The A640G CYBA polymorphism associates with subclinical atherosclerosis in diabetes

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TABLE OF CONTENTS
1. Abstract
2. Introduction
3. Materials and methods
   3.1. Participants and clinical studies
   3.2. Determination of superoxide anion production
   3.3. Genotyping
   3.4. Statistical analysis
4. Results
   4.1. Association of the A640G polymorphism with diabetes
   4.2. Association of the A640G polymorphism with clinical phenotypes
   4.3. Association of the A640G polymorphism with superoxide release in peripheral blood mononuclear cells
5. Discussion
6. Acknowledgements
7. References

1. ABSTRACT

Oxidative stress is implicated in diabetes. The NADPH oxidases are the main source of superoxide in phagocytic and vascular cells, and p22phox is a key subunit. Genetic variants of CYBA, the human p22phox gene, associate with cardiovascular disease. We investigated the association of the A640G polymorphism with diabetes and its impact on phagocytic NADPH oxidase-dependent superoxide production and subclinical atherosclerosis. We studied 1212 subjects in which clinical parameters including carotid intima-media thickness (cIMT) were assessed. The A640G polymorphism was genotyped by TaqMan probes. In 496 subjects, the NADPH oxidase-dependent superoxide production in peripheral blood mononuclear cells was assessed by chemiluminescence. The GG genotype prevalence was significantly higher in type 2 diabetic patients than in non-diabetic subjects. Peripheral blood mononuclear cells from diabetic GG patients presented higher NADPH oxidase-dependent superoxide production than those of diabetic AA/AG patients. Within the diabetic group, GG patients presented higher cIMT levels than AA/AG patients. The A640G CYBA polymorphism may be a marker of oxidative stress risk and may be indicative of subclinical atherosclerosis in type 2 diabetes.

2. INTRODUCTION

Type 2 diabetes mellitus is a metabolic disease characterized by the elevation of blood glucose concentration, lipid abnormalities and vascular complications. It is a major health problem worldwide, and its prevalence is on the rise (1). Diabetes is a multifactorial disease with both genetic and environmental causes. Although the mechanisms involved in the development and progression of diabetes and its complications are complex, oxidative stress, that is, the accumulation of reactive oxygen species (ROS) due to increased production and/or decreased detoxification by antioxidants, seems to play a critical role (2).

The generation of ROS in diabetes has important vascular consequences: it reduces nitric oxide bioavailability, thus favouring endothelial dysfunction, leukocyte adhesion, proliferation, migration and apoptosis of vascular cells, platelet aggregation and thrombus formation (3). All these mechanisms contribute to atherosclerosis and cardiovascular events, which are more frequent in diabetic patients (1). In this regard, the carotid intima-media thickness (cIMT) is a marker of subclinical atherosclerosis that predicts cardiovascular risk in the general population as well as in diabetic patients (4, 5).
It is noteworthy that the family of the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidases are key pathological pro-oxidants in diabetes, as observed both in clinical (6) and experimental studies (7, 8). Not only the vascular NADPH oxidases but also the NADPH oxidase of circulating white cells is altered in diabetes (9, 10).

The p22phox subunit is a common component of NOX1 to NOX4-dependent forms of the NADPH oxidase, and plays an essential role in NADPH oxidase activation (11). Interestingly, monocytic (10) and lymphocytic (12) p22phox levels are increased in human diabetes. One of the mechanisms that regulate p22phox levels and NADPH oxidase activity is the genetic component (13). Several allelic variants have been identified in CYBA, the gene encoding the human p22phox subunit, such as the A640G polymorphism, located in the 3´ untranslated region (UTR) of CYBA (14). Association studies of this variant with cardiovascular disease are somehow conflicting, with both positive (15) and negative (16, 17) results. In our study, we have analysed the potential association of the A640G polymorphism with diabetes. In addition, we have studied the impact of this polymorphism on superoxide release from peripheral blood mononuclear cells and its association with surrogate markers of cardiovascular risk in type 2 diabetes.

3. MATERIALS AND METHODS

3.1. Participants and clinical studies

The study population consisted of 1212 consecutive, asymptomatic subjects of Caucasian origin who attended the University Clinic of Navarra for a routine medical work-up. Subjects were confirmed as genetically unrelated through interviewing. Blood pressure was measured on three occasions using a mercury sphygmomanometer and the mean of these readings was recorded. None of the hypertensive patients presented echocardiography evidence of aortic stenosis or hypertrophic cardiomyopathy, clinical manifestations of heart failure. Type 2 diabetes was defined if the fasting glucose levels were above 125 mg/dL and/or if the patient was under hypoglycemic treatment. Obesity was defined if body mass index (BMI) was ≥ 30. Subjects were free from clinically apparent atherosclerotic disease based on: (1) absence of history of coronary disease, stroke, or peripheral artery disease; and (2) normal electrocardiogram and chest-x-ray results. Patients were excluded if they had advanced carotid atherosclerosis according to the cIMT measurements (>1.7 mm). Additional exclusion criteria were the presence of severely impaired renal function, arteritis, collagenosis, and a history of alcohol abuse. Patients with significant acute infection, according to clinical criteria by the attending physician, were also excluded.

To determine cIMT, ultrasonography of the common carotid arteries was performed with a 5- to 12-MHz linear-array transducer (ATL 500 HDI). The measurement of IMT was made 1 cm proximal to the carotid bulb of each common carotid artery at plaque-free sites. For each individual, the IMT was determined as the average of near wall and far wall measurements of each common carotid artery. Subjects were examined by the same 2 certified sonographers blinded to all clinical information. The reproducibility of IMT measurements between and within sonographers had previously been checked in individuals who returned 2 weeks later for a second examination. The intraobserver and interobserver coefficients of variation were 5% and 10%, respectively (18).

According to institutional guidelines, all subjects were aware of the research nature of the study and agreed to participate. The study was carried out in accordance with the Helsinki Declaration and the Ethics Committee of the University of Navarra approved of the protocol.

3.2. Determination of superoxide anion production

In 496 of our patients, the NADPH oxidase-dependent superoxide production was measured in peripheral blood mononuclear cells (monocytes and lymphocytes) isolated from blood samples with Lymphoprep (Axis- Shield) in response to stimulation with phorbol 12-myristate 13-acetate (PMA, 2 mg/L; Sigma) and using lucigenin (5 micromol/L; Sigma) in a chemiluminescent method that correlated well with the ferricytochrome C assay, as described previously (18, 19).

3.3. Genotyping

DNA was isolated from venous blood with the QIAamp DNA Blood Kit (Qiagen) according to the manufacturer. The A to G substitution at position 640 in the 3’ UTR (rs1049255) was genotyped by allelic discrimination, using the TaqMan probe (C_7516916_10) (Applied Biosystems) and the ABI PRISM 7000 Sequence Detector (Applied Biosystems).

3.4. Statistical analysis

Data were expressed as mean±SEM. Chi-square analyses were used to test for deviation of genotype distribution from Hardy-Weinberg equilibrium. Chi-square as well as binary logistic regression analyses were used to determine whether there were significant differences in genotype frequencies between cases and controls. In view of the results of the normality test (Shapiro-Wilks), variations in the clinical data were assessed either by Student’s t-test or a Mann-Whitney U-test Multivariate linear regression analysis was performed to evaluate factors related to the A640G polymorphism and the possibility of interactions. Statistical analyses were performed with SPSS for Windows, version 15.0 (SPSS Inc.). P<0.05 was considered statistically significant.

4. RESULTS

4.1. Association of the A640G polymorphism with diabetes

We genotyped A640G polymorphism of CYBA in 1212 subjects. The prevalence was: AA: 366 (30.2%), AG: 604 (49.8%), GG: 242 (20.0%). The distribution followed Hardy-Weinberg equilibrium law (Chi-
Table 1. Clinical parameters of the population in study

<table>
<thead>
<tr>
<th></th>
<th>Non-diabetic (n=1073)</th>
<th>Diabetic (n=139)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>54 +/- 1</td>
<td>60 +/- 1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Gender (m/f)</td>
<td>827/246</td>
<td>112/27</td>
<td>0.389</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>28.0 +/- 0.1</td>
<td>29.9 +/- 0.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>127 +/- 1</td>
<td>135 +/- 2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>81 +/- 1</td>
<td>81 +/- 1</td>
<td>0.190</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>96 +/- 1</td>
<td>154 +/- 4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Insulin (pmol/L)</td>
<td>70.2 +/- 1.4</td>
<td>112.5 +/- 7.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HOMA index</td>
<td>2.56 +/- 0.06</td>
<td>6.04 +/- 0.40</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>54 +/- 1</td>
<td>50 +/- 1</td>
<td>0.002</td>
</tr>
<tr>
<td>LDL (mg/dL)</td>
<td>143 +/- 1</td>
<td>134 +/- 4</td>
<td>0.009</td>
</tr>
<tr>
<td>Total Cholesterol (mg/dL)</td>
<td>219 +/- 1</td>
<td>210 +/- 4</td>
<td>0.005</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>112 +/- 2</td>
<td>134 +/- 6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>cIMT (mm)</td>
<td>0.687 +/- 0.006</td>
<td>0.766 +/- 0.19</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hypoglycemic treatment (%)</td>
<td>0</td>
<td>59</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Antihypertensive treatment (%)</td>
<td>29</td>
<td>48</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Statin treatment (%)</td>
<td>16</td>
<td>23</td>
<td>0.034</td>
</tr>
</tbody>
</table>

BMI: body mass index, DBP: diastolic blood pressure, SBP: systolic blood pressure, cIMT: carotid intima-media thickness.

Table 2. Prevalence of the A640G polymorphism in diabetes

<table>
<thead>
<tr>
<th></th>
<th>Non-diabetes</th>
<th>Diabetes</th>
<th>Chi-square</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA (n, %)</td>
<td>331, 31.0</td>
<td>35, 24.3</td>
<td>6.073</td>
<td>0.048</td>
</tr>
<tr>
<td>AG (n, %)</td>
<td>534, 50.0</td>
<td>70, 48.6</td>
<td>0.009</td>
<td></td>
</tr>
<tr>
<td>GG (n, %)</td>
<td>203, 19.0</td>
<td>39, 27.1</td>
<td>5.179</td>
<td>0.023</td>
</tr>
<tr>
<td>AA/AG (n%)</td>
<td>565, 81.0</td>
<td>105, 72.9</td>
<td>4.299</td>
<td></td>
</tr>
<tr>
<td>A allele frequency</td>
<td>0.5599</td>
<td>0.4861</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G allele frequency</td>
<td>0.4401</td>
<td>0.5139</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Comparison of all three genotypes. **Comparison of GG versus AA/AG.

Table 3. Logistic analysis of the association of the A640G polymorphism with diabetes

<table>
<thead>
<tr>
<th></th>
<th>Beta</th>
<th>P</th>
<th>R-square of model</th>
<th>Chi-square of model</th>
<th>P of model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>0.059</td>
<td>&lt;0.001</td>
<td>0.088</td>
<td>56.229</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sex (female vs male)</td>
<td>0.285</td>
<td>0.218</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A640G polymorphism</td>
<td>0.345</td>
<td>0.008</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

patients with GG genotype presented higher levels of glucose, insulin, HOMA and triglycerides, and lower HDL levels (Table 4). These data, together with the data in Table 3 suggest that the GG genotype may have deleterious effects; therefore, a recessive model in which GG patients were compared with AA/AG patients was used.

4.2. Association of the A640G polymorphism with clinical phenotypes

Since diabetes is a well established risk factor for atherosclerosis, we further investigated the effect of the polymorphism on cIMT, a surrogate marker of subclinical atherosclerosis. In the diabetic group, subjects with GG genotype presented significantly (P=0.040) higher cIMT than patients with AA/AG genotype, whereas no differences according to genotype were detected for the non-diabetic group (Figure 1).

4.3. Association of the A640G polymorphism with superoxide production in peripheral blood mononuclear cells

In a subpopulation representative of the whole of 496 patients, we were able to perform functional studies in circulating mononuclear cells. In diabetic patients, the A640G polymorphism altered the phagocytic NADPH oxidase-dependent superoxide production in response to PMA: there was a clear trend (P=0.055) towards higher superoxide anion production in diabetic patients with GG genotype, whereas there were no differences according to genotype in non diabetic subjects (Figure 2). What is more,
Table 4. General characteristics of the population in study according to the genotype for the A640G polymorphism of CYBA

<table>
<thead>
<tr>
<th>Age (y)</th>
<th>AA (n=366)</th>
<th>AG (n=604)</th>
<th>GG (n=242)</th>
<th>P</th>
<th>AA/GG (n=970)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (m/f)</td>
<td>54%+1</td>
<td>54%+1</td>
<td>54%+1</td>
<td>0.650</td>
<td>54%+1</td>
<td>0.869</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>28.2+/-0.3</td>
<td>28.1+/-0.2</td>
<td>28.4+/-0.3</td>
<td>0.736</td>
<td>28.1+/-0.1</td>
<td>0.484</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>129+/-1</td>
<td>128+/-1</td>
<td>129+/-1</td>
<td>0.365</td>
<td>128+/-1</td>
<td>0.488</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>81+/-1</td>
<td>81+/-1</td>
<td>81+/-1</td>
<td>0.929</td>
<td>81+/-1</td>
<td>0.759</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>102+/-1</td>
<td>101+/-1</td>
<td>107+/-2</td>
<td>0.083</td>
<td>107+/-2</td>
<td>0.034</td>
</tr>
<tr>
<td>Insulin (pmol/L)</td>
<td>76.4+/-2.8</td>
<td>77.1+/-2.8</td>
<td>85.4+/-3.5</td>
<td>0.011</td>
<td>77.1+/-2.1</td>
<td>0.003</td>
</tr>
<tr>
<td>HOMA index</td>
<td>2.82+/-0.12</td>
<td>2.95+/-0.13</td>
<td>3.29+/-0.17</td>
<td>0.006</td>
<td>2.90+/-0.09</td>
<td>0.002</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>54+/-1</td>
<td>54+/-1</td>
<td>51+/-1</td>
<td>0.057</td>
<td>54+/-1</td>
<td>0.019</td>
</tr>
<tr>
<td>LDL (mg/dL)</td>
<td>141+/-2</td>
<td>142+/-2</td>
<td>143+/-3</td>
<td>0.849</td>
<td>142+/-2</td>
<td>0.610</td>
</tr>
<tr>
<td>Total Cholesterol (mg/dL)</td>
<td>217+/-2</td>
<td>218+/-2</td>
<td>219+/-3</td>
<td>0.949</td>
<td>218+/-2</td>
<td>0.752</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>114+/-4</td>
<td>111+/-3</td>
<td>123+/-5</td>
<td>0.035</td>
<td>112+/-2</td>
<td>0.010</td>
</tr>
</tbody>
</table>

BMI: body mass index, DBP: diastolic blood pressure, SBP: systolic blood pressure, cIMT: carotid intima-media thickness.

1Comparison of all three genotypes. 2Comparison of GG versus AA/AG.

Table 5. Multivariate analysis of the phagocytic NADPH oxidase-dependent superoxide production

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Sex (female vs male)</th>
<th>Glucose (mg/dL)</th>
<th>Triglycerides (mg/dL)</th>
<th>A640G polymorphism (GG vs AA/AG)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta</td>
<td>P</td>
<td>R-square of model</td>
<td>F of model</td>
<td>P of model</td>
</tr>
<tr>
<td>-------------</td>
<td>----------------------</td>
<td>-----------------</td>
<td>------------</td>
<td>------------</td>
</tr>
<tr>
<td>0.083</td>
<td>0.069</td>
<td>0.044</td>
<td>4.493</td>
<td>0.001</td>
</tr>
<tr>
<td>0.057</td>
<td>0.207</td>
<td>0.044</td>
<td>4.493</td>
<td>0.001</td>
</tr>
<tr>
<td>0.083</td>
<td>0.080</td>
<td>0.044</td>
<td>4.493</td>
<td>0.001</td>
</tr>
<tr>
<td>0.081</td>
<td>0.075</td>
<td>0.044</td>
<td>4.493</td>
<td>0.001</td>
</tr>
<tr>
<td>0.093</td>
<td>0.038</td>
<td>0.044</td>
<td>4.493</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Figure 1. Carotid intima-media thickness in non-diabetic subjects (A) and in diabetic patients (B) according to genotype for the A640G polymorphism of CYBA. *P<0.05.

multivariate studies showed that the A640G polymorphism was a significant determinant of the NADPH oxidase-dependent superoxide production, after adjusting for confounding factors (Table 5).

5. DISCUSSION

The first finding of our study is the association of the A640G polymorphisms of CYBA with type 2 diabetes, an important risk factor for the development of atherosclerosis and cardiovascular disease. The prevalence of the A640G polymorphism in our population was similar to HapMap data (20). Genotyping studies showed an increased prevalence of the G allele in diabetic patients and this association was independent from age and sex. In agreement with this, Hodgkinson et al. (21) detected an association of the A640G polymorphism with diabetic
complications in which a combination of the A640G and
C242T polymorphisms of CYBA (T242/G640) was related to
greater nephropathy risk. Association studies of this
polyorphism with cardiovascular disease profiles have
been conflicting: Inoue and colleagues (16) did not find an
association of the variant with coronary artery disease
(CAD) in a Japanese population, nor did Zafari et al. (17)
in a white-American population. Conversely, Gardemann
et al. (15) detected a significant reduction of the G allele in
European patients with CAD. As we have detected that the
A640G polymorphism is associated with diabetes but not
hypertension or obesity, it can be speculated that the
diabetic status of the patients in the studies mentioned
above may have been a confounding factor.

The second result reported here is the association
of the A640G polymorphism with the NADPH oxidase-
dependent superoxide production in peripheral blood
mononuclear cells. Cells from diabetic patients with GG
genotype displayed higher production than those from
patients with AA/AG genotypes, whereas no differences
were found out in non diabetic subjects according to
genotype. It is known that the NADPH oxidase activation,
both in vascular (22) and circulating cells (23), is an
important mechanism in atherosclerosis and is enhanced in
diabetes (6). In our population, subjects with GG genotype
also presented higher levels of glucose and insulin, as well
as an altered lipid profile. There is evidence that glucose
(24, 25) and insulin (26, 27) can activate the NADPH
oxidase system and contribute to oxidative stress and a pro-
inflammatory state. In fact Guzik et al. (6) have shown that
the NADPH oxidase system is implicated in the superoxide
release that takes place in vessels from diabetic patients.

The cause of the increased NADPH oxidase-
dependent superoxide production of peripheral blood
mononuclear cells from GG patients may be a direct
genic effect driven by the polymorphism. Although the
functionality of the A640G polymorphism is currently
unknown, its location in the 3’ UTR suggests it may affect
mRNA processing and stability and, hence, transcriptional
rate. Studies in human neutrophils (28) and lymphoblasts
(29) show that the A640G polymorphism does not seem to
have an impact on the NADPH oxidase-dependent
superoxide production. However, the first work (28) was
carried out in young, healthy volunteers as opposed to our
study, in which subjects are older and may have diverse
risk factors. The second study (29) was performed in vitro
in cultured lymphoblastoids from patients with coronary
artery disease, rather than being a direct ex vivo
determination. Therefore, these two studies may lack the
setting in which the effect of the A640G polymorphism is
manifest. Nevertheless, further molecular studies would be
necessary to assess if the A640G polymorphism leads to
greater NADPH oxidase-dependent superoxide production
in mononuclear cells via an increased p22phox
transcription/translation.

On the other hand, the polymorphism may not be
active by itself but rather a marker of risk, maybe due to
other genetic causes. In this regard, preliminary linkage
disequilibrium studies of the A640G polymorphism with
other functional CYBA polymorphisms (namely the
-930A/G (19) and the C242T (30)) show that linkage is low
(data not shown). This suggests that, in our study, the pro-
oxidant profile associated with the A640G polymorphism is
not due to these other polymorphisms. We are aware that a
single variant can explain only a reduced part of the
phenotypic variability of a complex disease, and that the
environmental factors play a role, as well. Nevertheless,
a selection of markers in a certain context may help to single
out patients at risk.

The third finding of our study is that in our
population the A640G polymorphism shows clinical
relevance, as diabetic GG subjects present higher levels of
cIMT, a surrogate marker of subclinical atherosclerosis (4,
5). It is well known that one of the major complications of
diabetes is atherosclerosis (31-33) and that oxidative stress
is implicated (3). Our study shows that, in addition to the
biochemical alterations that contribute to atherosclerosis in
diabetes, the genetic component may further worsen the
clinical profile (34). In agreement with it, Hayashi-Okano
et al. (35) showed that another CYBA variant, the C242T
associates with cIMT. Therefore, genetic markers like the
A640G polymorphism may be useful to identify patients
with higher risk.

We have observed an association of the A640G
polyorphism with NADPH oxidase-dependent superoxide
production only in diabetic patients. Similarly, we have
detected an increased cIMT in diabetic patients with GG

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**Figure 2.** Phagocytic NADPH oxidase-dependent
superoxide production in non-diabetic subjects (A) and in
diabetic patients (B) according to the genotype for the
A640G polymorphism of CYBA. *P=0.055.
CYBA polymorphism associates with diabetes

genotype. Our findings are similar to that of Hayashi-Okano et al. (35) who detected an association of the CYBA C242T polymorphism with cIMT only in diabetic patients but not in controls. This observation in turn exemplifies the importance of the interaction between multiple environmental and genetic factors in complex diseases (34, 36).

Some limitations of the study should be acknowledged. First, the prevalence of diabetes in our population necessarily limits the statistical power; further studies including larger numbers of subjects should be performed to confirm the current results. Second, some of the subjects in our study were under treatment according to their cardiovascular profile (antihypertensive drugs, antiglycemic drugs and cholesterol-lowering drugs) and this may have been a confounding factor in our analysis. However, our multivariate study shows that the A640G polymorphism is a determinant of NADPH oxidase-dependent superoxide production, after correcting for glucose and triglyceride levels. Finally, no data have been presented regarding the antioxidant status. The literature suggests that, in addition to greater activity from pro-oxidant systems, diabetic patients present attenuated antioxidant defences (37, 38), which may worsen their oxidative stress status.

In summary, we have detected that the A640G polymorphisms of CYBA is associated with diabetes. Subjects with the GG genotype presented higher NADPH oxidase-dependent superoxide production by their peripheral blood mononuclear cells and subclinical atherosclerosis. Therefore, the A640G polymorphism may identify individuals at greater risk of developing vascular complications in the setting of type 2 diabetes mellitus.

6. ACKNOWLEDGMENTS

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**Key Words:** Atherosclerosis, Diabetes, Intima-Media Thickness, NADPH oxidase, oxidative stress, A640G polymorphism

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