

Maximal oxygen uptake is associated with the snp 13470 G>C polymorphism of the mitochondrial NADH dehydrogenase subunit 5 gene (*mtND5*) in caucasians from Poland

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Introduction. Physical performance displays a great inter-individual variance in both general population and among well-trained athletes. Genetic factor has an important contribution in this variance. The aim of this study was to examine the association between maximal oxygen uptake and genetic variants of mitochondrial NADH dehydrogenase subunit 5 gene (*mtND5*) in Caucasians from Poland. **Material and Methods.** The studies were carried out in a group of 154 men and 85 women, professional athletes representing various sports and fitness levels and students of the University of Physical Education in Poznań. Physiological and molecular procedures were used, i.e. direct measurement of maximal oxygen uptake ($VO_2\max$) and SNP 13470 G>C polymorphism of the mitochondrial NADH dehydrogenase subunit 5 gene (*mtND5*) was determined by restriction fragments length polymorphism (PCR-RFLP). **Results.** We have found that maximal oxygen uptake is associated with *BamHI*^{+/+} homoplasmic variant of the *mtND5* gene in Caucasians from Poland. We have also observed positive influence of *BamHI*⁺ allele on level of maximal oxygen uptake ($VO_2\max$).

KEY WORDS: *mtND5*, athletic performance, endurance, genetic polymorphism, energy efficiency.

Received: 1 June 2013

Accepted: 12 November 2013

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What is already known on this topic?

Physical performance displays a great inter-individual variance in both general population and among well-trained athletes. Genetic factor has an important contribution in this variance. Exercise performance is a multifactorial, quantitative trait resulting from interaction of genetic and environmental compound. Searching for genotypes favourable for physical fitness remains therefore difficult due to a great number of genes that may influence human performance.

Introduction

The NADH dehydrogenase subunit 5 gene is one of seven mtDNA-coded subunits, which contains about 41 polypeptides of the respiratory complex I. The MtND5 is coded by the H-strand of mitochondrial DNA rich in guanine, located at 12337-14148 bp. The gene comprises 1811 base pairs of uninterrupted coding sequence within the polycistronic H-strand transcript, and 521 base pairs of noncoding sequence at the 3' end, ended with a polyadenylation signal [1, 2, 3]. Complex I is the first link of the respiratory chain. It receives electrons from NADH and transfers them to ubiquinol (CoQ10) via a series of transmitters: flavin mononucleotide (FMN) and six iron-sulfur clusters (Fe-S). Complex I can be divided into three fragments: flavoprotein fragment, iron-protein fragment and hydrophobic fragment in which ND5 is located [4].

Studies following Dionne et al. [5] analyzing the frequency of two polymorphisms in the *mtND5* and one polymorphism within the D-loop in athletes and non-training controls did not confirm the existence of differences in the distribution of genotypes between the studied groups [6]. Also Brearley & Zhou [7] did not observe any influence of mtDNA polymorphisms on the level of maximal oxygen uptake ($VO_2\text{max}$) nor reveal differences in their distribution. On the other hand, Chen et al. [8] in their study of Chinese elite endurance athletes and non-training controls indicated differences in the frequency of D-loop polymorphisms. Ma et al. [9] noted differences in the maximal oxygen uptake for three polymorphisms studied earlier by Chen et al. Such divergent results can be related to different ethnicities in the studied samples, i.e. representations of different mtDNA haplogroups [7].

Due to their role in respiratory processes, mtDNA polymorphisms can be significant for determining differences in the maximal oxygen uptake levels and responses to endurance training. The present study attempted to analyze the *mtND5* restricted fragment length polymorphism (RFLP G→C) at 13470 bp, for which a higher $VO_2\text{max}$ was noted in individuals with an allele with restriction site recognized by *BamHI* [10].

Material and Methods

Study group

Studies were carried out on a group of professional athletes training in various sport disciplines, representing different sports classes, including representatives of Polish national teams and students of the University School of Physical Education in Poznań, both actively practicing sports, as well as those less active. The study was approved by the Poznań University of Medical Sciences Bioethics Committee, Poland, No 1060/05. Participants were informed about the aim and dangers of the analysis and each one signed written consent.

The group of 239 Caucasians (154 men and 85 women) aged 18-26 years was subjected to physiological and genetic analyses. All statistical analyses were performed separately for men and women.

In order to verify the effects of the analysed gene polymorphism on maximal oxygen consumption, depending on the level of physical activity, the participants of this study were then divided into a group of athletes (119 men and 37 women) and those who did not train in any sports (35 men and 48 women).

Additionally, athletes were then subdivided into three subgroups classified by the type of exercise metabolism predominating in the discipline they practice: (i) power oriented disciplines (disciplines with predominance of anaerobic energy metabolism) were denoted as POD, (ii) endurance-speed-strength disciplines (disciplines requiring both anaerobic and aerobic energy resources) were denoted as E-Sp-St, and (iii) endurance oriented disciplines (those predominating in aerobic energy metabolism) were denoted as EOD. The division of sport disciplines was based on the classification system developed by Bellotti et al. [11]. The POD subgroup contained individuals training in short-distance running, long jump, high jump, canoeing, discuss throw, the E-Sp-St subgroup comprised of individuals practising field hockey, tennis, rugby, football (soccer), volleyball, basketball, handball, boxing, kickboxing, rowing, while the EOD subgroup included triathlons, medium and long-distance runners, long-distance swimmers, race walkers, skiers, mountaineers.

VO₂max determination

Physiological analyses were conducted at the Laboratory of Functional Examinations at the University School of Physical Education in Poznań, certified by ISO 9001:2008 standards (no. 69178-2009-AQ-POL-RvA). In order to determine the maximal oxygen uptake of the participants the direct method during exercise tests on a treadmill (Woodway, USA) was used. During each test, the composition of air inhaled and exhaled (VO_2 , VCO_2) was analysed by Oxycon Mobile spiroergometer (Jaeger, Germany) and the heart rate (HR) was monitored using a pulsometer (Polar, Finland). The exercise tests were carried out on a treadmill with increasing load, starting from a running speed of 8 km/h, increasing the load by 2 km/h every 3 min, until the moment of maximum individual load was reached.

Genotyping

DNA for genetic analyses was isolated from 5 ml of peripheral blood collected from the participants onto anticoagulant (EDTA). DNA isolation was performed using guanidine isothiocyanate (GTC, Sigma) method. The SNP polymorphism 13470 G>C *BamHI* in *mtND5* was genotyped by polymerase chain reaction (PCR). DNA was amplified in a volume of 20 μ l. Genomic DNA from each examined individual was placed in a separate test tube in the amount of 4 μ l (200 ng) and 16 μ l reaction mixture was added, containing

50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 0.25 mM dNTP, 7.5 pmol each primer and 0.5 unit of Taq polymerase (Fermentas Life Sciences, Lithuania). Primers sequence was: Forward- Agg CgC TAT CAC CAC TCT GTT CG; and Reverse- GAA TTC CTG CGA ATA GGC TTC CGG CTG CC [10]. The 30 cycle reaction was run in a Biometra T-personal thermocycler. The cycle comprised initial denaturation at 95°C for 10 min, denaturation at 95°C – 30 s, annealing at 58°C – 30 s, synthesis at 72°C – 30 s and final synthesis at 72°C for 10 min.

Substitution 13470 G>C *BamHI mt ND5* gene was genotyped by PCR – RFLP method with *BamHI* enzyme in the condition recommended by the supplier (Fermentas Life Sciences, Lithuania). The digested products 294 base pairs (bp) and 259 bp long were then electrophoresed in 2% agarose gel. Electrophoresis was run at 100 V for 30 min in Biometra agagel mini horizontal apparatus (Germany). The results were visualized on a UV transilluminator with 2 µl of ethidium bromide (5mg/ml). Genetic analyses were conducted at the Laboratory of Genetic Analyses at the University School Physical Education in Poznań, certified by ISO 9001:2008 standards (no. 69178-2009-AQ-POL-RvA).

Statistical analysis

Statistical calculations were performed at the Computer Laboratory of the Faculty of Animal Breeding and Biology at the Poznań University of Life Sciences, with the use of SAS statistical software ver. 9.1 (USA).

The consistency of the maximal oxygen uptake values and variants distribution fit to the Hardy–Weinberg principle were verified with the χ^2 test. The Bartlett test was performed to determine the homogeneity of variance. The association between analysed polymorphisms and maximal oxygen uptake (VO₂max) was verified using the non parametric Kruskal-Wallis test.

Results

Characteristics of subjects

An analysis of association was carried out using results of physiological and genetic studies of 239 people. Smokers and subjects outside the age range of 18-26 years

and normal BMI range were excluded. Individuals for whom there were doubts as to whether their fitness test (treadmill test) results were not maximal due to their low motivation were also excluded from the study protocol.

The study sample consisted of 154 men (119 athletes, 37 non athletes) and 85 women (37 athletes, 48 non-athletes). All subjects were students of the University School of Physical Education, and thus even the non-athletes controls displayed a higher than average level of physical activity. The subjects trained endurance sports such as the marathon, rowing, and triathlon races; sports involving energy metabolism of aerobic/anaerobic character – field hockey, volleyball, football, handball, rowing; and speed-strength sports such, e.g. bodybuilding, sprints, kayaking, long jump, and high jump.

Results of exercise tests

The subjects performed a treadmill test to measure their maximal oxygen uptake levels (VO₂max) directly with the use of an Oxycon Mobile ergospirometer with constant data transfer from the analyzer to a PC registering changes of such physiological parameters as heart rate (HR), inhaled and exhaled air volume (VO₂, VCO₂, VE/MV) and respiratory exchange ratio (RER).

The division of the sample into subgroups of training and non-training subjects was justified by the different character of energy metabolism related to practicing individual sports. The mean VO₂max values are presented in Table 1 and 2. As expected the women and non-training subjects attained lower VO₂max levels than men and training subjects, respectively. Among the training subjects the highest maximal oxygen uptake levels were reached by athletes of endurance sports, and the lowest by athletes of speed and strength sports.

Table 1. Mean values of maximal oxygen uptake (VO₂max in ml/kg·min⁻¹) of both non-training and training groups

| | NT | | | | | | T | | | | |
|---|-----|----|-----------|------|-------|-------|-----|-----------|------|-------|-------|
| | Sex | N | \bar{x} | SD | Min. | Max | N | \bar{x} | SD | Min. | Max |
| F | | 48 | 42.29 | 5.16 | 30.60 | 58.40 | 37 | 49.91 | 5.85 | 33.80 | 59.80 |
| M | | 35 | 50.74 | 4.29 | 42.30 | 62.20 | 119 | 56.42 | 7.21 | 40.30 | 79.00 |

Note: NT – non-training group; T – training group; F – females; M – males

Table 2. Maximal oxygen uptake (VO_{2max} in $ml/kg \cdot min^{-1}$) of the subgroup having different energy metabolism characteristics

| | | POD | | | | E-Sp-St | | | | EOD | | | | NT | | | | | | |
|-----|----|-----------|------|-------|-------|---------|-----------|------|-------|-------|----|-----------|------|-------|-------|----|-----------|------|-------|-------|
| Sex | N | \bar{x} | SD | Min. | Max | N | \bar{x} | SD | Min. | Max | N | \bar{x} | SD | Min. | Max | N | \bar{x} | SD | Min. | Max |
| F | 11 | 49.45 | 4.65 | 39.30 | 56.10 | 9 | 47.54 | 4.51 | 40.20 | 52.00 | 17 | 51.46 | 6.90 | 33.80 | 59.80 | 48 | 42.29 | 5.16 | 30.60 | 58.40 |
| M | 24 | 54.88 | 5.88 | 41.10 | 71.50 | 62 | 53.84 | 4.24 | 40.30 | 62.00 | 33 | 62.37 | 9.01 | 42.30 | 79.00 | 35 | 50.74 | 4.29 | 42.30 | 62.20 |

Note: POD – power oriented disciplines; E-Sp-St – endurance–speed–strength disciplines; EOD – endurance oriented disciplines; NT – non-training group; F – females; M – males

Analysis of association of examined genes with VO_{2max}

The study examined the association of the SNP 13470 G>C *BamHI mtND5* polymorphism with the maximal oxygen uptake levels. An analysis of allele and genetic variants frequencies was performed against the distribution of subjects' VO_{2max} values. The χ^2 test did not reveal a normal distribution of the examined parameter, and the studied candidate gene was not in a genetic equilibrium ($\chi^2_{tab; n-1=2, \alpha=0,05} = 5,991$; $\chi^2_{tab; n-1=2, \alpha=0,01} = 9,21$, $\chi^2_{calc} = 164,040$). The homogeneity of variances was checked with Bartlett's test. Table 3 demonstrates descriptive statistics and a comparative analysis of VO_{2max} levels for polymorphic variants of studied gene.

The non-parametric Kruskal-Wallis analysis of variance revealed a statistically significant difference in VO_{2max} levels between men with the heterozygous genotype and others in the *mtND5* gene ($p = 0.047$).

The distribution of the genetic variants and VO_{2max} values (minimal, maximal, average) for individual polymorphisms in the subgroups of subjects is shown in Table 4.

The obtained results were also analyzed with regard to the VO_{2max} levels reached by subjects with different genotypes of the studied polymorphisms in relation to the character of energy metabolism prevalent in particular sports. Table 5 shows mean VO_{2max} values for the polymorphisms of the studied gene in the subgroups of subjects training speed-strength sports, endurance-

Table 3. Descriptive statistics and comparative analysis of maximal oxygen uptake (VO_{2max} in $ml/kg \cdot min^{-1}$) between genetic variants of the *bamhi* polymorphism of the *mtND5* gene

| mtND5 | | BamHI -/- | | | | BamHI +/- | | | | BamHI +/+ | | | | | |
|-------|-----|----------------|------|-------|-------|-----------|------------------|------|-------|-----------|---|----------------|------|-------|-------|
| Sex | N | \bar{x} | SD | Min. | Max | N | \bar{x} | SD | Min. | Max | N | \bar{x} | SD | Min. | Max |
| F | 75 | 45.78 | 6.68 | 30.60 | 59.80 | 2 | 44.15 | 1.06 | 43.40 | 44.90 | 8 | 44.34 | 7.26 | 38.50 | 57.70 |
| M | 142 | <u>54.82</u> a | 7.06 | 40.30 | 79.00 | 4 | <u>56.38</u> a A | 0.78 | 55.20 | 56.80 | 8 | <u>59.98</u> A | 7.44 | 53.60 | 74.90 |

Note: The statistically significant difference in maximal oxygen uptake between the given genetic variants was marked by underlining a – at $p \leq 0,05$, A – at $p \leq 0,01$

Table 4. Descriptive statistics and a comparative analysis of maximal oxygen uptake (VO_{2max} in $ml/kg \cdot min^{-1}$) between groups of different *BAMHI* polymorphism of the *mtND5* gene in training and non-training women and men

| mtND5 | | BamHI -/- | | | | | BamHI +/- | | | | | BamHI +/+ | | | | |
|-------|-------|-----------|-----------|------|-------|-------|-----------|-----------|------|-------|-------|-----------|-----------|------|-------|-------|
| Sex | Group | N | \bar{x} | SD | Min. | Max | N | \bar{x} | SD | Min. | Max | N | \bar{x} | SD | Min. | Max |
| F | NT | 41 | 42.53 | 5.53 | 30.60 | 58.40 | 1 | 43.40 | - | - | - | 6 | 40.48 | 1.05 | 38.50 | 41.20 |
| | T | 34 | 49.71 | 5.85 | 33.80 | 59.80 | 1 | 44.90 | - | - | - | 2 | 55.90 | 2.55 | 54.10 | 57.70 |
| M | NT | 34 | 50.65 | 4.32 | 42.30 | 62.20 | - | - | - | - | - | 1 | 53.60 | - | - | - |
| | T | 108 | 56.13 | 7.26 | 40.30 | 79.00 | 4 | 56.38 | 0.78 | 55.20 | 56.80 | 7 | 60.89 | 7.54 | 54.40 | 74.90 |

Note: NT – non-training group; T – training group

Table 5. Descriptive statistics and a comparative analysis of maximal oxygen uptake (VO₂max in ml/kg·min⁻¹) and different genetic variants of *BAMHI* polymorphism in the *mtND5* gene in the groups of women and men not involved in training and those training in various disciplines, subdivided by they energy metabolism characteristics

| mtND5 | | BamHI -/- | | | | | BamHI -/+ | | | | | BamHI +/+ | | | | |
|-------|---------|-----------|-----------|------|-------|-------|-----------|-----------|------|-------|-------|-----------|-----------|------|-------|-------|
| Sex | Group | N | \bar{x} | SD | Min. | Max | N | \bar{x} | SD | Min. | Max | N | \bar{x} | SD | Min. | Max |
| F | POD | 10 | 49.90 | 4.63 | 39.30 | 56.10 | 1 | 44.90 | - | - | - | - | - | - | - | - |
| | E-Sp-St | 9 | 47.54 | 4.51 | 40.20 | 52.00 | - | - | - | - | - | - | - | - | - | - |
| | EOD | 15 | 50.87 | 7.12 | 33.80 | 59.80 | - | - | - | - | - | 2 | 55.90 | 2.55 | 54.10 | 57.70 |
| | NT | 41 | 42.53 | 5.53 | 30.60 | 58.40 | 1 | 43.40 | - | - | - | 6 | 40.48 | 1.05 | 38.50 | 41.20 |
| M | POD | 20 | 54.66 | 6.43 | 41.10 | 71.50 | 3 | 56.23 | 0.90 | 55.20 | 56.80 | 1 | 55.20 | - | - | - |
| | E-Sp-St | 59 | 53.63 | 4.18 | 40.30 | 61.90 | - | - | - | - | - | 3 | 58.10 | 3.80 | 54.40 | 62.00 |
| | EOD | 29 | 62.23 | 9.15 | 42.30 | 79.00 | 1 | 56.80 | - | - | - | 3 | 65.57 | 9.78 | 55.40 | 74.90 |
| | NT | 34 | 50.65 | 4.32 | 42.30 | 62.20 | - | - | - | - | - | 1 | 53.60 | - | - | - |

Note: POD – power oriented disciplines; E-Sp-St – endurance–speed–strength disciplines; EOD – endurance oriented disciplines; NT – non-training group; F – females, M – males

speed-strength sports, endurance sports and in non-training controls. The analysis of variance revealed in some cases significant differences in the VO₂max level, depending on the genotype.

Polymorphism 13470 G>C *BamHI* *mt ND5* gene was genotyped by PCR – RFLP method with *BamHI* enzyme and digested products were separated in agarose (Fig. 1).

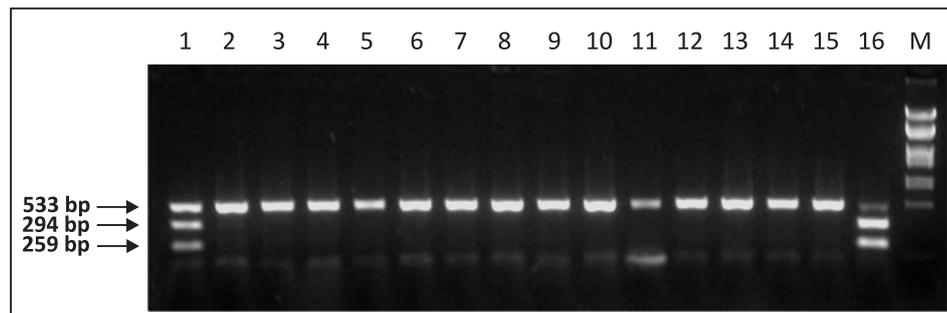


Figure 1. An example of the result for genotyping of restriction site for *BAMHI* within the *mtND5* gene using PCR–RFLP

Note: Electrophoresis of products was performed in 2% agarose gel.

Lanes 2-15 homoplasmic variant *BamHI*-/-; lane 1 – heteroplasmic variant *BamHI*+/-; lane 16 – homoplasmic variant *BamHI*+/+; M – size marker *EcoRI*+*HindIII*.

Discussion

The considerable variability of observed ontogenetic physical abilities in the general population, as well as in the population of athletes themselves, points to the need to describe factors that shape these abilities. The number

of such factors is undoubtedly high due to the complex character of physical fitness. They include environmental factors such as dietary habits, lifestyle or climate, as well as genetic factors in Polish athletes. While physiological or biochemical indices have been indispensable tools of assessment of physical fitness for a long time, genetic studies aimed at identification of genes responsible for the

development of physical abilities and determining patterns of inheriting predispositions to practice specific types of physical activities are relatively recent [14]. Nevertheless, the map of candidate genes that can potentially affect physical fitness becomes larger every year, and currently it contains more than 200 genes associated with such aspects as respiratory and cardiovascular stability; body build and composition – especially muscle mass and strength; carbohydrate and

lipid metabolism response to training; and exercise intolerance [14, 15].

The inclusion of the genetic component in physiological and biochemical studies would permit drawing a representation of predispositions for each individual interested in practicing high performance sports and

would be a valuable coaching aid in the process of training individualization. However, an analysis of complex traits being the sums of numerous genes with little individual effect is not easy, and interpretation of results of such an analysis may lead to false conclusions, e.g. due to the small size of the sample. The perplexity of analysis of association of candidate genes with a complex trait such as physical fitness can be illustrated by ambiguous or even strikingly different study results attained by various research teams examining the genetic profiles of physical abilities in groups of subjects with different levels of physical activity and of different ethnic background. The sporting character of the University School of Physical Education and the cooperation between its Department of Physiology and coaches representing different types of sports made it possible to collect biological material and conduct exercise tests on an interesting study sample. The gathered data may be used in future genetic research or expand into a comprehensive database for determining physical predispositions.

The results of physiological and genetic analysis were checked for conformity with the Hardy-Weinberg equilibrium model; however, the distributions of both $VO_2\max$ and the studied SNP did not conform with it. This discrepancy is rather difficult to explain. It is probably related to the non-Mendelian model of inheritance of mitochondrial DNA which follows a matrilineal descent pattern and is not subjected to segregation, unlike the core genome. The ethnic homogeneity of the study sample is also significant in this model of inheritance, thus a genotype of much higher frequency can be characteristic of the Polish population. This can be confirmed by the fact that the distribution of genetic variants and alleles did conform with the equilibrium model in other studies [10]. The significant difference in the numbers of *mtND5* observed and expected for particular genotypes may suggest that an allele of higher frequency is the so-called wild-type allele in which a substitution led to the formation of a more infrequent variant [10].

Another possibility can be the selection of the study sample, which comprised athletes and non-training controls; however, all of them were students of the University School of Physical Education who demonstrated a higher level of physical activity than the general population. The non-representativeness of the sample is, however, highly improbable since all the results of all our earlier, published [16, 17] and

unpublished studies showed that all polymorphisms examined by us conformed with the Hardy-Weinberg equilibrium model. A genotyping error seems also improbable since part of the collected material was genotyped twice.

Another important function in the energy processes is played by mtDNA encoded genes. They determine, to a great extent, the oxidative potential and efficiency of respiratory processes, which condition the maximal application of muscle abilities [18]. The search for differences in the mtDNA sequence impacting the level of maximal oxygen uptake was carried out by Dionne et al. [5]. Their research led to the identification of a G→C polymorphism in the NADH (ND5) dehydrogenase gene subunit 5 at bp 13 470 of H-strand as a factor with a significant impact on changes in $VO_2\max$ due to endurance training [5]. A similar study was conducted by Rivera et al. who confirmed higher an association of exercise-induced $VO_2\max$ levels with the 13 470 *BamHI*+ (G) allele in non-training subjects [10]. On the other hand, Murakami et al. did not observe an influence of polymorphisms of the mtDNA coding region on physical fitness [19]. The present study attempted to analyze this polymorphism as one of the few most promising genes in association with maximal oxygen uptake.

The only significant difference noted in the *mtND5* polymorphism was related to the *BamHI* -/- (GG) i *BamHI* +/+ (CC) genetic variants in the group of men, in which the highest $VO_2\max$ was observed in the *BamHI* -/- (CC) genetic variant and the lowest in the *BamHI*+/+ (GG) variant, with the number of CC genetic variant being disproportionately higher than the other genetic variants (142:8:4). The subdivisions into training and non-training subjects and subjects practicing sports of different character of exercise-induced metabolic changes resulted in the presence of many empty sets with no observations that rendered any conclusions useless. The analysis of data only from subgroups represented by all genetic variants revealed a positive impact of the *BamHI*+ (C) allele on the maximal oxygen uptake level, which corresponds with the results of other research teams, although our own study concerned only the baseline $VO_2\max$ and not its changes due to training [5, 10].

Dionne studied 25 polymorphic fragments of the mtDNA coding region and noted differences in baseline $VO_2\max$ and training-induced $VO_2\max$ changes [20]. The substitutions in the coding region can have

functional consequences for the building of proteins that form respiratory complexes. There is a supposition that mtDNA polymorphisms may depend on the climatic adaptation of favorable variants. Similar to uncoupling protein genes, polymorphic mtDNA variants can affect the coupling of respiratory processes, depending on the climatic demand for energy (ATP production) leading to generation of different amounts of reactive oxygen species (ROS). The warmer climate there is, the better coupling of energy processes can be observed: increased ATP production, lower heat losses, more efficient muscle metabolism but also more ROS. The mtDNA polymorphisms can also affect respiratory processes by regulating the quantity of mtDNA and mitochondria [21]. Studies of this sort involve the determination of a mtDNA haplogroup, i.e. a group of people sharing a common ancestor. The determination of the frequency of particular polymorphisms in mtDNA haplogroups in combination with exercise tests would surely give a better picture of the genetic aspects of physical fitness.

What this paper adds?

The present article demonstrates the results of association observed between the *mtND5 BamHI*+/+ homoplasmic variant with an elevated maximal oxygen uptake in men. A favorable influence of the *BamHI*+ allele on the VO_2max level was noted.

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