Association between the C242T polymorphism in the p22phox gene with arterial stiffness in the Brazilian population

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p22phox and TNF-α gene expression were quantified by real-time PCR in human arterial mammary smooth muscle cells. In both the entire and nonhypertensive groups: individuals carrying the TT genotype had higher PWV values and higher risk for increased arterial stiffness [odds ratio (OR) 1.93, 95% confidence interval (CI) 1.27–2.92 and OR 1.78, 95% CI 1.07–2.95, respectively] compared with individuals carrying CC+CT genotypes, even after adjustment for covariates. No difference in the p22phox gene expression according C242T genotypes was observed. However, TNF-α gene expression was higher in cells from individual carrying the T allele, suggesting that this genetic marker is associated with functional phenotypes at the gene expression level. In conclusion, we suggest that p22phox C242T polymorphism is associated with arterial stiffness evaluated by PWV in the general population. This genetic association shed light on the understanding of the genetic modulation on vascular dysfunction mediated by NADPH oxidase.

pulse wave velocity; NADPH oxidase; reactive oxygen species; coronary artery disease

NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE (NADPH) oxidase is a source of reactive oxygen species (ROS), including superoxide and hydrogen peroxide in vascular smooth muscle and endothelial cells (2, 19–21). The NADPH oxidase system is composed of multiple subunits of membrane (p22phox and gp91phox) and cytosolic (gp40 phox, gp47 phox, gp67 phox, and rac1) components that assemble on the cellular surface to generate ROS (13). Among these subunits the p22phox is highlighted as an essential membrane-associated factor that forms heterodimer with another membrane-integrated protein, gp91phox, or its homolog and plays a crucial role in the activation and stabilization of NADPH oxidase (4).

The C242T polymorphism in the p22phox gene, which histidine is replaced by a tyrosine at position 72 and located in the putative heme-binding site of the p22phox subunit, has been associated with NADPH oxidase activity and, in addition, with increased risk and progression for coronary atherosclerosis and thrombotic cerebral infarction (9, 18, 43, 48). Several epidemiological studies reported that increased arterial stiffness predicts mortality and morbidity, independently of other cardiovascular risk factors (6, 8, 35, 38). Decreased arterial elasticity may also be a consequence of various pathological processes associated with diabetes, hypertension, metabolic syndrome or chronic renal disease (15, 22, 49, 52).

Association studies between the C242T polymorphism and the arterial stiffness phenotype assessed by pulse wave velocity (PWV) are absent in the literature. In addition, findings about the protective or harmful effects of the T allele are still controversial (19). Our main aim was to assess the effect of p22phox C242T genotypes on arterial stiffness, a predictor of late morbidity and mortality, in individuals from the general population. We randomly selected 1,178 individuals from the general population of Vitoria City, Brazil. Genotypes for the C242T polymorphism were detected by PCR-RFLP, and pulse wave velocity (PWV) values were measured with a noninvasive automatic device Complior. p22phox and TNF-α gene expression level. In conclusion, we suggest that this genetic marker is associated with functional phenotypes at the gene expression level. In conclusion, we suggest that this genetic marker is associated with functional phenotypes at the gene expression level. In conclusion, we suggest that this genetic marker is associated with functional phenotypes at the gene expression level.
Demographic Data and Biochemical Measurement

Weight and height were determined according to a standard protocol, with participants wearing light clothing and no shoes. Height was measured in centimeters and weight in kilograms using a calibrated balance. The BMI (body mass index, weight in kilograms/height in meters^2) was calculated. Subjects were submitted to an ethnic classification according to a validated questionnaire for the Brazilian population and were classified as Caucasian descent, Mulattos (considered racially mixed subjects), and African descent (3, 36).

Blood triglycerides, total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and glucose were assayed by standard techniques in 12 h fasting blood samples (44, 47).

Blood Pressure Phenotypes and PWV Measurement

Blood pressure was measured in the sitting position with the use of a standard mercury sphygmomanometer on the left arm after 5 min rest. The first and fifth phases of Korotkoff sounds were used for systolic blood pressure (SBP) and diastolic blood pressure (DBP), respectively. The SBP and DBP were calculated from two readings with a minimal interval of 10 min apart. The mean blood pressure (MBP) was calculated as the mean pulse pressure (PP) added to one-third of the DBP, and PP was the difference between SBP and DBP. Hypertension was defined as mean SBP ≥140 mmHg and/or DBP ≥90 mmHg or use of antihypertension drugs.

Carotid-femoral PWV was analyzed with a noninvasive automatic device (Complior; Colson, Garges les Goness, France) by an experienced observer blinded to the clinical characteristics. Briefly, common carotid artery and femoral artery pressure waveform were recorded noninvasively using a pressure-sensitive transducer (TY-306-Fukuda; Fukuda, Tokyo, Japan). The distance between the recording sites (D) was measured, and PWV was automatically calculated as PWV = D/t, where t means pulse transit time. Measurements were repeated over 10 different cardiac cycles, and the mean was used for the final analysis. A dichotomic variable of increased arterial stiffness was defined as PWV ≥9.50 m/s (median value). The validation of this automatic method and its reproducibility has been previously described (7, 47).

Genotyping Protocol

Genomic DNA was extracted from leukocytes in samples of whole blood, following a standard salting-out technique (40). Genotypes for the C242T polymorphism (rs4673) in the CYBA gene (known also as p22phox) were detected by polymerase chain reaction (PCR) followed by restriction fragment length polymorphism analysis as previously described (29). Quality control was assessed by randomly selecting 60 samples to be genotyped by two independent technicians.

Primary Culture of Human Mammary Artery Smooth Muscle Cells

Human mammary artery smooth muscle cells were obtained from a sample of 86 individuals submitted to coronary artery bypass surgery at the Heart Institute (InCor), University of Sao Paulo Medical School. All individuals gave informed consent to participate in the study, which was reviewed and approved by the local Ethics Committee (SDC 2454/04/074-CAPesq 638/04). Cells were obtained by an explant protocol and cultured with Dulbecco’s modified Eagle’s medium containing 20% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin. Smooth muscle cells were characterized by hill-and-valley growth pattern and by immunofluorescence staining for α-smooth muscle actin (11).

p22phox and TNF-α Expression by Real-time RT-PCR

Quantitative RT-PCR was performed in all smooth muscle vascular primary culture, but expression data from 80 cells were available.

Total RNA was isolated with TRIzol Reagent according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). cDNA synthesis was performed using a commercial kit Superscript III (Invitrogen Technologies, Brazil) and 5 ng of cDNA were used for RT-PCR reaction (SYBR Green PCR Master Mix-PE Applied Biosystems). All samples were assayed in triplicate and the Cyclophilin gene was used to normalize the results. The comparative CT (threshold cycle) method was used for data analyses. CT indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold, and ΔCT is the difference in threshold cycle for target (p22phox and TNF-α) and reference (Cyclophilin). The primer sequences used were: p22phox, F: 5’CGCTTCAACCAATGTTACTT3’ and R: 5’GTGGAGCCCTTCTTCTTCTT3’, TNF-α, F: 5’AGGCAATAGGTITTTGAGGGCAT3’ and R: 5’ACACTCCCATCTCCCGCT3’; Cyclophilin, F: 5’ATGGTCAACCCCCACCGTGT3’ and R: 5’TCTGCTGTCCTTGACCTTGTC3’.

Statistical Analysis

Categorical variables were compared using χ² test and presented as percentage, while continuous variables were presented as means ± SD. Student t-test was performed for analysis of demographic, hemodynamic, and biochemical data according to C242T genotypes (CC+CT vs. TT). Multivariate linear regression models were created for adjustment for potential confounding variables. Biochemical and blood pressure data were adjusted for age and sex. PWV values were adjusted for age, sex, MBP, and ethnicity. Gene expression is presented as median 2^−ΔCT plus interquartile interval, and differences among C242T genotype groups were analyzed using the Mann-Whitney or Kruskal-Wallis tests since these data did have not a normal distribution in the studied sample. Logistic regression analysis was carried out to estimate the odds ratio (OR) of increased arterial stiffness according to C242T polymorphism. Statistical analyses were carried out using SPSS 16.0 software (IBM, New York, NY), with the level of significance set at p < 0.05.

RESULTS

Demographic, Hemodynamic, and Biochemical Data

Of the 1,178 individuals (mean age 45.1 ± 10.6 yr), 683 (58.0%) were female and 495 (42.0%) male. The studied sample was classified in two groups: nonhypertensive (n = 674, 57.2%) and hypertensive (n = 504, 42.8%). Genotypic frequencies (42.5% for CC, 45.9% for CT, and 11.6% for TT) were in accordance with the Hardy-Weinberg equilibrium (P = 0.64; X² = 0.22), and the frequency of allele T was of 34.6%.

Demographic, hemodynamic, and biochemical data according to C242T genotypes are shown in Table 1. There were no significant differences in age, sex, ethnicity, hypertension and diabetes frequencies, BMI, blood pressures (SBP, DBP, MBP, and PP), glucose, total cholesterol, LDL-C, HDL-C, and triglycerides among C242T genotypes in the general population (Table 1), in the nonhypertensive and hypertensive groups.

PWV Values and Arterial Stiffness Phenotype

General population. Individuals carrying the TT genotype had higher PWV mean values (P = 0.008) compared with CC+CT genotypes group. After adjustment for potential confounders (age, sex, mean arterial pressure, and ethnicity), PWV values remained significantly increased in the TT genotype group compared with CC+CT genotypes group (P = 0.02; 10.3 ± 2.1 m/s and 9.7 ± 2.1 m/s, respectively). In addition, the TT genotype group had higher frequency of individuals with increased arterial
Table 1. Demographic, hemodynamic, and biochemical data according to C242T polymorphism of the NADPH oxidase p22phox subunit

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>CC+CT (n = 1041)</th>
<th>TT (n = 137)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethnicity, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian descent</td>
<td>37.3</td>
<td>40.9</td>
<td></td>
</tr>
<tr>
<td>Mulatto</td>
<td>48.8</td>
<td>46.0</td>
<td></td>
</tr>
<tr>
<td>African descent</td>
<td>6.0</td>
<td>6.6</td>
<td>0.80</td>
</tr>
<tr>
<td>Other</td>
<td>8.0</td>
<td>6.6</td>
<td></td>
</tr>
<tr>
<td>Sex, male, %</td>
<td>42.1</td>
<td>41.6</td>
<td>0.50</td>
</tr>
<tr>
<td>Arterial stiffness, %</td>
<td>47.4</td>
<td>62.0</td>
<td>0.001</td>
</tr>
<tr>
<td>Diabetes, %</td>
<td>7.7</td>
<td>8.8</td>
<td>0.91</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>42.7</td>
<td>43.8</td>
<td>0.79</td>
</tr>
<tr>
<td>Age, yr</td>
<td>45.0 ± 10.6</td>
<td>45.4 ± 10.6</td>
<td>0.87</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>26.4 ± 5.0</td>
<td>26.4 ± 5.6</td>
<td>0.96</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>127.0 ± 21.3</td>
<td>128.8 ± 22.0</td>
<td>0.31</td>
</tr>
<tr>
<td>DBP, mmHg</td>
<td>82.4 ± 13.2</td>
<td>84.5 ± 12.9</td>
<td>0.07</td>
</tr>
<tr>
<td>PWV, m/s</td>
<td>9.7 ± 2.1</td>
<td>10.3 ± 2.1</td>
<td>0.02</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>71.8 ± 14.3</td>
<td>72.6 ± 14.2</td>
<td>0.91</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>105.6 ± 31.8</td>
<td>102.2 ± 20.9</td>
<td>0.23</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>217.7 ± 45.3</td>
<td>216.5 ± 45.2</td>
<td>0.75</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dl</td>
<td>145.7 ± 39.8</td>
<td>146.1 ± 36.2</td>
<td>0.91</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dl</td>
<td>45.9 ± 12.3</td>
<td>45.2 ± 11.3</td>
<td>0.56</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>132.7 ± 94.3</td>
<td>127.6 ± 101.8</td>
<td>0.54</td>
</tr>
<tr>
<td>Creatinine, mg/dl</td>
<td>0.97 ± 0.19</td>
<td>0.98 ± 0.18</td>
<td>0.45</td>
</tr>
</tbody>
</table>

*pEthnicity was categorized as Caucasian descent, African descent, Mulatto (person with admixture between Caucasian and African descent), and other (Amerindians and Asian descent). Increased arterial stiffness was defined as PWV ≥9.50 m/s. Adjusted for age and sex. bAdjusted for age, sex, mean blood pressure (MBP); and ethnicity. BMI, body mass index; CI, confidence interval; DBP, diastolic blood pressure; SBP, systolic blood pressure; PP, pulse pressure; PWV, pulse wave velocity; HDL, high-density lipoprotein; LDL, low-density lipoprotein. Values in boldface are significant.

stiffness (62.0%) compared with CC+CT group (47.4%) (P = 0.001) (Table 1).

In categorical analyses, higher increased arterial stiffness odds was associated with TT genotype (OR 1.93, P = 0.002) compared with CC+CT group, even after adjustment for covariates (Table 2).

Nonhypertensive individuals’ group. Higher PWV values were also found in TT genotype group (9.5 ± 1.8 m/s) associated with CC+CT genotypes’ group (9.0 ± 1.5 m/s) (P = 0.04, even after adjusted). And the frequency of increased arterial stiffness was associated with the TT genotype group (46.8%) compared with CC+CT group (33.5%) (P = 0.02).

The TT genotype was associated higher increased arterial stiffness risk (OR 1.78, P = 0.02), even after adjustment for covariates (Table 2).

Hypertensive individuals’ group. PWV values (P = 0.16), frequency of increased arterial stiffness (P = 0.47), and increased arterial stiffness risk (P = 0.63) were not different according to C242T genotypes.

Analysis stratified by ethnicity. In this subanalysis, the Caucasian descent and Mulatto groups presented results similar to the entire sample, but African descent individuals did not present a significant difference (P > 0.05). In the Caucasian descent group, individuals carrying the TT genotype had higher PWV mean values (P = 0.01, 10.2 vs. 9.6 m/s), higher frequency of individuals with increased arterial stiffness (P = 0.01, 64.3% vs. 46.4%), and higher increased arterial stiffness odds (OR 2.13, P = 0.03) compared with individuals carrying CC+CT genotypes, even after adjustment for potential confounders (age, sex, mean arterial pressure). In the Mulatto group, individuals carrying the TT genotype had higher frequency of individuals with increased arterial stiffness (P = 0.03, 60.3% vs. 46.3%), and higher increased arterial stiffness odds (OR 1.9, P = 0.02), but they did not have higher PWV mean values compared with individuals carrying CC+CT genotypes (P = 0.51, 10.0 vs. 9.7 m/s), after adjustment for potential confounders (age, sex, mean arterial pressure).

p22phox and TNF-α Gene Expression in Smooth Muscle Cells

RT-PCR expression assay was available for cells from 80 individuals. From these, 42 had CC genotype, 32 had CT genotype, and six had TT genotype.

No difference in p22phox expression among C242T genotypes was observed: in the model CC+CT (21 ± 104), interquartile range (IQR) 9 × 10−4–47 × 10−4 vs. TT (18 × 10−4, IQR 5 × 10−4–49 × 10−4) (P = 0.71) genotypes, or in the model CC (21 ± 104, IQR 9 × 10−4–37 × 10−4) vs. CT (21 ± 104, IQR 9 × 10−4–69 × 10−4) vs. TT (18 × 10−4, IQR 5 × 10−4–49 × 10−4) (P = 0.79).

For the TNF-α expression assay, cells carrying the T allele had higher TNF-α expression CC (8 × 10−4, IQR 4 × 10−4–24 × 10−4) vs. CT (29 × 10−4, IQR 13 × 10−4–50 × 10−4) vs. TT (22 × 10−4, IQR 18 × 10−4–23 × 10−4) (P = 0.006).

**DISCUSSION**

The main finding of the study was the association between the C242T polymorphism of the NADPH oxidase p22phox subunit with arterial stiffness in the general population and in a nonhypertensive individuals group. Here, the TT genotype was associated with higher PWV values and higher frequency and risk of increased arterial stiffness.

Corroborating with our findings, Shimo-Nakanishi et al. (48) demonstrated an association of the T allele with increased NADPH oxidase activity suggesting higher ROS production in individuals harboring this variant. Castejón et al. (12) reported lower nitric oxide (NO) metabolites levels in individuals with the TT genotype, and they concluded that increased NADPH oxidase-dependent superoxide production could affect NO bioactivity. Kals et al. (31) showed the relationship between high-grade oxidative stress and increased arterial stiffness. In a recent study, Kuznetsova et al. (33) reported the association of pathological changes in the carotid with the T allele of C242T polymorphism (OR 1.70, P = 0.04). Our study did not measure NADPH oxidase activity and ROS levels, but our main find-

Table 2. Analysis of the arterial stiffness risk according to TT genotype

<table>
<thead>
<tr>
<th>Group</th>
<th>OR</th>
<th>95% CI</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>General population total</td>
<td>1.93</td>
<td>1.27–2.92</td>
<td>0.002</td>
</tr>
<tr>
<td>Nonhypertensive individuals</td>
<td>1.78</td>
<td>1.07–2.95</td>
<td>0.02</td>
</tr>
<tr>
<td>Hypertensive individuals</td>
<td>1.18</td>
<td>0.60–2.31</td>
<td>0.63</td>
</tr>
</tbody>
</table>

Logistic regression analysis was carried out to estimate the odds ratio (OR) for increased arterial stiffness according to C242T polymorphism (TT genotype vs. CC+CT genotypes). Adjusted values for age, sex, MBP, and ethnicity. Values in boldface are significant.
ings (TT genotype associated with higher PWV values and higher risk of increased arterial stiffness) could be explained, at least partially, from the studies above.

In contrast, some studies reported no significant association or association of the T allele with a protective effect for cardiovascular disease phenotypes (17, 24, 25, 32, 37, 50, 53). Thus, a recent meta-analysis was developed with 15 studies including 6,273 cases (coronary artery disease) and 5,045 controls (19). It was concluded that the T allele for the C242T polymorphism had significant protective effect only in Asian populations and absence of effect in Caucasian populations suggesting heterogeneity for a modulating role across ethnicities (19). It is worthy to note that the T allele frequency in the Asian population (between 0.13 and 0.15) is significantly lower than that in non-Asian populations (~0.35) (19). Our T allele frequency (34.6%) was similar to non-Asian populations described in previous studies.

NADPH oxidase is involved in the ROS production mainly in the vascular tissue (42), and ROS have an important role in the vascular remodeling through intracellular signaling of transcription factors responsible for expression of proinflammatory genes such as TNF-α, AP-1, and NF-κB (10). In addition, higher ROS levels lead to decreased NO bioavailability triggering the endothelial dysfunction process (30).

In this study, no difference in p22phox gene expression among C242T genotypes was found in vascular smooth muscle cells. Similar to our data, Mehranpour et al. (39) demonstrated that p22phox expression according to genotypes was not affected as determined by RT-PCR and Western blot analysis. Nevertheless, cells carrying the T allele had increased TNF-α expression levels. TNF-α is a cytokine with pleiotropic functions, and it is widely associated with the development of atherosclerosis and vascular calcification process (5, 28, 46, 51, 55). To clarify the possible mechanisms involved, De Keulen et al. (16) reported that TNF-α is able to stimulate the NADPH oxidase-mediated ROS production by increasing the p22phox expression in smooth muscle cells. In addition, Hirota et al. (26) demonstrated a positive correlation between circulating TNF-α levels and PWV values in Japanese patients with Type 2 diabetes; and Picchi et al. (45) reported that TNF-α can impair endothelial function leading to functional stiffness in the vessel wall due to reduced NO bioavailability. Our findings suggest the hypothesis that the C242T polymorphism could affect TNF-α level and the TNF-α level could affect NADPH oxidase function, in a positive feedback of repetitive cycles. However, two issues should be kept in mind as potential limitations of our functional analyses. First, the influence of cardiovascular risk factors on gene expression data according to genotypes can’t be fully excluded by maintaining these cells in culture for at least three passages. Second, we only obtained six cell lines from individuals of the TT genotype, and although this number is expected based on the genotype distribution from the general population, the small sample size precludes a more detailed analysis off the behavior of gene expression data on this group.

The present study demonstrates that both the entire and nonhypertensive group individuals carrying TT genotype present higher PWV values and an increased arterial stiffness phenotype. In the hypertensive group, the PWV values were not significantly different according to the C242T polymorphism. Rather than believing that statistical significance was not reached because of an underpowered sample size, we suggest a different behavior is present in the hypertensive strata (Table 2). Some possible explanations may be operant at this point. Once you have hypertension it is such an important factor in the determination of PWV that it reduces the importance, and consequently the capacity to detect, genetic and other factors modulating the PWV phenotype (47).

PWV is a noninvasive method widely accepted and validated to assess cardiovascular risk and atherosclerosis through arterial distensibility and stiffness. The arterial stiffness literature indicate the PWV measure as a continuous variable as the gold-standard method for analyzing this phenotype. Dichotomization of the measure is also an optional, though one should keep in mind that the cut-off point for defining an increased arterial stiffness is a controversial matter. Some authors have used the 10 or 12 m/s as an arbitrary point, but the majority use the median value of their sample as a cut-off. Here, significant differences were identified in both continuous and categorized data, which indicates a consistency of the results.

Conceivably, the PWV values and arterial stiffness phenotype will result from multiple factors, such as known covariates age, sex, blood pressure, and ethnicity (23, 27, 41, 47). Our analysis remains significant even after adjustment for these potential confounders; however, it is difficult to completely exclude the participation of these variables in the observed results. Related to ethnicity, in our stratified subanalysis, the significant results found in Caucasian descent and Mulatto groups show that the C242T polymorphism can be associated with arterial stiffness in a multiethnic Brazilian population sample as Mulatto group. However, the nonsignificant result found in African descent group can be a result of the small sample size of this group or, indeed, a real absence of association. Indeed, ours and other results suggest that population structure may be a major confounder of the association between C242T genotype and cardiovascular phenotypes, and this should be further explored.

Our data and some other studies report the existence of significant genetic contribution to arterial stiffness; however, they have not yet identified an exact signaling pathway associated with this phenotype of polygenic nature (14, 28, 34, 54). Nonetheless, in the described scenario, our investigation encourages replication studies and provides evidence that the C242T polymorphism of the NADPH oxidase p22phox subunit may indicate a pivotal via involved in arterial stiffness development.

In conclusion, we suggest that p22phox C242T polymorphism is associated with arterial stiffness evaluated by PWV in the general population. This genetic association shed light in the understanding of the genetic modulation on vascular dys-function mediated by NADPH oxidase.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).


