

ANALYSIS OF THE *PPARD* GENE EXPRESSION LEVEL CHANGES IN FOOTBALL PLAYERS IN RESPONSE TO THE TRAINING CYCLE

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ABSTRACT

The *PPARD* gene codes protein that belongs to the peroxisome proliferator-activated receptor (PPAR) family engaged in a variety of biological processes, including lipid metabolism in muscle cells. In this study, we assess the relationship between *PPARD* gene expression lipid metabolism parameters and the variation of the *PPARD* gene expression before (T_1) and after 12 hours of training (T_2) sessions in a group of football players. Peripheral blood lymphocytes were obtained from 22 football players (17.5±0.7 years, 178±0.7 cm, 68.05±9.18 kg). The *PPARD* gene expression, analyzed by quantitative polymerase chain reaction (qPCR), was significantly higher after T_2 ($p = 0.0006$). Moreover, at the end of the training cycle, there was a significant decrease in relative fat tissue (FAT) (%) ($p = 0.01$) and absolute FAT (kg) ($p = 0.01$). A negative correlation was observed between absolute FAT (kg) and *PPARD* gene expression level in T_2 ($p = 0.03$). The levels of cholesterol and triglyceride (TG) fractions were not significantly different ($p > 0.05$) before and after training. No significant relationship between *PPARD* expression and

cholesterol or TG levels was found. We found that physical training affects *PPARD* expression. Moreover, the negative correlation between *PPARD* expression and absolute FAT (kg) level may be indicative of the contribution of *PPARD* in metabolic adaptation to increased lipid uptake that can be used to control the body composition of athletes.

Keywords: Body fat; Exercise; Gene expression; Lipid metabolism; Peroxisome proliferator-activated receptor (PPAR); Sports.

INTRODUCTION

The peroxisome proliferator-activated receptor (PPAR) belongs to a ligand-dependent family that comprises three subtypes: PPAR α , PPAR γ and PPAR β/δ , each of which is related to different tissues or cells. In the present study, we examined the *PPARD* gene, located on the short arm of the chromosome 6 (6p21.1-p21.2), that encodes a member of the PPAR family. This transcription factor gene encompasses an 85 kb section of DNA and consists of nine exons and eight introns [1].

The highest expression level of *PPARD* mRNA is observed in Sertoli cells, intestine, heart, liver, brain and kidney tissues [2-5]. The most popular ligands of PPAR β/δ are: unsaturated fatty free acid (FFA) and their metabolites [1,2,5,6], GW0742 [7-9], ezetimibe [7], GW501516 [6,10], 15-HETE, 4-HNE, 4-HDDE and ATRA [2]. The PPAR activation plays a vital role in regulation of many physiological and pathological processes, such as energy consumption, inflammation, tissue repair, proliferation and differentiation of various types of cells [1-3].

Mammalian skeletal muscles are a major site of fatty acid catabolism in the fasting state. Thus, factors affecting

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this process, such as PPAR β/δ , modulate homeostasis at the whole-body level [11,12]. The PPAR β/δ receptor exerts a pleiotropic impact on skeletal muscle physiology and metabolism. The use of cell cultures and animal models has revealed that PPAR β/δ activation results in a metabolic shift promoting lipid utilization (increased fatty acid uptake and oxidation) and reduced carbohydrate oxidation [6,11,13]. These modifications occur through upregulation of the expression of proteins implicated in myocyte energetic substrate preference, such as fat tissue (FAT)/CD36 [13,14], lipoprotein lipase, PDK4 or CPT1 [2,4,6,14]. The PPAR β/δ also promotes mitochondrial biogenesis, angiogenesis and changes in fiber type composition from glycolytic to slow/fast oxidative fibers [11,13,15,16]. Moreover, mice fed with a high fat diet and treated with PPAR agonist showed decreased progress of obesity as well as upgraded glucose tolerance and insulin sensitivity [2,17].

Nutritional status and physical activity are well-known causative agents of alteration in *PPARD* expression level in skeletal muscle [2,12,18]. Analysis of the mRNA expression in skeletal muscles obtained from males proved that during recovery from endurance exercise (in the first 3 hours) it can be observed in transient elevated *PPARD* expression level (2.6 \pm 0.6-fold) [19]. Therefore, we performed this study to evaluate if there is any relationship between training-induced changes in *PPARD* gene expression level and lipid metabolism parameters.

MATERIAL AND METHODS

The study was approved by the Medical University of Lodz Ethics Committee (RNN/157/16/KE). All participants gave full written informed consent prior to commencement of the study.

Twenty-two young male football players (17.5 \pm 0.7 years, 178 \pm 0.7 cm, 68.05 \pm 9.18 kg) participated in the study. Before the experiment, all the players took part in the 2 months of preliminary training. This experiment took place during 2 months training cycle (from middle of April to middle of June 2016). All the players were subjected to the same football training that consisted of strength, speed and endurance exercises. The typical weekly training load during the experiment involved different training drills in the work week (two mornings and five afternoons during the week) and the competition game on Saturday. Training drills included: interval run, small-sided games and plyometric, speed, technical, coordination, tactical and aerobic exercises. Small-sided games were carried out on the field (44 \times 33 m) on Tuesdays with 120 square meters per football player. The subjects played four games, 4 min.

each with 3 min. active break that consisted of walking and muscle relaxing exercises. The intensity of the training was imposed by the heart rate (HR) that was equal or higher than anaerobic threshold (AnT) value but did not exceed 90.0% HR_{max} value. Individual maximal intensity run and run at lactate threshold of the player was determined on a synthetic field at the beginning of an experiment. The test protocol included 3.5-5.0 min. running stages separated by a 1 min. rest, during which a capillary blood sample was taken from the fingertip. The initial speed was set at 2.8 m/s and increased by 0.4 m/s after each stage until exhaustion [20].

Collection of Biological Material. Blood samples were collected before (T_1) and 12 hours after training (T_2). Before blood collection, the players had been resting in the supine position for 10 min. Blood was aspirated into 5 mL EDTA-containing tubes. For lymphocyte isolation, a density gradient cell separation solution Histopaque $^{\text{®}}$ -1077 (Sigma-Aldrich Co., St. Louis, MO, USA) was used. Blood needed for determination of lipid profiles was collected into the serum separator tubes.

Gene Expression Analyses. RNA isolation was performed using the mirVana $^{\text{TM}}$ miRNA Isolation Kit (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's protocol. The quality and quantity of isolated RNA was spectrophotometrically assessed (Eppendorf BioPhotometr $^{\text{TM}}$ Plus; Eppendorf, Hamburg, Germany). The purity of total RNA (ratio of 16S to 18S fraction) was determined by automated electrophoresis using the RNA Nano Chips LabChipplates in Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Complementary DNA (cDNA) was transcribed from 100 ng of total RNA, using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA) in a total volume of 20 μ L, according to manufacturer's protocol. The relative expression analysis was performed in 7900HT Fast Real-Time PCR System (Applied Biosystems) using TaqMan probes for the study gene *PPARD* (Hs00987008_m1) and *ACTB* gene (Hs99999903_m1) used as an endogenous control. The PCR mixture contained cDNA (1-100 ng), 20 \times TaqManR Gene Expression Assay, 2 \times KAPA PROBE Master Mix (2 \times) ABI PRISM $^{\text{®}}$ Kit (Kapa Biosystems, Wilmington, MA, USA), and RNase-free water in a total volume of 20 μ L. The expression levels relative quantification (RQ) values of the studied gene were calculated using the $\Delta\Delta$ CT method, with the adjustment to the β -actin expression level and in relation to the expression level of calibrator, for which RQ value was equal to 1.

Lipid Profile Analyses. The concentration of chosen plasma lipids was determined using a high performance

Table 1. The median values (mmol/L) and interquartile range (IQR) of the lipid profile parameters in both time points.

	Median Concentration in T ₁ (mmol/L) (IQR)	Median Concentration in T ₂ (mmol/L) (IQR)	p Value (Wilcoxon signed-rank test)
Total cholesterol	4.26 (Q1=3.89; Q3=4.73)	4.11 (Q1=3.82; Q3=4.63)	0.695
High-density lipoprotein	1.30 (Q1=1.16; Q3=1.54)	1.27 (Q1=1.20; Q3=1.41)	0.658
Low-density lipoprotein	2.39 (Q1=1.97; Q3=2.85)	2.27 (Q1=2.04; Q3=2.74)	0.778
Triglycerides	0.85 (Q1=0.60; Q3=1.36)	1.09 (Q1=0.70; Q3=1.46)	0.235

T₁: before training; T₂: 12 hours after training; IQR: interquartile values; Q1: quartile 1; Q3: quartile 3.

Table 2. The results of statistical analysis regarding correlations between *PPARD* gene expression level (median RQ value) and the other studied parameters (Spearman's rank correlation coefficient).

	<i>PPARD</i> Gene Expression	
	RQ Value in T ₁	RQ Value in T ₂
Total cholesterol (mmol/L)	rho = 0.24; p = 0.289	rho = 0.43; p = 0.070
High-density lipoprotein (mmol/L)	rho = 0.25; p = 0.257	rho = 0.45; p = 0.052
Low-density lipoprotein (mmol/L)	rho = -0.35; p = 0.111	rho = -0.21; p = 0.401
Triglycerides (mmol/L)	rho = 0.12; p = 0.600	rho = 0.18; p = 0.470
Absolute fat tissue (kg)	rho = 0.24; p = 0.285	rho = -0.46; p = 0.031
Relative fat tissue (%)	rho = 0.18; p = 0.418	rho = -0.32; p = 0.147

T₁: before training; T₂: 12 hours after training; RQ: relative quantification of *PPARD* gene expression.

laboratory analyzer (Olympus AU680; Beckman Coulter, Atlanta, GA, USA). The analytical enzymatic methods used in our study were: method with esterase and cholesterol oxidase for total cholesterol, enzymatic method with esterase and cholesterol oxidase after prior forming an immunological complex with other lipoproteins for high-density lipoprotein (HDL) cholesterol and the enzymatic method with phosphoglycerol oxidase (determination of H₂O₂ using peroxidase) for triglycerides (TGs). The low-density lipoprotein (LDL) cholesterol concentration was calculated.

Body Fat Analyses. The body FAT data, absolute FAT (kg) and relative FAT (%), were determined using an electrical bioimpedance method with mode for athletes (Tanita MC-980 MA, Abdominal Fat Analyzer AB-140; Tanita, Tokyo, Japan). The football players were tested in the morning (in a fasting state), 24 hours after training.

Statistical Analyses. A Shapiro-Wilk test was carried out to assess the normal distribution. The Wilcoxon signed rank test was used to compare the levels of relative expression values (RQs) in both time points. Spearman's rank correlation coefficient was used to assess the correlation between gene relative expression level and cholesterol concentration or fat mass in both time points. Outcomes of $p < 0.05$ were considered to be statistically significant. Calculations were based on the Statistica for Windows, version) 13.0 program.

RESULTS

Relative Expression Levels of the *PPARD* Gene.

The median relative expression levels (RQ values) 0.02 in T₁ (Q1 = 0.009, Q3 = 0.073) and 0.75 (Q1 = 0.061, Q3 = 5.065) in T₂. Significant differences in RQ values were found between T₁ and T₂ ($p = 0.001$; Wilcoxon signed rank test), with higher gene expression level in T₂ (Figure 1).

Analysis of Lipid Profile Parameters Before and After Training. The median values (mmol/L) and interquartile range (IQR) obtained in the quantitative measurements of the parameters of the lipid profile are presented in Table 1. We did not observe any statistically significant differences between the median values of total cholesterol ($p = 0.695$), HDL ($p = 0.658$), LDL ($p = 0.778$) and the TGs concentration ($p = 0.235$) (Wilcoxon signed rank test) before and after training.

Analysis of Absolute FAT (kg) and Relative FAT (%) Parameters Before and After Training. Significant differences were found between the median FAT value [absolute FAT (kg), $p = 0.006$; relative FAT (%), $p = 0.009$] before and after training, with lower mean values of FAT (kg) and FAT (%) observed in T₂ (Figures 2 and 3).

Analysis of the Reciprocal Relationship Between the Expression of the *PPARD* and Absolute FAT (kg), Relative FAT (%) and Lipid Profile Parameters Before and After Training. Finally, we assessed the reciprocal

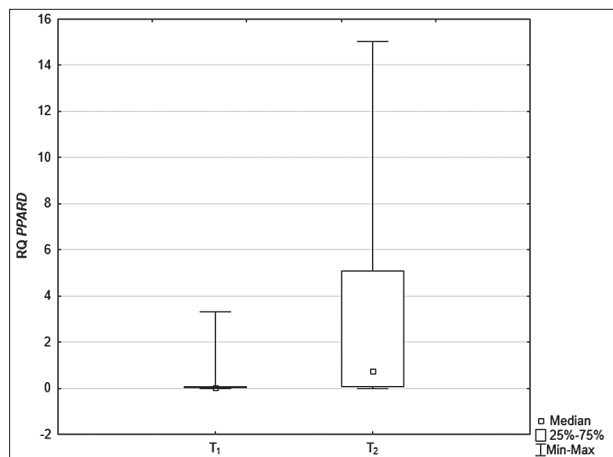


Figure 1. Box-and-whisker plots, representing PPARD expression levels (median RQ values) before and after training.

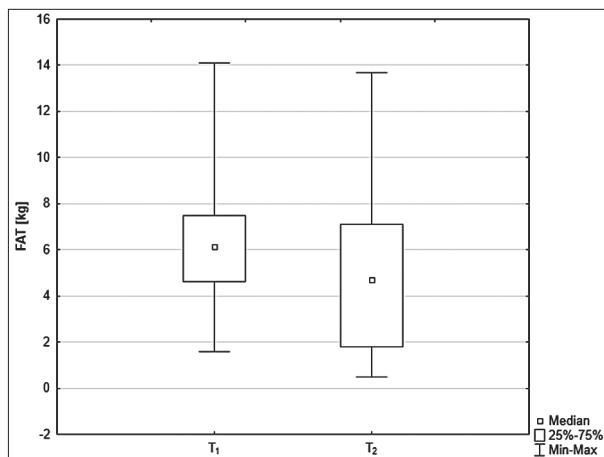


Figure 2. Box-and-whisker plots, representing relative FAT (%) levels before and after training.

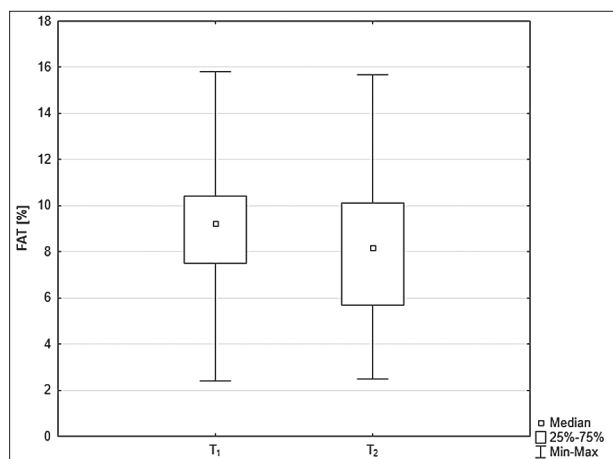


Figure 3. Box-and-whisker plots, representing absolute FAT (kg) levels before and after training.

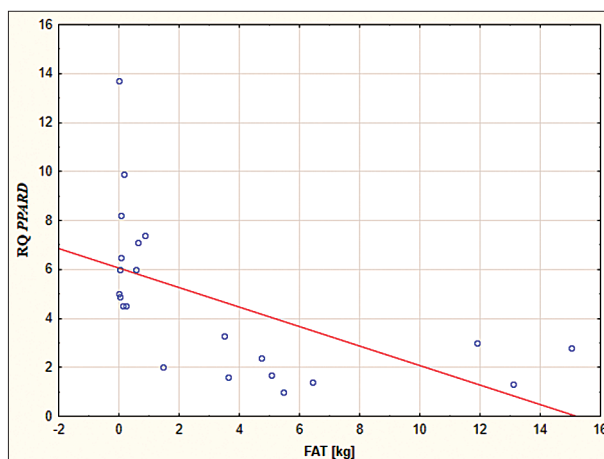


Figure 4. Negative correlation between *PPARD* gene expression level (RQ values) and the absolute FAT (kg) level in T_2 .

relationship between the expression level of *PPARD* gene (median RQ values) and the other examined parameters. The results are presented in Table 2. Significant negative correlation was found between the expression level of *PPARD* gene and the absolute FAT (kg) value after training ($\rho = -0.46, p = 0.031$) (Figure 4).

DISCUSSION

In physical activity, PPAR β/δ acts as a key regulator of fuel metabolism, promoting a shift from glucose to lipid as the main energy substrate. It promotes cellular lipid uptake, activation of fatty acids by fatty acylCoA synthetase and their mitochondrial uptake and β -oxidation. This mechanism decreased glucose oxidation as a consequence, which mimics caloric restriction and physical exercise conditions. Peroxisome proliferator-activated receptors

play a regulatory role in preventing metabolic disorders and in muscle adaptation to fasting and physical exercise [10,21-24]. The studies performed in mice showed that activation of PPAR β/δ in skeletal muscle results in enhanced lipid metabolism as an adaptive response to external stimuli such as food availability and prolonged physical activity [2,25-27]. This activation of PPAR β/δ in skeletal muscle enhances lipid use for energy expenditure, which is preferred to glucose and allows glucose to become more available for peripheral organs.

Physical exercise enhances *PPARD* expression, improves cardio-respiratory fitness and decreases circulating lipids levels [13]. In parallel with decreased liver fat accumulation and inflammatory markers, enhanced glucose uptake associated with physical exercise is also observed [11,13]. Moreover, the type and duration of exercise determines muscle mass or hypertrophy [28,29].

In our study, we found significantly increased expression of *PPARD* after a training cycle of 2 months. Our results are consistent with other authors' results of research on animals models [30-32]. Mice undergoing endurance exercise showed an accumulation of PPAR β/δ protein in muscle [15]. Further, muscle-specific over expression of *PPARD* in mice enhanced muscle metabolism (fatty acid in flux and β -oxidation) and remodeled muscle fiber type to increase oxidative type 2a but not type 1 fibers. Those mice also showed decreased body fat mass and thus, had smaller fat cells. Interestingly, *PPARD* transgenic mice additionally displayed increased glucose metabolism. Together, these results obtained by Luquet *et al.* [15] in 2003, implicated *PPARD* in muscle development and adaptive response to exercise training. Another model, a mouse engineered to express a constitutively activated form of PPAR β/δ (VP16-PPAR β/δ) in skeletal muscle, showed that a *PPARD*-mediated transcriptional pathway can regulate muscle fiber specification, enabling the generation of a strain of mice with a "long-distance running" phenotype [16,33].

As we mentioned above, our study has provided information about training-induced changes in the expression level of the *PPARD* gene in peripheral blood. It is worth emphasizing that our results are compatible with those obtained for mRNA expression level analyses performed in human skeletal muscle samples [19,34].

Regular physical activity induces desirable changes in plasma levels of HDL and LDL, respectively, and TGs. Physical exercise helps in maintaining lipid homeostasis, enhances glucose uptake and expenditure and also leads to changes in fiber type composition from glycolytic (type II b/x) to slow/fast oxidative (types I and IIa) fibers [35]. Positive effects of exercise are also seen on blood TGs, but little specific effect is seen on LDL and total cholesterol (TC). Abundant evidence supports the benefits of exercise on levels of certain blood lipids (namely HDL-C and TG) [36]. During the 8-week long training cycle we also observed changes in lipid profile, confirming the results of other researchers [37,38]. However, we did not find any statistically significant differences before and after the training cycle.

We have also documented that 8-week long training cycles lead to changes in the absolute FAT (kg) level (decreased). The studies of other authors also showed the decrease in absolute FAT (kg) under the influence of physical activity [39,40].

Many researchers showed that PPAR β/δ has been associated with the development of obesity. In mechanism of obesity development, PPAR β/δ activation leads to loss of adipose mass in different mouse models of obe-

sity (stimulating fatty acid oxidation) [33]. Moreover, the same effects on fatty acid oxidation have been observed in heart muscle (improved muscle contraction) [41]. Research suggested that high-fat-diet-induced adiposity was strongly inhibited by activation of PPAR β/δ in adipose tissue. Moreover, *in vitro* model activation of PPAR β/δ in adipocytes and skeletal muscle cells promotes fatty acid oxidation and utilization. The study showed that PPAR β/δ served as a widespread regulator of fat burning and identified PPAR β/δ as a potential target in treatment of obesity [33]. It is very possible that the expression of this gene may affect the body composition analysis.

Our research may provide a starting point for further investigating interactions between *PPARD* gene expression level and the lipid profile parameters. However, our examination has some limitations caused partly by a small study group and biological material we had at our disposal. Due to the fact that muscle biopsy is an invasive procedure (especially in young people), we did not have the opportunity to directly measure mRNA expression level in skeletal muscles. Additionally, there is only limited knowledge about circadian clock gene regulation by *PPARD* and about its role in epigenetically modified regulation of skeletal muscle metabolism and function. Ultimately, much work remains to be done before any clinical PPAR β/δ -based interventions will be possible in analysis of body composition in athletes.

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The experiment complied with the current laws of the country in which it was performed (Poland).

Declaration of Interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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