The *NOS3* G894T (rs1799983) and -786T/C (rs2070744) polymorphisms are associated with elite swimmer status

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ABSTRACT: Endothelial nitric oxide synthase (*NOS3*) generates nitric oxide in blood vessels and is involved in the regulation of vascular function, metabolism and muscle fibre type transformations. Evidence suggests that the *NOS3* G894T (rs1799983) and -786T/C (rs2070744) polymorphisms are associated with athletic performance. The purpose of this study was to determine the association between the *NOS3* G894T and -786T/C polymorphisms with elite swimmer status in Polish athletes. One hundred and ninety-seven Polish swimmers (104 males and 93 females), who competed in national and international events, and 379 healthy control subjects (222 males and 157 females) were recruited for this study. The swimmers were divided into two groups: short distance swimmers (SDS; n=147; 50-200 m) and long distance swimmers (LDS; n=49; more than 500 m). As expected, the frequencies of the -786T/C T allele (77.0 vs. 63.1%, p = 0.0085) and G-T haplotype (63.7 vs. 52.0, p=0.025) were significantly higher in the LDS group in comparison with controls. Compared with the -786T/C CC genotype, the chance of being a long distance swimmer was 8.49 times higher (CI=1.14-62.78, p=0.023) for the carriers of -786T/C T allele than in control subjects. On the other hand, the Asp allele frequency was significantly higher in the female SDS group compared with controls (34.3 vs. 18.5%, p=0.00043). In conclusion, our results demonstrate that the T allele and the G-T haplotype of the -786T/C and G894T polymorphisms may be beneficial for long distance swimmers.

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INTRODUCTION

Nitric oxide (NO) is a gaseous free radical that is the most potent endothelium-derived relaxation factor [1]. NO is synthesized from arginine by a family of three distinct isoforms of nitric oxide synthase (NOS) enzymes.. Both neuronal NOS (nNOS, NOS1) and endothelial NOS (eNOS, NOS3) are constitutively expressed, while inducible NOS (iNOS, NOS2) is not expressed under normal circumstance although it may be induced under stress conditions [2]. A growing body of evidence suggests that NO is involved in glucose metabolism (human skeletal muscle glucose uptake during exercise), control of skeletal muscle structure and function, skeletal muscle fiber type conversion as well as mitochondrial ATP production and oxygen consumption in skeletal muscles [3,4] which are all crucial for aerobic and anaerobic performance. NO also plays an important role in repairing and regeneration of myocardium and in the modulation of oxygen consumption in the myocardium [5].

There are two NOS isoforms that have been identified in skeletal muscle – nNOS and eNOS. nNOS is the primary isoform to be found in skeletal muscle, whereas eNOS is mainly expressed in endothelial cells and mostly contribute to control of vascular tone.The endothelial nitric oxide synthase (eNOS or NOS3) is encoded by *NOS3* gene that is located on chromosome 7 (7q36). The gene spans roughly 21 kb of genomic DNA and comprises 26 exons that encode

a 1203 amino acids protein [6]. The *NOS3* has been extensively screened for polymorphisms and several polymorphic sites have been identified. The most examined and functionally related common variants of the *NOS3* are single nucleotide polymorphisms (SNP): promoter -786T/C (rs2070744), G894T (Glu298Asp or, E298D or rs1799983) in exon 7, as well as the variable number tandem repeats (VNTR, microsatellite (CA)n repeats in intron 13 and 27 bp repeats in intron 4) [2].

Several investigations have shown that *NOS3* polymorphic variants can lead to altered transcription of eNOS and/or processing rates, and in that way interfering with normal enzyme function [7]. On the basis of these facts, the eNOS gene variants have been extensively investigated to determine their relevance in various diseases such as myocardial infarction, hypertension, coronary artery disease, stroke, and evidence supports a major role of these variants in increasing susceptibility to cardiovascular disease [2].

Numerous candidate gene studies that have been performed also indicate that *NOS3* gene -786T/C, G894T and VNTR polymorphisms are associated with several health/fitness, training or exercise response phenotypes, e.g. adaptation of parasympathetic modulation response to exercise training [8], cardiovascular traits such as blood pressure [9] and heart rate responses [10], cardio-biochemical parameters [11], vascular reactivity [12], exercise-induced adaptation to hypoxia [13] and aerobic capacity of athletes [14]. Collectively, these data suggest that *NOS3* and its polymorphisms are logical candidates for association with athlete status and physical performance.

In fact, -786T/C and G894T variants of *NOS3* have been associated with endurance performance, elite endurance athlete [13,15], power athlete [16,17] and soccer player's [18] statuses, as well as with the differentiation of elite power from endurance athletes [19]. More specifically, significant differences in the frequency of the *NOS3* 786T/C T allele (75.4% vs. 65.0%) between endurance-oriented Ukrainian athletes and controls has been found [13]. However, Gómez-Gallego et al. did not find any differences in the frequency of the *NOS3* 786T/C T allele between Spanish world-class endurance athletes and controls [16]. As to the power athlete status, Drozdovska et al. (2012) have found that the frequency of the *NOS3* 786T/C T allele was significantly higher in Ukrainian power-oriented athletes compared to controls [20]. These results were confirmed in two independent studies of Spanish elite power-oriented athletes and non-athletic controls [16] and Italian power-oriented athletes [17].

With respect to the *NOS3* G894T polymorphism and its relation to athletic performance and/or athletes' status, results across various studies have been inconsistent. Saunders et al. reported an excess of the G894T G allele (combined with a *BDKRB2*-9/-9 genotype) in the fastest finishing Caucasian Ironman triathletes when compared with control subjects [15]. However, Wolfarth et al. in the Genathlete study found no difference in allele and genotype frequencies of the G894T polymorphism between elite endurance athletes and controls [21]. Likewise, no differences in allele and genotype frequencies of the G894T were found by Buxens et al. between elite Spanish Caucasian elite endurance and power-oriented athletes [22]. On the other hand, Sessa et al. have shown that the G894T G allele was over-represented in Italian power-oriented athletes compared to controls [17].

Taken together, although inconsistent results exist, previous findings indicate that more common *NOS3* alleles (i.e. -786T/C T with frequency of ~ 56% in European populations and G894T G with frequency of ~ 66% in European populations) may be favorable for both power and endurance performance [23].

There are some behavioral variability and motor performance differences as an effect of practice specialization in swimming between competitive short distance swimming vs. long distance swimmers [24]. However regular swimming modulate oxidant-antioxidant balance via the involvement of the endothelial NO system [25] where vascular adaptive response involved an increase in endothelial nitric oxide is induced by swimming exercises [26]. Previously we suggested that the response to long-term exercise training could be modulated by the *BDKRB2* polymorphism in competitive male swimmers [27]. In this article we hypothesize that the *NOS3* G894T and -786T/C polymorphisms may influence swimming performance in Polish competitive swimmers.

The purpose of this study was to determine the association between the *NOS3* G894T and -786T/C polymorphisms with elite swimmer status in Polish athletes.

MATERIALS AND METHODS

Ethics Committee

The Pomeranian Medical University Ethics Committee, Poland, approved the study and an informed consent form was completed by each participant. The study complied with the guidelines set out in the Declaration of Helsinki.

Participants

One hundred and ninety-six Polish swimmers (104 males and 93 females; 20.31 ± 2.67 years), who competed in national and international events, were recruited for this study. All participants were Caucasians to minimize the influence of racial genetic skew and to remove any potential population stratification problems. The swimmers were divided into two groups, based on their competitive distance and values of relative contribution of the aerobic or/anaerobic energy systems: long distance swimmers (LDS; n=49; 24 males, 25 females), more than 500 m (mainly aerobic events) and short distance swimmers (SDS; n=147; 80 males, 67 females), between 50 and 200 m (mainly anaerobic events).

All investigated swimmers had been finalists of the Polish National Championships. Additionally, 7 of them had participated in the Olympic Games and 48 of them had taken part in the World Championships or European Championships. The whole group of swimmers included 8 World Championship medalists, 15 European Championship medalists and 128 Polish Championship medalists.

TABLE 1. Frequencies of the *NOS3* gene G894T and -786T/C genotypes, alleles and haplotypes in Polish swimmers and controls.

Group	n	*NOS3* G894T genotypes			p	HWE	Glu298 allele, %	p	*NOS3* -786T/C genotypes			p	HWE	T allele, %	p	*NOS3* haplotypes				p§
		GG (%)	GT (%)	TT (%)					TT (%)	TC (%)	CC (%)					G-T (%)	G-C (%)	T-C (%)	T-T (%)	
MALES																				
Controls	222	113 (50.9)	89 (40.1)	20 (9.0)	1.000	0.744	71.0	1.000	92 (41.4)	87 (39.2)	43 (19.4)	1.000	0.011*	61.0	1.000	47.4	23.6	15.4	13.6	1.000
SDS	80	46 (57.5)	27 (33.8)	7 (8.7)	0.575	0.376	74.4	0.408 0.469	38 (47.5)	29 (36.3)	13 (16.2)	0.624	0.085	65.6	0.305 0.351	59.2 (0.036†)	15.2 (0.087†)	19.2 (0.596†)	6.4 (0.042†)	0.005
LDS	24	14 (58.3)	9 (37.5)	1 (4.2)	0.649	1.000	77.1	0.371 0.467	12 (50.0)	11 (45.8)	1 (4.2)	0.181	0.636	72.9	0.107 0.145	59.3 (0.110†)	17.7 (0.347†)	9.3 (0.232†)	13.6 (0.990†)	0.453
FEMALES																				
Controls	157	104 (66.2)	48 (30.6)	5 (3.2)	1.000	1.000	81.5	1.000	63 (40.1)	81 (51.6)	13 (8.3)	1.000	0.077	65.9	1.000	58.7	22.8	11.3	7.2	1.000
SDS	67	30 (44.8)	28 (41.8)	9 (13.4)	0.001	0.590	65.7	0.00027 0.00043	25 (37.3)	30 (44.8)	12 (17.9)	0.109	0.613	59.7	0.209 0.250	48.3 (0.025†)	17.4 (0.198†)	22.9 (0.001†)	11.4 (0.062†)	0.005
LDS	26	17 (65.4)	7 (26.9)	2 (7.7)	0,526	0.288	78.9	0.647 0.790	16 (61.5)	10 (38.5)	0 (0.0)	0.071	0.545	80.8	0.033 0.049	67.6 (0.195†)	11.3 (0.035†)	7.9 (0.427†)	13.2 (0.112†)	0.079
TOTAL																				
Controls	379	217 (57.3)	137 (36.1)	25 (6.6)	1.000	0.582	75.3	1.000	155 (40.9)	168 (44.3)	56 (14.8)	1.000	0.377	63.1	1.000	52.0	23.3	13.6	11.1	1.000
SDS	147	76 (51.7)	55 (37.4)	16 (10.9)	0.209	0.235	70.4	0.103 0.120	63 (42.9)	59 (40.1)	25 (17.0)	0.647	0.111	62.9	0.964 0.975	54.3 (0.811†)	16.1 (0.032†)	21.0 (0.015*†)	8.6 (0.602†)	0.007
LDS	50	31 (62.0)	16 (32.0)	3 (6.0)	0.815	0.680	78.0	0.559 0.645	28 (56.0)	21 (42.0)	1 (2.0)	0.020*	0.419	77.0	0.0061 0.0085	63.7 (0.025†)	14.3 (0.032†)	8.7 (0.129†)	13.3 (0.418†)	0.064

p values were calculated by χ^2 test and χ^2 test with Yates' correction for comparisons between groups of athletes and control group HWE – χ^2 test for Hardy-Weinberg equilibrium, §global score statistic, additive haplotype effect, † versus *Controls*.

A control group of healthy individuals (n = 379; 222 males and 157 females; 22.6 ± 2.8 years) was also selected from the Polish population (college students) with no background in swimming.

Genetic analyses

The buccal cells donated by the participants were collected in Resuspension Solution (GenElute Mammalian Genomic DNA Miniprep Kit, Sigma, Germany) using sterile foam-tipped applicators (Puritan, USA). DNA was extracted from the buccal cells using a GenElute Mammalian Genomic DNA Miniprep Kit according to the manufacturer's protocol.

All samples were genotyped using an allelic discrimination assay on a CFX96 Touch™ Real-Time PCR Detection System (Biorad, USA) with TaqMan probes. For discrimination between the *NOS3* G894T (rs1799983) G and T as well as *NOS3* -786T/C **(**rs2070744) T and C alleles, TaqMan Pre-Designed SNP Genotyping Assays were used (Applied Biosystems, USA), including primers and fluorescently labelled (FAM and VIC) MGB probes. The Glu298Asp and rs2070744 T/C genotypes were determined for 197 out of 198 DNA samples of athletes and 379 out of 381 DNA samples of controls. Genotyping error was assessed as 1%, while the call rate (the proportion of samples in which the genotyping provided unambiguous reading) was above 95%.

Statistical analysis

The STATISTICA statistical package (version 8.0, StatSoft Inc., Tulsa, Oklahoma, USA) was used to perform all analyses. The Hardy-Weinberg equilibrium (HWE) for *NOS3* G894T and -786T/C genotypes was assessed separately in swimmers and control subjects with a χ^2 test. Genotype distributions as well as allele frequencies were determined and χ^2tests were used to compare the *NOS3* G894T and -786T/C alleles and genotypes frequencies between the groups of athletes and control participants. The analysis of the individual effects of these variants was based on three genetic models: general, dominant and recessive. The odds ratio (OR) with 95% confidence intervals were calculated. For haplotype analysis, haplo.stats package for R (http://cran.r-project.org) was used. Global and haplotype-specific tests for associations were conducted using haplo.score function, while haplo.group function was used to estimate haplotype frequencies. Bonferroni's correction for multiple testing was applied, and the alpha level for determining statistical significancewas set at $P< 0.025$.

RESULTS

Genotype distributions of all athletes including LDS group and SDS group as well as sedentary controls met Hardy-Weinberg equilibrium (all $p > 0.05$). The results of the distribution of alleles and genotypes for both the *NOS3* G894T and -786T/C polymorphic sites in Polish athletes compared with the non-athletic controls (all subjects stratified by gender) are presented in Table 1.

The genotype as well as allele distributions of the G894T polymorphic site were not significantly different when long distance swimmers and short distance swimmers were compared to sedentary controls (Table 1). However, a sex-specific analysis indicated significant differences in genotype distribution between female athletes and female controls. The data showed that the genotype distribution and allele frequencies amongst the female SDS (n=67): 44.8% GG, 41.8% GT, 13.4% TT, the Asp allele frequency 34.3%, differed significantly from female control subjects (n=157): 66.2% GG, 30.6% GT, 3.2% TT, the T allele frequency 18.5%; p=0.0013 (Chi2=13.2, df=2), p=0.00043 (Chi2=12.4, df=1), for genotype and allele frequencies respectively (Table 1).

There were significant *NOS3* G894T genotype-dependent differences in the likelihood of being classified as a short distance swimmer under general, dominant and recessive models in female SDS group. Assuming general model, relative to female control subjects, carriers of the GG genotype of G894T polymorphism were less likely to be found in the SDS group than TT homozygotes (OR 0.16 [CI=0.05-0.514], p=0.0021). On the anther hand, the chance of being a female short distance swimmer was 6.24 times higher (CI=1.94-20.03, p=0.0021) for the TT than in a female control. These results indicate that the T allele of the G894T may be beneficial for short distance swimmers compared to control subjects.

In reference to the second SNP analyzed in the presented study, a χ^2 test revealed that the genotype as well as allele frequencies of the -786T/C polymorphic site were significantly different when long distance swimmers group (n=50): 56.0% TT, 42.0% TC, 2% CC, the T allele frequency 77.0%, was compared to sedentary controls (n=379): 40.9% TT, 44.3% TC, 14.8% CC, the T allele frequency 63.1%; p=0.019 (χ^2=7.8, df=2), p=0.0085 (χ^2=6.9, df=1), for genotype and allele frequencies respectively (Table 1). Further analyzes showed that the frequency of the T allele of the -786T/C SNP was higher, however not statistically significant, in the female LDS group than in the female controls (80.8% vs. 65.9%); p=0.049 (χ^2=3.7, df=1, Table 1). The athletes from LDS group were more likely than controls to possess TT genotype (TT genotype compared to the CC [OR 10.11, CI=1.34–76.10], p=0.026; TT compared to both TC and CC genotypes [OR 1.84, CI=1.01–3.33], p=0.047, both p values statistically not significant). It was also observed that the T allele carriers (TT+TC) were over-represented in LDS swimmers group. Specifically, when compared with the CC genotype, the chance of being a long distance swimmer was 8.49 times higher (CI=1.14-62.78, p=0.023) for the carriers of T allele than in control subjects. Further data analyzes showed that the allele frequency amongst the female LDS (n=26), the T allele frequency 19.2%, differed from female control subjects (n=157), the T allele frequency 34.1%; p=0.049 (Chi2=3.9, df=1), (Table 1). There was also *NOS3* -786T/C genotype-dependent differences, however not statistically significant, in the likelihood of being classified as a long distance swimmer under dominant mode in female LDS group. It was observed that the chance of being a long distance swimmer was 2.39 times higher (CI=1.02-5.60, p=0.045) for the holders of TT genotype than in a female control subjects. Presented results

demonstrate that the T allele of the -786T/C may be beneficial for long distance swimmers compared to control subjects.

The haplotype analysis revealed that in presented study four haplotypes (G-T, G-C, T-C and T-T) occur in both the athletes and sedentary controls (Table 1). The linkage disequilibrium between analysed loci was D'=0.287 and D'=0.474 for control subjects and all athletes, respectively. A statistically significant difference was observed in the frequency of the all four haplotypes between SDS (both men and women) and control (p=0.007), with excess of the T-C haplotype observed in the SDS subgroup compared with the controls (21.0% vs. 13.6%, p=0.015). A sex-specific analysis indicated, that haplotype frequencies amongst the female SDS differed significantly from female control subjects (p=0.005), and T-C haplotype was over-represented in the female SDS compared to the female controls (22.9% vs. 11.3%, p=0.001). We also identified that the G-T haplotype was over-represented in the LDS group compared with controls (63.7 vs. 52.0, p=0.025).

DISCUSSION

Previously we observed a significant over-representation of the GG genotype and the G allele of the G894T polymorphic site irrespective of athletes' status, i.e. type and intensity of exercise performed (power-oriented, endurance-oriented, or "mixed") [28]. Comparison of sedentary control subject and whole athlete cohort led us to the conclusion that G894T polymorphism may be associated with overall physical fitness and physical ability, no matter what type of sports activity the athletes are involved in [28]. Additionally, we observed a tendency towards an increase in both the GG genotype and the G allele frequency in relation to a smaller aerobic component of physical ability. We interpret it that the G allele promotes power-oriented sport events.

In this paper the G894T genotype was determined for 197 simmers and 379 control individuals. HWE tested for genotype distributions of LDL and SDL athletes for both the *NOS3* G894T polymorphic site in Polish athletes compared with the non-athletic controls allow to state there is no divergence from the expected values (in all $p > 0.05$.) One could expect this as the Poland become still highly genetically constant and migration as well as transnational marriage is still seldom also among athletes.

In this study we observed that the G894T G allele is disadvantageous in female SDS swimmers since the chance of being a female short distance swimmer was 6.24 times higher for the TT than in a female control. Additionally, GG homozygotes were significantly under-represented in female SDS swimmers group.

Previous studies delivered inconsistent results on the association between the G894T *NOS3* polymorphism and athletic performance. Until today some research group do not confirm such dependencies. Among them, the Genathlete study did not find any differences in allele and genotype frequencies of the G894T between elite endurance athletes and controls [21]. Also when we examined elite Polish rowers we found no evidence of association between the G894T and endurance performance [29]. Likewise, no differences in allele and genotype frequencies of the G894T were found between elite Spanish Caucasian elite endurance and power-oriented athletes [22]. The missense type G894T polymorphism within exon 7 *NOS3* gene is one of the most analyzed polymorphism within this gene among several others like, microsatellite (CA)n repeats in intron 13, and 27-bp repeats in intron 4 (4B/4A) variations [30].

The promising expectations are based on facts that *NOS3* G894T is associated with the BP [9], HR, and stroke volume responses to submaximal and maximal aerobic exercise [10], the no exercising muscle vasodilation response to isometric handgrip exercise [12], and parasympathetic modulation response to submaximal aerobic exercise [8].

Physical training causes changes of arterial tension and increasing the cross section area of the medium and small arteries [31]. In large vessels a vascular resistance to blood flow is small. In the aorta, adaptive changes associated with training are less visible than in the smaller arterioles located in skeletal muscle [32]. Physical training causes an increase in blood flow in the vessel and a consequent increase shear stress, causing deformation of the mechanosensitive channel of endothelial cells [33]. Following separation cascade is initiated a series of compounds that affect the size of the local movement. Nitric oxide is one of the most important factors dishes diastolic produced by the endothelium [34]. Adjusting the flow of blood by increasing the production of NO increases the speed of transport endothelium, and thus supply the necessary ingredients myocytes [35]. Remodeling of the arteries in athletes, is also largely related with increased release of NO by the endothelium [36].

For the -786T/C polymorphic site genotypes and allele frequencies were significantly different when long distance swimmers vs. short distance swimmers where compared. Frequency of the T allele of the -786T/C SNP and TT genotype was over-represented in the female LDS group. It means that the T allele of the -786T/C may be beneficial for long distance swimmers compared to control subjects since chance of being a long distance swimmer was 2.39 times higher for the TT genotype than in a female control subjects. In the opposite, T-C haplotype was overrepresented in the female SDS compared to the female controls. Interesting conclusions may be brought in studies by Gomez-Gallego et al., who found a significant association between an intronic polymorphism -786T/C, and elite performance in strength/ power sports [16]. Expression of eNOS is subject to fluctuations in response to growth factors, hormones, cytokines released during exercise [37]. Depending on the intensity and applied volume of physical exercise, they are induced in a vessel different pattern of shear forces and transient hypoxia [36].

It was shown that shear forces vary depending on whether the physical effort is of the aerobic or anaerobic, and which ultimately results in specific changes among the endothelial function [34]. Adaptive changes in endothelial function associated with athletic training are dependent on the activity of endothelial nitric oxide synthase [33] and environmental redox prevailing in the vessel. The

level of oxidative damage to cells, production of free radicals or the efficiency of the antioxidant system [34].

Increased blood flow in a blood vessel associated with physical training exercises while induced increase in the level of mRNA and protein expression, and therefore an increase in eNOS activity and production of nitric oxide [38]. Increase in eNOS activity also occurs after the training [39], but is independent of intracellular Ca^{2+} and triggered by some phosphorylation of the enzyme molecule.

It can therefore be assumed that the increased trend to reduce the activity of eNOS was associated with decreased antioxidant capacity of blood plasma [40] pointed out that the very intense, long-lasting training can cause adverse changes in endothelial function correlated with lower levels of antioxidant system and the increase in oxidative stress.

CONCLUSION

In conclusion, although our study is restricted to two SNPs and the statistical power suffers from low sample size, it offers suggestion that the Glu allele of the *NOS3* G894T polymorphic site is disadvantageous in female SDS swimmers and the T allele of the -786T/C polymorphism may be beneficial for long distance swimmers.

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