97; $P<0.001$) per 1-standard deviation increase in TB. The corresponding pooled relative risks for CHD and stroke were 0.95 (0.92-0.99; $P=0.018$) and 0.93 (0.88-0.98; $P=0.006$), respectively. Results remained consistent across several clinically relevant subgroups and at different levels of risk factors.[25] TB levels have been shown to be inversely associated with the Framingham Risk score and with the prevalence of metabolic syndrome.[26,27] A small number of studies have also suggested that higher TB is associated with a lower risk of prevalent vascular disease. For example, data from the National Health and Nutrition Examination Survey 1999-2004 suggested that increased TB is associated with decreased peripheral arterial disease prevalence.[28] Increased TB is also associated with decreased prevalence and incidence of stroke,[29,30] and bilirubin might confer some protective function against stroke risk in men.[30] TB has also been shown to be negatively correlated with arterial stiffness in men with established CHD,[31] and it has been

suggested that lower TB is associated with increased risk of coronary artery calcium (CAC).[32,33] In 2012, further evidence for a beneficial role of TB in protecting against CVD events was provided by a large UK primary care based study. After conventional risk factors were accounted for, the regression models predicted that, compared with patients with a TB level of 0.70 mg/dL, those with a similar CVD risk profile, but a TB level of only 0.35 mg/dL had an 18% higher risk of any CVD event, a 34% higher risk of myocardial infarction, and a 33% higher risk of death resulting from any cause.[34]

In healthy European populations, common genetic variation of the *UGT1A1* promoter region explains ≈45% to 50% of the total variability in TB and conjugated bilirubin levels.[35] From the results of Mendelian randomization studies, it is uncertain whether genetic variation in *UGT1A1* typical of Gilbert syndrome is associated with variable risk of CHD. In support of there being a causal link between higher TB levels and reduced CHD risk, a prospective study involving 1,780 individuals from the Framingham Heart Study Offspring cohort found that *UGT1A1* polymorphism resulting in higher bilirubin levels was associated with lower risk of CHD. Homozygote UGT1A1*28 allele carriers with higher bilirubin had a lower risk of CHD.[36] However, in contrast, some other studies have failed to show the same association between *UGT1A1* polymorphism and CHD.[37-39] Whether these smaller prospective studies were lacking power to detect an association is uncertain, but we suggest that larger Mendelian randomization studies are now needed to test whether common genetic variation of the *UGT1A1* promoter region (that explains close to half of variability in conjugated bilirubin levels) predicts CHD risk. The precise relationship between polymorphisms in the *UGT1A1* gene and TB levels is also unclear. In a small study of Korean individuals, the effect of *UGT1A1* polymorphisms on TB has been reported. These data showed that a threshold TB level of >1.3 mg/dL was found in approximately 5% of the Korean population and levels of TB >1.3 mg/dL were caused by two of the 10 haplotypes based on different combinations of three polymorphisms of the *UGT1A1* gene.[40] However, it should be noted that bilirubin metabolism is subtly different in Asians compared with Caucasians, because both ethnic groups tend to have different *UGT1A1* polymorphisms.[41]

CAC scoring with cardiac computed tomography is a sensitive method to demonstrate the presence of subclinical atherosclerosis and to identify individuals at increased risk of CHD.[42] We have reported that the relationship between conjugated bilirubin and CAC score is as strong as any relationship between total/unconjugated bilirubin and CAC score.[43] Consequently, in attempting to better understand the nature of any causal relationship between CVD and serum TB levels, it remains uncertain whether conjugation of bilirubin or absence of conjugation of bilirubin is the more important in conferring any protection against CVD. Recent data suggest that addition of TB concentration to conventional risk factors does not significantly improve CVD risk prediction in the general population.[25]

Potential mechanisms linking bilirubin and CVD risk

Bilirubin is a known potent anti-oxidant,[44] and although both unconjugated and conjugated bilirubin are both effective as anti-oxidants, it has been suggested that unconjugated bilirubin is a stronger anti-oxidant than conjugated bilirubin.[45] Bilirubin modulates signaling pathways regulating inflammation and affects apoptosis, cell proliferation, and immune responses;[46] and for over 50 years, it has been known that

bilirubin powerfully scavenges peroxyl radicals generated under low oxygen tension, which often occurs in pathophysiological states.[47] Although the precise mechanisms by which bilirubin might confer benefit for CVD are still a subject for debate, the most frequently postulated mechanisms of benefit are bilirubin-mediated inhibition of lipid oxidation, bilirubin-mediated inhibition of immune reactions and inflammatory processes, and bilirubin as a marker reflecting enhanced heme oxygenase-1 activity.[48,49]

Aminotransferases and alkaline phosphatase (ALP)

What are aminotransferases and ALP?

Serum levels of ALT, AST and ALP are common liver enzymes that are frequently increased with liver injury. ALT and AST are transaminases that catalyze the transfer of amino groups to generate products in gluconeogenesis and amino acid metabolism. ALP is a glycosylphosphatidylinositol-anchored ectophosphomonoesterase that is mainly expressed in liver, bone and intestine. ALP is capable of hydrolytic phosphatase and transphosphorylase activity on host-derived nucleotides, such as adenosine triphosphate, adenosine diphosphate and uridine diphosphate.[50]

Associations between aminotransferases and CVD risk

The association between serum aminotransferases and the risk of CVD events appears somewhat weaker than that observed for GGT. Data from the Framingham Offspring Heart Study showed that 1-standard deviation higher log ALT at baseline was associated with an increased risk of CVD events in age/sex-adjusted models after 20 years of follow-up (HR 1.23, 1.12-1.34; P<0.0001), but this was attenuated in multivariable adjusted models (adjusted-HR 1.05, 0.96-1.16; P=0.27). AST was not associated with an increased risk of CVD.[51]

Among the US adult participants in the Third National Health and Nutrition Examination Survey, there was no significant association between ALT and the risk of all-cause and CVD mortality over the 12-year follow-up period in multivariate-adjusted analyses.[52] Similar findings were observed using data from the Busselton Health Study in Western Australia.[53] Conversely, mildly elevated ALT was independently associated with increased CVD mortality in a cohort of 37,085 Korean individuals, who were followed for a median period of 5 years.[54] Again, among the 1,439 Hoorn Study participants, the association between ALT and CHD events remained significant after adjustment for traditional risk factors and metabolic syndrome traits.[55]

Recently, a comprehensive systematic review and meta-analysis of 29 population-based cohort studies with aggregate data on over 1.23 million participants and 20,406 CVD outcomes has confirmed that there was no strong evidence for any associations of serum aminotransferases with CVD events.[56] However, stratified analysis by cause-specific CVD endpoints showed that ALT was somewhat inversely associated with CHD and positively associated with stroke.[56] This observation might be due to different effects of ALT levels on CVD risk factors (since vascular outcomes may somewhat have diverse etiologies) or to limited statistical power to detect cause-specific CVD endpoints. Subgroup findings were also suggestive of a positive association of ALT

with CVD events in Asian populations, and possible negative associations in North American and European populations.[56]

Associations between ALP and CVD risk

In a prospective study of 3,381 older British men without history of myocardial infarction or stroke, total ALP but not serum phosphate was associated with an 11-year increased risk of CHD events, which persisted after adjustment for traditional risk factors and inflammatory biomarkers and after exclusion of men with chronic kidney disease [adjusted-HR per 1-standard deviation increase in log baseline levels of ALP, 1.10 (1.01, 1.21); P=0.04].[57]

Similarly, multivariable-adjusted associations between higher total ALP and risk of all-cause and CVD mortality were observed both in the general population and in survivors of myocardial infarction.[58] Again, higher total ALP was an independent predictor of mortality, myocardial infarction, and stent thrombosis in CHD patients who underwent percutaneous coronary intervention with drug-eluting stent.[59]

Notably, the above-mentioned meta-analysis of 29 population-based cohort studies with aggregate data on over 1.23 million participants and 20,406 CVD outcomes (but that included only 4 prospective studies with ALP measurements available) confirmed that higher baseline ALP levels were independently associated with CVD events in a log-linear manner; the pooled fully adjusted relative risk for CVD was 1.08 (1.03-1.14) per 1-standard deviation increase in log baseline levels of ALP.[56] This large-scale data suggests that circulating ALP level is modestly and log-linearly associated with first-ever CVD outcomes in the general population.

Potential mechanisms linking aminotransferases and ALP to CVD risk

Mechanisms postulated for the increased risk of CVD mortality with elevated aminotransferase levels include the presence of unrecognized liver diseases (mainly NAFLD), which increase the risk of CVD mortality, as liver diseases are generally asymptomatic until there are complications of advanced disease.[60-62] To date, NAFLD is becoming one of the most common causes of chronic liver disease worldwide, and is now a major cause of liver-related morbidity and mortality. However, it has been recently shown that the clinical burden of NAFLD is not only confined to liver-related morbidity and mortality, but there is now growing evidence that NAFLD is also associated with an increased risk of developing CVD, type 2 diabetes and other important extra-hepatic complications.[60-62] However, it is important to note that most patients with NAFLD have fairly normal serum aminotransferases.[61,62] This suggests that serum aminotransferase levels are insensitive markers for the detection of NAFLD and that the "normal" reference values for serum aminotransferases (and other LFTs) currently used to exclude NAFLD need to be challenged and revised.[61,62] To date, limited research has deeply examined how the coexistence of NAFLD may affect the relationship between the levels of aminotransferases (or other LFTs) and the risk of CVD events. Elevated aminotransferase levels have also been implicated with increased risk of CVD via underlying endothelial dysfunction, inflammation and impaired hemostasis.[56,61] Indeed, studies have shown that aminotransferase levels, albeit within the reference range, are associated with both circulating biomarkers of inflammation and markers of subclinical atherosclerosis, such as increased CAC

score, increased carotid intima-media thickness and circulatory endothelial dysfunction, independently of conventional risk factors. However, no data are available about the potential impact of changes (induced by lifestyle modification or drugs) in circulating levels of aminotransferases and other LFTs *per se* on these CVD endpoints. The mechanisms for the inverse associations between ALT and CHD risk are not clear, but reduced functionality of the liver in the presence of low ALT levels has been postulated or it could be that ALT may simply be a marker of an underlying aetiology.[61,63]

The excess CVD risk associated with total ALP has been suggested to be via mechanisms correlated to vascular calcification through increased bone metabolism and impaired vascular homoeostasis.[56,58] However, it has been also postulated that the association between total ALP and CVD is unrelated to mineral metabolism, but instead represents confounding by another characteristic that increases CVD mortality, such as decreased kidney function, inflammation or subclinical liver dysfunction.[58,61] Further studies are needed to better elucidate these mechanisms.

Conclusions

Mildly elevated GGT levels are independently associated with an increased risk of future CVD events in most published studies and it is most likely from the available evidence that mildly elevated GGT levels are a marker not only of an unrecognized liver disease but also of increased oxidative stress. Further work is needed to understand better the relationship between TB levels and CVD risk, and also to elucidate whether there is a beneficial effect of increased levels of glucuronidation, a process that also affects many small bio-active molecules that influence vascular function and thereby potentially influence develop of CVD. Nonetheless, irrespective of whether GGT, TB and other LFT concentrations are simple markers (or ephiphenomena) of coexisting CVD risk factors or have a causal role in the aetiology of CVD (**Figure** 1), recent data from some population-based cohort studies suggest that addition of information on either GGT or TB to conventional risk factors provide no improvement in CVD risk prediction.[14,15,25] If confirmed by further prospective, well-designed studies, these findings would support the notion that concentrations of TB and liver enzymes have limited clinical utility for improving current CVD risk prediction models in the general population. Although concentrations of TB and liver enzymes do not identify high risk subgroups (with liver disease) for CVD (*e.g.,* patients with NAFLD), we suggest further research is needed to assess whether in patients with diagnosed NAFLD, serum TB and liver enzyme concentrations may have any clinical utility to improve CVD risk prediction.

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SIGNIFICANCE (word count: 145)

Serum measurements of various liver-derived enzymes and bilirubin (colloquially known as 'liver function tests' or LFTs) are frequently measured in clinical practice. This review focuses on the associations between abnormal levels of LFTs and risk of cardiovascular disease (CVD). Mildly elevated gamma-glutamyltransferase levels are independently associated with future CVD events in most published studies. The association between serum aminotransferases and CVD risk appears somewhat weaker than that observed for gamma-glutamyltransferase. Increased levels of alkaline phosphatase are modestly associated with first-ever CVD outcomes in some studies. Lower levels of total bilirubin are independently associated with an increased risk of CVD events. It is unclear whether abnormal levels of these LFTs are simply markers or causal risk factors for CVD. Recent data from population-based studies suggest that addition of information on either gamma-glutamyltransferase or other LFT concentrations to traditional risk factors provide no improvement in CVD risk prediction.

FIGURE LEGEND

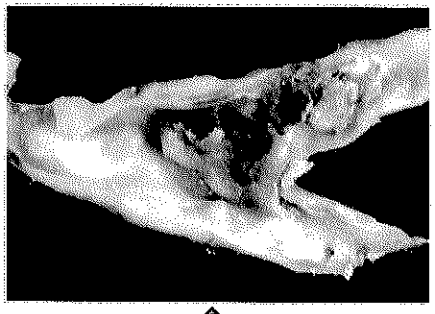
FIGURE 1. Schematic representation of the putative biological mechanisms by which lower levels of total bilirubin and higher levels of aminotransferases (AST and ALT), alkaline phosphatase (ALP) and gamma-glutamyltransferase (GGT) are associated with an increased risk of future CVD events.

- Marker of underlying liver disease (e.g. NAFLD)
- Marker of decreased kidney function
- Marker of vascular calcification

↑ ALP

- Marker of underlying liver disease and increased liver fat (e.g. NAFLD)
- Marker of metabolic syndrome traits

↑ ALT
↑ AST



↑ GGT

- Marker of underlying liver disease (e.g. NAFLD)
- Marker of metabolic syndrome traits
- Marker of systemic oxidative stress
- GGT activity colocalizes with oxidized LDL within atherosclerotic plaques

↓ Bilirubin

- Marker of coexisting cardiovascular risk factors
- ↓Bilirubin may favour lipid oxidation, inflammatory response and immune reactions