TOTAL HEMOGLOBIN MASS, AEROBIC CAPACITY AND THE *HBB* GENE IN POLISH ROAD CYCLISTS

ABSTRACT

The relationships between genes, amount of hemoglobin and physical performance are still not clearly defined. The aim of this study was to examine the association between - 551C/T and intron 2, +16 C/G polymorphisms in the *HBB* gene and total hemoglobin mass (tHb_{mass}) and aerobic capacity in endurance athletes. tHb_{mass} and aerobic capacity indices, i.e. maximal oxygen uptake (VO_2max), oxygen uptake at anaerobic threshold (VO_2AT), maximal power output (Pmax), and power at anaerobic threshold (PAT), were determined in 89 young road cyclists, female (n=39) and male (n=50), who were genotyped for 2 polymorphisms in the *HBB* gene. The relative values of aerobic capacity indices differed significantly among intron 2, +16 C/G polymorphisms of the *HBB* gene only in female cyclists; athletes with GG genotype had significantly higher values of VO_2max (P=0.003), VO_2AT (P=0.007), PAT (P=0.015) and Pmax (P=0.004) than did C carriers. No relationships were found between the C-carrier model (CC+CG vs GG in the case of intron 2, +16 C/G and CC+CT vs TT for -551 C/T polymorphisms of the *HBB* gene) and relative values of tHb_{mass}. Our results demonstrated that the *HBB* gene could be related to aerobic capacity, but it seems that it does not result from an increase in the amount of hemoglobin in the blood.

KEY WORDS: genetic polymorphism, *HBB* gene, hemoglobin, aerobic capacity, athletes

INTRODUCTION

In endurance athletes the most important factor of success is aerobic capacity, which is mainly expressed by maximal oxygen uptake (VO_2max) (17,30). The important factors affecting VO_2max are cardiac output, O_2 carrying capacity and oxygen utilization by muscle tissue (30). However, among endurance athletes the main limiting factor of VO_2max is oxygen supply (5). Hemoglobin is a protein responsible for efficient transport of oxygen to the tissues; therefore it is an important factor contributing to aerobic capacity (25,30). Although it has been estimated that an increase in total hemoglobin mass (tHb_{mass}) by 1 g causes a rise in VO_2max of approximately 4 ml/min (25), it is worth emphasizing that the total hemoglobin mass, rather than its concentration, shows a strong correlation with maximal oxygen uptake (24,25). Variation in the amount of hemoglobin and indices of aerobic capacity is dependent on many factors, e.g. iron status, illness, period of inactivity, altitude exposure as well as length, duration, type, intensity and age of initiation of the training stimulus (10,15,17,20,25,29), but may also be influenced by genetic parameters (8,15,17,26). Schmidt and Prommer (24,25) reported that tHb_{mass} may be relatively stable in healthy adults (mostly competitive athletes) over a very long period, despite changes in training and lifestyle.

To date, researchers have described over 300 genes that could be related to predisposition to physical fitness and sports results (2, 7). One of them could be the beta hemoglobin (*HBB*) gene (18). The impact of the *HBB* gene on physical performance is not well documented, because so far this gene has been studied mainly in the context of genetic diseases (11). An association between the *HBB* gene polymorphisms and running economy in the untrained state and in response to aerobic training was described only in one study with recruits from the Chinese military police (13).

Many authors have emphasized that genetic predisposition seems to be a prerequisite for high tHb_{mass} and high endurance performance, but despite the many excellent scientific papers

about tHb_{mass} and performance parameters (15,24,25) the tHb_{mass}–performance–gene relationship has not been clearly defined. Although Ahmetov et al. (3) showed recently that the rs157231 CC genotype of the *NFIA-AS2* gene (involved in the regulation of expression of the erythropoiesis inducing nuclear factor I A) was associated with high VO_2max and high hemoglobin concentration, as well as a high number of reticulocytes and erythrocytes in endurance athletes, they did not assess the total amount of hemoglobin. Therefore the aim of our study was to examine the association between 2 polymorphisms of the *HBB* gene and tHb_{mass} and indices of aerobic capacity in endurance athletes.

METHODS

Experimental approach to the problem

To the best of our knowledge, there is still no study concerning the association between the *HBB* gene, amount of hemoglobin and aerobic capacity. To elucidate whether having specific polymorphisms of the *HBB* gene could exert a positive effect on the amount of hemoglobin in the blood and aerobic capacity, we analyzed relationships between *HBB* gene intron 2, +16C/G and -551C/T polymorphisms and tHb_{mass} as well as maximal oxygen uptake (VO_2max), oxygen uptake at anaerobic threshold (VO_2AT), maximal power output (Pmax), and power at anaerobic threshold (PAT) in endurance athletes.

Subjects

Ninety-two road cyclists (male and female), aged 16-28 years, participated in the study. Most of the study participants were members of national junior or senior teams. In order to exclude individuals with symptoms of infectious or cardiovascular diseases, latent iron deficiency (n=3) or iron deficiency anemia, the subjects were given a medical and biochemical examination. Finally, the results obtained from 89 athletes (39 females and 50 males) were

analyzed. The physical characteristics of subjects, separated by gender, as well as basic data concerning sports experience and training load, are shown in Table 1. (table 1 about here) The results concerning *HBB* genotyping obtained in athletes were compared with those observed in 119 Polish untrained persons (59 females and 60 males) aged 20-25 years (control group). All athletes and untrained persons were Caucasians. The study was approved by the Institute of Sport Committee of Ethics, and written informed consent was obtained from all individual participants of the study.

Design

The study consisted of three steps performed on two days in the following order: first day – 1) venous blood sampling and anthropometric measurements, 2) evaluation of aerobic capacity, 3) measurements of tHb_{mass}; second day – 1) measurement of body mass and venous blood sampling, 2) measurements of tHb_{mass}.

Procedures

Blood collection and analysis

The blood samples were withdrawn from the cephalic vein in the morning in a preprandial state after remaining for at least 15 min in a sitting position.

Indices of iron status

Hemoglobin concentration (Hb), hematocrit (Hct), and erythrocyte count (RBC) were assessed using an ADVIA 120 hematological analyzer (Siemens, Germany). In serum the following indices were measured: soluble transferrin receptor (sTfR) concentration by using immunoenzymatic commercial kits (Ramco, USA); ferritin concentration by using the immunoturbidimetric method (Pentra, USA), total iron binding capacity (TIBC) by using the colorimetric method (BioMaxima, Poland), and C-reactive protein (CRP) by using the immunoturbidimetric method (Pentra, USA).

DNA isolation and *HBB* polymorphism typing

Genomic DNA from athletes and untrained person was extracted from whole blood using the GeneMATRIX Quick Blood DNA Purification Kit (Eurx, Germany). *HBB* gene intron 2, +16C/G and -551C/T polymorphisms were analyzed as described previously (19) using pairs of primers specific to DNA fragments containing the polymorphic site (13). Genotyping of two SNPs was performed using the RFLP technique with 2 U of AvaII and 2 U of RsaI restriction enzyme (Fermentas, USA) for intron 2, +16C/G and -551C/T typing, respectively. All restriction cutting was performed for 2.5 h at 37°C and digested products were electrophoresed on 3% agarose gel.

Determination of tHb_{mass}

tHb_{mass} was measured using a modified version of the CO rebreathing procedure, according to Schmidt and Prommer (23). Briefly, the subjects inhaled a bolus of 99.9% chemically pure CO (Linde Gas) in a dose of 1.0 ml/kg body mass for males and 0.8 ml/kg body mass for females and rebreathed in a closed system (spirometer, SpiCo, Bayreuth, Germany) for 2 min. The samples of the arterialized capillary blood were taken from the earlobe three times: directly before the test and in the 6th and 8th minute after the respiration through the spirometer was started. Analysis of the percentage value of carboxyhemoglobin (HbCO%) (ABL 80 Flex, Radiometer, Denmark) was performed in triplicate samples before and in the 8th minute and in duplicate samples in the 6th minute of the study. A detailed description of this method has been provided in publications by its authors (22,23). Based on the results of tHb_{mass}, Hb and Hct, the blood (BV) and plasma volumes (PV) were also computed. In all participants measurements of tHb_{mass} were made in duplicate. The typical error (TE) in our laboratory with duplicated measures (24-48 h time lag between tests) in the cyclist group was 1.85%.

Aerobic capacity

A graded exercise test to exhaustion was performed on a cycle ergometer (Cyclus2, Leipzig, Germany) to determine maximal aerobic capacity (VO_2max), maximal power output (Pmax), as well as anaerobic threshold (AT). The tests were performed using the participant's personal bike. The test started at workload 1.50 W/kg of body mass and was increased every 3 minutes by 0.75 W/kg for males and 0.70 W/kg for females. The test was terminated when the subject could no longer complete the desired workload despite verbal encouragement. Additional maximal exercise performance criteria were: a heart rate close to age predicted maximum, respiratory exchange ratio (RER) value of >1.1, blood lactate concentration >10 mmol/L. The test was preceded by a 10-minute warm-up at workload of 1 W/kg and thereafter a 5-minute rest.

During the exercise test expiratory air was analyzed using a portable measuring system (MetaMax, Cortex, Germany). Prior to each test this system was calibrated with a known volume syringe and gas concentration (O_2, CO_2). Heart rate was monitored using the Polar Sports Tester device.

At the end of each workload capillary blood samples were taken from the fingertip in order to determine changes in lactate concentration (Super GL2 analyzer, Dr. Muller, Germany).

The anaerobic threshold was assumed as power output (PAT) and corresponding oxygen uptake (VO_2AT) at threshold (4 mmol/L) blood lactate concentration (14) and was estimated by the method of interpolation.

Anthropometric measurements

Anthropometric measurements comprising assessment of body height, body mass and skinfold thickness were performed. The percentage of body fat was calculated using the equation of Durnin and Womersley (9).

Statistical analysis

All the data are presented as means and standard deviations, and were analyzed using the Statistica 10 software package (StatSoft Inc. Tulsa, USA).

Owing to the low number of CC homozygotes for intron 2, +16 C/G and of -551 C/T polymorphisms, they were combined with heterozygotes (C-carrier model) and compared to GG and TT homozygotes, respectively. Differences between mean values of tHb_{mass} in groups of athletes (males and females separately) possessing different genotypes of the *HBB* gene were tested by the Kruskal-Wallis test, whereas the Mann-Whitney U test was used for comparison of mean values of tHb_{mass}, oxygen consumption, and power output in groups distinguished according to genotype variants. The significance of differences in genotype and allele frequencies as well as conformity with the Hardy–Weinberg principle was estimated using the χ^2 test. A Pearson correlation test was used to analyze the relationship between two quantitative variables. The statistical significance was set at $P<0.05$.

RESULTS

Both polymorphisms were in Hardy-Weinberg equilibrium in male and female athletes and controls. No differences were found in the *HBB* genotype and allele frequencies between male and female athletes, as well as between athletes and controls (Table 2). (table 2 about here)

The *HBB* genotypes had no significant effect on relative values of tHb_{mass} both for female and male athletes (Table 3).

Moreover, there were no associations between PV, BV and Hb concentrations and genotype variants of the *HBB* gene (data not shown). Also no relationships were found between genotype models, i.e. CC+CG vs GG in the case of intron 2, +16 C/G polymorphism and CT+CC vs TT for -551 C/T polymorphism of the *HBB* gene and relative values of tHb_{mass} (Table 3). (table 3 about here)

196 The relative values of aerobic capacity indices differed according to intron 2, +16 C/G
197 polymorphism of the *HBB* gene in female cyclists; athletes with GG genotype had
198 significantly higher values of VO_2max (P=0.003), VO_2AT (P=0.007), Pmax (P=0.004) and
199 PAT (P=0.015) than did C carriers (CC + CG genotypes) (Table 4).
200 Among the male athletes these indices did not differ significantly between the C-carrier model
201 and GG genotypes in intron 2, +16 C/G polymorphism of the *HBB* gene (Table 5).
202 The -551 C/T polymorphism of the *HBB* gene had no significant effect on relative values of
203 VO_2max, VO_2AT, Pmax and PAT in both female and male athletes (Tables 4 and 5). (tables 4
204 and 5 about here)
205 In female athletes there was an association between tHb_{mass} and VO_2max (P=0.00002),
206 VO_2AT (P=0.00000), Pmax (P=0.00001) and PAT (P=0.00000) in relative values. In men a
207 relationship was observed between relative values of tHb_{mass} and VO_2max (P=0.00008),
208 VO_2AT (P=0.0006) and PAT (P=0.0012) (Figure 1). (figure 1 about here) Additionally, there
209 was a significant association between absolute values of tHb_{mass} and VO_2max, VO_2AT, Pmax,
210 and power output at 4 $mmol \cdot l^{-1}$ blood lactate concentration in both male and female athletes
211 (data not shown).
212

213 **DISCUSSION**

214 In athletes, hematological traits are important not only in the clinical and health aspect but
215 also with respect to their physical performance. The regulation of erythropoiesis takes place
216 on several levels and depends on many factors such as cytokines, hormones, transcription
217 factors, and miRNA, which in turn have an effect on gene expression (12), while training has
218 only small effects on the total amount of hemoglobin in the blood (24). On the other hand,
219 many studies, including the one presented here, indicate a strong relationship between tHb_{mass}
220 and maximal oxygen uptake (15,25). Moreover, very high values of tHb_{mass} were observed in

elite Polish endurance athletes, as well as in young athletes who had just begun professional training (unpublished results). These results confirm that hemoglobin is the principal transporter of oxygen, and therefore a high total amount of it could, to a large extent, determine aerobic capacity (1,25). One of the genes responsible for the production of red blood cells and hemoglobin is the *HBB* gene. It should be noted that hundreds of variations have been identified in the *HBB* gene, and many polymorphisms may be related to hematological traits (11). For example, Auer et al. (4) reported that one polymorphism of the *HBB* gene (rs33971440) was associated with lower hemoglobin concentration, hematocrit level and clinical anemia. It is more likely that several polymorphisms of the *HBB* gene are responsible for the amount of hemoglobin and hence for aerobic capacity. In addition, it is often emphasized that genetics is an important factor influencing physical performance, although it is still not known which gene variants have an impact on it (2,3,16,21). So far in sport genetics the *HBB* gene has been examined only for three polymorphisms (intron 2+16 C/G, -551 C/T and +340 A/T polymorphisms) (13). He et al. (13) observed the relationship between homozygosity for the C allele of -551C/T and intron 2, +16 C/G (rs10768683) polymorphisms and running economy training response, but not with VO_2max. In our study in male athletes there was no relationship between the *HBB* gene polymorphisms and VO_2max, as well as other aerobic capacity indices. However, in female athletes we observed a strong relationship between relative values of VO_2max, Pmax and PAT and the *HBB* gene variants, but only in the case of G homozygotes of the intron 2, +16 C/G polymorphism. One might suggest that the same association was not replicated in male athletes due to relatively small sample size, differences in factors affecting hemoglobin levels between genders and the fact that within-person variation from day to day of hemoglobin values are higher in men than in women (6). However, the results of our study show no differences in the *HBB* genotype and

allele frequencies between male and female athletes, which is in accordance with earlier results obtained in Polish cross-country skiers and runners (19).

Because both tHb_{mass} and *HBB* genotypes (13) demonstrated relationships with indices of aerobic capacity, it was suggested that tHb_{mass} may depend on the *HBB* gene. However, we did not confirm this hypothesis, because regardless of gender none of the *HBB* variants (genotypes and genotype models) showed an association with tHb_{mass} or Hb concentration. Similar results were observed in Polish cross-country skiers and middle and long distance runners (19). In accordance with this, the *HBB* gene effect is opposite to other genes, because higher hemoglobin and hematocrit levels were observed in some polymorphisms of *EPO* (erythropoietin), *TFR2* (transferrin receptor 2), *NFIA-AS2* (nuclear factor I A antisense RNA 2) and *HIF1A* (hypoxia-inducible factor 1 alpha) genes (3,4,27). Despite the fact that the *HBB* gene is one of the primary genes in hemoglobin synthesis (28), there is still too little information concerning relationships of this gene's polymorphisms with amount of hemoglobin, so this issue requires further investigations.

Moreover, we did not find any differences in the *HBB* genotype distribution and allele frequencies between athletes and control groups. However, such a phenomenon has been observed for other "sport genes", and it has been suggested that genetic factors may predispose to successful sport performance (7). There is no study concerning the distribution of genotypes of the *HBB* gene among athletes and control groups, so we cannot compare our results with others.

The only study on this issue was carried out on Chinese non-athletes (13). However, comparing the frequencies of genotypes in Polish and Chinese populations can be difficult due to the ethnic origin, because certain alleles could be overrepresented in some ethnic groups (32). This is especially evident in the frequency of CC genotype for intron 2 +16 C/G polymorphism, which in the Polish male population was 6.0% and 2.0% in athletes and

controls, respectively, in contrast to 24.5% in the Chinese male population (13). Moreover, the racial differences in impact of specific polymorphisms on exercise capacity is strongly suggested (31,32). As described by He et al. (13), in the Chinese cohort +16CC genotype was associated with better physical performance, while in Polish athletes GG genotype benefits endurance capacity. It seems that this discrepancy is due to ethnic origin rather than selection for endurance disciplines, which is confirmed by similar results obtained in our earlier study (19), as well as the lack of differences in distribution of both polymorphisms between athletes and the control group, regardless of sex, in the present study. Therefore, we cannot clearly determine whether this gene may be considered as a "sports gene" and be helpful in the selection of athletes for sport.

To our knowledge this study is the first to determine the association between the *HBB* gene, tHb_{mass} and parameters of aerobic capacity in athletes. The main finding of our study was the significant correlation of aerobic capacity indices with one polymorphism of the *HBB* gene intron 2, +16 C/G in the female group, so the impact of the *HBB* gene on aerobic capacity may be connected with gender. We also found that neither of the studied polymorphisms of the *HBB* gene was associated with total hemoglobin mass.

PRACTICAL APPLICATIONS

Our results suggest that the *HBB* gene intron 2, +16 C/G polymorphism may be related to aerobic performance, but it seems that it is not due to an increase in the amount of hemoglobin in the blood. Therefore, the *HBB* GG genotype can be considered as one of the genetic markers associated with predisposition to endurance performance in females. However, further research including tHb_{mass}, genes and aerobic performance indices on a larger population of athletes and using different ethnic cohorts is necessary to better

294 understand the relationship between hemoglobin amount, genetic predisposition and physical
295 performance.

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Figure legend

Figure 1

Relationships between relative values of total hemoglobin mass (tHb_{mass}) and (A) relative values of maximal oxygen uptake (VO_2max), (B) oxygen uptake at anaerobic threshold (VO_2AT), (C) maximal power output (Pmax), and (D) power output at anaerobic threshold (PAT) in female and male cyclists; circles – females, triangles – males.

Titles of tables:

Table 1. Characteristics of study participants (mean ± SD)

Table 2. Genotype and allele frequencies of intron 2,+16 G/C and -551C/T polymorphisms of *HBB* gene in male and female athletes

Table 3. Relative values of total hemoglobin mass according to *HBB* genotypes in male and female athletes (mean ± SD)

Table 4. Aerobic capacity indices according to *HBB* genotypes in female athletes (mean ± SD)

Table 5. Aerobic capacity indices according to *HBB* genotypes in male athletes (mean ± SD)