Original Research

# Common Single Nucleotide Polymorphisms of the p22phox NADPH Oxidase Subunit do not Influence Aortic Stiffness in Young, Healthy Adults

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**Introduction:** Aortic stiffness is a valuable biomarker for stratifying cardiovascular risk. NADPH oxidase regulates oxidative status in vessels; its single nucleotide polymorphisms (SNPs) modify the redox state of carriers and may lead to noxious structural alterations and affect the vasomotor properties of arteries. We hypothesized that genetic variability of NADPH oxidase would be accompanied by differences in aortic stiffness; to this end, we explored the interplay of pulse wave velocity (PWV), a measure of aortic stiffness, with common SNPs of the CYBA gene that encodes the p22phox subunit of NADPH oxidase.

**Methods:** 289 young, healthy adults were studied. The -930A/G, A640G and C242T CYBA SNPs were genotyped and PWV was measured. Differences in PWV across genotypes were examined in unadjusted models and after adjustment for confounders.

**Results:** Genetic variability of the examined SNPs did not result in changes of aortic stiffness. In unadjusted models, PWV did not differ across genotypes for the -930A/G (p=0.20), A640G (p=0.65) or C242T SNP (p=0.50). In stepwise multiple linear regression analysis only sex, age and systolic blood pressure emerged as independent predictors of PWV.

**Conclusions:** Common genetic variants of NADPH oxidase do not influence aortic stiffness in young, healthy adults.

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A large body of evidence has associated arterial structure and function with future cardiovascular outcomes, not only in the setting of hypertension, but also in the general population.[1-6] Aortic stiffness can be assessed in a noninvasive manner by measuring pulse wave velocity (PWV); this biomarker depends on distending aortic pressure and on classical risk factors.[5] Leaving aside the impact of pathological processes that pave the way for loss of elasticity in arterial walls, stiffness has been shown to have a genetically inherited component.[7,8] Genetic variability, in the form of single nucleotide polymorphisms (SNPs) of genes that regulate the composition of the arterial intima, media and adventitia, can influence PWV values.[9,10] Moreover, SNPs of enzymes partaking in inflammatory, neurohumoral and oxidative pathways have an impact on arterial stiffness.[11-15] The observation that common SNPs of the CYBA gene that encodes an essential subunit of NADPH oxidase lead to differential central and peripheral blood pressures raises the possibility of a similar influence of these SNPs on aortic stiffness.[16]

## Methods

### *Study population*

We enrolled 289 Greek Caucasians, who were randomly selected from the employ-

ees of two companies. They were free from cardiovascular/systemic inflammatory disease, hypertension, diabetes mellitus, and dyslipidemia, and were not taking any medication that could alter oxidative status or arterial stiffness.

### *Arterial stiffness measurement and genotyping*

Carotid-femoral PWV, the gold-standard index of large artery stiffness, was measured noninvasively (Complior®, Artech Medical, Pantin, France), as previously described.[17] Measurements were made in the morning; participants refrained from caffeine, alcohol, smoking, and food consumption for at least 12 hours prior to the study. Blood samples were obtained after a 12-hour fast for biochemical screening and genotyping of -930A/G, A640G and C242T CYBA SNPs using restriction fragment length polymorphism, as previously described.[18] Genotypes were determined by two independent investigators according to the pattern of the bands on electrophoresis. Participants gave informed consent to their participation in the study, which was approved by the local Ethics Committee.

### *Statistical analysis*

The Hardy-Weinberg equilibrium was tested using the $\chi^2$-test. Categorical variables are presented as frequencies and group percentages. Statistical normality was checked using the Kolmogorov-Smirnov test. Normally distributed continuous variables are presented as mean ± standard deviation (SD). Non-normally distributed variables are presented as median (25th-75th percentile) and were log-transformed for multiple linear regression analyses. Differences across genotypes were examined using the Kruskal-Wallis test. In order to explore the relationship of PWV and genotypes independently of confounders, we performed stepwise multiple linear regression analysis. Three models were constructed with PWV as dependent variable and genotypes for the -930A/G, A640G and C242T SNPs as independent variables in each model, respectively. Sex, smoking status, age, body mass index, total cholesterol, estimated glomerular filtration rate, C-reactive protein, heart rate, and systolic blood pressure were covariates. The SPSS 13.0 statistical software (SPSS, Chicago IL, USA) was used for the analyses. A two-tailed p-value $<0.05$ was considered significant.

Sample size calculation was based on the hypothesis that a difference of 0.3 m/s in PWV would be detected across genotypes. Previous studies by our group have shown an SD for PWV of 0.3 m/s. Therefore, a total sample size of 190 subjects was required to provide the study with 80% power at the 5% level of significance. We recruited additional subjects (n=289) for confidence.

## Results

Baseline characteristics are presented in Table 1. Successful genotyping was possible in 76.5% (n=221) of cases for -930A/G, in 94.8% (n=274) for A640G and in 87.9% (n=254) for the C242T SNPs. The distribution of alleles was consistent with the Hardy-Weinberg equilibrium ($\chi^2=3.29$, p=0.07 for -930A/G; $\chi^2=0.23$, p=0.63 for A640G; $\chi^2=1.18$, p=0.28 for C242T).

For -930A/G, G allele frequency was 0.50 and the prevalence of AA, AG and GG genotypes was 27.6%, 43.9% and 28.5%, respectively. PWV values did not differ across genotypes; AA: 6.0 (5.5, 7.0) m/s, AG: 6.2 (5.6, 7.0) m/s, GG: 6.6 (5.8, 7.5), p=0.20. For A640G, G allele frequency was 0.49 and the prevalence of AA, AG and GG genotypes was 26.3%, 48.5% and 25.2%, respectively. All A640G genotypes had similar PWV values; AA: 6.4 (5.7, 6.8) m/s, AG: 6.0 (5.4, 7.2) m/s, GG: 6.2 (5.5, 7.1), p=0.65. For C242T, T allele frequency was 0.41 and the preva-

**Table 1.** Baseline characteristics of the study population. Continuous variables are presented as mean ± SD or median (25th, 75th percentile), while categorical variables are presented as percentages.

| | |
|---|---|
| Age, years | 40 (33, 47) |
| Men, % | 63.7 |
| Smokers, % | 51 |
| Pack years | 18 (10, 30) |
| BMI, kg/m$^2$ | 25.1 (23, 28.1) |
| eGFR, mL/min/1.73 m$^2$ | 106 ± 26 |
| Total cholesterol, mg/dL | 193 ± 40 |
| LDL cholesterol, mg/dL | 127 (107, 150) |
| HDL cholesterol, mg/dL | 46 (40, 54) |
| Triglycerides, mg/dL | 79 (57, 109) |
| Glucose, mg/dL | 87 (81, 94) |
| CRP, mg/dL | 0.9 (0.5, 1.6) |
| PWV, m/s | 6.2 (5.5, 7.0) |

BMI – body mass index; eGFR – estimated glomerular filtration rate; LDL – low-density lipoprotein; HDL – high-density lipoprotein; CRP – C-reactive protein; PWV – carotid-femoral pulse wave velocity.

lence of CC, CT and TT genotypes was 33.5%, 51.6% and 15%, respectively. Again, PWV values did not differ; CC: 6.3 (5.7, 7.2) m/s, CT: 6.2 (5.5, 6.9) m/s, TT: 6.4 (5.7, 7.3), p=0.50.

In all multiple linear regression analyses only sex, age and systolic blood pressure emerged as independent predictors of PWV (Table 2).

C-reactive protein values did not differ across genotypes for all three SNPs (-930A/G: $\chi^2$=0.05, p=0.97; A640G: $\chi^2$=0.99, p=0.61; C242T: $\chi^2$=0.05, p=0.98).

## Discussion

PWV is the gold-standard index of aortic stiffness. It has emerged as an easily measured, noninvasive, yet robust marker that can predict adverse cardiovascular sequelae.[3] Having been introduced in the recommendations for the workup of hypertension, its predictive role now further extends to various diseases, including renal disease, diabetes mellitus, inflammatory states and, finally, in the general population setting.[2,19] Oxidative burden holds a pivotal role in the progression of such disease entities. Elevated levels of reactive oxygen species (ROS) produced by enzymic complexes predispose to the progression of atherosclerotic processes in vessel walls, and superoxide generation in vessel walls has a functional relationship with aortic elasticity.[20,21] The interplay between genes that regulate ROS production and arterial stiffness forms a novel, yet complex field of study. Recent data point to the existence of gene-environment interactions. Indeed, Mayer and coworkers have reported an impact of endothelial nitric oxide synthase SNPs on PWV only in smokers.[22]

The NADPH enzyme consists of six subunits. The p22phox subunit is ubiquitous in all NADPH isoforms. SNPs of the CYBA gene that encodes p22phox can lead to differential ROS production.[23,24] Our working hypothesis was that genetic variability of the CYBA gene would be reflected in a marker of vascular function such as PWV. This is the first study to explore the interconnection between aortic stiffness and SNPs of NADPH oxidase, which is the major source of vascular ROS. Our main finding is that the -930A/G, A640G, and C242T SNPs of the CYBA gene are not associated with alterations in aortic stiffness. This holds true in young, healthy Caucasians with no history of cardiovascular comorbidities. Moreover, the lack of association remains even after controlling for confounding factors that have a known impact on PWV levels.

Candidate gene analyses for arterial stiffness, like our study, focus on distinct SNPs that regulate a specific, pathophysiologically relevant pathway that can alter downstream arterial properties. A number of SNPs have been reported to influence PWV;[25] notably, genes of the renin-angiotensin-aldosterone system (RAAS), matrix proteins/metalloproteinases, nitric oxide pathway, β-adrenergic/endothelin receptors, and inflammatory cascade.[12,22,26-28] On the other hand, genome wide association (GWA) studies, having the benefit of examining the genome as a whole without *a priori* hypotheses, have reported intriguing correlations between genes and arterial stiffness. Notably, in the Framingham Heart Study a number

**Table 2.** Multiple linear regression analysis for pulse wave velocity. In all models $\log_{10}$ pulse wave velocity was the dependent variable and sex, smoking status, $\log_{10}$ age, $\log_{10}$ body mass index, total cholesterol, $\log_{10}$ estimated glomerular filtration rate, $\log_{10}$ C-reactive protein, $\log_{10}$ heart rate and $\log_{10}$ systolic blood pressure were forced as covariates; stepwise selection was used. For model 1 ($R^2$=0.320), the -930A/G genotype, for model 2 ($R^2$=0.377), the A640G genotype and for model 3 the C242T genotype ($R^2$=0.368) was used as an independent variable.

| Model | Independent variable | Unstandardized coefficient $\beta$ (s.e.) | Standardized coefficient $\beta$ | 95% confidence interval | p |
|---|---|---|---|---|---|
| 1 | Sex (female) | -0.027 (0.013) | -0.162 | -0.053 to -0.002 | 0.036 |
| | $\log_{10}$ SBP | 0.436 (0.122) | 0.283 | 0.195 to 0.677 | 0.001 |
| | $\log_{10}$ age | 0.321 (0.064) | 0.363 | 0.194 to 0.447 | 0.001 |
| 2 | Sex (female) | -0.039 (0.012) | -0.225 | -0.062 to -0.016 | 0.001 |
| | $\log_{10}$ SBP | 0.443 (0.115) | 0.274 | 0.217 to 0.670 | 0.001 |
| | $\log_{10}$ age | 0.360 (0.060) | 0.387 | 0.242 to 0.478 | 0.001 |
| 3 | Sex (female) | -0.038 (0.012) | -0.225 | -0.061 to -0.015 | 0.001 |
| | $\log_{10}$ SBP | 0.399 (0.109) | 0.260 | 0.185 to 0.614 | 0.001 |
| | $\log_{10}$ age | 0.363 (0.058) | 0.403 | 0.249 to 0.478 | 0.001 |

SBP – systolic blood pressure

of genes not previously regarded as relevant to arterial stiffness have attracted attention, while the importance of RAAS genes has been downplayed.[29] Importantly, SNPs of NADPH oxidase have not been examined to date in either candidate gene or GWA studies.

The lack of association of CYBA SNPs with PWV can be attributed to a number of reasons. Change in arterial properties, as manifested by a loss of elasticity, is a cumulative process. It reflects the impact of numerous pathways including, but not limited to oxidative status, such as extracellular matrix composition, vascular smooth muscle cell properties and inflammatory alterations. Change of only one parameter would have to be of significant magnitude to result in PWV changes, especially in the absence of comorbidities. Given the fact that our cohort was young and healthy, it can be postulated that changes in oxidative status across genotypes would not alone suffice to make arteries stiffer. Nevertheless, we have previously reported that this can be the case for G-allele carriers of the -930A/G regarding peripheral and aortic blood pressures.[16] Taken together, these results build on previous knowledge stating that PWV is a cumulative marker of arterial properties reflecting structure and smooth muscle tone; it is thus less amenable to short-term changes when compared to peripheral/aortic blood pressures.[30]

Certain limitations should be taken into account when interpreting the results of our study. Only young, healthy Caucasians were enrolled; a different effect of the studied SNPs may be observed in cohorts of various ethnic backgrounds. Furthermore, the role of the CYBA SNPs may differ in the presence of comorbidities that are closely linked to redox status, such as hypertension, diabetes mellitus and dyslipidemia, through upregulation of gene transcription and expression. Since only SNPs of the CYBA gene were typed, possible synergistic effects with other polymorphisms were not taken into account.[14]

In conclusion, our findings suggest that common polymorphisms of the CYBA gene that encodes the essential p22phox subunit of NADPH oxidase do not influence aortic stiffness, as measured in young, healthy individuals. Whether this finding is applicable to cohorts with hypertension or other diseases that are linked to an elevated redox state is a vexing issue that needs to be addressed in the future. Further studies are warranted to elucidate the role of CYBA SNPs in diverse populations both with and without cardiovascular disease.

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