NOS3 Variants, Physical Activity, and Blood Pressure in the European Youth Heart Study

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BACKGROUND
In this study, we examined the influence of genetic variation in NOS3 on resting blood pressure (BP) in children and adolescents from the European Youth Heart Study (EYHS). Because the NOS3 gene expression is altered by physical activity (PA), we also tested for interaction between habitual PA and NOS3 variants on BP.

METHODS
A cross-sectional, random sample of 8–10-year old children (n = 1,214) and 14–16-year old adolescents (n = 1,141) from Denmark and Estonia were genotyped for four NOS3 tagging polymorphisms (rs1800783, rs1799983 (Glu298Asp), rs3918227, rs743507). PA was measured objectively using a hip-mounted accelerometer and through self-reported bicycling and TV-viewing. Permutation testing was used to correct for multiple testing, yielding an α level of 0.006.

RESULTS
Glu298Asp showed age-group-dependent associations with BP. In adolescents, Asp298 allele homozygotes had 0.19 s.d. (95% confidence interval (CI): 0.06; 0.13, P = 0.004) higher diastolic BP (DBP) and 0.25 s.d. (95% CI: 0.05; 0.46, P = 0.015) higher systolic BP (SBP), compared to Glu298 allele carriers. None of the three other single-nucleotide polymorphisms (SNPs) were associated with BP in adolescents. In children, none of the SNPs were associated with BP. No evidence of interaction between Glu298Asp and objectively measured PA was observed. Both self-reported bicycling and TV-viewing nominally modified the association between Glu298Asp and BP in adolescents (P < 0.05), the genetic effect being most apparent in inactive individuals. However, none of the interactions persisted after correcting for multiple testing.

CONCLUSIONS
The NOS3 Glu298Asp variant may associate with resting BP in adolescence but not in childhood, an effect that could be modified by PA.

Keywords: adolescents; blood pressure; children; epidemiology; genetics; hypertension; NOS3; physical activity

Primary hypertension is a major risk factor for various cardiovascular diseases such as myocardial infarction, stroke, congestive heart failure, end-stage renal disease, and peripheral vascular disease.1 Heritability studies suggest that variation in blood pressure (BP) between individuals is, in part, explained by genetic factors (40–60%).2 One reason why success has been limited in detecting specific molecular variants may be that genes for primary hypertension interact with environmental parameters, thus potentially masking main genetic effects.3 Human and animal studies point to a number of candidate genes, which may interact with environmental parameters. These genes include the endothelial nitric oxide synthase gene (NOS3) which encodes the enzyme (eNOS) that synthesizes endothelium-derived nitric oxide.

Both exercise training and forced inactivity alter the vascular expression of eNOS.4,5 These observations have been associated with the exercise- and inactivity-induced effect on endothelium-dependent vasodilation.4,5 Thus, genetic variation in NOS3 may be important for BP regulation and for the development of hypertension, effects which may be modified by habitual physical activity (PA), as previously indicated in adults.6

In this study, we tested two hypotheses: (i) common variants in the NOS3 gene are associated with BP during rest and (ii) PA modifies these potential associations. Our study population comprised children and adolescents from Denmark and Estonia who had participated in the European Youth Heart Study (EYHS).

METHODS
Design. This is a cross-sectional study of children and adolescents from the Danish and Estonian part of the EYHS. Eight-to-ten-year old children and 14–16-year old adolescents were recruited from two European cities: the city of Odense,
which is the third-largest city of Denmark, and from the city and surrounding rural areas of Tartu, the second-largest city of Estonia. The sampling procedure and methods have been described in detail elsewhere. Ethical approval for the study was obtained from the local research ethics committee in each study region.

**Anthropometry and maturity.** Height and weight were measured using standard anthropometric procedures, with the participants wearing light clothing and no shoes. Sexual maturity was assessed by trained research assistants using Tanner classification, of breast development in girls and pubic hair growth in boys. Maturity stage among the 8–10-year olds was almost exclusively stage 1 or 2 in both Danish and Estonian children and almost all adolescents were categorized as Tanner stage 3, 4, or 5. Thus, we collapsed maturity to a 3-point ordinal variable (Tanner 1, Tanner 2–3, and Tanner 4–5).

**Habitual PA.** Habitual PA and sedentary behavior was assessed with an accelerometer (ActiGraph, model 7164; ActiGraph, Walton Beach, FL), self-reported bicycling to school and TV-viewing. The participants were asked to wear the accelerometer on the right hip using an elastic band during the whole day (except while sleeping) for five consecutive days. Accelerometer data were processed using custom-written software (available from the authors upon request) which scans individual data files for abnormal values and periods with zero activity to generate an individual average PA value (counts/min) for the time the monitor was worn. Children and adolescents who did not accumulate at least 600 registered min/day for at least 3 days were excluded from further analysis. The accelerometer method has been validated in both children and adolescents against various criteria and found to be a valid method (r = 0.54–0.66) for assessment of habitual PA in epidemiological studies. Because the sample size was markedly reduced due to missing or noncomplete objective PA data, we also included information on self-reported bicycling to school and time spent viewing TV. We have previously shown that these PA measures are associated with fitness and obesity in earlier reports from EYHS. Responses on usual travel to school, the amount of time spent watching TV before and after school and eating while viewing TV were obtained using a computer-based questionnaire. Commuting to school was asked as follows: “How do you usually travel to school?” and a binary variable were generated for cycling to school. Two questions were asked about the time spent viewing TV: “How many hours of TV do you usually watch before school?” and “How many hours of TV do you usually watch after school?” The data were subsequently summarized into one variable (h/day). Frequency of eating meals while viewing TV was obtained by asking: “How often do you eat meals while watching TV?” yielding an ordinal variable with five categories which was collapsed into a binary response: most days/less than twice a week.

**BP.** BP was measured with a Dinamap pediatric and adult neonatal vital signs monitor (model XL; Critikron, Tampa, FL) with appropriate cuff size. Five measurements were taken at 2-min intervals with the mean of the final three measurements used in all analyses. Diastolic BP (DBP) and systolic BP (SBP) were measured in the sitting position after 5 min of rest by trained researchers. The Dinamap monitor has been validated in children against direct radial artery readings (mean error 0.24 mm Hg SBP, 1.28 mm Hg DBP). BP measurements were converted to age-, height-, and sex-specific z-scores according to National High BP Education Program (NHBPEP) Working Group on Children and Adolescents. This allows adjusting for the effect of growth during childhood and adolescence on BP.

**SNP selection and genotyping.** Blood samples were obtained after an overnight fast and stored at −80°C before analysis. DNA from white blood cells was extracted and genotyped using the Illumina high-throughput mass-array platform (Leveraging BeadArray technology). Single-nucleotide polymorphisms (SNPs) were selected to capture the common allelic variation in NOS3. Accordingly, we derived SNPs within NOS3 for genotyping from the HapMap phase II CEU panel. From HapMap release 19, five SNPs were reportedly located within ±5 kb of the NOS3 locus. Tagger, as implemented in HaploView, was then used to identify tagging SNPs. Minor allele frequency and r² thresholds were set at ≥5% and ≥0.80, respectively. We force-included the nonsynonymous rs1799983 located in exon 7 (yielding a glutamate >aspartate amino acid change at residue 298 (Glu298Asp)). Altogether, four NOS3 SNPs were genotyped: rs1800783 (intronic), rs1799983, rs3918227 (intronic), and rs743507 (intronic) with minor allele frequency of 0.33, 0.28, 0.10, and 0.21 respectively. All SNPs conformed Hardy–Weinberg equilibrium (P >0.05). The genotyping success rate was >0.989. In combination, these four SNPs captured 100% of the common allelic variation. Linkage disequilibrium between SNPs (r²) is presented in Figure 1. Participants who were not European white were excluded from the analyses (n = 120) to minimize confounding by population stratification.
Statistical analyses. Multiple linear regression models were used to analyze the main effect of each NOS3 SNP on BP. Adjustments were made for age, gender, and sexual maturation in order to reduce the residual (non-genetic) variance in BP. To test whether PA modifies the association between genotype and BP, an interaction term between genotype and the PA variable was included in a separate model containing both the main effects and interaction term. Because TV-viewing has been associated with frequency of eating meals while watching TV in a previous report from EYHS, we additionally adjusted for eating while watching TV in this analysis. We employed additive inheritance models (per allele effect) in all analyses, with common allele homozygous as the reference group. However, owing to the results of a recent meta-analysis involving 12,142 participants of the Glu298Asp variant on ischemic heart disease, which suggested this variant may have a recessive effect, we also tested the recessive model for this SNP. Standard linear regression diagnostics were performed, including examining linearity and normality of residuals.

As previous studies investigating NOS3 genotype association with BP have reported age- and gender-specific associations, we separately tested whether these variables modified the genotype association with BP by including interaction terms in the main effect analyses. Likewise, we crudely evaluated potential population stratification by testing the significance of the interaction term between NOS3 SNPs and country in the main effects models. Stratified analyses by these variables were conducted when a significant interaction had been detected. Otherwise, adjustments for these covariates were made to the models.

In order to correct for multiple testing, we used the minimum P value permutation-based approach. Specifically, we randomly permuted BP levels 10,000 times and for each permutation analyses were rerun to generate null distributions, against which the observed test statistics were compared. On the basis of these multiple permutations, statistical significance was set at an alpha level of 0.006 to ensure an experiment-wise type I error rate of 0.05.

All statistical analyses were performed with Stata 9 (Stata, College Station, TX), except power calculations which was done using Quanto version 1.2.3 (http://hydra.usc.edu/GxE). With a sample size of n = 2,355 and an alpha level of 0.006, we have 80% power to detect main effects between 0.07–0.11 s.d. for DBP and 0.1–0.16 s.d. for SBP, depending on the allele frequency of the SNPs (0.10–0.33) and assuming Hardy–Weinberg equilibrium. This corresponds to a per allele effect of ~0.8–1.2 mm Hg DBP and 1.2–1.8 mm Hg SBP.

RESULTS

We observed no gender- or country-specific genotype associations with DBP or SBP (P > 0.4) but there was a significant age group—Glu298Asp interaction on SBP (P = 0.005) and a borderline significant interaction on DBP (P = 0.03). Thus, all results are stratified by age group. The characteristics of the study population by age group are summarized in Table 1.

Association between NOS3 variants and BP

The rs1799983 (Glu298Asp) was significantly associated with DBP and SBP variation in adolescents under a recessive model of inheritance (Table 2). Minor allele homozygotes (TT (Asp/Asp)), had 0.19 s.d. (95% confidence interval (CI) 0.06;
0.13, $P = 0.004$) higher DBP and 0.25 s.d. (95% CI: 0.05; 0.46, $P = 0.015$) higher SBP compared to carriers of the common G allele (Glu), corresponding to ~2.0 mm Hg higher DBP and 2.6 mm Hg higher SBP. None of the other SNPs showed any significant interaction with any of the three PA phenotypes.

However, this additional adjustment did not materially change the results. None of the other SNPs showed any significant interaction between Glu298Asp and obesity. Estimates (per allele) with nominal $P$ values and 95% CI are from multiple linear regression adjusted for age, gender, country (0/1) and maturity (0/1/2). The experiment-wise significance threshold required to keep type I error rate at 0.05 is 0.006. Both interactions were not significant after correcting for multiple testing. A significant interaction between TV-viewing and Glu298Asp was observed within adolescents for DBP ($P = 0.04$) and SBP ($P = 0.04$) in adolescents (Figure 2) but not in children. However, the significance of these interactions did not persist after correcting for multiple testing (a level of 0.006). Comparing adolescent carriers of the common G allele (Glu) with TT (Asp/Asp) homozygotes reporting not cycling to school, the TT (Asp/Asp) homozygotes had 0.31 s.d. higher DBP (95% CI: 0.12; 0.49, $P = 0.001$) and 0.45 s.d. higher SBP (95% CI: 0.16; 0.74, $P = 0.003$), whereas no association were observed in adolescents who reported cycling to school.

A significant interaction between TV-viewing and Glu298Asp was observed within adolescents for DBP ($P = 0.0057$) but no interaction was observed for SBP ($P = 0.11$). Figure 3 illustrates a positive association between TV-viewing and DBP in individuals homozygous for the minor T (Asp) allele ($\beta = 0.16$ (95% CI: 0.04; 0.28, $P = 0.009$)) but not in individuals carrying the common Glu298 allele ($\beta = 0.01$ (95% CI: ~0.02; 0.03, $P = 0.74$)). No interaction between TV-viewing and Glu298Asp on BP was observed in children.

Since PA is correlated with adiposity, we considered whether the interaction with TV-viewing and bicycling was independent of a possible interaction between Glu298Asp and obesity. However, this additional adjustment did not materially change the results. None of the other SNPs showed any significant interaction with any of the three PA phenotypes.

### Interaction between PA and NOS3 genotype on BP

We further investigated whether habitual PA modified the association between the Glu298Asp variant (recessive model) and BP. No indication of interaction between Glu298Asp and objectively measured habitual PA on DBP or SBP in children or adolescents was observed ($P > 0.26$). We observed nominally significant interactions between cycling to school and Glu298Asp for DBP ($P = 0.04$) and SBP ($P = 0.04$) in adolescents (Figure 2) but not in children. However, the significance of these interactions did not persist after correcting for multiple testing (a level of 0.006). Comparing adolescent carriers of the common G allele (Glu) with TT (Asp/Asp) homozygotes reporting not cycling to school, the TT (Asp/Asp) homozygotes had 0.31 s.d. higher DBP (95% CI: 0.12; 0.49, $P = 0.001$) and 0.45 s.d. higher SBP (95% CI: 0.16; 0.74, $P = 0.003$), whereas no association were observed in adolescents who reported cycling to school.

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Recent independent meta-analyses of variants in NOS3 and hypertension and BP suggest no association between Glu298Asp and BP but reveals significant heterogeneity among results, possibly owing to gene–environment interactions, ethnic diversity, population admixture, and publication bias.24 Subsequent to these meta-analyses, one study reported a positive association26 between the Glu298Asp and BP/hypertension but we and others did not detect any significant association in adult populations. Our present results support the notion that the Glu298Asp variant may be involved in the regulation of BP in European adolescents. Although evidence from mechanistic studies of functional consequence of the amino acid change for glutamate to aspartate at residue 298 remains controversial, two recent studies have shown that the Asp298 allele alters eNOS function.29,30

The detrimental effect of inactivity and the response to chronic exercise on BP varies greatly between individuals which may be partly attributable to genetic variation.31 Previous studies have indicated that genetic variants in NOS3 may interact with PA.32 We suspect that the Glu298Asp genotype effect on BP may become more prominent with increasing exposure to inactivity. Forced physical inactivity in mice results in impaired endothelium-dependent vasodilation through reduced vascular expression of eNOS.5 Thus, the reduction in nitric oxide bioavailability, as a consequence of being sedentary, may depend on the NOS3 genotype. In line with this, we observed a positive association between TV-viewing and DBP within Asp298 homozygote adolescents, whereas there was no association between TV-viewing and DBP in carriers of the common Glu298 allele. In addition, adolescents not commuting to school by bicycle who are homozygous for the Asp298 allele had a 0.31 s.d. higher DBP and 0.45 s.d. higher SBP compared to common allele carriers. This corresponds to an absolute difference of ~3–5 mm Hg and may be of clinical relevance because small reductions in BP levels may have considerable impact on cardiovascular disease incidence and mortality.33 Again, the statistical evidence of these interactions were weak and only the Glu298Asp interaction with TV-viewing on DBP persisted after correcting for multiple testing.

Although TV-viewing may displace some PA, this sedentary behavior has also been associated with snacking.13 Especially intake of energy-dense foods high in added sugars and fats have been associated with TV-viewing in youth.34 The interaction between the Glu298Asp and TV-viewing was independent of eating while watching TV which indicates that the association between the Glu298Asp and TV-viewing was independent of school commute, and may depend on the acceleration behavior, although residual confounding caused by imperfect measurement of diet cannot be ruled out. A high fat/high sucrose diet reduces the vascular expression of eNOS5 and Nos3−/− mice fed a high-fat diet develops exaggerated arterial hypertension compared to wild-type mice.36 Accordingly, the observed interaction between Glu298Asp and TV-viewing on BP needs further attention in future studies since this may be partly confounded by dietary factors.

Interestingly, it was the self-reported measure of active commuting and TV-viewing and not the accelerometric...
measure of nondomain-specific PA which showed evidence of interaction, albeit only nominally for bicycling. Although this may be explained by the considerably lower sample size for the accelerometer analysis, an alternative explanation may be that commuting to school and TV-viewing are more stable behaviors and therefore more reliably captured, compared to general PA for which the accelerometry method, despite being objective, does have its limitations. To address the matter of statistical power, we repeated the analyses of interaction for bicycling to school and TV-viewing with valid accelerometer data in post-hoc analyses. In these analyses, we observed small attenuations of the interaction effect estimates but these were no longer nominally significant. These observations support the notion that statistical power is at least part of the explanation for absence of effect in the accelerometer analysis.

It is widely believed that the penetrance of certain genetic variants is age-dependent. This has previously been suggested attributable to factors such as differences in the duration of environmental exposures37 and epigenetics.38 Our results indicate that the effect of Glu298Asp variant may be dependent on chronological or biological age, because there was no effect of genotype on BP in children.

There are several limitations of this study. Measurement error in independent variables can introduce bias in the regression coefficients.39 The error associated with measurement of PA is probably nondifferential across genotypes and will therefore only dilute the interaction between genotype and PA on BP. Thus, together with the smaller sample size for the NOS3 × accelerometry interaction we cannot rule out type-II error as an explanation for this null finding. In addition, it is possible that the derived tagSNPs did not capture the full allelic variation in NOS3. We replicated our SNP selection in the more recent HapMap release 27, force including only the four SNPs genotyped in the present study. This post-hoc analysis indicated that we only tagged four of nine additional SNPs, therefore only capturing 57% of the total allelic variation. Thus, it is not unlikely that our characterization of allelic variation in the NOS3 locus may have missed common variants of functional importance.

Our results suggest that the Glu298Asp polymorphism may play a role in the regulation of BP early in life in European whites, possibly more so in adolescents who are more sedentary. These observations could advance the understanding of why some individuals are more prone to the health risks of physical inactivity. The results should be included with other pediatric population data in future meta-analyses and ideally replicated in trials manipulating both PA and inactivity.

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