Gamma Glutamyltransferase (GGT) as a Biomarker of Atherosclerosis

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Abstract

Gamma-glutamyltransferase (GGT) is an ubiquitous enzyme in human tissues that recycles precursors to the antioxidant and metabolic substrate, glutathione (GSH). GSH is critical in the dynamic preservation of antioxidant balance and in the elimination of xenobiotic substrates. When the total GSH pool becomes limited, either due to increased exposure to oxidative challenges or demand for detoxification, GGT activity increases. Related to atherosclerosis, GSH demand increases with exposure to dietary and environment exposures that have been implicated in vascular inflammation and subsequent atherosclerosis including dietary iron, lipid peroxides, advanced glycation end (AGE) products, and reduced antioxidant intake. Additional stressors on GSH balance include environmental contaminants such as persistent organic pollutants and heavy metals, which also contribute to vascular inflammation directly and indirectly due to metabolic disruption. The results of examinations of several cross-section and longitudinal cohort studies, including our results in the Multi-Ethnic Study of Atherosclerosis (MESA), demonstrate strong associations with: individual risk factors, composite cardiometabolic conditions, mechanistic atherosclerotic biomarkers, and cardiovascular events. Evaluated in totality, the existing evidence strongly suggests GGT activity is a biomarker of systemic oxidative demand indicative of active vascular inflammation, metabolic compromise, and atherosclerosis.

Keywords

Oxidative stress • Inflammation • Oxidized LDL • Endothelial dysfunction • Atherosclerosis • Adhesion molecules • IL-6 • sICAM-1 • CRP

Abbreviations

4-HNE	4-Hydroxynonal
AGE	Advanced glycation end products
CARDIA	Coronary Artery Risk Development in Young Adults CARDIA
CRP	C-Reactive protein
FBG	Fasting blood glucose
FI	Fasting insulin
FMD	Flow-mediated dilation
GGT	Total serum γ-glutamyltransferase activity
GSH	Glutathione
HbA1c	Hemoglobin A1c
HOMA-IR	Homeostasis assessment index
IL-6	Interleukin 6
MESA	Multi-Ethnic Study of Atherosclerosis
NAC	n-Acetylcysteine
oxLDL	Oxidized LDL
sICAM-1	Soluble intercellular adhesion molecule

Key Facts of GGT in Relation to Atherosclerosis and Cardiovascular Disease

- GGT appears to represent total body demand for a key antioxidant molecule called glutathione.
- Studies in large groups of adults performed across the world in many ethnic groups suggest GGT activity is related to increased risk for cardiovascular disease, including male gender, higher risk ethnic subgroups, age, smoking history, waist circumference, LDL, triglycerides, blood pressure, and use of medications for diabetes, hypertension, and cholesterol.
- GGT is associated with multiple indicators of atherosclerosis, including oxidized LDLs, markers of inflammation, markers of immune system contribution to plaque development, and binding molecules that indicate atherosclerosis.
- GGT activity is also associated with conditions that are a significant risk for developing cardiovascular disease, specifically type 2 diabetes and the metabolic syndrome.
- Individual risk factors for developing diabetes are also associated with GGT, including blood glucose (FBG); insulin; average blood sugar, measured by hemoglobin A1c (HbA1c); and models of insulin resistance (HOMA-IR).
- Significant trends for increased prevalent metabolic disease were evident in all ethnic groups (i.e., White, Black, and Hispanic), except Chinese.
- Age greater than 65 years reduced the strength of the associations between GGT activity and disease risk.

Definitions

Advanced glycation end (AGE) products Chemical modification of food that forms from the nonenzymatic reaction between carbohydrates and proteins during cooking at high temperatures in the presence of oxygen.

C-reactive protein (CRP) An acute phase reactant associated with immune stimulation and increased risk of cardiovascular events.

Glutathione An antioxidant peptide consisting of glycine, cysteine, and glutamate that serves as a substrate for the enzyme glutathione peroxidase (GPx) in the reduction of lipid peroxides, and the enzyme glutathione-s-transferase (GST), which conjugates glutathione to xenobiotic compounds for elimination.

HOMA-IR Homeostasis assessment index of insulin resistance, a mathematical model to estimate in vivo insulin resistance based on fasting glucose and fasting insulin or c-peptide.

Interleukin-6 (IL-6) Immune cytokine that triggers acute phase inflammation and the release of acute phase reactants in the liver.

Lipid peroxides Chemical modification of fatty acids, especially unsaturated fatty acids, when cooked at high temperatures. Lipid peroxides are substrates of glutathione-s-transferase, and thus require glutathione for elimination.

***N*-acetylcysteine (NAC)** An antioxidant that provides cysteine for the production of glutathione.

Oxidized LDL (oxLDL) Oxidized low density lipoproteins are chemically oxidized lipoprotein particles that bind receptors and contribute to the formation of atherosclerotic plaques.

Soluble intracellular adhesion molecules (sICAM-1) Endothelial receptors that bind white blood cells during the process of atherosclerosis.

γ–Glutamyltransferase (GGT) An enzyme, previously considered a liver enzyme, that helps recycle precursors to the antioxidant peptide, glutathione.

Introduction

There has been a resurgence of interest in the enzyme γ-glutamyltransferase (GGT) due to the results of several observational studies identifying associations between graded elevations in its activity in serum and increased risk of adverse cardiovascular and metabolic outcomes, including metabolic syndrome (Liu et al. 2012b), type 2 diabetes (Andre et al. 2006; Lim et al. 2007; Meisinger et al. 2005; Nguyen et al. 2011; Onat et al.; Fraser et al. 2009), hypertension (Onat et al.; Liu et al. 2012a), congestive heart failure (Wannamethee et al.), and vascular events (Meisinger et al. 2006; Fraser et al. 2007), plus increased mortality from cardiovascular disease and diabetes (Ruhl and Everhart 2009; Lee et al. 2009). The known physiologic function of GGT is to contribute to in vivo antioxidant homeostasis through recycling extracellular glutathione (GSH), and its precursor amino acids, for intracellular reconversion to reduced GSH (Dickinson and Forman 2002). The tripeptide reduced glutathione (GSH) is a critical antioxidant defense in human tissues; in the absence of adequate GSH, elevations in superoxide, peroxide, and peroxynitrite free radicals persist causing lipid peroxidation, protein modification, and DNA adduct formation with varying consequences on membrane receptor and gene functioning, including impaired endothelial-mediated vasodilation (Franco et al. 2007). Vascular inflammation and oxidative stress have been implicated in the origins of endothelial dysfunction, which contributes to the microvascular complications of metabolic disease and atherosclerotic disease of the macro-vasculature (Evans et al. 2002; Zambon et al. 2005; Touyz 2005; De Mattia et al. 2008). Endothelial dysfunction is a cumulative process secondary to increased concentrations of, and variability in, blood glucose and lipids, with subsequent redox dysregulation (Ceriello 2000; Ceriello et al. 2002). Relevant biomarkers of oxidation, immune activation, and

subclinical inflammation include malondialdehyde modified low-density lipoproteins (commonly referred to as "oxidized" LDL or oxLDL), cytokines such as interleukin-6 (IL-6), elevations in acute phase inflammatory biomarkers including C-reactive protein (CRP), and increased soluble vascular adhesion molecule (sICAM-1) expression- biomarkers which have all demonstrated increased risk prediction beyond traditionally established risk factors (Pereira et al. 2008). However, biomarkers of oxidative-inflammatory stress have limitations in clinical research due to the need for careful sample handling, instrumentation requirements, and the high costs of measurement- factors which limit investigation of these processes in population-based studies of human disease, and create barriers to translating basic science evidence into clinical research (Mayne 2003). GGT provides: a rapid, inexpensive, clinically available biomarker to assess physiologic demand for antioxidant substrates; an indicator of composite dietary and environmentally associated disease risk; and direct insight into the risk for adverse cardiovascular and metabolic disease outcomes in at-risk populations.

The Physiologic Action of γ–Glutamyltransferase (GGT)

The primary action of GGT in vivo is to facilitating transmembrane transport of cysteine and other precursors of the antioxidant peptide glutathione for reconversion into intracellular glutathione (GSH) (Dickinson and Forman 2002). Intracellular glutathione is a critical antioxidant defense; in the absence of adequate glutathione lipid peroxidation, protein modification and DNA adduct formation occur with varying consequences on membrane receptor and gene function. Intrahepatic glutathione also serves as an important conjugation substrate for the elimination of xenobiotics. The specific relationship between GGT and in vivo redox balance has been extensively reported by Whitfield (2001), is summarized in our Multi-Ethnic Study of Atherosclerosis (MESA) findings (Bradley et al. 2013).

The Validity of GGT Measurement

There is no "gold-standard" biomarker for systemic oxidative stress in human populations. The evidence available on GSH status and cardiovascular risk is limited to patients with type 2 diabetes, and demonstrates lower concentrations of GSH (measured as erythrocyte GSH) in those with diabetes compared to those without diabetes, and diabetics with microvascular complications appear to have still lower concentrations, suggesting the importance of GSH status in complication development (De Mattia et al. 2008; Ahmadpoor et al. 2009; Thornalley et al. 1996). Although these relatively small case–control studies have demonstrated differences in GSH status, the validity of erythrocyte GSH as a biomarker remains mostly unknown in large population-based studies of cardiovascular disease. Unfortunately, the need to preserve reduced GSH in stored specimens at the time of collection impedes post-hoc measurement of erythrocyte GSH in stored

samples, and thus limits evaluations of GSH in cardiovascular disease cohorts (Tietze 1969).

Alternatively, the case for the validity of GGT as a clinically significant biomarker of oxidative stress has gained strength. In order for a biomarker of systemic (i.e., multiple tissues) oxidative stress to be valid in metabolic disease one would expect the biomarker to: (1) Have supporting mechanistic data that supports the role of the biomarker in relation to oxidative stress development, (2) Correlate to existing gold-standard biomarkers of biomolecule oxidation (e.g., F2-isoprostanes) for lipid peroxidation, (3) Correlate with known risk factors for metabolic disease including lifestyle factors, (4) Increase with increasing risk for metabolic disease, and (5) Continue to correlate with oxidation-linked complications in relevant disease states.

Several available research results provide a convincing rationale that increased GGT activity represents increased "oxidative stress" via increased demand for GSH. Findings supportive of this role include: (1) GSH depletion appears to be a prerequisite condition to induce GGT (Braide 1989); (2) NADPH oxidase-produced ROS, and reactive nitrogen species both induce GGT expression (Huseby et al. 2003; Ravuri et al. 2011); (3) mitochondria of GGT-knock-out mice have depleted GSH, increased reactive oxygen species (ROS) formation, depleted energy stores, and impaired oxidative phosphorylation (thus impaired ATP production), which can be attenuated by *N*-acetylcysteine (NAC), a GSH precursor (Will et al. 2000); and (4) GGT-knock-out mice die prematurely with complications associated with increased oxidative stress- similar to complications in type 2 diabetes (e.g., cataracts and microvascular compromise) (Chevez-Barrios et al. 2000). Additional animal in vivo data support the relationship between GGT activity and oxidative stress induction. Watkins et al. reported increased GGT activity in streptozotocin (STZ)-induced diabetic rats (Watkins et al. 1998). STZ is a potent alkylating agent that induces diabetes in the rat in part through depletion of pancreatic glutathione concentration. In addition, GGT knock-out mice have reduced glutathione levels compared to controls, develop cataracts and die prematurely unless treated with glutathione precursors (Chevez-Barrios et al. 2000). These data provide mechanistic support for the role of GGT in offsetting damage caused by oxidative stress. Translating this hypothesis to research in humans, and further supporting a relationship between GGT and GSH, Sedda et al. demonstrated inverse associations between GGT activity and plasma total GSH concentration in people with established cardiovascular risk, and after multivariate adjustment for individual risk factors, plasma total GSH remained the only independent variables associated with GGT activity (Sedda et al. 2008).

Regarding the relationship between GGT and "gold-standard" markers of oxidative stress in humans, lipid peroxidation- both endogenous and exogenous-contributes to glutathione demand. GSH is required for the elimination of lipid peroxides, and thus increases requirements for GSH. Peroxidation is thought to contribute to vascular injury and endothelial dysfunction. Positive associations were found between biomarkers of lipid peroxidation and GGT in the Coronary Artery Risk Development in Young Adults (CARDIA) cohort (Lee et al. 2003). Specifically, increasing percentiles of GGT (although still within the normal

range) were associated with F2-isoprostanes, in both men and women after adjustment for study center, race, age, and sex. In addition to positive correlations with isoprostanes, positive associations were also found with C-reactive protein, a recognized measure of systemic inflammation. Additional support for the validity of GGT as a marker for systemic oxidative stress in humans comes from the Tromso cohort study, which measured GGT in over 21,000 men and women and evaluated population determinants of GGT activity (Nilssen et al. 1990). Positive associations were found between GGT activity and alcohol, body mass index, and total cholesterol. Inverse associations were found between GGT activity and increased physical activity and the consumption of coffee; as mentioned coffee is known to increase erythrocyte glutathione levels (Esposito et al. 2003).

Laboratory Measurement of GGT

GGT (as activity) is measured in human plasma or serum. Measurement of GGT has been used clinically for many years as a sensitive indicator of hepatobiliary disease, including alcoholism and bile duct obstruction. Because of its role in clinical diagnostic testing, Clinical Laboratory Improvement Amendments (CLIA) and International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) testing standards are in place and CLIA/IFCC-certified automated, multitest instruments that test GGT are readily available in clinical laboratories and exhibit good quality control. Published day-to-day coefficients of variability for example multitest instruments are <5 % (Forouhi et al. 2007; Steinmetz et al. 2007).

The assay used to measure GGT is technically a measure of GGT activity, not concentration or expression; tissue GGT expression has been measured using northern and western blot (Watkins et al. 1998). Figure 1 outlines the chemical reaction used to measure GGT activity. In brief, an amino acid residue is transferred by GGT from gamma-glutamyl-p-nitroanilide to a small peptide, producing p-nitroaniline, which is measured using a visible spectrum photometer at 405 nm. The resulting absorbance is then compared to known GGT standards for report of GGT activity (Lee et al. 2003).

The Reliability of GGT Measurement

One of the earliest reported reliability studies on GGT was performed by Rhone and White in 1976 (Rhone and White 1976). In their study, they collected blood from 10 participants and split the samples, storing half of the sample at 4 °C with GGT

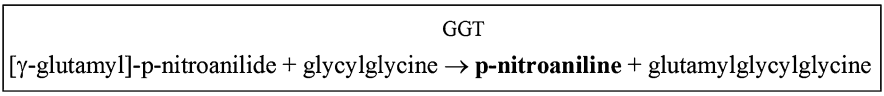

Fig. 1 Chemical reaction used to measure GGT in blood samples

measurement at 1, 2, 5, and 9 weeks. The other aliquot they stored at −6 °C with thawing and GGT measurement at 1, 2, 6, 10, and 40 weeks. Although the authors did not calculate reliability coefficients, they report that of the 50 GGT measurements from samples stored at 4 °C, only 15 showed a 10 % or greater change in GGT activity. For those samples stored at −6 °C, no statistically different changes in GGT activity were measured using paired analyses, however a reliability coefficient was not calculated. Although the data presented by Rhone and White suggest low variability in GGT activity with proper samples storage (freezing), their data is not completely interpretable due to the lack of reporting of reliability coefficients.

Better data regarding the reliability of GGT is available from the CARDIA cohort (Lee et al. 2003). CARDIA followed 5,115 adults between 18 and 30 years of age for 15 years. GGT was measured at year 0 and year 10. Because of differences in instrumentation between year 0 and year 10, the investigators remeasured GGT in 103 year 0 samples. This remeasurement happened after 17 years of sample storage at −70 °C. They reported a reliability coefficient of 0.995 between year 0 measured samples and the 103 remeasured year 0 samples. This high reliability coefficient supports high intra-sample and inter-method reliability for GGT. In addition it suggests the effects of storage were negligible. The number of freeze-thaw cycles for the samples in the interim period, i.e., year 0 to year 17, was unstated. In addition, the investigators calculated a reliability coefficient of 0.67 between year 0 samples and year 10 samples, suggesting good inter-sample reliability.

GGT as a "Liver Enzyme"

Commonly classified as a "liver enzyme" associated with gall bladder disease, alcoholism, and frank hepatitis, liver tissue GGT expression does not always correlate with *serum* GGT activity (Satoh et al. 1980; Selinger et al. 1982). GGT is expressed in multiple human tissues other than the liver, with total serum GGT activity corresponding to the sum of activity from at least four fractions (Franzini et al. 2008, 2009, 2012, 2013a; Paolicchi et al. 2006). Methods for measuring subfractions of total GGT activity (i.e., *s-GGT, m-GGT, b-GGT, and f-GGT* for "small," "medium," "big" and "free," respectively) have been reported by Franzini et al. (2008), and disease-specific patterns of GGT subfractions and ratios of subtypes are emerging, e.g., increased *b-GGT* has been recently associated with cardiovascular risk (Franzini et al. 2013a), whereas *s-GGT* was increased in alcoholics (Franzini et al. 2013b). Subfractionation should be applied in future studies to better differentiate subgroups within larger cohorts by disease status, including those with isolated hepatic disease and those with multiorgan disease, i.e., concomitant nonalcoholic fatty liver disease, metabolic disease, *and* cardiovascular disease. The innovative method of GGT subfractionation will also be critical in future determinations of the organ-specific mechanisms responsible for observed increases in serum GGT activity. Despite the potential to improve upon our methods by subfractionating GGT, the associations demonstrated here with total serum GGT

activity remain valid. Other studies have demonstrated that participants with increased *total* serum GGT activity have increased GGT subfractions more associated with disease (i.e., non-*f-GGT fractions*); total serum GGT activity increased with increases in each subfraction (i.e., except *f-GGT)*; and total serum GGT activity remains the most accessible biomarker to clinicians (Franzini et al. 2012, 2013a, b). However, despite the insights gained from fractionating GGT, the relationship between *tissue* GGT activity and *serum* GGT activity remains under active investigation.

GGT Activity and Atherosclerosis

GGT Activity and Individual Risk Factors for Cardiovascular Disease

Our investigations in the Multi-Ethnic Study of Atherosclerosis (MESA) began with detailed evaluations of potential cross-sectional associations between GGT and established risk factors for atherosclerosis and cardiovascular disease, including demographics, lifestyle variables, and clinical/laboratory risk factors (Bradley et al. 2014). The results are consistent, demonstrating GGT is strongly and positively associated with nearly all established risk factors (Table 1), including male gender, at-risk ethnic subgroups, smoking status, increased alcohol intake, increased LDL, reduced HDL, elevated triglycerides, increased insulin resistance, and both increased systolic and diastolic blood pressures. The consistency of these findings suggests a common mechanism underlying these associations, such as vascular inflammation secondary to oxidative stress, immune activation, and inflammation.

GGT Activity and Increased Risk for Adverse Metabolic and Cardiovascular Outcomes

The results of numerous observational studies, e.g., NHANES III, CARDIA, EPI-Norfolk, have identified associations between graded elevations in its activity in serum and increased risk of adverse cardiovascular and metabolic outcomes, including metabolic syndrome (Liu et al. 2012b; Bradley et al. 2013), type 2 diabetes (Andre et al. 2006; Lim et al. 2007; Meisinger et al. 2005; Nguyen et al. 2011; Onat et al.; Fraser et al. 2009; Bradley et al. 2013), hypertension (Onat et al.; Liu et al. 2012a), congestive heart failure (Wannamethee et al.), and vascular events (Meisinger et al. 2006; Fraser et al. 2007), plus increased mortality from cardiovascular disease and diabetes (Ruhl and Everhart 2009; Lee et al. 2009).

In order to connect GGT activity specifically with increased vascular risk, our work tested a mechanistic conceptual model in the MESA cohort hypothesizing GGT activity would be independently associated with biomarkers of oxidation, immune activation, and atherosclerosis (Bradley et al. 2014). We measured significant, positive associations between GGT activity and increased oxidized LDL (oxLDL), C-reactive protein (CRP), interleukin-6 (IL-6), and intravascular adhesion molecule-1 (ICAM-1);

Table 1 Associations between GGT, demographic variables, and cardiometabolic risk factors

Baseline characteristic (n = 6,446)	GGT-Q1 <24.5 U/l *n* (%) or Mean (SD)	GGT-Q2 24.5–29.3 U/l *n* (%) or Mean (SD)	GGT-Q3 29.3–35.1 U/l *n* (%) or Mean (SD)	GGT-Q4 35.1–45.2 U/l *n* (%) or Mean (SD)	GGT-Q5 >45.2–99.7 U/l *n* (%) or Mean (SD)	*P*-value for trend
Age (year)	62.4 (10.9)	63.5 (10.3)	62.8 (10.0)	62.1 (10.0)	61.0 (9.7)	<0.0001
Gender: Male	325 (25.2 %)	523 (40.7 %)	635 (49.1 %)	724 (52.5 %)	840 (65.1 %)	<0.0001
Race White Chinese Black Hispanic	665 (30.0 %) 194 (21.9 %) 226 (12.9 %) 204 (14.5 %)	543 (24.5 %) 173 (19.6 %) 308 (17.6 %) 262 (18.6 %)	465 (20.9 %) 164 (18.6 %) 389 (22.3 %) 274 (19.5 %)	435 (19.6 %) 123 (13.9 %) 430 (24.6 %) 301 (21.4 %)	408 (18.4 %) 119 (13.5 %) 395 (22.6 %) 368 (26.2 %)	Referent 0.91 <0.0001 <0.0001
Smoking status Never Current Past	746 (22.9 %) 105 (14.9 %) 432 (18.2 %)	663 (20.4 %) 135 (19.2 %) 483 (20.4 %)	695 (21.4 %) 159 (22.6 %) 435 (18.3 %)	615 (18.9 %) 172 (24.4 %) 498 (21.0 %)	533 (16.4 %) 228 (32.4 %) 525 (22.1 %)	Referent <0.0001 <0.0001
Pack years (year)	21.1 (23.2)	21.6 (21.6)	24.8 (27.1)	23.4 (25.5)	24.5 (33.7)	0.021
Current alcohol use (%)	699 (10.3 %)	669 (9.8 %)	672 (10 %)	719 (10.6 %)	782 (11.5 %)	0.34
Current drinks/week	2.4 (3.7)	3.5 (5.1)	3.5 (5.1)	4.4 (6.8)	5.9 (8.0)	<0.0001
Physical activity (total MET-min/Week)	1,499 (2,131)	1,529 (2,286)	1,548 (2,196)	1,558 (2,580)	1,609 (2,841)	0.75

Diabetes medications None Insulin Oral	1,218 (94 %) 11 (1 %) 60 (5 %)	1,184 (92 %) 19 (2 %) 83 (6 %)	1,159 (89 %) 20 (2 %) 113 (9 %)	1,150 (89 %) 21 (2 %) 118 (9 %)	1,124 (87 %) 33 (3 %) 133 (10 %)	Referent <0.0001 <0.0001
Total cholesterol (mg/dl)	192.0 (32.6)	192.8 (35.7)	193.5 (35.3)	195.3 (34.8)	196.8 (37.6)	<0.0001
LDL-C (mg/dl)	114.1 (29.4)	116.6 (31.1)	118.3 (31.0)	119.6 (31.9)	118.7 (32.5)	<0.0001
HDL-C (mg/dl)	56.7 (15.3)	52.4 (15.5)	49.4 (14.1)	48.4 (13.3)	48.0 (13.8)	<0.0001
Triglycerides (mg/dl)*	94.0 (68–127)	104.0 (74–146)	111.0 (78–158)	119.0 (83–174)	132.0 (89–191)	<0.0001
Lipid-lowering medications	181 (14 %)	203 (16 %)	213 (16.5 %)	230 (18 %)	222 (17 %)	0.036
SBP (mm Hg)	121.9 (21.6)	125.7 (22.0)	127.7 (21.7)	128.1 (21.0)	129.2 (20.5)	<0.0001
DBP (mm Hg)	68.3 (10.0)	70.4 (9.9)	72.4 (10.1)	74.4 (10.1)	73.9 (10.2)	<0.0001
Current HTN	466 (36 %)	531 (41 %)	591 (46 %)	642 (50 %)	650 (50.4 %)	<0.0001
HTN medications	395 (31 %)	454(35 %)	485 (38 %)	537 (42 %)	517(40 %)	<0.0001
Waist circumference (cm)	91.9 (14.3)	96.2 (14.3)	99.0 (14.0)	101.1 (13.8)	101.4 (13.4)	<0.0001
Glucose (mg/dl)*	85.0 (80–91)	88.0 (82–95)	91.0 (84–100.5)	92.0 (85–103)	93.0 (86–106)	<0.0001
Insulin (μU/l)*	3.8 (2.7–5.5)	4.8 (3.3–7.2)	5.6 (3.8–8.6)	6.2 (4.1–9.4)	7.1 (4.4–11.1)	<0.0001

*denotes a skewed variable; descriptive statistics reported as median and inter-quartile range (IQR).
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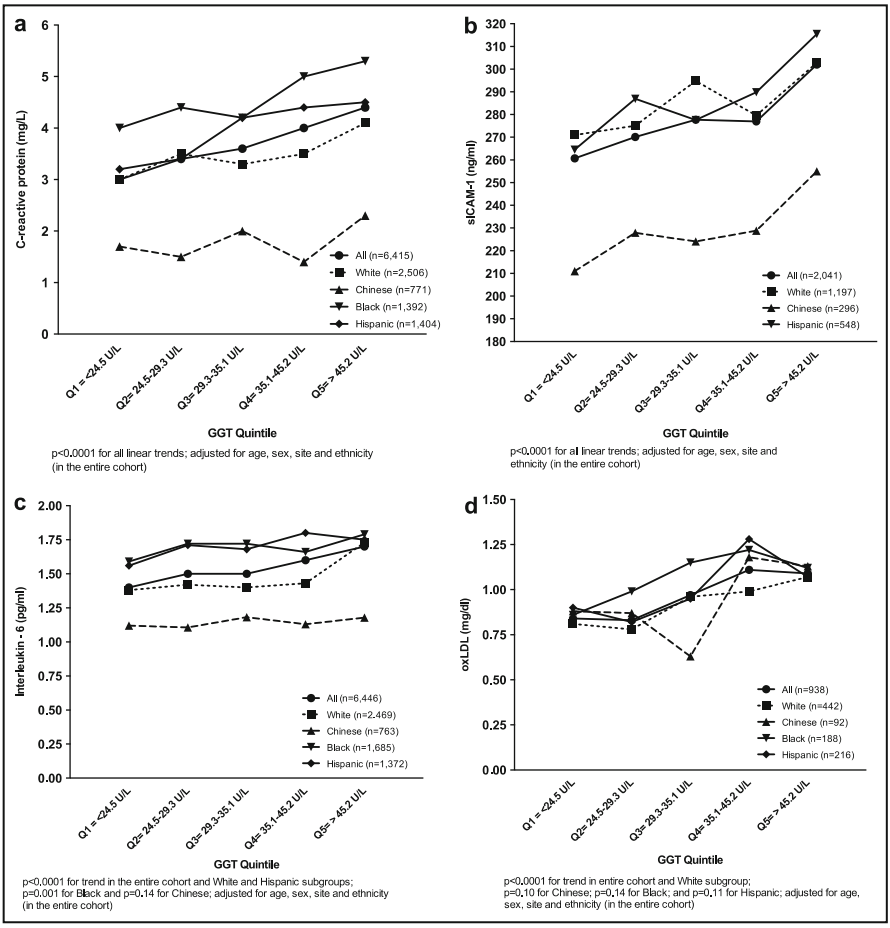

Fig. 2 Associations between GGT and biomarkers of atherosclerosis. Associations between GGT activity and biomarkers of atherosclerosis including: C-reactive protein (**a**), soluble intravascular adhesion molecules (**b**), interleukin-6 (**c**), and oxidized LDL (**d**) (Reprinted with permission from Elsevier Publishing)

see Fig. 2 and Table 2. The overall relationship identified was consistent across ethnicities, with rare exceptions. Specifically, we saw evidence of several significant associations between γ-glutamyltransferase (GGT), traditional cardiovascular risk factors, and biomarkers of oxidative stress, immune activation, acute phase response, and endothelial dysfunction. Specifically, we saw evidence of strong associations for increasing trends in oxLDL, IL-6, CRP, and sICAM-1 with graded increases in GGT in the entire cohort. Continuous associations between GGT and all biomarkers of interest were significant after adjustment for age, race, gender, and study site, and remained significant for IL-6, CRP, and sICAM-1 after adjustment for risky lifestyle factors and

Table 2 Adjusted associations between GGT and biomarkers of atherosclerosis: CRP, IL-6, oxLDL, and sICAM-1

CRP = $\beta_{Adj.}$*GGT + $\beta_n X_n \ldots + \beta_0$, n = 6,415	$\beta_{Adj.}$[a]	95 % CI	p-value for GGT
M1 (n = 6,415)	0.042	0.03–0.05	<0.0001
M2 (n = 3,819)	0.032	0.02–0.04	<0.0001
M3 (n = 3,774)	0.025	0.01–0.04	<0.0001
M4 (n = 3,772)	0.017	0.005–0.03	0.006
IL-6 = $\beta_{Adj.}$*GGT + $\beta_n X_n \ldots + \beta_0$, n = 6,289	$\beta_{Adj.}$	95 % CI	p-value for GGT
M1 (n = 6,289)	0.0069	0.005–0.009	<0.0001
M2 (n = 3,745)	0.0065	0.004–0.009	<0.0001
M3 (n = 3,701)	0.0060	0.003–0.009	<0.0001
M4 (n = 3,699)	0.0036	0.001–0.006	0.007
oxLDL = $\beta_{Adj.}$*GGT + $\beta_n X_n \ldots + \beta_0$, n = 935	$\beta_{Adj.}$	95 % CI	p-value for GGT
M1 (n = 935)	0.005	0.002–0.008	0.004
M2 (n = 583)	0.006	0.003–0.010	0.001
M3 (n = 573)	0.001	−0.001–0.004	0.41
M4 (n = 572)	0.0007	−0.002–0.004	0.64
sICAM-1 = $\beta_{Adj.}$*GGT + $\beta_n X_n \ldots + \beta_0$, n = 938	$\beta_{Adj.}$	95 % CI	p-value for GGT
M1 (n = 938)	0.87	0.68–1.06	<0.0001
M2 (n = 585)	0.68	0.45–0.91	<0.0001
M3 (n = 575)	0.68	0.45–0.93	<0.0001
M4 (n = 574)	0.51	0.27–0.75	<0.0001

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M1 adjusts for age (years), gender, ethnicity, and study site

M2 equals M1 + alcohol use (current/former/never and drinks/week) + exercise (MET-min/week) + smoking (current/former/never and pack years)

M3 equals M2 + lipids (LDL-C, HDL-C, triglycerides in mg/dl), lipid-lowering medications, systolic and diastolic blood pressures (mmHg), antihypertensive medications, diabetes medications, and family history of heart attack or stroke

M4 equals M3 + waist circumference (cm), fasting blood glucose (mg/dl), and fasting insulin (μU/l)

[a]Corresponding units for regression coefficients are: CRP: mg/L/unit GGT activity; IL-6: pg/ml/unit GGT activity; oxLDL: mg/dl/unit GGT activity; and sICAM-1: ng/ml/unit GGT activity

traditional risk factors. Although the strength of all associations was attenuated by adjustment for metabolic status, most associations remained significant and independent. Associations between GGT and oxLDL were significant in the entire cohort after adjustment for demographics and risky lifestyle factors, but not following adjustment for traditional risk factors, likely secondary to moderate colinearity between LDL and oxLDL (i.e., correlation coefficient = 0.58 between LDL-C and ln(oxLDL) in MESA). Our findings are strengthened by the use of the MESA cohort for our analyses, which is unique amongst population cohorts in its excellent ethnic representation and

its collection of emerging biomarkers like oxLDL. No known prior studies have evaluated associations between GGT and oxLDL, IL-6, and sICAM-1, nor have any prior studies evaluated associations with CRP in ethnic subgroups.

These findings are supported by those of prior investigations in the CARDIA cohort study which demonstrated similar associations between GGT and CRP and fibrinogen, as well as additional support for GGT associations with lipid peroxidation products, e.g., F2-isoprostanes (Lee et al. 2003). Combined with our findings confirming associations with CRP, and demonstrating novel associations with oxLDL, IL-6, and sICAM-1, these data support the hypothesis that increased GGT activity represents immune stimulation (i.e., IL-6) of hepatic acute phase response (i.e., CRP and fibrinogen), possibly due to increased lipid peroxidation products (i.e., oxLDL and F2-isoprostanes) (Il'yasova et al. 2008; Zhang et al. 2012). Our findings extended this conceptual model to include vascular endothelial involvement, i.e., sICAM-1.

Because nonalcoholic fatty liver disease and other hepatic disease are known to be associated with adverse cardiovascular outcomes, at first glance findings appear limited in that we do not adjust for liver function, i.e., serum AST or ALT, in our analyses. Unfortunately, neither AST nor ALT data are available in the MESA cohort for inclusion. While adjustment for AST and ALT would have assisted in reducing the contribution of hepatic disease to our findings, as suggested in our prior work (Bradley et al. 2013), serum GGT activity is not correlated with hepatic expression of GGT (Selinger et al. 1982). However, we partially accounted for liver disease in two ways: by restricting our analyses to the lower 95th percentile of the GGT activity range (thus likely eliminating severe hepatic disease) and by adjusting for continuous alcohol intake. We were not interested in adjusting our results for fatty liver disease, as fatty liver is highly correlated with hepatic insulin resistance, and thus adjusting for it would have eliminated the influence of metabolic risk fundamental to our hypothesis.

In addition to our research in MESA developing a mechanistic model connecting GGT to atherosclerosis, we also evaluated the relationship between GGT and clinical models of extreme oxidative stress and vascular risk – namely metabolic syndrome and type 2 diabetes (Bradley et al. 2013). GGT activity is strongly associated with individual and composite cardiovascular and metabolic risk factors in the MESA cohort (Fig. 3 and Tables 3 and 4). The observed increases in prevalent metabolic diseases across GGT quintiles are highly consistent with our unadjusted analyses demonstrating positive associations between GGT and "riskier" profile for nearly every individual risk variable (Table 1). The results of our multivariable analysis suggest the associations between GGT and individual metabolic risk factors, especially fasting insulin and HOMA-IR, are very stable and remain independent of standard clinical measures and lifestyle variables. Similar to previous reports, we measured a significant interaction between GGT, BMI, and HOMA-IR, and weaker associations in adults over 65 years of age (Lim et al. 2007; Lee et al. 2009). Because these associations are so consistent, strongly significant, and relatively independent of ethnic group, we suggest total serum GGT activity is a continuous generalizable biomarker of composite metabolic risk in those adults without clinically evident vascular

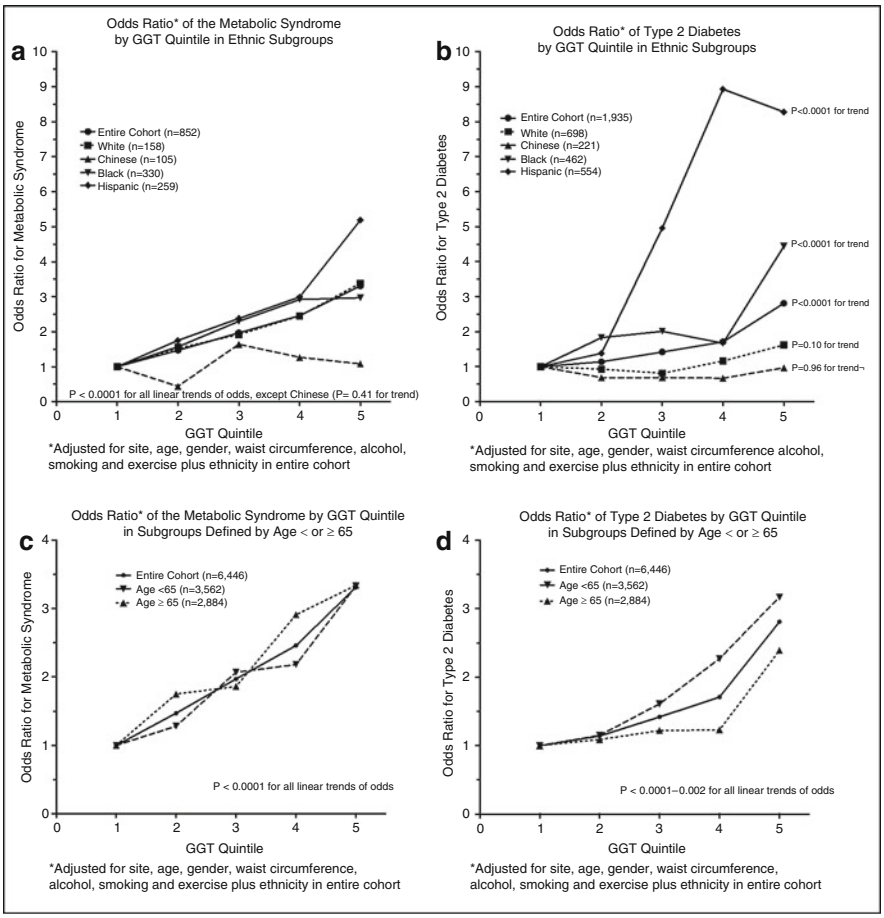

Fig. 3 Odd ratios of metabolic disease by GGT quintile in subgroups defined by ethnicity and age. Demonstration of increased risk of type 2 diabetes and metabolic syndrome by ethnic and age subgroups

disease, with particular utility in people under age 65 years, even after considering differences in established demographic, behavioral, and clinical risk factors.

GGT Activity and Dietary Patterns Associated with Cardiovascular Disease and Diabetes

One explanation regarding sources of increased oxidative stress and endothelial dysfunction in the general population is the diet, and specifically dietary characteristics, including nutritional composition, as well as, preparation methods.

Table 3 Adjusted odds ratios of cardiometabolic disease by GGT quintile in the entire MESA cohort and stratified by ethnic groups

Cardiometabolic subgroup	GGT-Q1 <24.5 U/l Referent group Prevalence	GGT-Q2 24.5–29.3 U/l OR[a] (95 % CI) *P*-value[b] Prevalence	GGT-Q3 29.3–35.1 U/l OR[a] (95 % CI) *P*-value[b] Prevalence	GGT-Q4 35.1–45.2 U/l OR[a] (95 % CI) *P*-value[b] Prevalence	GGT-Q5 45.2–99.7 U/l OR[a] (95 % CI) *P*-value[b] Prevalence	*P*-value for trend	*P*-value for interaction with ethnicity
Metabolic syndrome All ethnicities ($n = 1{,}151$)	Referent $n = 154$	1.47 (1.09–1.99) 0.01 $n = 210$	1.97 (1.47–2.65) <0.0001 $n = 246$	2.46 (1.83–3.30) <0.0001 $n = 256$	3.31 (2.46–4.46) <0.0001 $n = 285$	<0.0001	–
White ($n = 466$)	Referent $n = 76$	1.53 (1.03–2.29) 0.04 $n = 97$	1.92 (1.27–2.91) 0.002 $n = 97$	2.45 (1.63–3.70) <0.0001 $n = 90$	3.39 (2.24–5.14) <0.0001 $n = 106$	<0.0001	Referent
Chinese ($n = 127$)	Referent $n = 22$	0.44 (0.12–1.56) 0.21 $n = 25$	1.64 (0.52–5.17) 0.40 $n = 32$	1.27 (0.39–4.19) 0.69 $n = 26$	1.08 (0.30–3.87) 0.90 $n = 22$	0.41	0.34
Black ($n = 229$)	Referent $n = 24$	1.57 (0.76–3.26) 0.22 $n = 29$	2.30 (1.16–4.56) 0.017 $n = 56$	2.92 (1.50–5.69) 0.002 $n = 70$	2.97 (1.46–6.03) 0.003 $n = 50$	<0.0001	0.83
Hispanic ($n = 329$)	Referent $n = 32$	1.75 (0.88–3.49) 0.11 $n = 59$	2.38 (1.23–4.63) 0.01 $n = 61$	2.99 (1.53–5.85) 0.001 $n = 70$	5.19 (2.68–10.03) <0.0001 $n = 107$	<0.0001	0.30

Type 2 diabetes All ethnicities ($n = 781$)	Referent $n = 73$	1.14 (0.70–1.86) 0.61 $n = 120$	1.42 (0.86–1.86) 0.15 $n = 168$	1.71 (1.09–2.70) 0.02 $n = 182$	2.81 (1.80–4.39) <0.0001 $n = 238$	<0.0001	–
White ($n = 158$)	Referent $n = 22$	0.93 (0.46–1.92) 0.84 $n = 26$	0.81 (0.37–1.77) 0.60 $n = 28$	1.16 (0.57–2.38) 0.68 $n = 35$	1.62 (0.80–3.25) 0.18 $n = 38$	0.10	Referent
Chinese ($n = 105$)	Referent $n = 17$	0.69 (0.17–2.78) 0.60 $n = 23$	0.69 (0.18–2.61) 0.59 $n = 17$	0.67 (0.18–2.49) 0.55 $n = 17$	0.97 (0.28–3.34) 0.96 $n = 22$	0.96	0.46
Black ($n = 330$)	Referent $n = 15$	1.83 (0.75–4.44) 0.18 $n = 45$	2.01 (0.85–4.71) 0.11 $n = 74$	1.67 (0.72–3.91) 0.23 $n = 65$	4.44 (1.93–10.21) <0.0001 $n = 104$	<0.0001	0.21
Hispanic ($n = 259$)	Referent $n = 19$	1.38 (0.24–7.90) 0.72 $n = 26$	4.96 (1.09–22.6) 0.04 $n = 49$	8.93 (2.02–39.38) 0.004 $n = 65$	8.28 (1.87–36.54) 0.005 $n = 74$	<0.0001	0.09

[a]Adjusted for age, gender, waist circumference, study site, alcohol, smoking, and exercise plus ethnicity in the entire cohort
[b]*P*-value corresponds to the point estimate for odds of disease within each quintile

Table 4 Adjusted associations between GGT and metabolic risk factors, overall and in metabolic disease subgroups

	Metabolic subgroup							
Risk variable	Entire (n = 6,446) $\beta_{Adj.}$* ln (GGT)$_{(95\%CI)}$	*P*-value	Normal (n = 3,783) $\beta_{Adj.}$* ln (GGT)$_{(95\%CI)}$	*P*-value	MetS (n = 1,935) $\beta_{Adj.}$* ln (GGT)$_{(95\%CI)}$	*P*-value	Diabetes (n = 852) $\beta_{Adj.}$* ln (GGT)$_{(95\%CI)}$	*P*-value
Glucose, fasting (FBG, mg/dl)								
M1	0.11 (0.10–0.13)	<0.0001	0.03 (0.026–0.04)	<0.0001	0.04 (0.03–0.06)	<0.0001	0.13 (0.07–0.20)	<0.0001
M2	0.11 (0.10–0.13)	<0.0001	0.03 (0.02–0.04)	<0.0001	0.04 (0.02–0.06)	0.001	0.13 (0.02–0.23)	0.02
M3	0.07 (0.05–0.09)	<0.0001	0.03 (0.02–0.04)	<0.0001	0.05 (0.02–0.07)	<0.0001	0.05 (−0.05–0.15)	0.34
Insulin, fasting (FI, mU/l)								
M1	0.53 (0.49–0.57)	<0.0001	0.39 (0.34–0.44)	<0.0001	0.38 (0.31–0.45)	<0.0001	0.42 (0.28–0.55)	<0.0001
M2	0.55 (0.50–0.60)	<0.0001	0.40 (0.38–0.47)	0.0001	0.38 (0.28–0.47)	<0.0001	0.45 (0.25–0.65)	<0.0001
M3	0.39 (0.34–0.44)	<0.0001	0.33 (0.27–0.39)	<0.0001	0.38 (0.28–0.47)	<0.0001	0.47 (0.27–0.67)	<0.0001

Hemoglobin A1c (HbA1c, %)								
M1	0.06 (0.05–0.07)	0.0001	0.02 (0.008–0.02)	0.0001	0.04 (0.02–0.05)	<0.0001	0.03 (−0.01–0.08)	0.13
M2	0.06 (0.05–0.08)	<0.0001	0.02 (0.008–0.02)	<0.0001	0.02 (0.003–0.04)	0.02	0.04 (−0.02–0.11)	0.18
M3	0.04 (0.03–0.05)	<0.0001	0.01 (0.004–0.02)	0.004	0.02 (0.002–0.04)	0.03	0.04 (−0.03–0.11)	0.26
HOMA-IR								
M1	0.64 (0.60–0.69)	<0.0001	0.43 (0.37–0.48)	0.0001	0.42 (0.35–0.50)	<0.0001	0.55 (0.41–0.69)	<0.0001
M2	0.66 (0.60–0.72)	<0.0001	0.44 (0.37–0.51)	<0.0001	0.42 (0.31–0.52)	<0.0001	0.57 (0.36–0.79)	<0.0001
M3	0.47 (0.41–0.52)	<0.0001	0.36 (0.29–0.43)	<0.0001	0.42 (0.32–0.53)	<0.0001	0.52 (0.30–0.73)	<0.0001
$M4_{HOMA}$	0.37 (0.32–0.42)	<0.0001	0.28 (0.22–0.34)	<0.0001	0.39 (0.29–0.48)	<0.0001	0.43 (0.24–0.62)	<0.0001

FBG, FI, HbA1c, HOMA-IR, and GGT are log transformed. All models exclude insulin users

Regression coefficient units for FBG, FI, HbA1c, and HOMA-IR are: 10*mg glucose/unit GGT activity; mU insulin/unit GGT activity

% HbA1c/unit GGT activity and 1/unit GGT activity, respectively

M1 adjusts for age, gender, ethnicity, and study site

M2 equals M1 + alcohol use (current/former/never and drinks/week) + exercise (MET-min/week) + smoking (current/former/never and pack years)

M3 equals M2 + lipids (LDL-C, HDL-C, triglycerides), lipid-lowering medications, systolic and diastolic blood pressures, antihypertensive medications, diabetes medications, and family history of heart attack or stroke

$M4_{HOMA}$ equals M3 + waist circumference

The Postprandial State, Oxidative Stress, and Endothelial Dysfunction

Ceriello et al. have conducted trials in multiple populations, including healthy humans and patients with type 2 diabetes, demonstrating acute increases in biomarkers of oxidative stress (oxidized LDL and nitrotyrosine) and impaired flow-mediated dilation (FMD) following experimentally-induced hyperlipidemia and hyperglycemia following high fat and high glycemic index control meals (Dickinson et al. 2008; Ceriello et al. 2002, 2008; Ceriello 2000, 2003). Other example meals that have been administered as positive control meals to induce short-term reductions in FMD include fast food dishes (e.g., Big Mac®, Egg McMuffin®, hash browns), corn oil, pizza, and whipped cream (Carroll and Schade 2003; Kay and Holub 2002; Bogani et al. 2007; Esmaillzadeh et al. 2007). The control meals used by Ceriello et al. resulting in short-term endothelial dysfunction included a 70 g fat bolus as whipped cream +/− a 75 g oral glucose tolerance test or a standardized, high-fat breakfast meal (Ceriello et al. 2002). These important trials have provided detailed proof-of-concept data regarding the interrelations between metabolic status, dietary intake, postprandial oxidative stress, and subsequent acute changes in vascular response.

Glutathione Requirements for Metabolism of Dietary Iron, Lipid Peroxides, and AGE Products

Many compounds found in food, and especially cooked food, require GSH for either metabolism or elimination, providing a mechanistic rationale for how GGT may be associated with dietary characteristics. Three of these compounds include dietary iron present in mainly animal foods, lipid peroxides created as a consequence of cooking fatty acids at high temperatures, and advanced glycation end (AGE) products formed from aerobic cooking of protein and carbohydrates together.

Iron intake requires glutathione and has been associated with increased GGT activity. Serum ferritin (an iron storage protein) as well as heme iron consumption from meat have been both positively associated with GGT activity in large cohorts including the EPI-Norfolk cohort and CARDIA (Forouhi et al. 2007; Lee et al. 2004).

Lipid peroxides (LPx) induce glutathione-s-transferase (GST) and require glutathione for metabolism. Human liver microsome ex vivo and animal in vivo research has established lipid peroxidation product 4-hydroxynonal (4-HNE) induces glutathione-s-transferase, and a glutathione conjugate is formed during its metabolism (Awasthi et al. 2005; Sharma et al. 2004; Yang et al. 2003; Fukuda et al. 1997; Huang et al. 2012; Prabhu et al. 2004; Luckey and Petersen 2001; Tjalkens et al. 1999). Specific to human vascular disease, 4-HNE is known to directly increase endothelial permeability and dysfunction, and induce NRF-2 to upregulate glutathione-s-transferase (Yang et al. 2004, 2008; Siow et al. 2007; Usatyuk et al. 2006). Although the induction of NRF-2 by 4-HNE has been argued as “cardioprotection” in cardiac

myocytes (Zhang et al. 2010), sustained exposure of the endothelium to lipid peroxides and 4-HNE appears causative of endothelial dysfunction (Yang et al. 2004, 2008; Siow et al. 2007; Usatyuk et al. 2006) – an exposure we hypothesize is represented by a chronically increased fasting GGT activity compared to those with less exposure to lipid peroxides.

AGE products require glutathione for metabolism via glyoxalase enzyme activity. Metabolism to AGE products requires the availability of reduced glutathione in several known tissues, including the vascular endothelium, and the renal epithelium. Cai et al. elegantly demonstrated incubation of human endothelial cells with AGE immediately caused depletion of reduced glutathione and increase glutathione peroxidase activity (Cai et al. 2002). Notably, hepatic glutathione depletion is known to cause hepatic AGE accumulation and reduced phase 2 conjugation (Masterjohn et al. 2013) because cellular AGE metabolism is facilitated by the glyoxalase enzyme system, which also requires reduced glutathione (Wu et al. 2002).

The Relationship Between GGT and Diet

Support for the validity of GGT as a biomarker of dietary factors comes from multiple cohorts, including the TromsØ cohort study, NHANES, CARDIA, and EPI-Norfolk. The TromsØ cohort measured GGT in over 21,000 men and women and evaluated population determinants of GGT activity (Nilssen et al. 1990). Inverse associations were found between GGT activity and the consumption of coffee, which is known to increase erythrocyte glutathione levels (Esposito et al. 2003). Data from both NHANES III and CARDIA support inverse associations between dietary antioxidant intake (vitamin C, vitamin E, and beta-carotene) and increasing deciles of GGT activity. Increasing quartiles of estimated antioxidant consumption (vitamin C, vitamin E, and beta-carotene) was inversely associated with GGT activity in CARDIA, while in NHANES similar associations were found between increased *serum* concentrations of dietary antioxidant and GGT (Lee et al. 2004; Lim et al. 2007). The cumulative data support *lower* GGT activity with a dietary pattern generally considered "cardiovascularly protective" including: high intakes of vegetables, whole grains, legumes, plus raw, steamed, and poached foods, and lower intakes of refined carbohydrates, saturated fat, animal-based proteins, plus fried, grilled, and broiled foods.

Serum GGT Activity as a Postprandial Indicator

As proof-of-concept of postprandial GGT elevations, we measured GGT in 47 metabolically healthy adult volunteers following a mixed-macronutrient, cooked fast-food meal containing 1,420 kcal, including 74 g of fat (22 g saturated) and 89 g of sugar. Participants were required to consume the entire meal within 30 min. Serum was collected in the fasting state and 1, 2, and 4 h after meal administration. Figure 4a below demonstrates changes in GGT following the meal, including elevation

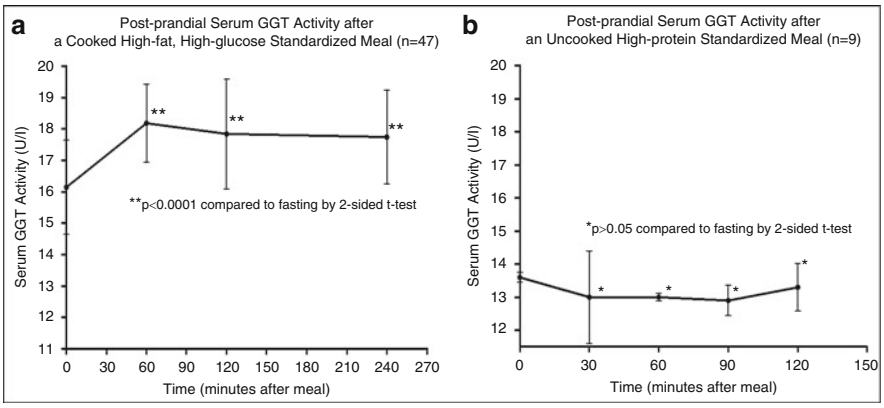

Fig. 4 (**a**, **b**) Postprandial changes in GGT. Changes in serum GGT activity acutely following: cooked high fat/glucose (**a**) versus uncooked high protein mixed-meals in healthy human volunteers (**b**)

compared to baseline persisting until at least 4-h postprandial; the total area under the curve was 371.4 U/l/t. Although these data support GGT elevations occur in the postprandial state, even in insulin-sensitive humans, we were unable to determine the dietary components responsible for this elevation because of the mixed-nutrient nature of the meal administered. We considered the postprandial increase we observed may be a generalized response to eating and therefore conducted an experiment to measure GGT activity following a very different standardized meal, i.e., a body-weight adjusted, uncooked, high-protein shake and snack bar, which led to the data in Fig. 4b, demonstrating no significant increases in postprandial GGT, and in fact a minor reduction; the total area under the curve was −61.50 U/l/t.

Ongoing and Future Research

Although the preliminary data support an interesting potential relationship between serum GGT activity and dietary characteristics – a relationship that, if proven, could provide enormous insight into the mechanisms of diet-induced cardiometabolic disease – there are several research gaps that must be filled. Three research studies are underway to determine: (1) whether acute intake of iron, lipid peroxides, and/or AGE products cause elevations in postprandial serum GGT activity; (2) whether postprandial serum GGT activity correlates to physiologically-measured endothelial function; (3) whether postprandial serum GGT activity varies in metabolic subgroups according to insulin resistance status; and (4) determine whether preprandial administration of NAC modifies any observed increases in GGT.

Our preliminary research (Fig. 4) demonstrates GGT activity increases in the postprandial state *in metabolically healthy humans* following acute intake of a cooked meal containing sugar, fat, iron, lipid peroxides, and AGE products, but does not increase significantly following an uncooked protein meal, leading to our

hypothesis that either the nutritional content of the meal itself, e.g., iron, or by products of the cooking process, i.e., lipid peroxides (LPx) and AGE products, are responsible for the observed increase. The basic science evidence for increases in GGT activity being coupled to glutathione demand in vivo and the dependency on glutathione for the metabolism of iron, lipid peroxides, and AGE products justify the need to systematically evaluate these dietary components individually to determine which components are most highly correlated to acute increases in GGT activity. Our current research is comparing a control meal low in iron, LPx, and AGE products to standardized test meals designed to differentially increase iron, LPx, and AGE products.

Our preliminary data in the MESA cohort (Table 1) demonstrates the very strong correlations between GGT activity and biomarkers of endothelial dysfunction and atherosclerosis (Bradley et al. 2014). These data combined with findings from other groups demonstrating reduced postprandial endothelial function after the acute administration of meals very similar to the meal after which we measured increased GGT activity led to our hypothesis that postprandial increases in GGT activity are concurrent with reductions in endothelial function. As endothelial dysfunction can be caused by increased oxidative stress (with subsequent decline in endothelial nitric oxide synthase product of nitric oxide), and glutathione serves as a critical keystone of in vivo antioxidant defense, it is feasible that increased GGT activity represents acute demand for glutathione in vivo, and if not available in adequate supply, endothelial function may be compromise (if only transiently). This hypothesis will be tested in proposed research by simultaneously measuring postprandial endothelial function via peripheral tonometry and then correlating observed changes between fasting and the postprandial period with the area under the GGT activity curve for the same time period. Independent of our findings related to correlations with GGT activity, the results of the proposed research will provide important data on potentially differential endothelial responses to acute intake of dietary iron, lipid peroxide, and AGE products.

Additionally, our preliminary data in the MESA cohort (Fig. 3) demonstrate the very strong correlations between GGT activity and insulin resistance state, including composite cardiometabolic risk conditions (i.e., the metabolic syndrome) (Bradley et al. 2013). Our data in MESA also demonstrate associations between GGT activity and biomarkers of atherosclerosis (Fig. 2) are attenuated with adjustment for insulin resistance (Bradley et al. 2014). These observations, combined with the findings of Ceriello et al. demonstrating that baseline oxidative stress is higher in insulin resistance participants and that postprandial reductions in endothelial function are greater in insulin resistance participants (Ceriello 2000, 2003), led to our hypothesis that the insulin resistance status of the individual may mediate the amplitude and duration of postprandial increases in GGT activity. This hypothesis will be tested by recruiting three subgroups of human volunteers who vary by degree of insulin resistance (i.e., metabolically normal, prediabetes, and type 2 diabetes) and comparing the area under the postprandial GGT activity curve for each standardized test meal compared to the control meal *within and between each participant subgroup*.

Our preliminary clinical data (Fig. 5) in humans with type 2 diabetes, and the findings of other groups, provide rational for collecting additional data on the impact

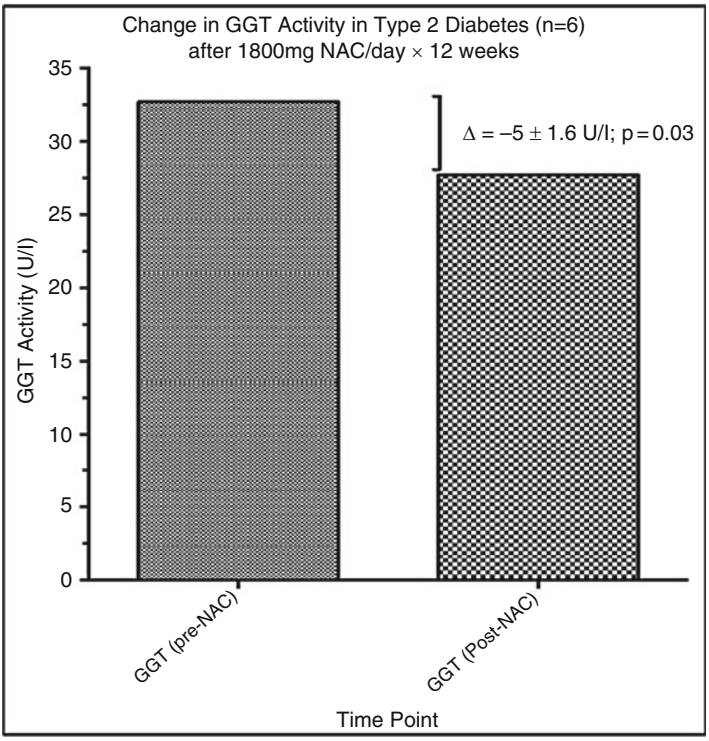

Fig. 5 Changes in GGT activity following treatment with 1,800 mg NAC in humans with type 2 diabetes ($n = 6$). Changes in fasting GGT activity in participants with type 2 diabetes, before and after treatment with 1,800 mg/day of NAC for 12 weeks

of n-acetylcysteine (NAC) on GGT activity. In GGT-knockout animals, treatment of these animals with NAC prevents the development of complications *and* prevents their premature death (Chevez-Barrios et al. 2000). Further support that GGT activity may be modified by NAC, which can contribute to clinically significant changes in metabolism, is represented by our preliminary data in people with type 2 diabetes (see Fig. 4a, b). Specific to our proposal, but untested in humans, pretreatment of human umbilical vein endothelial cells with NAC obliterated the glutathione depleting effects of AGE products (Cai et al. 2002).

Potential Application to Prognosis, Other Diseases, or Conditions

One of the most clinically relevant applications of GGT activity is its potential contribution to the identification of "fat but fit" individuals, or those patients who, despite being obese, are metabolically fit and are at lower risk for developing type

2 diabetes. This concept is supported by the research of Lim et al. in NHANES that demonstrates an important interaction between GGT, BMI, and subsequent risk of developing type 2 diabetes (Lim et al. 2007). Specifically, for those with a BMI between 30 and 34.9, the odds ratios for developing type 2 diabetes according to GGT were: GGT <22 U/l: 1.3; GGT 23–35 U/l: 7.1, and GGT >35: 15.4. For those with a BMI > 35, the odds ratios for developing type 2 diabetes according to GGT were: GGT <22 U/l: 2.4; GGT 23–35 U/l: 11.3, and GGT >35: 19.3. These findings suggest a strong interaction that could be used immediately in clinical practice to identify individuals at potentially extreme risk of developing diabetes.

Additional applications include the emerging utility of GGT subfractionation. Should GGT subfractionation become available for clinical use, the applications include: identification of alcoholism through the measurement of s-GGT, localization of tissue-specific glutathione demands (which could be used to guide route of administration of NAC or other antioxidants), and the identification of glutathione demand especially significant to cardiovascular disease through the measurement of b-GGT. Theoretically, the measurement of GGT subfractions could be used very similarly to current isozyme measurement of other routine clinical lab measures, like alkaline phosphatase, in order to identify its tissue of origin.

Conclusion

The data presented in this chapter, combined with the accumulation of past evidence, support total serum GGT activity as a significant biomarker of cardiovascular and metabolic risk, including strong associations with established individual risk factors for vascular disease, plus composite risk conditions including metabolic syndrome and type 2 diabetes. The established role of GGT in GSH homeostasis supports the need for translational investigations of the behavioral and environmental causes of increased GGT activity in humans, including triggers, regulators, and more precise understanding of its relationship to GSH status in single- and multiorgan pathology.

Our findings in the MESA cohort support the hypothesis that increases in serum GGT activity occur in parallel with increases in oxidative stress, immune activation, acute phase response, and vascular inflammation, and serum GGT activity may represent the influence of metabolic status on vascular injury. These results are consistent with in vivo findings that increased GGT activity represents a reduction in available GSH (Sedda et al. 2008), potentially leaving substrates, e.g., LDL, vulnerable to oxidative modification, which then activate immune targets. Based on the strength and consistency of the associations demonstrated here, combined with current understanding of the oxidative/inflammatory mechanisms of endothelial dysfunction, we conclude serum GGT activity should be considered a continuous biomarker of increased glutathione demand relevant in the development of endothelial dysfunction and subsequent arteriosclerosis.

Although more experimental research is needed, the available research supports a relationship between GGT elevation and dietary patterns long known to be associated with cardiovascular disease. Our group and others are closer to determining dietary characteristics which directly influence GGT activity, which may provide a pathway to more precise research regarding diet-induced "oxidative stress" and the relative contributions of different food types and preparation methods to cardiovascular disease.

Given the importance of redox balance to cellular metabolism in all tissues, it is conceivable that total GGT activity is a biomarker of multiorgan GSH depletion, whereas increases in tissue-specific GGT activity subfractions suggested chronic tissue-specific GSH depletion. Extending this research may even result in the ability to differentiate isolated liver, kidney, vascular disease, as well as, chronic disease with multiple affected organs, i.e., type 2 diabetes. If this differentiation is confirmed by additional experimental research, it would fill an important research gap in population-based cohort studies (Mayne 2003).

The low cost of measuring GGT and its stability in stored samples makes it ideal for continued confirmatory research in large population-based cohort studies. Yet, given the accumulation of population-based research findings suggesting cross-sectional associations with established cardiometabolic risk factors, as well as increased cardiometabolic event prediction from graded elevations in GGT activity, we suggest translational clinical research is now needed to determine the mechanistic determinants of serum GGT activity.

Summary Points

- Increased GGT activity is associated with riskier cardiovascular risk profile, including male gender, smoking, increased alcohol use, hyperlipidemia, hypertension, and insulin resistance.
- Literature supports both epidemiological associations between increased GGT activity (within the normal range) and cardiovascular events, as well as mechanistic biomarkers like C-reactive protein, vascular adhesion molecules, inflammatory cytokines, and oxidized LDLs.
- GGT activity is associated with metabolic risk, including individual risk factors, including elevated BMI and waist circumference, lifestyle factors, as well as clinical risk factors including hemoglobin A1c, fasting glucose, and fasting insulin.
- The odds of prevalent metabolic syndrome and type 2 diabetes increases with elevations in GGT activity.
- Dietary components requiring glutathione for metabolism, including iron, lipid peroxides, and advanced glycation end (AGE) products, are candidate exposures to evaluate for the acute induction of GGT activity and its relationship to atherosclerosis.

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