Published in final edited form as:

Atherosclerosis. 2009 April ; 203(2): 459–465. doi:10.1016/j.atherosclerosis.2008.07.018.

Association of Polymorphisms in Cyclooxygenase (COX)-2 with Coronary and Carotid Calcium in the Diabetes Heart Study

Megan E. Rudock¹, Yongmei Liu^{2,3}, Julie T. Ziegler², Stewart G. Allen³, Allison B. Lehtinen^{1,5}, Barry I. Freedman³, J. Jeffrey Carr⁴, Carl D. Langefeld², and Donald W. Bowden^{1,3,5}

¹ Center for Human Genomics, Wake Forest University School of Medicine, Winston-Salem, North Carolina, 27157, USA

² Dept. of Public Health Sciences, Wake Forest University School of Medicine, Winston-Salem, North Carolina, 27157, USA

³ Dept. of Internal Medicine, Wake Forest University School of Medicine, Winston-Salem, North Carolina, 27157, USA

⁴ Dept. of Radiology, Wake Forest University School of Medicine, Winston-Salem, North Carolina, 27157, USA

⁵ Dept. of Biochemistry, Wake Forest University School of Medicine, Winston-Salem, North Carolina, 27157, USA

Abstract

BACKGROUND—Cardiovascular Disease is the leading cause of death among Americans. Inflammation is a hallmark of the development of atherosclerosis and is mediated by prostaglandins, catalyzed by cyclooxygenase (COX)-2. We sought to determine if variants in the COX-2 gene were associated with subclinical measures of cardiovascular disease in a primarily type 2 diabetic population.

METHODS—Eight polymorphisms in COX-2 were genotyped and vascular calcified plaque measured in the coronary, carotid, and aortic arterial beds in 977 Caucasian siblings (83% with T2DM) from 369 Diabetes Heart Study families. Tests for single SNP and haplotypic association were performed using SOLAR and QPDT, respectively (results adjusted for age, gender, diabetes affection status, smoking, and use of lipid altering medications).

RESULTS—All eight SNPs genotyped were found to be in strong pair-wise linkage disequilibrium ($D' = 1.0$). Three SNPs (rs689466, rs2066826 and rs20417) are associated with either coronary or carotid calcified plaque. Subjects carrying the G allele of rs689466 ($n = 31$) or the A allele of rs2066826 ($n = 16$) had significantly lower coronary calcified plaque ($p = 0.02$ and 0.04 , respectively). Subjects homozygous for the C allele of rs20417 ($n = 22$) or the A allele of rs2066826 ($n = 16$) had increased carotid calcified plaque ($p = 0.011$, $p = 0.014$). In addition, multiple 2-SNP and 3-SNP haplotypes were associated with CorCP with p -values ranging from $P = 0.002$ to $P = 0.035$.

Corresponding Author: Donald W. Bowden, Ph.D., Department of Biochemistry, Wake Forest University School of Medicine, Medical Center Blvd. Winston-Salem, NC 27157, USA, Telephone: 336-713-7507, FAX: 336-713-7566, EMAIL: E-mail: dbowden@wfubmc.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

CONCLUSIONS—Polymorphisms in COX2 were associated with significant changes in coronary and carotid calcified plaque. Diabetic individuals with these variants may be at higher risk for developing cardiovascular disease.

Keywords

Type 2 Diabetes Mellitus; Cardiovascular Disease; Polymorphisms; Inflammation; Vascular Calcification; Cyclooxygenase

Introduction

More than 70 million Americans currently live with cardiovascular disease (CVD) and almost 1 million people die from related events each year. It is estimated that 1 in 3 adult Americans have some form of cardiovascular disease, including high blood pressure, coronary heart disease (myocardial infarction and/or angina pectoris), heart failure, or stroke¹. Inflammation is a hallmark in the development and progression of cardiovascular disease and is mediated by prostaglandins, produced by the enzyme prostaglandin endoperoxide H synthase (more commonly known as cyclooxygenase)². Alterations in prostaglandin expression and/or activity can significantly modify cardiovascular disease risk^{3–5}.

There are at least two COX genes encoding two enzymes: COX-1 and COX-2. COX-1 is expressed constitutively and is involved in production of prostaglandins for cellular housekeeping functions. COX-2 is an inducible form and is associated with injury, inflammation and proliferation^{6,7}. COX-2 derived PGE has been shown to increase in subclinical atherosclerosis and promotes release of matrix metalloproteinases, MMPs, contributing to plaque rupture and CVD events⁸. It has also been shown that COX2 levels are increased in symptomatic vs asymptomatic plaques⁹.

Cipollone et al. found the COX2-765G>C (rs20417) promoter polymorphism to be an inherited protective factor against MI and stroke in 1728 Italian unrelateds¹⁰. However, the same polymorphism was identified in the Atherosclerosis Risk in Communities Study to increase the risk of incident stroke in African Americans^{11,12}. Additionally, COX2 rs20417 has been reported to be associated with reduced COX-2 expression, decreased atherosclerosis and systemic inflammation¹³, as well as T2DM¹⁴. Konheim et al. found intron 6 SNP rs2066826 to be associated with T2DM as well¹⁴.

There is a well known connection between CVD and T2DM, however the mechanism is poorly understood. Type 2 diabetes is a major risk factor in developing cardiovascular disease. With 7–10% of the American population being affected with T2DM and 30% or more affected by CVD, there is a strong need to study this interaction further. The focus of the Diabetes Heart Study is to identify genes that contribute to the development of cardiovascular disease in a diabetes enriched population in order to gain a better understanding of the interaction between these two diseases. COX-2 is a gene that may link these two diseases through the underlying inflammatory pathway involved in their development. While there is little or no literature to suggest a direct role for COX2 in calcification, calcification is widely regarded as evidence of systemic vascular disease²⁰, a primary mediator of which is inflammation, thus in the pathway of COX2 action. The goal of this study is to help further delineate the relationship between polymorphisms in COX-2 and markers of cardiovascular disease.

Subjects and Methods

Study subjects

The study sample consisted of 977 European-American siblings (83% with T2DM) from 369 families in the Diabetes Heart Study. Ascertainment and recruitment have been described previously^{15,16}. Briefly, siblings concordant for T2DM without renal insufficiency were recruited. T2DM was clinically defined as diabetes developing after the age of 35 yr and treated with insulin and/or oral agents, in the absence of historic evidence of ketoacidosis. The general absence of nephropathy was defined as a serum creatinine concentration less than or equal to 1.3 mg/dl (women) or less than or equal to 1.5 mg/dl (men) after a minimum diabetes duration of 5 yr. Metabolic Syndrome was defined using criteria established in the Third Report of the National Cholesterol Education Program Expert Panel Detection, Evaluation and Treatment in Adults (ATP III) as the presence of three or more of the following risk factors: waist circumference > 88 (102) cm in women (men); triglycerides $\geq$ 150 mg/dL; HDL < 50 (40) mg/dL in women (men); blood pressure $\geq$ 130/85 mmHg; and fasting glucose $\geq$ 110 mg/dL or a diagnosis of diabetes¹⁷.

All protocols were approved by the Institutional Review Board of Wake Forest University School of Medicine, and all participants gave informed consent. Participant examinations were conducted in the General Clinical Research Center of the Wake Forest University Baptist Medical Center and included interviews for medical history and health behaviors, anthropometric measures, resting blood pressure, fasting blood sample, and spot urine collection. Laboratory assays included urine albumin and creatinine, total cholesterol, low-density lipoprotein-cholesterol (calculated), high-density lipoprotein-cholesterol, triglycerides, hemoglobinA1c, fasting glucose, calcium, and inorganic phosphate.

Coronary and carotid calcified plaque, CorCP and CarCP respectively, were measured under a standardized protocol¹⁸ as described by Wagenknecht et al.¹⁹. Over the course of this study, three versions of the General Electric computed tomography system (CTi, LightSpeed QXi, and Pro16; General Electric Medical Systems, Waukesha, Wisconsin) capable of 520-, 520-, and 244-millisecond temporal resolutions, respectively, when operating in the cardiac mode have been used. The robustness of the plaque score using various computed tomography systems with different temporal resolutions in this range has been established in standardized protocols used at this and other institutions^{18,20}. In brief, calcified plaque was measured in each of the epicardial coronary arteries (right, left main, left anterior descending and posterior descending) and summed to create the total CorCP burden. Two cardiac scans were performed sequentially, and the average of the two measurements was used. For the carotid examination, an unenhanced computed tomography scan was performed through the right and left carotid bifurcations, and CarCP was measured in four vascular segments: common, bulb, internal, and external. Specifically, the carotid bifurcation level was identified, and the 15 mm of internal and external carotid above the bifurcation and 30 mm of carotid bulb and common carotid artery below the bifurcation were measured. Computed tomographic examinations were analyzed by experienced analysts producing an Agatston score corrected for slice thickness on a GE Advantage Windows Workstation using the Smart-Scores software package (General Electric Medical Systems). The slice thickness was 2.5–3.0 mm for all scans, and the number of adjacent pixels used to define a calcified plaque was one, resulting in a minimum lesion size of 1 mm². The reproducibility of calcified plaque scores in the coronary and carotid arteries was assessed by obtaining duplicate scans. These methods in our hands have a high reproducibility of the calcium score with $r = 0.98$ (Spearman correlation coefficient)¹⁵ and measured inter-reader and intra-reader coefficients are 0.96 or greater.

Genetic analysis

Total genomic DNA was purified from whole-blood samples obtained from subjects using the PUREGENE DNA isolation kit (Gentra, Inc., Minneapolis, MN). DNA was quantitated using standardized fluorometric readings on a Hoefer DyNA Quant 200 fluorometer (Hoefer Pharmacia Biotech, Inc., San Francisco, CA). Each sample was diluted to a final concentration of 5 ng/μl.

Single nucleotide polymorphisms (SNPs) in the COX-2 gene were chosen from public databases to comprehensively cover the 18.56-kb genomic region containing COX-2. Polymorphisms affecting the protein coding sequence were preferentially selected for evaluation. Of the 8 SNPs evaluated, seven were selected from the HapMap database. All of the HapMap SNPs evaluated in this study had a minor allele frequency (MAF) more than or equal to 0.12 in the Centre d'Etude du Polymorphisme Humain Utah residents with ancestry from northern and western Europe (CEU population). The remaining SNP (rs20432) was selected in an effort to maintain consistent SNP spacing across the gene region.

Genotypes were determined using a MassARRAY SNP Genotyping System (Sequenom, Inc., San Diego, CA) as previously described²¹. This genotyping system uses single-base extension reactions to create allele-specific products that are separated automatically and scored in a matrix-assisted laser desorption ionization/time of flight mass spectrometer. Primers for PCR amplification and extension reactions were designed using the MassARRAY Assay Design Software (Sequenom, Inc.).

Statistical analysis

Allele and genotype frequencies for each SNP were calculated from unrelated probands and tested for departure from Hardy-Weinberg equilibrium using a chi square test. Estimates of linkage disequilibrium (LD) between SNPs were determined by calculating pair-wise D' and r^2 statistics in unrelated individuals. To test for an association among the polymorphisms of each individual SNP and each trait, a series of variance component measured genotype (VCMG) models as implemented in SOLAR²² were computed. Likelihood ratio tests based on the correlation structure suggested by the familial relationships was used²². For each SNP, the 2 degree of freedom (df) test of genotypic association with each phenotype was performed. If there was evidence of a significant association ($P < 0.05$), three individual contrasts defined by the *a priori* genetic models (dominant, additive, and recessive) were computed. This is consistent with the Fisher's protected least significant difference multiple comparison procedure. All effects were estimated while adjusting for age, gender, diabetes affection status (yes/no), smoking status (current/past/never), and use of lipid-lowering medications (yes/no). Phenotypes included in these analyses were transformed to approximate conditional normality and to reduce heterogeneity of residual phenotypic variance across SNP genotypes. The quantitative pedigree disequilibrium test (QPDT)²³ using one-, two-, and three-marker moving windows was performed to assess for association between alleles (or haplotypes) and CorCP or CarCP. Haplotype frequencies were estimated using the expectation-maximization algorithm to account for phase uncertainty and the corresponding likelihood ratio test was computed. The QPDT and VCMG models included the clinical covariates age, gender, smoking status, and use of lipid-lowering medications. Unlike the population-based VCMG approach, the family-based QPDT analysis is modestly conservative in the presence of population stratification and is generally valid even under population admixture. These exhaustive haplotype analyses were not corrected for multiple comparisons.

Results

The clinical characteristics of the 977 European-American subjects are presented in Table 1. The average age of the diabetes-affected participants was 62 yr and 51% were female. The non-diabetic subjects had an average age of 59.6 with 62.3% females. Metabolic syndrome¹⁷ affects 89.6% of the diabetes-affected subjects and 49.1% of the non-diabetic subjects, thus showing significant risk factors even in non-diabetic relatives of the T2DM subjects. Of the diabetes-affected subjects, 94.8% have CorCP greater than 0 and 78.2% have CarCP greater than 0. CorCP and CarCP are less frequent in the non-diabetic subjects, with 84.4% and 58.7% respectively having scores greater than 0. The general phenotypic correlation ($r=0.562$), as well as the corresponding genetic correlation ($r_g=0.52 \pm 0.11$) and environmental correlation ($r_e=0.37 \pm 0.07$), between CorCP and CarCP are all statistically significant ($P < 0.05$).

Power calculations assuming 700 individuals with complete genotypic and phenotypic data suggest that a SNP with MAF of 0.20 provides approximately 80% power at $\alpha = 0.05$ to observe a 0.22 change of a standard deviation under a dominant genetic model in unrelated individuals. This analysis suggests that the DHS has the power to detect clinically meaningful differences in quantitative measures of vascular function.

DNA from the DHS participants was genotyped for eight SNPs that were chosen across the 18.65kb genomic region containing *COX-2* (Figure 1). Of the eight SNPs, seven were selected from the HapMap database (<http://www.hapmap.org>)²⁴ and have a MAF ≥ 0.12 in the HapMap CEU population. The ability of these SNPs to capture genotypic and haplotypic variation was assessed using the greedy pairwise tagging algorithm implemented in the Tagger program²⁵ of Haploview²⁶. The seven HapMap SNPs capture 0.67 of the genetic variation within this region as defined by r^2 .

The mean genotyping success rate for the 8 SNPs evaluated was 98.4%, with rs20417 having the lowest success rate (97.3%) and rs2066826 having the highest success rate (99.2%). The genotyping consensus rate for duplicate DNA samples within and across DNA plates was 100% for all SNPs. Allele and genotype frequencies were consistent with those expected under Hardy-Weinberg equilibrium. All eight of the SNPs genotypes were contained in a single block of LD (Figure 2) as defined by the D' statistic using confidence interval²⁷, 4-gamete rule²⁸ and solid spine algorithms implemented in Haploview²⁶.

Initially genotype data were analyzed for association with measures of subclinical cardiovascular disease, including both CorCP and CarCP, using the 2df test for overall genotypic association (Table 2a). These data show evidence of association between CorCP and rs689466 ($P=0.046$) and rs2066828 ($P=0.057$), and among CarCP and rs20417 ($P=0.039$) and rs2066826 ($P=0.049$). None of these results remain statistically significant after adjusting for a sequential Bonferroni multiple comparison adjustment.

The three significant, and one near-significant, associations by the 2df test were further evaluated using the dominant, additive and recessive genetic models in both SOLAR (Table 2b) and GEE1 (data not shown). The promoter SNP (rs689466) was associated with CorCP under the dominant ($P = 0.017$) and additive ($P=0.013$) genetic models, while the intron 6 SNP (rs2066828) was most strongly associated under the dominant model only ($P = 0.037$). The mean CorCP scores support the dominant model for rs2066826 because only the minor allele homozygotes (AA) have significantly increased mean CorCP scores (2086), while the other two genotypes (GG/GA) have similar means (1235 and 952 for rs2066826). The strongest evidence for association of the promoter SNP rs20417 was obtained under the recessive model with respect to the minor allele (C; $P = 0.014$). Likewise the intron 6 SNP rs2066826 was most strongly associated with CarCP under the recessive model with respect to the minor allele (A; $P = 0.014$). In both cases, the presence of the minor allele homozygote corresponded to a

significant increase in mean carotid calcified plaque score. We note that although the number of homozygotes of the minor allele in these two tests provides an appropriate test, some caution is merited.

To test whether a haplotype would provide increased evidence of association over the individual SNP associations with CorCP and CarCP, QPDT was used to evaluate one-, two-, and three-marker moving windows across the 8 SNPs in *COX-2*. Three single SNPs and three 2-SNP haplotypes were associated with CorCP with p-values ranging from $P = 0.002$ to $P = 0.032$ (Table 3.). CorCP was also associated with one 3-SNP haplotype and trending towards association with another. The rs5275+rs2745557 2-SNP haplotype was associated with CorCP ($P = 0.005$). The GA haplotype for these SNPs was associated with an increase in CorCP (Z-score = 2.419; $P = 0.016$), while the GG haplotype was associated with decreased CORCP (Z-score = -2.929; $P = 0.003$). A similar association was seen with the rs20417+rs689466 haplotype ($P = 0.002$). Here the GA haplotype was associated with an increase in CorCP (Z-score = 3.126; $P = 0.002$) and the GG haplotype associated with decreased CorCP (Z-score = -2.896; $P = 0.004$). Similar results were seen with three SNP haplotypes including rs5277+rs2745557+rs20417 or rs2745557+rs20417+rs689466. For the haplotype rs5277+rs2745557+rs20417, individuals with a GGG genotype had decreased CorCP (Z-score = -2.109; $P = 0.035$) and individuals with the genotype GAG had a significant increase in CorCP (Z-score = 2.436; $P = 0.015$). Interestingly, this suggests that the haplotypic association of these three SNPs is being driven by the rs2745557, which was not individually associated with CorCP. Furthermore, the strong haplotypic associations remain significant after adjusting for multiple tests using the sequential Bonferroni. QPDT was also used to evaluate SNP association with CarCP, however there were no significant associations (data not shown).

Discussion

Genetic evaluation of 8 SNPs in the *COX2* gene in the Diabetes Heart Study European-American families enriched for T2DM reveals association between SNPs in *COX2* and CorCP, independent of the clinical covariates age, gender, smoking status, and use of lipid lowering medications (Table 1). The SNP tagging algorithm implemented in this study suggests that the 8 SNPs evaluated capture a fairly high proportion of the HapMap variation within this region (mean $r^2 = 0.666$). The 8 SNPs that were evaluated in *COX2* are in high LD (Fig 2.). One SNP was marginally associated with CorCP ($P = 0.046$) and another was trending toward significance ($P = 0.057$) and two SNPs were modestly associated with CarCP ($P = 0.039$, $P = 0.049$). No single multiple comparison approach is generally accepted for *a priori* candidate genes with multiple correlated SNPs and correlated traits. Since this study was based on an *a priori* hypothesis of *COX2* association with measures of subclinical CVD, we report the unadjusted results. The single tagging SNP results provide evidence of association that would not survive a multiple comparison adjustment, but the haplotypic associations provide strong evidence that would survive multiple comparison adjustment. This is not surprising since the tagging SNPs were selected to capture the haplotypic information within the gene and not due to *a priori* functional relevance. Again, we note that the results from the association analysis with carotid calcified plaque analyses which suggest a recessive mode of inheritance for these two SNPs are based upon a relatively small number of minor allele homozygotes (22 for rs20417 and 16 for rs2066826) and should be viewed with caution.

Genetic association of *COX2* has been reported with diabetes¹⁴, measures of atherosclerosis and cardiovascular disease¹³, myocardial infarction¹⁰, stroke^{10,11}, and asthma^{29,30}, as well as several types of cancer^{31–33}. Papafili et al. genotyped the *COX2* rs20417 (-765G>C) polymorphism in patients that were subjected to coronary artery bypass graft surgery and healthy UK controls and found that the level of C-reactive protein was strongly genotype dependent. Compared with the GG homozygotes, patients carrying the C allele had

significantly lower plasma CRP levels ($P < 0.05$)³⁴. The evaluation of CRP levels in the DHS population was unable to replicate this finding. Orbe et al., examined the rs20417 (G-765C) polymorphism in asymptomatic subjects free from CVD in relation to atherosclerosis and inflammatory markers¹³. Subjects that were hypercholesterolemic and carriers of the rs20417 C allele had lower *COX2* expression ($p < 0.05$), reduced carotid IMT ($p < 0.01$), and diminished levels of inflammatory markers (CRP, vWF and IL-6; $p < 0.05$) compared with GG homozygotes. The rs20417 polymorphism was also examined in African Americans of the ARIC study¹¹. Here carriers of the C allele had a hazard rate ratio of 1.34 for developing stroke, when compared to the GG homozygotes ($P = 0.03$). Furthermore, the association of *COX2* rs20417 with MI and stroke was investigated in a matched case-control study of patients with first MI or atherothrombotic ischemic stroke¹⁰. The C allele was more prevalent among controls than cases, with prevalence ratios for MI or stroke equal to 0.48 for GC genotypes and 0.33 for CC genotypes. Here the C allele of rs20417 was associated with decreased risk of MI and stroke. However, Hegener et al. was unable to replicate any association of rs20417 with MI or stroke in a group of 600 males with incident myocardial infarction (MI) or ischemic stroke and 600 age/smoking matched male controls from the Physicians' Health study³⁵. Current results regarding the *COX2* polymorphism rs20417 are inconsistent across studies. We were unable to detect an association between *COX2* SNP rs20417 and coronary or carotid calcified plaque in the Diabetes Heart Study population (although modest association is seen within a haplotype and with CarCP). There is little reported regarding association of other variants in this gene, warranting further investigation of *COX2* variants.

To date, no studies have examined the association of *COX2* variants with measures of vascular calcification. Of the three polymorphisms associated with coronary and/or carotid calcium in our study, only two were associated with other phenotypes in other studies. The *COX2* promoter polymorphism rs20417 (also reported as G-765C and G926C) was significantly associated with CRP levels in coronary bypass graft patients from the UK³⁴, T2DM in Pima Indians¹⁴, carotid IMT, CRP, and IL-6 in hypercholesterolemic patients from Spain¹³; as well as stroke and MI in African Americans¹¹ and Italians¹⁰. To our knowledge this study is the first examination of the *COX2* using a tagging SNP approach. While the design, sample size, and measures examined in this study are largely different from previous studies, we observe evidence for association of measures of subclinical CVD with *COX2* SNPs consistent with prior observations that genetic variability in this gene contributes to variation in bio-medically important traits.

Haplotype analysis as implemented in QPDT provides us with evidence of association due to inheritance within families. Two SNPs, rs5277 and rs274557, were marginally associated with CorCP in QPDT ($P = 0.034$ and $P = 0.024$, respectively), but not using the variance components models implemented in SOLAR. Furthermore, the SNP rs2066826 was associated with CorCP in SOLAR, but not in QPDT. This is likely due to methodological differences between these two analytical tests. It is also necessary to note that the genotypic means for the minor allele homozygotes of rs689466 (GG) and rs2066826 (AA), as seen in the CorCP association results (Table 2b) are inconsistent with the results from analysis using both QPDT and VCMG in SOLAR. We have reported that the G allele of rs689466 and the A allele of rs2066826 are associated with a decrease in coronary calcified plaque under the dominant genetic model. Evidence for the direction of the association is consistent between the z-scores produced with QPDT, the beta values in SOLAR, as well as the genetic models. The inconsistency of the means is likely due to the small number of individuals contributing to the mean. However, it is encouraging that the median CorCP scores are significantly decreased (AA = 247 vs. GG = 151 for rs689466), and are consistent with the direction of the association.

The two SNPs rs2066826 and rs20417 were associated with an increase in CarCP, as seen in the recessive model. This is inconsistent with current literature and opposite of the direction

of the association with CorCP. Due to the low number of minor allele homozygotes for these two SNPs and the recessive mode of inheritance, this data must be viewed with extreme caution. The genotypic means are consistent with a recessive genetic model; however the standard deviations are quite large. Therefore we choose to place more emphasis on the CorCP results, as they provide stronger evidence of association with COX-2.

Overall, the association results as seen from analysis in SOLAR are broadly consistent with QPDT. Evidence of association with COX2 SNPs with measures of subclinical cardiovascular disease is broadly consistent with the extensive literature in this area of investigation. The direction of association may be inconsistent with previous studies for several reasons. Many of the previous investigations of COX2 association with cardiovascular measures were in significantly smaller populations and may be under-powered. Furthermore, most of the prior studies examined a very limited number of SNPs and did not use tagging SNPs to capture a significant portion of the genotypic and haplotypic variation in this gene.

As with any study of this type, there are limitations in addition to those summarized above. Although the body of literature showing that vascular calcified plaque is an independent predictor of CVD events (e.g. Detrano et al. 2008) continues to increase, we have not assessed prevalent disease in this study. It should be emphasized that this study is focused on subclinical measures of vascular disease rather than prevalent clinical disease. While there advantages of this study design, such as power, these are surrogate measures of CVD.

In summary, the association of COX2 single nucleotide polymorphisms with measures of subclinical cardiovascular disease has been investigated in the Diabetes Heart Study population. Several SNPs have proven to be associated with both coronary and carotid calcified plaque. The direction of the association appears to be inconsistent with current literature. We were unable to replicate previous association of rs20417 with measures of cardiovascular disease. However, our findings are consistent across two distinct tests of association, the population-based VCMG approach implemented in SOLAR and the family-based QPDT analysis. Furthermore, the strong haplotypic association from QPDT analysis is robust to sequential Bonferroni correction for multiple comparisons. It is known that the actions of COX2 are varied. Here we have shown that variants of COX2 are associated with measures of subclinical cardiovascular disease, specifically coronary and carotid calcified plaque, in a type 2 diabetes population. The complex involvement of COX2 in inflammation and CVD truly warrants further investigation.

References

1. Rosamond W, Flegal K, Friday G, Furie K, Go A, Greenlund K, Haase N, Ho M, Howard V, Kissela B, Kittner S, Lloyd-Jones D, McDermott M, Meigs J, Moy C, Nichol G, O'Donnell CJ, Roger V, Rumsfeld J, Sorlie P, Steinberger J, Thom T, Wasserthiel-Smoller S, Hong Y. Heart disease and stroke statistics--2007 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation* 2007;115:e69–171. [PubMed: 17194875]
2. Belton O, Byrne D, Kearney D, Leahy A, Fitzgerald DJ. Cyclooxygenase-1 and -2-dependent prostacyclin formation in patients with atherosclerosis. *Circulation* 2000;102:840–845. [PubMed: 10952950]
3. Davidge ST. Prostaglandin H synthase and vascular function. *Circ Res* 2001;89:650–660. [PubMed: 11597987]
4. Grosser T, Fries S, FitzGerald GA. Biological basis for the cardiovascular consequences of COX-2 inhibition: therapeutic challenges and opportunities. *J Clin Invest* 2006;116:4–15. [PubMed: 16395396]
5. Antman EM, DeMets D, Loscalzo J. Cyclooxygenase inhibition and cardiovascular risk. *Circulation* 2005;112:759–770. [PubMed: 16061757]

6. Hla T, Neilson K. Human cyclooxygenase-2 cDNA. *Proc Natl Acad Sci U S A* 1992;89:7384–7388. [PubMed: 1380156]
7. Tazawa R, Xu XM, Wu KK, Wang LH. Characterization of the genomic structure, chromosomal location and promoter of human prostaglandin H synthase-2 gene. *Biochem Biophys Res Commun* 1994;203:190–199. [PubMed: 8074655]
8. Cipollone F, Fazia M, Iezzi A, Ciabattoni G, Pini B, Cuccurullo C, Uchino S, Spigonardo F, De Luca M, Prontera C, Chiarelli F, Cuccurullo F, Mezzetti A. Balance Between PGD Synthase and PGE Synthase Is a Major Determinant of Atherosclerotic Plaque Instability in Humans. *Arterioscler Thromb Vasc Biol* 2004;24:1259–1265. [PubMed: 15155382]
9. Cipollone F, Prontera C, Pini B, Marini M, Fazia M, De Cesare D, Iezzi A, Uchino S, Boccoli G, Saba V, Chiarelli F, Cuccurullo F, Mezzetti A. Overexpression of Functionally Coupled Cyclooxygenase-2 and Prostaglandin E Synthase in Symptomatic Atherosclerotic Plaques as a Basis of Prostaglandin E2-Dependent Plaque Instability. *Circulation* 2001;104:921–927. [PubMed: 11514380]
10. Cipollone F, Toniato E, Martinotti S, Fazia M, Iezzi A, Cuccurullo C, Pini B, Ursi S, Vitullo G, Aversa M, Arca M, Montali A, Campagna F, Uchino S, Spigonardo F, Taddei S, Virdis A, Ciabattoni G, Notarbartolo A, Cuccurullo F, Mezzetti A. A polymorphism in the cyclooxygenase 2 gene as an inherited protective factor against myocardial infarction and stroke. *JAMA* 2004;291:2221–2228. [PubMed: 15138244]
11. Kohsaka S, Volcik KA, Folsom AR, Wu KK, Ballantyne CM, Willerson JT, Boerwinkle E. Increased risk of incident stroke associated with the cyclooxygenase 2 (COX-2) G-765C polymorphism in African-Americans: The Atherosclerosis Risk in Communities Study. *Atherosclerosis*. 2007
12. Lee CR, North KE, Bray MS, Couper DJ, Heiss G, Zeldin DC. Cyclooxygenase Polymorphisms and Risk of Cardiovascular Events: The Atherosclerosis Risk in Communities (ARIC) Study. *Clin Pharmacol Ther*. 2007
13. Orbe J, Belouqui O, Rodriguez JA, Belzunze MS, Roncal C, Paramo JA. Protective effect of the G-765C COX-2 polymorphism on subclinical atherosclerosis and inflammatory markers in asymptomatic subjects with cardiovascular risk factors. *Clin Chim Acta* 2006;368:138–143. [PubMed: 16458279]
14. Konheim YL, Wolford JK. Association of a promoter variant in the inducible cyclooxygenase-2 gene (PTGS2) with type 2 diabetes mellitus in Pima Indians. *Hum Genet* 2003;113:377–381. [PubMed: 12920574]
15. Wagenknecht LE, Bowden DW, Carr JJ, Langefeld CD, Freedman BI, Rich SS. Familial aggregation of coronary artery calcium in families with type 2 diabetes. *Diabetes* 2001;50:861–866. [PubMed: 11289053]
16. Lange LA, Bowden DW, Langefeld CD, Wagenknecht LE, Carr JJ, Rich SS, Riley WA, Freedman BI. Heritability of carotid artery intima-medial thickness in type 2 diabetes. *Stroke* 2002;33:1876–1881. [PubMed: 12105369]
17. Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III). *JAMA* 2001;285:2486–2497. [PubMed: 11368702]
18. Carr JJ, Nelson JC, Wong ND, McNitt-Gray M, Arad Y, Jacobs DR Jr, Sidney S, Bild DE, Williams OD, Detrano RC. Calcified coronary artery plaque measurement with cardiac CT in population-based studies: standardized protocol of Multi-Ethnic Study of Atherosclerosis (MESA) and Coronary Artery Risk Development in Young Adults (CARDIA) study. *Radiology* 2005;234:35–43. [PubMed: 15618373]
19. Wagenknecht LE, Langefeld CD, Freedman BI, Carr JJ, Bowden DW. A Comparison of Risk Factors for Calcified Atherosclerotic Plaque in the Coronary, Carotid, and Abdominal Aortic Arteries: The Diabetes Heart Study. *Am J Epidemiol*. 2007
20. Detrano RC, Anderson M, Nelson J, Wong ND, Carr JJ, McNitt-Gray M, Bild DE. Coronary calcium measurements: effect of CT scanner type and calcium measure on rescan reproducibility--MESA study. *Radiology* 2005;236:477–484. [PubMed: 15972340]
21. Bensen JT, Langefeld CD, Hawkins GA, Green LE, Mychaleckyj JC, Brewer CS, Kiger DS, Binford SM, Colicigno CJ, Allred DC, Freedman BI, Bowden DW. Nucleotide variation, haplotype structure, and association with end-stage renal disease of the human interleukin-1 gene cluster. *Genomics* 2003;82:194–217. [PubMed: 12837270]

22. Almasy L, Blangero J. Multipoint quantitative-trait linkage analysis in general pedigrees. *Am J Hum Genet* 1998;62:1198–1211. [PubMed: 9545414]
23. Dudbridge F. Pedigree disequilibrium tests for multilocus haplotypes. *Genet Epidemiol* 2003;25:115–121. [PubMed: 12916020]
24. A haplotype map of the human genome. *Nature* 2005;437:1299–1320. [PubMed: 16255080]
25. de Bakker PI, Yelensky R, Pe'er I, Gabriel SB, Daly MJ, Altshuler D. Efficiency and power in genetic association studies. *Nat Genet* 2005;37:1217–1223. [PubMed: 16244653]
26. Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 2005;21:263–265. [PubMed: 15297300]
27. Gabriel SB, Schaffner SF, Nguyen H, Moore JM, Roy J, Blumenstiel B, Higgins J, DeFelice M, Lochner A, Faggart M, Liu-Cordero SN, Rotimi C, Adeyemo A, Cooper R, Ward R, Lander ES, Daly MJ, Altshuler D. The structure of haplotype blocks in the human genome. *Science* 2002;296:2225–2229. [PubMed: 12029063]
28. Wang N, Akey JM, Zhang K, Chakraborty R, Jin L. Distribution of recombination crossovers and the origin of haplotype blocks: the interplay of population history, recombination, and mutation. *Am J Hum Genet* 2002;71:1227–1234. [PubMed: 12384857]
29. Szczeklik W, Sanak M, Szczeklik A. Functional effects and gender association of COX-2 gene polymorphism G-765C in bronchial asthma. *J Allergy Clin Immunol* 2004;114:248–253. [PubMed: 15316498]
30. Shi J, Misso NL, Duffy DL, Thompson PJ, Kedda MA. A functional polymorphism in the promoter region of the cyclooxygenase-2 gene is not associated with asthma and atopy in an Australian population. *Clin Exp Allergy* 2004;34:1714–1718. [PubMed: 15544595]
31. Zhang X, Miao X, Tan W, Ning B, Liu Z, Hong Y, Song W, Guo Y, Zhang X, Shen Y, Qiang B, Kadlubar FF, Lin D. Identification of functional genetic variants in cyclooxygenase-2 and their association with risk of esophageal cancer. *Gastroenterology* 2005;129:565–576. [PubMed: 16083713]
32. Kang S, Kim YB, Kim MH, Yoon KS, Kim JW, Park NH, Song YS, Kang D, Yoo KY, Kang SB, Lee HP. Polymorphism in the nuclear factor kappa-B binding promoter region of cyclooxygenase-2 is associated with an increased risk of bladder cancer. *Cancer Lett* 2005;217:11–16. [PubMed: 15596291]
33. Panguluri RC, Long LO, Chen W, Wang S, Coulibaly A, Ukoli F, Jackson A, Weinrich S, Ahaghotu C, Isaacs W, Kittles RA. COX-2 gene promoter haplotypes and prostate cancer risk. *Carcinogenesis* 2004;25:961–966. [PubMed: 14754878]
34. Papafili A, Hill MR, Brull DJ, McAnulty RJ, Marshall RP, Humphries SE, Laurent GJ. Common promoter variant in cyclooxygenase-2 represses gene expression: evidence of role in acute-phase inflammatory response. *Arterioscler Thromb Vasc Biol* 2002;22:1631–1636. [PubMed: 12377741]
35. Hegener HH, Diehl KA, Kurth T, Gaziano JM, Ridker PM, Zee RY. Polymorphisms of prostaglandin-endoperoxide synthase 2 gene, and prostaglandin-E receptor 2 gene, C-reactive protein concentrations and risk of atherothrombosis: a nested case-control approach. *J Thromb Haemost* 2006;4:1718–1722. [PubMed: 16879213]

Figure 1.

Genomic map of the *COX-2* gene with locations of the 8 genotyped SNPs. The *full-length shaded regions* are exons, numbered 1–10. The *half-length shaded regions* represent the 5'- and 3'-untranslated regions. The *ruler* along the *bottom* represents the location and spacing of exons and SNPs in kilobases within the 18.65-kb region containing *COX-2*. The *arrow* above *COX-2* indicates the direction of transcription.

Figure 2.
LD (D') between the 8 SNPs in *COX-2* from Haploview.

Table 1

Demographic characteristics of DHS European-American participants.

Phenotype	Diabetes-affected subjects	Nondiabetic subjects
<i>n</i>	810	167
Sex (% female)	51.0	62.3
Age (years)	62.0 ± 9.3	59.6 ± 10.0
Metabolic syndrome affected (%) (n = 808)	89.6	49.1
Self-reported prevalent CVD (CVD1) (%) (n = 393)	46.4	23.7
Self-reported prevalent CVD (CVD2) and/or CCP >400 (%) (n = 589)	67.2	41.8
CCP >0 (%) (n = 731)	94.8	84.4
CarCP >0 (%) (n = 667)	78.2	58.7
Hypertension (hypertensive or taking medication) (%)	71.0	44.2
Duration of diabetes for affected (years)	10.4 ± 7.4	NA
BMI (kg/m ²)	32.4 ± 6.8	28.8 ± 5.1
Waist circumference (cm)	107.6 ± 17.9	95.8 ± 13.6
Total cholesterol (mg/dl)	187.1 ± 43.5	195.0 ± 34.7
Non-HDL cholesterol (mg/dl)	144.9 ± 42.3	146.9 ± 33.1
HDL cholesterol (mg/dl)	42.2 ± 12.0	47.9 ± 13.7
LDL cholesterol (mg/dl)	103.8 ± 31.6	114.1 ± 29.3
Triglycerides (mg/dl)	213.9 ± 142.2	165.0 ± 83.4
HbA1C (%)	7.7 ± 1.8	5.6 ± 0.51
Fasting glucose (mg/dl)	150.8 ± 58.1	95.0 ± 14.8
C-reactive protein (mg/dl)	1.10 ± 0.30	1.02 ± 0.21

Data are means ± SD or means.

Table 2
a) Association analysis: *P* values for overall genotypic associations of *COX-2* SNPs with Coronary and Carotid calcified plaque in European-American DHS participants. b) Association analysis: untransformed, unadjusted summary statistics of *COX-2* SNPs with Coronary and Carotid calcified plaque in European-American DHS participants.

A								
SNP	Chromosomal position (bp)	Location in gene	Alleles (major/minor)	Minor allele frequency	CCP <i>P</i> ^a	CarCP <i>P</i> ^a		
rs689466	Chr1:183382408	Promoter	A/G	0.18	0.046	0.450		
rs20417	Chr1:183381844	Promoter	C/G	0.32	0.583	0.039		
rs2745557	Chr1:183380878	Intron 1	G/A	0.12	0.163	0.770		
rs5277	Chr1:183379854	Intron 3	G/C	0.14	0.569	0.110		
rs20432	Chr1:183377980	Intron 5	T/G	0.15	0.114	0.186		
rs2066826	Chr1:183377584	Intron 6	G/A	0.18	0.057	0.049		
rs5275	Chr1:183374715	Exon 10	T/C	0.15	0.865	0.180		
rs10911902	Chr1:183363974	Downstream of COX-2	C/T	0.22	0.505	0.582		
B								
p-values*								
SNP	Genotype	n	Mean	SD	Overall	Dominant	Additive	Recessive
Coronary Calcified Plaque								
rs689466	AA	449	1188	2478	0.046	0.0170	0.0134	0.2371
	AG	260	1143	2395				
	GG	31	1701	2530				
rs2066826	GG	584	1235	2463	0.057	0.0372	0.1041	0.5477
	GA	142	952	1772				
	AA	16	2086	5515				
Carotid Calcified Plaque								
rs20417	GG	593	329	702	0.039	0.9236	0.4756	0.0141
	GC	188	256	483				
	CC	22	649	1117				
rs2066826	GG	643	320	680	0.049	0.6751	0.2604	0.0143

B						
SNP	Genotype	n	Mean	SD	p-values*	
					Overall	Dominant Additive Recessive
	GA	156	260	511		
	AA	16	850	1257		

P values in *bold* indicate significant association at the $P \leq 0.05$ level.

^a Adjusted for age, gender, diabetes affection status, smoking status, and use of lipid-lowering medications.

Table 3

QPDIT haplotype analysis of COX-2 SNPs with CorCP.

	Haplotype	Frequency	Z score ^a	P value ^b	Global P value
1-SNP					
	rs5277				0.034
	G	0.845	-2.126	-	
	C	0.156	2.126	-	
rs2745557					0.024
	G	0.800	-2.262	-	
	A	0.200	2.262	-	
rs689466					0.002
	A	0.786	3.029	-	
	G	0.214	-3.029	-	
2-SNP					
	rs5277+rs2745557				0.005
	GG	0.693	-2.929	0.003	
	GA	0.176	2.419	0.016	
	CG	0.131	1.137	0.256	
rs2745557+rs20417					0.032
	GG	0.656	-1.627	0.104	
	GC	0.143	-0.506	0.613	
	AG	0.197	2.478	0.013	
	AC	0.003	-1.640	0.101	
rs20417+rs689466					0.002
	GA	0.687	3.126	0.002	
	GG	0.193	-2.896	0.004	
	CA	0.12	-0.864	0.387	
3-SNP					
	rs5277+rs2745557+rs20417				0.029
	GGG	0.555	-2.109	0.035	
	GGC	0.144	-0.397	0.691	
	GAG	0.180	2.436	0.015	
	CGG	0.118	0.534	0.593	

	Haplotype	Frequency	Z score ^a	P value ^b	Global P value
rs2745557+rs20417+rs689466	GGA	0.521	0.644	0.520	0.059
	GGG	0.162	-2.018	0.044	
	GCA	0.130	-0.346	0.729	
	AGA	0.185	2.028	0.043	

Ten 2-SNP haplotypes and eight 3-SNP haplotypes spanning the genomic region from the promoter to intron 3 were observed in European-American subjects, seven of which were associated with CorCP. P values in *bold* indicate significant association at the $P \leq 0.05$ level. P values in *italics* indicate haplotypes trending toward association at the $0.05 < P \leq 0.07$ level.

^a A positive Z score reflects an increase in the mean CorCP score, whereas a negative Z score reflects a decrease in the mean CorCP score.

^b Adjusted for age, gender, diabetes status, smoking status, and use of lipid lowering medications.