**Title:** *PGC-*related gene variants and elite endurance athletic status in a Chinese cohort: a functional study

**Authors:** ZI-HONG HE1, YANG HU2\*, YAN-CHUN LI2, LI-JING GONG2, PAWEL CIESZCZYK3, AGNIESZKA MACIEJEWSKA-KARLOWSKA4, AGATA LEONSKA-DUNIEC4, CARLOS A. MUNIESA5, MARÍA MARÍN-PEIRO5, CATALINA SANTIAGO5, NURIA GARATACHEA6,7, NIR EYNON8,9, ALEJANDRO LUCIA5,7

**Affiliations:**

1Biology Centre, China Institute of Sport Science, Beijing, China

2Science and Research Centre of Beijing Sports University, Beijing, China

3 Department of Sport Education, Academy of Physical Education and Sport, Gdansk, Poland

4 Department of Physical Culture and Health Promotion, Szczecin University, Szczecin, Poland

5 European University, Madrid, Spain

6 Faculty of Physical Activity and Sports, University of Zaragoza, Spain

7 Research Institute *Hospital 12 de Octubre* (i+12), Madrid, Spain

8 Institute of Sport, Exercise and Active Living (ISEAL), Victoria University, Australia

9 Murdoch Childrens Research Institute, Royal Children's Hospital, Melbourne, Australia

**Correspondence:** Yang Hu, Science and Research Centre of Beijing Sports University, Beijing, 100084 China

**Telephone:** 81(10)13391925625, **Fax:** 861062989208, **Email:** bsugene@yahoo.com

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# Conflicts of Interest

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**ABSTRACT**

**Purpose**: To examine the association between proliferator-activated receptor γ (PGC)-gene family related single nucleotide polymorphisms (SNPs) and elite endurance runners’ status in a Chinese cohort, and to gain insights into the functionality of a subset of SNPs.

**Methods**: Genotype distributions of 133 SNPs in *PPARGC1A, PPARGC1B, PPRC1, TFAM, TFB1M, TFB2M, NRF1, GABPA, GABPB1, ERRα* and *SIRT1* genes were compared between 235 elite Chinese (Han) endurance runners (127 women) and 504 healthy non-athletic controls (237 women). Luciferase gene reporter activity was determined in 20 SNPs.

**Results**: After adjusting for multiple comparisons (in which threshold *P*-value was set at 0.00041), no significant differences were found in allele/genotype frequencies between athletes and controls (when both sexes were analysed either together or separately). The lowest *P*-value was found in *PPARGC1A* rs4697425 (*P*=0.001 for the comparison of allele frequencies between elite female endurance runners and their gender-matched controls). However, no association (all *P*>0.05) was observed for this SNP in a replication cohort from Poland (194 endurance athletes and 190 controls). Using functional genomics tool, the following SNPs were found to have functional significance: *PPARGC1A* rs6821591, rs12650562, rs12374310, rs4697425, rs13113110 and rs4452416; *PPARGC1B* rs251466 and rs17110586; and *PPRC1* rs17114388 (all *P*<0.001).

**Conclusion:** This study found no significant association between PGC-related SNPs and elite endurance athlete status in the Chinese population, despite some SNPs showing potential functional significance and the strong biological rationale to hypothesise that this gene pathway is a candidate to influence endurance exercise capacity.

**Key words:** elite endurance athlete; single nucleotide polymorphism; association study; gene expression; exercise

**Introduction**

The peroxisome proliferator-activated receptor γ (PGC) family members PGC-1α, PGC-1β and PGC-1-related coactivator (PGC-1-PRC) share several structures and functions (Matos et al. 2011). Of the three family members, PGC-1α has been the most widely investigated and is known to regulate a myriad of cell processes including metabolic control, transcriptional activity and muscle respiratory function. Together with PGC-1β, PGC-1α plays a key role in exercise training adaptations and mitochondrial biogenesis (Baar 2004; Koulmann & Bigard 2006; Mortensen et al. 2006; Shao et al. 2010; Yan et al. 2011; Zechner et al. 2010).

PGC-1α has also been shown to interact with the oestrogen-related receptor (ERRα) and nuclear respiratory factors 1 (NRF1) and 2 [NRF2, also known as GA-binding protein alpha chain (GABP)], and to up-regulate the expression of nuclear-encoded mitochondrial genes. PGC-1α mediated co-activation of NRF1 and 2 induces the transcription of mitochondrial transcription factor A (TFAM), which is the final activator of mitochondrial DNA replication (Lehman et al. 2000; Scarpulla 2002; Yan et al. 2011). PGC-1β stimulates mitochondrial biogenesis by co-activating NRF-1 and ERRα (Shao et al. 2010); whereas both PGC-1α and PGC-1β up-regulate citrate synthase expression (Mortensen et al. 2006) and mitochondrial β–oxidation (Huss et al. 2002; Lin et al. 2005; Vega et al. 2000), and are important factors in muscle adaptation to exercise training.

The signalling protein 5' AMP-activated protein kinase (AMPK) is often referred to as the master indicator of cellular energy balance. Several studies have shown the existence of indirect mechanisms of AMPK control on PGC-1α activity during endurance exercise (Lira et al. 2010; Little et al. 2010; Winder et al. 2006). AMPK enhances sirtuin 1 (SIRT1) activity by increasing intra-cellular NAD+ levels (Canto et al. 2009). Once activated, SIRT1, a type III deacetylase, modulates the activity of PGC-1α and its downstream targets, which in turn control oxidative phosphorylation transcriptional activity by acetylation/deacetylation (Canto et al. 2009).

Several genetic variants (*i.e.* polymorphisms) within the PGC-1-related gene pathway have been associated with elite athletic performance and the response to aerobic exercise training. A particularly strong candidate to influence athletic performance, at least in Caucasians, is the common missense polymorphism (Gly482Ser, rs8192678) at exon 8 of the *PGC-1* (also termed *PPARGC1A*) gene. This polymorphism has been associated with endurance athletic status; the minor Ser482 allele (linked to an increased risk of chronic diseases such as type 2 diabetes, insulin resistance, obesity, dyslipidemia or hypertension) is less frequent in athletes compared to controls (Eynon et al. 2010; Lucia et al. 2005; Maciejewska et al. 2012). However, many other variants exist in the PGC-related gene pathway and their influence, if any, on athletic performance is currently unknown.

Given the above-mentioned evidence suggesting that PGC-related genes influence mitochondrial biogenesis and adaptations to endurance exercise, there is a biological rationale in hypothesising that variants in these genes are associated with elite endurance athletic status. The majority of previous studies in the field have only focused on one or a handful of specific variants in the PGC-related genes. However, it remains to be determined if elite endurance athletic status is not only associated with previously studied candidate variants, but also with other unstudied variants. For this reason, we adopted a ‘general’ or ‘massive’ approach: the main purpose of the present study was to determine the association between 133 PGC-related genetic variants, *i.e.* all tag single nucleotide polymorphisms (SNPs) in the gene region showing a minor allele frequency (MAF) ≥ 0.1, and elite endurance athletic status in a cohort of Chinese (Han) athletes. To gain insight into the functionality of these variants as well as to provide information potentially valuable for future research in the field, our second purpose was to determine the luciferase activity in a subset of SNPs (*i.e.* those for which a *P*-value ≤ 0.05 was obtained in the allele/genotype frequency comparisons between athletes and controls -see Methods section). Finally, to replicate the association between the main SNPs and athletic status, we tested two independent cohorts of elite endurance athletes from different ethnic and geographic background. We hypothesized that some of the studied variants would be associated with elite endurance athletic status and/or would have functional significance.

**Methods**

**Participants**

*The Chinese cohort*

Written informed consent was obtained from each participant prior to commencing the study. The study protocol received local institutional (China Institute of Sport Science) review board approval. All subjects were unrelated and of Chinese descent (Han origin for 3+ generations) and were recruited from mainland China. The Chinese cohort included:

 (i) 235 elite endurance runners (108 men, 127 women). We selected all (n=59) the *international master* category endurance runners [*i.e.* best performance in 3000 m steeplechase, 5000 m, 10000 m and marathon equal or better than 8 min 23 s (men) and 10 min (women), 13 min 32 s (men) and 15 min 30 s (women), 28 min 19 s (men) and 32 min 30 s (women) and 2 h 13 min (men) and 2 h 34 min (women), respectively] as well as the *national master* category endurance runners (n=176) in China [*i.e.* best performance in 3000 m steeplechase, 5000 m, 10000 m and marathon equal or better than 8 min 47 s (men) and 10 min 8 s (women), 14 min 10 s (men) and 15 min 45 s (women), 29 min 45 s (men) and 33 min (women) and 2 h 20 min (men) and 2 h 45 min (women), respectively]. Mean ages were 23±4 years (men) and 21±4 years (women).

(ii) 504 healthy control subjects (267 men, 237 women, all non-athletes with no self-reported family history of competitive sports participation). These individuals (age=20±1 years) were undergraduate students of the China Agricultural University.

*The Polish cohort*

Written informed consent was obtained from each subject prior to commencing the study. The study was approved by the review board of the Pomeranian Medical University. All subjects enrolled for the replication study were unrelated Caucasians. The Polish cohort included:

(i) 194 unrelated elite endurance athletes (152 men, 42 women). The athletes were rowers [n=62 (59 men), including 14 Olympic/World champions and 22 medallists in the Olympic Games or World/European championships], endurance road cyclists [n=26 (23 men), including 7 medallists in the Olympic Games or World/European championships], 3000 m runners [n=36 (16 men), including one Olympic medallist and two European Championship medallists], 1500 m runners [n=9 (7 men), including two European Championship medallists], marathon runners [n=18 (15 men)], 800-1500 m swimmers [n=15 (12 men), including two medallists in the Olympic Games or World/European championships], 15-50 km cross-country skiers [n=2 (one man), both Olympic champions], walkers (n=2 men, including an Olympic champion), canoeists [n=10 (7 men)], and triathletes [n=14 (10 men), including 3 European championship medallists].

(ii) 190 unrelated healthy sedentary controls (104 men, 86 women) aged 19-24 years who were students of the University of Szczecin and did not undertake regular physical activity.

*The Spanish cohort*

Written informed consent was obtained from each participant prior to commencing the study. The study was approved by the institution’s (European University, Madrid, Spain) review board. The Spanish cohort included:

(i) 190 unrelated elite endurance athletes (138 men, 52 women). Men athletes were runners (mostly 5000 m and over, n=25), professional road cyclists (n=49), and rowers (n=64). Inclusion criteria were: finalist in at least one Olympic Game or World/European championship, except for the cyclists. The inclusion criterion for the latter was to have won one stage in one of 3 major races (*Vuelta a España*, *Giro d’ Italia* or *Tour de France*). Women athletes were top Spanish female marathon runners.

(ii) 360 control, non-athletic subjects (292 men, 68 women, 20-30 years) who did not undertake regular physical exercise.

**Chinese cohort: genotyping and functional analysis**

*Selection of tag SNPs*

A list of SNPs for the Han population of Beijing, China (CHB) was obtained from the International HapMap Project database ([www.hapmap.org](http://www.hapmap.org)). We then used haploview to select all tag SNPs in the gene region with a Minor Allele Frequency (MAF) ≥ 0.1. A total of 11 genes and 133 SNPs were studied as follows:

. 41 SNPs in the *PPARGC1A* gene(positions 23, 402, 743 to 23, 500, and 798 in chromosomal region 4p15.1)

. 43 SNPs in the *PPARGC1B* gene(positions 149, 088, 057 to 149, 209 and 460 in 5q32)

. 4 SNPs in the peroxisome proliferator-activated receptor gamma, coactivator-related 1 gene (*PPRC1*, positions 103, 880, 777 to 103, 902, 078 in 10q24.32)

. 3 SNPs in the *TFAM* gene (positions 59, 813, 182 to 59, 827, 902 in 10q21)

. 7 SNPs in mitochondrial transcription factor 1 gene (*TFB1M*, positions 155, 618, 490 to 155, 679, 310 in 6q25.1-q25.3)

. 3 SNPs in the mitochondrial transcription factor 2 gene (*TFB2M*, positions 244, 768, 491 to 244, 798, 186 in 1q44)

. 14 SNPs in the *NRF1* gene (positions 129, 036, 791 to 129, 186, 156 in 7q32)

. 2 SNPs in the *NRF2* gene (official name: *GABP*, positions 26, 027, 570 to 26, 068, 210 in 21q21.3)

. 5 SNPs in the beta 1 subunit of the GABP gene (*GABPB1,* positions 48, 354, 682 to 48, 436, 687 in 15q21.2)

. 4 SNPs in the oestrogen-related receptor alpha gene (*ERRα*, positions 63, 827, 620 to 63, 842, 786 on chromosome in 11q13)

. 7 SNPs in the *SIRT1* gene (positions 69, 310, 433 to 69, 348, 147 in 10q21.3).

The selected SNPs and their MAFs are listed in **Supplementary** **Table 1**.

*Genotyping*

Peripheral whole blood samples were obtained from the elite athletes (in 2003-2004 and 2010) and the control participants (in 2004 and 2011). Genomic DNA was extracted using a Wizard Genomic DNA Purification Kit (Promega, Madison, Wisconsin, USA). Genotype analysis was performed in 2011-2012 at Shanghai Benegene Biotechnology, LTD (Shanghai, China). For high-throughput genotyping of all SNPs, we used a matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) platform (Sequenom, San Diego, CA, USA). Primers for the polymerase chain reaction (PCR) and single base extension were designed using the Assay Designer software package (Sequenom, San Diego, CA, USA). Genotyping was performed as described elsewhere (He et al. 2011).

*Functional analysis: luciferase reporter gene*

We examined the luciferase reporter gene for 20 SNPs that yielded one or more *P*-values ≤ 0.05 when comparing genotype and/or allele frequencies in the Chinese cohort (see Results section). The construct was PCR-generated using DNA from two homozygous subjects. Amplicons were cloned directionally into the pGL3-promoter luciferase expression vector at the restriction recognition sites indicated in **Supplementary Table 2.** Mice skeletal muscle C2C12 cell lines were used to represent muscle-specific expression. Cell cultures, transfections and dual-luciferase reporter assays were performed as previously described (He et al. 2011). We used the pRL-SV40 vector as an internal control for variations in transfection efficiency. The pGL3-promoter vector without an insert was used as a negative control. The transfected cells were harvested after 48h, and assayed for firefly luciferase activity and renilla luciferase activity using the dual-luciferase reporter assay system (Promega Biotech, Beijing, China), as suggested by the manufacturer using a luminometer (Tecan Genios Pro, Männedorf, Switzerland). From each measurement, relative luciferase activity was calculated by dividing the firefly luciferase activity reading by the renilla luciferase activity reading. Experiments were performed in triplicates. Relative luciferase activity values are expressed as the means±SD of the three different experiments.

*Genotyping in the Polish and Spanish cohorts*

*PPARGC1A* rs4697425 analysis reached the lowest *P*-value in the Chinese cohort (–see Results section), and was therefore replicated in the two independent cohorts. Genomic DNA was isolated over the period of 2008-2010 from buccal epithelium using GenElute Mammalian Genomic DNA Miniprep Kit (Sigma, Germany) (the Polish cohort), and over 2009-2013 according to standard phenol/chloroform procedures followed by alcohol precipitation (the Spanish cohort). All samples were genotyped in duplicate in 2012-2013 using an allele discrimination assay in a StepOne (the Polish cohort) or StepOne Plus Real-Time PCR instrument (Applied Biosystems, USA) with Taqman® probes (the Spanish cohort). Custom Taqman® SNP Genotyping Assay Service (Applied Biosystems, USA) was used [custom assay IDs: AHGJ3Y8 (Polish samples) and AHN1TVT (Spanish samples)]. All assays included appropriate primers and fluorescently labelled (FAM and VIC) MGB™ probes.

**Statistical Analysis**

In the Chinese cohort, the Hardy-Weinberg Equilibrium (HWE) was tested in the control group for all SNPs using a χ2 test. For SNPs that met HWE expectations, we performed between-group comparisons of genotype/allele frequencies using the χ2 test. All individual comparisons were corrected for multiple comparisons using the Bonferroni method. The threshold *P*-value was achieved by dividing 0.05 by the number of SNPs that met HWE expectations as follows: *P*= 0.05 / (133-10) = 0.00041. For SNPs in which between-gender differences were found in allele/genotype in athletes or controls, comparisons between athletes and controls were performed separately for each sex. The HWE was also tested in the Polish and Spanish controls, and between-group comparisons (two sexes combined and separately) were performed using the χ2 test. One way ANOVA was used to compare the relative luciferase activity in the different plasmids of each SNP, using a threshold *P*-value of 0.0025 (= 0.05 divided by number of SNPs studied, *i.e.* 20). All statistical tests were performed using the Statistical Package for Social Sciences software, version 16.0 (SPSS Inc, Chicago, IL).

**RESULTS**

*Genotype results for the Chinese cohort*

In the athletes group,thegenotype success rate was 100% for all SNPs except 9 in which the success rate was 99.6%. In the control group, a genotype success rate of ≥ 98% was recorded for 43 SNPs and in the remaining SNPs, the genotype success rate was 100%. In the control group, genotype distributions for the following 10 SNPs did not meet HWE expectations: rs12500214, rs2970869, rs1833661, rs251460, rs11746929, rs1937, rs2402970, rs2402976, rs12594956 and rs12440811. These SNPs were therefore omitted from further analysis. We observed significant between-gender differences in 28 SNPs (9 in *PPARGC1A*, 12 in *PPARGC1B*, 2 in *PPRC1*, and one in *TFAM*, *TFB2M*, *NRF1*, *GABPA* and *SIRT1*). Accordingly, genotype/allele frequencies for these SNPs were compared separately for each sex (**Table 1**). With respect to the remaining SNPs, comparisons between elite endurance athletes and controls were performed together for both sexes (**Table 2**).

In 20 SNPs, the obtained *P*-value was <0.05 for genotype and/or allelic frequency comparisons between the athletes and controls: 7 SNPs are located in *PPARGC1A* (rs6821591, rs12650562, rs4697045, rs12374310, rs4697425, rs13113110, rs4452416), 5 SNPs are in *PPARGC1B* (rs251466, rs17110586, rs32579, rs2161257, rs17600568), 2 SNPs are located in *PPRC1* (rs4917960, rs17114388) 1 SNP is located in *TFAM* (rs2306604), 1 SNP is located in *TFB2M* (rs10802426), 2 SNPs are located *NRF1* (rs1984823, rs7794909) and 2 SNPs are located in *SIRT1* (rs11596401, rs4746720) (**Tables 1 and 2**). However, after adjusting for multiple comparisons, no significant association was found, *i.e.* all *P*-values were higher than the stringent *P*=0.00041 threshold. The SNP for which a lowest *P*-value was found in the analyses was *PPARGC1A* rs4697425, with *P*=0.001 for the comparison of allele frequencies between elite female endurance runners and their gender-matched controls.

*Functional analysis*

The results of luciferase report analyses are presented in **Table 3**. The following 9 SNPs had functional significance, as determined by different luciferase activity between the constructs:

. *PPARGC1A* rs6821591, rs12650562, rs12374310, rs4452416, rs13113110 and rs4697425

. *PPARGC1B* rs251466 and rs17110586

. *PPRC1* rs17114388

These SNPs were associated with up-regulation of relative luciferase activity (*P*<0.001). Except only for rs13113110, the genotypes that tended to be more frequent in the endurance athletes than in the control group (*P*<0.05 in Tables 1 and 2) demonstrated significantly higher relative luciferase activity (*P*<0.001).

*Genotype results for the Polish and Spanish cohort*

Genotype success rates for *PPARGC1A* rs4697425 was 96.9% in the Spanish cohort and 100% in the Polish cohort. Genotype distributions met HWE expectations in the Polish, but not in Spanish controls (**Table 4**). This SNP was therefore removed from the analysis in the Spanish cohort. No significant allele/genotype frequencies differences were observed in the Polish cohort.

**DISCUSSION**

Using comprehensive SNP analysis, we found no significant association between PGC-related SNPs and elite endurance athlete status. Some of these SNPs (namely; rs6821591, rs12650562, rs12374310, rs4452416, rs13113110, rs4697425, rs251466, rs17110586 and rs17114388) showed potential functional significance, *i.e.* up-regulation of luciferase activity. Among these SNPs, *PPARGC1A* rs4697425 yielded the lowest *P*-value for the analyses in the Chinese cohort (*P*=0.001 for the comparison of allele frequencies in female athletes vs. gender-matched controls). However, the stringent *P*-value=0.00041 was not reached for *PPARGC1A* rs4697425, and our replication analysis in a Polish cohort supported the lack of association.

Several studies have previously shown that the *PPARGC1A* gene is up-regulated following endurance exercise training. This type of training increases *PPARGC1A* mRNA levels in both animals (Baar et al. 2002; Goto et al. 2000; Terada et al. 2002; Terada & Tabata 2004) and humans (Norrbom et al. 2004; Pilegaard et al. 2003; Russell et al. 2003; Short et al. 2003; Tunstall et al. 2002). Furthermore, transgenic over-expression of *PPARGC1A* mRNA has been shown to increase muscle resistance to fatigue (Lin et al. 2002). A specificGly482Ser polymorphism (rs8192678) in the *PPARGC1A* gene has been associated with elite endurance athlete status in Israeli (Eynon et al. 2010), Polish/Russian (including some of the athletes in the present study) (Maciejewska et al. 2012), and Spanish cohorts (same as above) (Lucia et al. 2005), with the Ser482 allele being less frequent in athletes compared to controls. However, we were unable to detect an association between the Gly482Ser variant and elite endurance athletic status in the Chinese cohort. This discrepancy between our findings and those of previous studies is not surprising and suggests that the genes that influence athletic performance vary across ethnicities. This hypothesis is supported by the fact that the minor *PPARGC1A* 482Ser-allele is more frequent in Caucasian elite endurance athletes (~45%) (Lucia et al. 2005) compared to Asian elite endurance athletes (~29%). Further, no association was previously observed between training adaptations and *PPARGC1A* Gly482Ser in non-athletic Chinese individuals, also of a Han origin (He et al. 2008). On the other hand, the fact that the lowest *P*-values were observed only in Chinese women suggests that the genetic influence on athletic performance may also vary by gender. Gender differences have usually been ignored in studies into the influence of genomics on athletic performance. Our findings indicate that these differences should be taken into account in future studies in the field. In fact, the strongest genetic candidate showing an influence on athletic performance (at least in sprint/power oriented events) to date, the R577X SNP in the α-actinin-3 (*ACTN3*) gene (Eynon et al. 2013), seems to affect athletic performance differently in males and females (Yang et al. 2003).

In the present study, we have shown that 9 SNPs up-regulated the relative luciferase activity. The fact that a homozygous plasmid for a given SNP has higher relative luciferase activity than the opposite homozygous plasmid might reflect that changes in gene expression are genotype-dependent. With regard to this, except for rs13113110, the genotypes that up-regulated gene expression also tended to show higher frequencies in athletes than in their controls, (*P*<0.05 in Tables 1 and 2). For instance, the TT genotype in rs12650562 might potentially result in a ~2-fold greater expression compared with the CC genotype (Table 3). Previous research has shown that the mRNA expression is positively correlated with protein levels (Ling et al. 2004). Thus, it could be speculated that those genotypes that might affect protein levels and influence biological function could also be potentially influential to endurance exercise performance. This hypothetical ‘cause and effect’ influence of genomic markers on athletic performance warrants future research. Interestingly, the 9 SNPs that showed potential functional significance are located in non-coding gene regions (**supplementary Table 1**). Although intron cis-elements, such as enhancers or repressors, have been reported for various genes (Gregori et al. 1998; Hwang et al. 2002), it remains to be determined whether the intron region of a gene that is associated with elite athletic status might function as an enhancer or repressor, regulating the expression of the gene. Although the molecular mechanisms mediating the functional significance of SNPs outside coding DNA regions are still to be clearly established, non-coding SNPs might regulate the alternative splicing of mRNA, leading to changes in gene expression (Mercado et al. 2005), or phenotype traits (Sasabe et al. 2007; Saxena et al. 2007), and may also potentially influence the binding of transcription factors (Knight 2005).

We believe that the approach we have taken is in accordance with recent recommendations for studying the influence of genomic markers on athletic performance (Pitsiladis et al. 2013). First, we have comprehensively analysed all tagged SNPs with a MAF ≥ 0.1 in 11 candidate genes. Second, we have functionally analysed 20 SNPs. Finally, we have attempted to replicate our main findings in independent cohorts of elite athletes with different ethnic background, and used the very stringent Bonferroni method to avoid statistical type I errors (*i.e.* false significant associations) due to multiple comparisons. A further strength of our work is the relatively large sample of endurance athletes – both male and female– and the study of Asian (Chinese, Han) athletes. On the other hand, a limitation of our study stems from the relative heterogeneity of Spanish/Polish athletic cohorts (composed by endurance athletes of different sports disciplines) *vs.* the Chinese cohort (composed solely by endurance runners), yet we believe this is justified by the difficulty of gathering top-competition athletes.

In conclusion, we did not find significant associations between PGC-related SNPs and elite endurance athlete status in the Chinese population, despite some SNPs showing potential functional significance and the strong biological rationale in postulating this gene pathway as a candidate to influence endurance exercise capacity.

**Perspective**

The fact that no actual significant association was detected despite the large number of studied SNPs and the positive results arising from the luciferase-based functional analyses, illustrates the difficulty in revealing genes that influence the status of being a world-class elite athlete. There are indeed numerous potential contributors to the ‘complex trait’ of being an athletic champion (*e.g.* gene-gene or gene-environment interactions) which are difficult to identify. Future replication studies in different cohorts are however needed to determine whether the functional PGC-related SNPs contribute to the large variability in athletic performance and adaptation to endurance exercise training.

# Conflicts of Interest

None of the authors have professional relationships with the companies or manufacturers mentioned in this paper.

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