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Are *Glutathione S*-Transferase Null Genotypes "Null and Void" of Risk for Ischemic Vascular Disease?

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The major risk factors for cardiovascular disease (CVD) relate to intrinsic changes in cholesterol metabolism or blood pressure regulation or to diseases such as diabetes. In addition, it has been found that chemical and pollutant exposure has adverse effects on cardiovascular health. Although we have known about the harmful effects of smoking on heart disease for a long time, recent research shows that exposure to other environmental pollutants such as ambient air particles, automobile exhaust, and pesticides can also increases CVD risk.[1] It is therefore reasonable to expect that CVD risk could be modified by metabolic processes that detoxify foreign chemicals (xenobiotics) or even endogenous toxins (eg, products of lipid peroxidation). The concept that the mechanisms of detoxification regulate disease susceptibility is one of the cornerstones of cancer research; however, only recently have we begun to appreciate the cardiovascular implications of this concept.

For the most part, the relationships between CVD and detoxification mechanisms remain unknown. Glutathione *S*-transferases (GSTs), however, are one exception. These enzymes catalyze the conjugation of glutathione with electrophilic xenobiotics.[2] In Phase II detoxification reactions, they transform xenobiotics into usually less reactive, water-soluble compounds that are excreted in urine or bile. Genetic variations and deletion genotypes of GSTs are relatively prevalent in human populations. Several studies have suggested that variations in GSTs alter CVD risk particularly in smokers, but the data are inconsistent. Some studies report strong interactions between GST genotypes and smoking, whereas others have found no significant association. The current study by Norskov et al,[3] published in this issue of *Circulation: Cardiovascular Genetics*, is the most highly powered epidemiological study to date that investigates the contribution of GSTs to CVD risk specifically for ischemic heart disease (IHD) and cerebrovascular disease.

Norskov et al studied the gene copy number variation (CNV) for 2 GSTs, namely *GSTM1* and *GSTT1*, in 4 different cohorts representing a predominant Caucasian population of Danish descent. Because GSTM1 and GSTT1 proteins are widely expressed throughout the body and occur frequently as null gene polymorphisms in this population, it was hypothesized that null alleles decrease overall GST activity and thus compromise

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detoxification potential, thereby increasing CVD risk. Regression modeling was conducted and hazard ratios for cardiovascular diseases were calculated for all genotypes and for potential interactions between *GSTM1*- and *GSTT1*-null genotypes. Included in these analyses was additional screening for the *GSTP1* polymorphisms (ie, I105V and A114V), albeit with less extensive data. Surprisingly, there were no significant associations between genotypes and CVD risk observed in any of the 4 cohorts alone (or combined) or when adjusted for smoking pack-years (and other risk factors), and similarly negative results were found in a meta-analysis performed using a total of 33 228 control subjects and 13 196 cases. Moreover, as appropriate internal validation controls, the authors observed increased IHD risk with smoking and independently for the expected increase in bladder cancer risk in *GSTM1*-null subjects who smoked.

Although this is a highly powered prospective study, can we conclude that GST proteins do not affect CVD? There are several reasons why we should be cautious in inferring that GSTs (in general) have a limited role in CVD: (1) CNV provides a reliable quantification of gene copies but cannot provide qualitative data regarding protein expression, protein modification, or protein function (eg, activity). Obviously, if a gene is absent (null), then no protein is made, but partial changes in gene expression may not necessarily lead to loss of function. (2) One measure of GST activity is the rate of conjugation of reduced glutathione (GSH) with an electrophilic substrate, for example, an unsaturated aldehyde such as 4-hydroxynonenal (4HNE), yet GST activity varies between substrates. Moreover, there are 16 GST genes coding for GSTs in 6 subclasses: A, alpha, α; Z, zeta, ζ; T, theta, θ; M, mu, μ; P, pi, π; and O, omega, ω; and a few GST gene subclasses such as A and M have multiple gene members, for example, A1-A5 and M1-M5.[2] What is more intriguing is that there is substrate preference within a subclass but also generous substrate overlap between classes (ie, substrate promiscuity). Because we do not know how much either GSTM1 or GSTT1 contributes to GST activity in cardiovascular tissue or which specific substrates confer increased IHD risk, we are hard-pressed to formulate plausible hypotheses from GST-null genotypes.

What can we conclude from these negative findings? The established *GSTM1*-null homozygous genotype in Danish population perhaps indicates a general lack of importance of endogenous M1 protein and/or activity. Similarly, is GSTT1 also unimportant to endogenous metabolism? Although we do not know the effect of these null genotypes on overall GST activity (phenotype) in cardiovascular tissues, we should consider how, when, and why *GSTM1*- and *GSTT1*-null genotypes became fixed in the human genome. There is little question of the integrity of the GST genotyping data and genotype percentages reported are within previously described ranges for *GSTM1*- and *GSTT1*-null genotypes in Caucasian/northern Europeans.[2] In fact, the *GSTM1*-null allele is quite frequent, with 52% homozygous null and 40% heterozygous indicating 92% of Danish subjects lack at least 1 allele. Although GSTT1-null allele is not as frequent, the high frequency of GSTM1- and GSTT1-null alleles does beg the question whether these null genotypes confer any major loss of GST function in cardiomyocytes or coronary vasculature (or anywhere else that matters).

Although previous epidemiological studies show statistically significant associations between *GSTM1*- and -*T1*-null genotypes, environmental/tobacco smoke exposures and CVD risk, the current study does not, but it is internally validated through an observed association between *GSTM1*-null genotype and the risk of urinary bladder cancer in smokers. Interestingly, we observed that the GSTM protein is quite abundant (and probably accounts for high GST activity) in the mouse bladder,[4] and thus GSTM1 is probably important in urinary bladder protection because it is highly expressed in the bladder. Yet, in the current study, no association between either *GSTM1* or *GSTT1* (or genotype interactions) and IHD was observed even among smokers. The explanation for these findings could

simply be that in this cohort the *GSTM1* and *GSTT1* genes are not highly expressed in cardiovascular tissue, and thus variations in the activity of these enzymes do not affect the process of atherogenesis or the frequency of adverse cardiovascular events. It could also be that the chemicals that cause cancer (which are metabolized by GSTs) are not the same ones that cause cardiovascular injury or that the cardiovascular tissues can upregulate other local detoxification processes that the bladder cannot. Clearly, additional work is required to identify the individual role of GSTs in tissue-specific and systemic detoxification and to identify the mechanisms underlying the differential effects of the same insult on different tissues.

How can we reconcile the results of the current study with previously published positive associations between GSTs and CVD risk? Because, in addition to detoxification of chemicals present in tobacco smoke, GSTs also participate in the metabolism of several dietary constituents (eg, isothiocynates in cruciferous vegetables) and environmental pollutants, the role of GSTs varies within the same population as a result of different environmental exposures and dietary habits. Differences between different ethnic populations living in different environments are likely to be even greater sources of variation. In contrast to recently published meta-analysis,[5] which showed positive associations, the meta-analysis presented by Norskov et al revealed no associations. However, the results of their analysis were driven largely by the three studies from Copenhagen, and therefore it remains likely that the lack of association seen in one population in one specific environment may not be seen with another populations living in a different environment.

Meta-analyses of gene-environment interactions with variable outcomes and population demographics are likely to be confounded by the complexities of these relationships. For instance, some studies report that the GST-null genotype is associated with a decrease in myocardial infarction,[6] whereas others find a greater risk associated with this phenotype.[7] Although such inconsistencies could be ascribed to lack of power, differences in study design or exposure misclassification, it is also likely that the differences are real. GSTs are multifunctional proteins, and whereas in one environment they could protect against disease by removing harmful pollutants and toxins, in another environment they could diminish the effects of beneficial food substances. Additionally, even though conjugation by GSTs is generally considered to reduce xenobiotic toxicity, glutathiolation could also activate endogenous electrophiles by imparting to them new biological activities,[8] which in turn could be either beneficial by triggering inflammation or harmful by supporting chronic nonresolving inflammation. Given this complexity, it is important to consider the results of each study in its own context and to design future studies that could address at least some aspects of this complexity. Moreover, a lack of association between specific GSTs and CVD, even if universally applicable, does not rule out the involvement of these enzymes in regulating the disease process or disease risk. Because GSTs are inducible enzymes, induction of one GST could offset the contribution of the other GST-null or polymorphic allele.

Hence, to design more informative population-based studies in future, it may be important to evaluate specific environmental exposures and dietary pattern as well as more quantifiable cardiovascular end points indicative of disease mechanism. In addition, better exposure assessments are required. In their study, Norskov et al calculated maximal accumulated smoking exposure from questionnaires. Smokers who smoked 1 pack of cigarette per day for <1 year were classified as nonsmokers. No direct measurements of cotinine levels were obtained and exposure to secondhand smoke was not considered. Although this classification yielded a significant association between smoking and IHD (hazard ratios between 1.4 to 2.0) and a strong association of *GSTM1*-null genotype with bladder cancer in

smokers, more subtle associations with CVD disease may have been missed because of exposure misclassification.

It is well known that the dose-response relationship between smoking and CVD is nonlinear.[9] Substantial risk is associated with even low-dose exposure. The risk of IHD in light-smokers (1 to 4 cigarettes per day) is 3 times that of a nonsmoker,[10] and even secondhand exposure to smoke is associated with a risk ratio between 1.45 to 1.57, or in other words, passive smoking is associated with 68% to 86% of the risk of light smoking.[11] In comparison, the dose-response relationship between cigarette smoking and cancer shows no threshold level and the risk is monotonically distributed. For instance, for those smoking >20 cigarettes per day, the risk of dying from lung cancer is >23 times higher, whereas those who smoke 1 to 4 cigarette per day have a 3-fold higher risk than nonsmokers.[10] Moreover, the CVD risk associated with smoking occurs rapidly and is diminished quickly on cessation, whereas the cancer risk varies more slowly. Thus, misclassification of brief, low-dose smoke exposures could obscure cardiovascular effects without affecting the robust linear association with cancer. This may be particularly problematic when studying multiple relationships (between smoking, CVD, and CNV) in a population treated with multiple medications. Because xenobiotics affect inflammatory processes, it is difficult to correct statistically the confounding effects of drugs such as statins and aspirin that also affect inflammation.

Designing more informative population studies will also require a better understanding of the metabolic role of GSTs in cardiovascular function. For this, additional well-designed animal studies are needed to identify which specific GSTs are involved in protecting cardiovascular tissues from environmental toxins and pollutants and to delineate the individual contribution of these enzymes to CVD and its clinical complications. Furthermore, we must identify the contexts within which these enzyme could prevent xenobiotic or metabolic injury and when they can activate inert chemicals and metabolites to cause tissue injury and dysfunction. Clearly, the current findings of Norskov et al, which represent a true tour de force, serve as a wake-up call for additional studies to interrogate the role of these fascinating enzymes that can lead us to a better understanding of the mechanisms by which reactive environmental chemicals or endogenous metabolites contribute to heart disease.

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