Association Between A Leukotriene C₄ Synthase Gene Promoter Polymorphism and Coronary Artery Calcium in Young Women

The Muscatine Study

David M. Iovannisci, Edward J. Lammer, Lori Steiner, Suzanne Cheng, Larry T. Mahoney, Patricia H. Davis, Ronald M. Lauer, Trudy L. Burns

Objective—A majority of the recognized risk factors for atherosclerosis and the development of cardiovascular disease have been derived from the study of older populations who have already manifested clinical symptoms. If risk factors can be identified earlier in life, such as genetic variation, preventive measures may be taken before overt symptoms of pathology have manifested, and when treatments may be most effective.

Methods and Results—In an effort to identify individuals at increased risk for cardiovascular disease, we genotyped 732 members of the Muscatine Study Longitudinal Adult Cohort for candidate genetic markers associated with several pathogenetic processes. We identified age-adjusted increased risks for coronary artery calcium (OR 4.29; 95% CI 1.78, 10.31) and increased mean carotid artery intimal-medial thickness associated with the (−444)A>C promoter polymorphism of Leukotriene C₄ Synthase (LTC₄S) in women. There were no similar associations in men.

Conclusions—LTC₄S plays a key role in the process of inflammation as the rate limiting enzyme in the conversion of arachidonic acid to cysteinyl-leukotrienes, important mediators of inflammatory responses. The (−444)C variant upregulates LTC₄S mRNA expression, increasing the synthesis of proinflammatory leukotrienes. Our results support genetic variation modifying inflammatory pathways as an important mechanism in the development of atherosclerosis.

Key Words: atherosclerosis ■ epidemiology ■ coronary artery calcification ■ calcification ■ leukotriene

Most of the risk factors for atherosclerosis and the development of cardiovascular disease (CVD) have been derived from the study of older populations who have already manifested clinical symptoms. Therefore, there is a need to identify younger subjects at risk for CVD so that preventive measures may be instituted before occlusive vascular disease occurs. A current goal of The Muscatine Study, initiated in 1970,¹ is to identify early predictors of coronary artery calcium (CAC) and carotid artery intimal-medial thickness (IMT). A representative subset (Muscatine Study Longitudinal Adult Cohort; n=866) of the 11 377 children who participated in the Muscatine Study school surveys conducted between 1971 and 1981 is currently being followed with cardiovascular risk factors measured during childhood and at several stages during adulthood, along with measures of subclinical CVD (CAC and IMT). Risk factors include anthropometric and blood pressure measurements, serum lipid profiles and other biochemical indices, dietary surveys, and behavioral questionnaires.²⁻⁵

Underlying genetic susceptibilities, as well as environmental factors, are involved in the initiation and progression of complex diseases such as atherosclerosis. Therefore it is important to add an assessment of the genetic contribution to the CVD risk for the Muscatine Study Longitudinal Adult Cohort. We have augmented the Muscatine Study data set by incorporating information on genetic polymorphisms within candidate genes associated with a variety of pathogenetic processes that include oxidative stress, lipid metabolism, thrombosis/coagulopathies, blood pressure regulation, inflammation, and cell adhesion,⁶⁻⁸ to investigate the association of these candidate gene polymorphisms with assessments of subclinical CVD. Here we report on the associations between CAC, IMT, and a promoter polymorphism of Leukotriene C₄ Synthase (LTC₄S), the rate limiting enzyme in the production of the potent proinflammatory cysteinyl-leukotriene metabolites of arachidonic acid. Because CAC and IMT are strongly associated with the amount of coronary atherosclerotic plaque,⁹⁻¹¹ either the amount or presence of CAC or

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the increase in mean IMT can be used as an early measure of coronary atherosclerosis.

Methods

The University of Iowa Institutional Review Board approved the Muscataine Study protocol and informed consent was obtained from all subjects. Both the University of Iowa, and Children’s Hospital Oakland Institutional Review Boards approved protocols for genotyping subjects.

Study Population

Between 1971 and 1981, 11,377 school children 8 to 18 years of age from Muscataine, Iowa underwent 26,919 total examinations in 6 biennial school surveys. Between 1981 and 1991, 2,547 of the school survey participants (67% of those eligible) who were then 20 to 37 years of age were examined (anthropometric, blood pressure, lipid measurements, and health history questionnaires) 1 or 2 times during the Young Adult Follow-Up phase of The Muscataine Study. Between 1992 and 1996, a representative subset of the Young Adult Follow-Up participants was invited to undergo a clinic examination and electron beam computed tomographic (EBCT) scanning for the measurement of coronary artery calcium (CAC). More than 95% of those invited became members of the Longitudinal Adult Cohort (n=866, 29 to 43 years of age; 98% non-Hispanic white). Childhood risk factor levels (adjusted for age, gender, and survey year) of the 866 cohort members did not differ significantly from the entire group of childhood participants with respect to height, weight, blood pressure, triceps skinfold thickness, total cholesterol, or triglycerides at the time of their childhood examinations (data not shown).

Beginning in 1996, Cohort members were invited to return for a clinic examination and an ultrasound examination for measurement of IMT. Data from the first EBCT examination for CAC and the first ultrasound examination for IMT, along with risk factor measurements obtained throughout the period of their participation in The Muscataine Study, are reported herein. At the time of the CAC examination, 2 men and 1 woman had been diagnosed with diabetes, and 1 man and 3 women were taking lipid-lowering medications. None of these individuals had evidence of coronary artery calcium.

Measurement of Coronary Artery Calcium

Calcium examinations for the 866 cohort members were performed using an Imatron C-150 ultrafast electron beam computed tomographic (EBCT) scanner (Imatron) as previously described. Briefly, 40 contiguous 3-mm slices were acquired during a single breath hold. Total radiation dose to the skin was estimated to be 10 mGy (1.0 rad). For this analysis, CAC was defined as a focus located within 5 mm of the arterial midlines, with at least 3 contiguous pixels (area 1.03 mm²) having a density of at least 130 Hounsfield units. Image-processing software computed the area of all pixels meeting these criteria within the regions of interest. For the analyses reported here, only the presence or absence of CAC was evaluated, because of the relatively low prevalence of CAC in this age group. Subjects with duplicate scans (approximately 50% of the cohort members) were classified as having CAC if either scan was positive.

Measurement of Carotid Artery Intimal-Media! Thickness

Carotid artery ultrasound examination was performed as previously reported. For each participant, the maximum carotid IMT was measured for the near and far wall of each common carotid artery, carotid bifurcation, and internal carotid artery. The mean of the maximum carotid IMT measured at the 12 locations (3 sites × 2 sides × 2 walls) was the variable used for analysis. The mean of non-missing walls was used for participants with missing data for one or more of the 12 locations. Approximately 55% of the participants had a maximum IMT for each of the 12 locations; 35% had a maximum IMT for 9 to 11 of the 12 locations; 98.5% had a maximum IMT for at least 6 of the 12 locations. The minimum number of measurements was 3.

Genotyping

DNA was available for 756 of the 866 cohort members. We used a multilocus allele-specific hybridization assay as previously described for genotyping. Briefly, the first step consisted of a multiplex polymerase chain reaction amplification using a primer blend containing a biotinylated primer pair for each polymorphic site. Next, the biotin-tagged amplification products were hybridized to a linear array of immobilized oligonucleotide probes specific for the alternate alleles of each polymorphic site. After a stringent wash, chromogenic genetic probes were used to visualize the biotin-tagged amplicons that remained hybridized. After color development, arrays were scanned for archiving, and genotypes were interpreted by two independent observers. All genotyping was performed blinded to participant’s identity and CAC status. The (−444)A>C polymorphism of LTC4S corresponds to rs730012 (www.ncbi.nlm.nih.gov). LTC4S genotyping was successful for 725 (96%) of the cohort members.

Results

At the time of the EBCT examination to assess CAC, the 341 men with genotype data ranged in age from 29 to 37 years; the 391 women ranged in age from 29 to 43 years. The wider age range for women was by design because the prevalence of calcium among women 29 to 37 years of age is considerably lower than in men. One hundred one (13.8%) of the 732 cohort members with genotype data had evidence of CAC.
(20.5% of men and 7.9% of women). Among the women, 6% of those 29 to 37 years of age had CAC, compared with 16% of those 40 to 43 years of age.

The distribution of LTC4S (−444)A>C genotypes was consistent with Hardy–Weinberg expectations; χ²=0.55 (P>0.40) for men and women combined (P>0.10 for women; P>0.60 for men). Table 1 shows the LTC4S genotype distribution by CAC status for men and women. LTC4S shows a strong association with CAC status for women, with 77% of the women with CAC having at least one copy of the variant allele compared with 46% of the women without CAC. In contrast, there is no association between CAC status and LTC4S genotype for men.

Comparing risk factor levels for men and women by their CAC status, we found that those with CAC tend to be slightly older, with lower HDL-cholesterol, higher systolic and diastolic blood pressures, and higher body mass indices (data not shown). Table 2 shows several logistic regression models for risk factor levels for men and women, which also includes an indicator variable for LTC4S genotype. The age-risk factor-adjusted odds of CAC relative to those with the AA genotype of 4.29 (95% CI 1.78, 10.31). Three of the load variables were also consistently significant in the logistic models: body mass index, diastolic blood pressure, and Apolipoprotein A1. Models 2 through 5 show the results from inclusion of various combinations of these three risk factor loads. The genotype-specific odds ratios are greater than those of Model 1, indicating that the association identified between the LTC4S genotype and CAC in women is not explained by an association between LTC4S and any of these recognized cardiovascular risk factors. The odds of CAC increase with increasing BMI and DBP loads and decrease with increasing Apolipoprotein A1 load. The age-BMI load-DBP load-Apolipoprotein A1 load-adjusted odds of CAC are almost 7-fold higher for women who carry at least one LTC4S C allele relative to women who are AA homozygotes.

Carotid IMT was available for these women from an examination 4 to 5 years after the EBCT examination, which allowed a further investigation of the association between the LTC4S and subclinical cardiovascular disease in a different vascular bed. Table 3 displays the results from including CRF loads in a general linear model along with an indicator variable for LTC4S genotype. The age-risk factor-adjusted

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**TABLE 1. Distribution of LTC4S Genotypes in Women and Men With (CAC+) and Without (CAC−) Coronary Artery Calcium**

<table>
<thead>
<tr>
<th></th>
<th>CAC− (n%)</th>
<th>CAC+ (n%)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA</td>
<td>AC</td>
<td>CC</td>
</tr>
<tr>
<td>Women</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>191</td>
<td>144</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>54%</td>
<td>40%</td>
<td>6%</td>
</tr>
<tr>
<td></td>
<td>49%</td>
<td>41%</td>
<td>10%</td>
</tr>
<tr>
<td>Men</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>133</td>
<td>110</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>49%</td>
<td>41%</td>
<td>10%</td>
</tr>
</tbody>
</table>

**LTC4S** indicates leukotriene C4 synthase.

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**TABLE 2. Logistic Regression Models of the Association Between Coronary Artery Calcium Status and Risk Factor Loads, and LTC4S Genotypes in Women**

<table>
<thead>
<tr>
<th>Model</th>
<th>V1 CAC Age</th>
<th>BMI Load</th>
<th>DBP Load</th>
<th>Apo A1 Load</th>
<th>LTC4S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model 1</td>
<td>1.16 (1.05, 1.28)*</td>
<td>2.64 (1.73, 4.04)</td>
<td>1.90 (1.02, 3.53)</td>
<td>—</td>
<td>4.29 (1.78, 10.31)</td>
</tr>
<tr>
<td>Model 2</td>
<td>1.18 (1.07, 1.31)</td>
<td>2.26 (1.44, 3.55)</td>
<td>2.16 (1.15, 4.06)</td>
<td>—</td>
<td>5.21 (2.05, 13.26)</td>
</tr>
<tr>
<td>Model 3</td>
<td>1.18 (1.06, 1.31)</td>
<td>2.53 (1.64, 3.92)</td>
<td>2.11 (1.33, 3.34)</td>
<td>0.57 (0.34, 0.97)</td>
<td>5.64 (2.45, 13.26)</td>
</tr>
<tr>
<td>Model 4</td>
<td>1.19 (1.08, 1.33)</td>
<td>2.11 (1.33, 3.34)</td>
<td>2.11 (1.33, 3.34)</td>
<td>0.57 (0.34, 0.97)</td>
<td>6.64 (2.45, 18.02)</td>
</tr>
<tr>
<td>Model 5</td>
<td>1.20 (1.08, 1.33)</td>
<td>2.11 (1.33, 3.34)</td>
<td>2.11 (1.33, 3.34)</td>
<td>0.57 (0.34, 0.97)</td>
<td>6.64 (2.45, 18.02)</td>
</tr>
</tbody>
</table>

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*Odds Ratio (95% Confidence Interval), P value.

— indicates that the variable was not included in the model; DBP, diastolic blood pressure; BMI, body mass index; Apo A1, apolipoprotein A1; c, the area under the receiver operating characteristic (ROC) curve.

The odds ratios associated with BMI Load, DBP Load, and Apo A1 Load represent z-score units, eg. participants whose BMI Load was 1.0, ie, one standard deviation above the gender-specific survey-specific mean throughout their lifetime, relative to participants whose BMI load was 0.0, ie, at the age-gender-specific survey specific-mean throughout their lifetime. Apo A1 was not measured until the Young Adult Follow-up examination.
TABLE 3. Association Between \( \text{LTC}_4 \text{S} \) and Carotid Artery Intimal–Medial Thickness (IMT) in Women (n=371)

<table>
<thead>
<tr>
<th>Predictor Variable</th>
<th>( P ) Value†</th>
<th>( \text{LTC}_4 \text{S} ) Genotype</th>
<th>Mean IMT±SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMT age</td>
<td>&lt;0.0025</td>
<td>AA</td>
<td>0.702±0.007</td>
</tr>
<tr>
<td>Systolic BP load</td>
<td>&lt;0.01</td>
<td>AC and CC</td>
<td>0.733±0.007</td>
</tr>
<tr>
<td>Cholesterol load</td>
<td>&lt;0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chol/HDL load</td>
<td>&lt;0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI load</td>
<td>&lt;0.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \text{LTC}_4 \text{S} ) C allele</td>
<td>&lt;0.0025</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

†P values are based on Type III Sums of Squares from a general linear models analysis.

**Discussion**

Based on measurements that assess the early atherosclerotic process in two different vascular beds, we found evidence for a significant association between \( \text{LTC}_4 \text{S} \) and subclinical disease for women, but not for men in The Muscatine Study. In women, the age-adjusted odds ratio for CAC+ associated with the \((-444)A>C\) promoter region polymorphism of \( \text{LTC}_4 \text{S} \) was 4.29 (95% CI 1.78, 10.31). This increased risk is higher than that associated with elevated levels of established CVD risk factors in the Muscatine Study.12 We also identified an association between the \( \text{LTC}_4 \text{S} \) polymorphism and carotid artery IMT measured in the same women 4 to 5 years later. At the time of the carotid examination, 136 women had a concurrent repeat CAC examination. Davis et al22 identified a strong age-adjusted association between tertile of IMT and prevalence of CAC in this subset of women from the Muscatine Study Longitudinal Adult Cohort.

Our study represents one of the first attempts to assess the risk of CVD in a cohort of young subjects having no clinical symptoms of overt disease, by combining genetic, physical, and biochemical risk factor measurements with noninvasive indicators of subclinical atherosclerotic CVD. Previously thought to be a passive accumulation of lipids within artery walls, atherosclerosis is now understood to be a disease characterized by endothelial injury with active recruitment of inflammatory cells, the accumulation of lipids, and the formation of a fibrous cap to form complex plaque structures on the vessel walls.16–19 The resulting chronic inflammatory process involving the arterial endothelium may be caused by a response to lipid free radicals, lipid hydroperoxides, lysophospholipids, carbonyl compounds, and other biologically active moieties.20 \( \text{LTC}_4 \text{S} \) plays a key role in the process of inflammation as the rate limiting enzyme in the conversion of arachidonic acid to cysteinyl-leukotrienes, important mediators of the inflammatory response.21 In this pathway (Figure), arachidonic acid is first converted to the epoxide intermediate leukotriene A4 by the coordinated activity of Arachidonate 5-Lipoxygenase and Arachidonate 5-Lipoxygenase Activating Protein.23–24 Additional leukotrienes are subsequently generated through the specific conjugation of leukotriene A4 with glutathione by \( \text{LTC}_4 \text{S} \), leading to the formation of leukotriene C4.21 Sequential removal of the glutamic acid and glycine residues from the glutathione moiety of leukotriene C4 leads to the formation of leukotriene D4 and leukotriene E4, respectively. Leukotriene C4, -D4, and -E4, which together compose the cysteinyl-leukotrienes, are potent mediators of inflammation by their abilities to stimulate sustained smooth muscle contraction, mucus hypersecretion, airway edema,25 and eosinophil recruitment.26

Previously, studies of cysteinyl-leukotrienes have focused on their role in asthma where it has been firmly established that both LTD4 and LTE4 can induce bronchospasm when taken by inhaler.27,28 In addition, a substantial body of evidence demonstrates that the cysteinyl-leukotrienes are increased in the sputum,29,30 lung lining fluid,31 blood, and urine32 of asthmatics compared with healthy individuals, thus associating these compounds with the pathophysiology of bronchial asthma. The \((-444)A>C\) SNP within the \( \text{LTC}_4 \text{S} \) gene has been found to be associated with an increased risk for aspirin intolerant asthma.33 The \((-444)C\) promoter region sequence alteration results in upregulation of \( \text{LTC}_4 \text{S} \) mRNA expression attributable to the generation of a putative H4TF-2 transcription factor binding site, and subsequently increased levels of the inflammatory molecules LTD4, LTE4.34 Increased bronchial expression of \( \text{LTC}_4 \text{S} \), resulting in marked overproduction of cysteinyl-leukotrienes and subse-
quent bronchoconstriction, has been confirmed in aspirin intolerant asthmatics relative to aspirin tolerant asthmatics, or when compared with normal individuals.35 Thus the role of the LTC4S (−444)C allele in the inflammatory process of asthma, likely through enhanced synthesis of inflammatory leukotrienes, has clearly been established. However, to our knowledge no previous reports have provided direct evidence for a role of LTC4S in the development of atherosclerosis.

Recently several studies have linked atherosclerosis risk with genetic variants of two other proteins participating in the leukotriene B arm of the 5-Lipoxygenase metabolic pathway: Arachidonate 5-Lipoxygenase Activating Protein and Leukotriene A4 Hydrolase (Figure).36–38 Several of the associations, however, were limited to males with advanced stages of disease that included at least one cardiovascular event such as stroke or myocardial infarction. Furthermore, the association with Leukotriene A4 Hydrolase was for African Americans with genetic variants of two other proteins participating in the risk factors. Recently, Dwyer et al39 has correlated ALOX5 with evidence of recent Caucasian admixture. In addition, this work supports our observation that alterations in the genes encoding the arachidonate metabolic pathway correlate with the development of cardiovascular disease in subclinical individuals, to our knowledge our data represent the first report of an association with a LTC4S DNA sequence alteration and two early measurements of cardiovascular disease development, CAC, and carotid IMT.

There is no universally accepted definition for the presence of CAC. However, the more widely used definition is at least one focus with three contiguous pixels, each with a pixel density ≥130 Hounsfield units (HU). Several reports have demonstrated that foci with three or four pixels are not reproducible on duplicate scans.40–41 This lack of reproducibility has been attributed to patient positioning differences between the duplicate scans, partial volume effect, breath holding, and artifact, among other possible explanations, and it was observed among the duplicate scans in the present study. Therefore, we classified women as having CAC only if they had at least one focus with five or more contiguous pixels ≥130 HU; 19 of the women were classified with CAC using this more conservative definition. The distribution of LTC4S genotypes among these 19 women was compared with the remaining 368 women (those with no evidence of CAC or with foci smaller than five contiguous pixels) and the association remained (79% of those with CAC versus 47% of those without CAC have at least one copy of the variant C allele $P<0.025$). The age-adjusted odds of CAC for women with at least one copy of the variant C allele were 4.66 times higher than the odds of CAC for women with the AA genotype (OR=4.66; 95% CI 1.49, 14.55). Therefore the association is slightly stronger when a more conservative definition of coronary artery calcium is applied.

Whether the definition of a focus is three or five contiguous pixels, the number of women with CAC in the present study is small because the prevalence of coronary artery calcium is this age group is low. Ideally, an observed genetic association such as this, especially one based on a small sample size, should be replicated in another population. Because we did not have access to a second population of women in this age group who had been examined for CAC, we chose to replicate the observed CAC association by investigating the association in a different vascular bed (carotid artery IMT) in the same group of women.

Whereas women tend to develop atherosclerosis about a decade later than men, cardiovascular disease remains the leading cause of death for women, claiming more lives than the next six causes of death combined and totaling approximately 500,000 women’s lives each year. The effect of genetic predisposition to develop early atherosclerosis, which LTC4S may contribute to, may be more apparent earlier in the course of the disease before the overwhelming effects of environmental and pathophysiologic influences (CRFs) obscure it. Whether this explains our observed association in women remains to be investigated. Therefore improved methods are needed to identify those women most at risk for the development of CVD early in life, before overt symptoms of pathology have manifested and at a time when preventive interventions may be most effective.

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Disclosures

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References


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