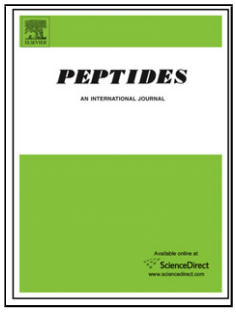

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Title: Adropin and apelin fluctuations throughout a season in professional soccer players: Are they related with performance?

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Abstract

Myokines are likely to be involved in the whole-body metabolic adaptive changes that occur in response to regular exercise. We aimed to investigate the association of the two myokines (adropin and apelin) with physical performance in professional soccer players. To this purpose, we analyzed the fluctuations of circulating levels of both adropin and apelin in professional soccer players during a season and evaluated the possible association of these myokines with the performance level. *Creatine kinase* (CK) and *lactate dehydrogenase* (LDH) activity as well as iron, transferrin and high-sensitivity C-Reactive protein (hsCRP), ferritin, soluble transferrin receptor (sTfR), free testosterone/cortisol ratio (FTCR), total iron binding capacity (TIBC) were also determined. Fifteen male professional soccer players from an Italian Serie A team were included in this study. Regarding the results of the biochemical analyses, the patterns of changes in the biomarkers of fatigue and inflammation, i.e., HsCRP, CK and LDH reflected the effects of the training throughout the season. No significant changes were observed in adropin, while apelin exhibited variations that seem not to be related with performance. In addition, both adropin and apelin did not represent valuable strategy to assist in the performance assessment of professional soccer players.

Keywords: skeletal muscle; performance; cytokines.

Introduction

Myokines are cytokines produced by skeletal muscles, especially induced by exercise, modulating different metabolic processes [6]. By influencing metabolism locally in the muscles, myokines are thought to be involved in the whole-body metabolic adaptive changes that occur in response to regular exercise like, for example, attenuation of fat accumulation [2]. Skeletal muscle and pancreas act in a synergistic manner to monitor systemic glucose homeostasis, and it has been suggested that myokines mediate the cross-talk between insulin-sensitive tissues [17]. Striated skeletal muscle is one of the body's largest tissues. However, it is unclear how contracting skeletal muscles transmit metabolic positive effects on health. One of the possible explanations for the health benefit of exercise can be that regular muscle contractions produce important messengers such as myokines [5]. Released circulating myokines may explain how normal muscle activity influences mood, physical performance and cognitive function [14].

It has been shown that exercise up-regulates the expression of the newly described myokine apelin in patients with type 2 diabetes [11]. In addition, apelin expression is induced by exercise and secreted in vitro in human primary myotubes, and may behave as a novel exercise-regulated myokine with autocrine/paracrine action [4]. Apelin is also up-regulated by insulin, contributing thus to glucose homeostasis [19]. Finally, apelin is highly implicated in cardiovascular function [10].

Adropin is also a recently described myokine involved in the regulation of lipid metabolism. It was first isolated in 2008 by Kumar et al. in liver and brain tissues [12]. In mice, adropin regulates physical activity (locomotion and coordination) via the *NB-3/Notch* signaling pathway [20]. Elevated circulating levels of adropin reduce insulin

resistance and glucose intolerance that arise in response to metabolic stress [7]. In this case, there is no clear evidence about whether exercise can regulate circulating levels of this myokine.

Therefore, because myokines are clearly involved in exercise-associated metabolic and cardiac changes, and hence could be potentially implicated in performance improvements throughout a soccer season, we aimed to analyze the fluctuations of circulating apelin and adropin levels in professional soccer players during a season. In addition, we also evaluated the possible association of both myokines with the performance level.

Material and methods

Subjects

Fifteen male professional soccer players from an Italian Serie A team (age (mean±SD) 27±5 years, weight 76.9±4.1 kg, height 1.82±0.05 m, body fat 8.7±2.4 %) were included in this study. Goalkeepers were not considered in this study since their physical load during soccer games is different from the other field players and as such their training programs are also different. All participants were informed of the purpose, protocol, and procedures of the study before agreeing to participate. The study complies with the World Medical Association Declaration of Helsinki regarding ethical conduct of research involving human subjects and/or animals and was approved by the ethics committee of University of Valencia, and by the soccer clubs involved.

Experimental Protocol

The players were sampled 3 times during the last part of a competitive season (in January, in March, in May). The competitive season finished at the end of May.

Thereafter, all players took a vacation and returned to the team discipline at the beginning of July, when the players included in the study were sampled again (just before preseason training beginning). At all sampling points, both mokines were assessed along with the physical performance determinations. An extensive biochemical and hematological profile study was also performed at all time-points but at May.

Physical performance determinations

At all time-points, the players were subjected to three physical performance tests, a continuous running test (Mognoni's Test) [8], a high-intensity intermittent test (HIT) [18] and a counter-movement jump (CMJ) test.

During the Mognoni's Test, blood lactate (La^-) concentration was determined immediately after a single 6-min run at 13.5 $km \cdot h^{-1}$ while the mean heart rate (HR) of the last minute of running was considered for the analysis. After 10-min of passive recovery, subjects completed, following an acoustic signal, a HIT protocol (total duration = 5 min) consisting of 10 x 10 s shuttle running at 18 $km \cdot h^{-1}$ 'over a 25-m course with an 180° direction change from run to run and 20s of passive recovery between runs. Immediately after the HIT protocol, blood La^- concentration was determined and the mean HR of 5-min run was considered for the analysis.

During both tests, blood La^- accumulation was measured using a portable amperometric microvolume lactate analyzer (Lactate Plus, Nova Biomedical, Waltham, MA, USA). Capillary blood samples (0.7 µl) were collected from the earlobe. Before the tests, the analyzer was calibrated following the instructions of the manufacturer. HR data was collected using Polar Team^2 Pro system (Kempele, Finland).

125 The CMJ test was performed using a portable force platform (Quattro Jump, Kistler,
126 Switzerland). In short, after a standardized warm-up, the subjects performed 6 single
127 CMJ and jump height and peak power output (PPO), averaged from the 3 best jumps
128 were recorded.

129 In addition, the body fat percentage was estimated at all time points by the skin-fold
130 technique, based on the Jackson and Pollock formula [9].

131 *Blood sampling*

132 Blood collection and sample management before analysis was carried out strictly
133 following the good laboratory practice for pre-analytical phase of sports biochemistry
134 and hematology tests [3]. The samples were drawn by venipuncture in the antecubital
135 vein in fasting conditions. For hematological determinations, blood was collected in
136 K_3EDTA vacuum tubes (Vacutainer, BD, Franklin Lakes, NJ, USA) and for
137 biochemical tests, in vacuum plain tubes without additives (Vacutainer). The former
138 were allowed to coagulate, then centrifuged at 3000 g for 10 min at room temperature
139 and after centrifugation, serum was separated into aliquots. Whole blood tubes were
140 immediately kept refrigerated at 4° C and assayed within 24 hours. Serum aliquots were
141 frozen at -80 °C until the assay.

142 *Laboratory Methods*

143 Adropin and apelin were determined in serum samples using available commercial
144 competitive enzyme-linked immunosorbent assay kits (CSB-EL007669HU, Cusabio,
145 Wuhan, China and EIA-APC, RayBiotech, Norcross, GA, USA; respectively). Both
146 assays were performed in duplicate following manufacturer's instructions. Intra-assay
147 coefficients of variation were 13.92% for adropin and 4.52% for apelin determinations.

Full blood cell count was carried out on the automated analyzer XE-2100L (Sysmex, Kobe, Japan). *Creatine kinase* (CK) and *lactate dehydrogenase* (LDH) activity as well as iron, transferrin and high-sensitivity C-Reactive protein (hsCRP) concentration were determined on the automated clinical chemistry platform ADVIA 1800 (Siemens Healthcare Diagnostics, Erlangen, Germany), employing proprietary reagents. Cortisol and testosterone were immunoassayed on the automated analyzer Elecsys 1010 (Roche Diagnostics, Mannhein, Germany) using the dedicated electro-chemiluminescence immunoassay kits. Ferritin was assayed on automated immunoassay system (ADVIA Centaur) and soluble transferrin receptor (sTfR) on a fully automated immunonephelometer (BN ProSpec), both provided by Siemens Healthcare Diagnostics and by using proprietary immunoassays. For calculation of the testosterone/cortisol ratio (FTCR), free testosterone was assumed as 2% of the total testosterone, and the formula previously validated was adopted [1]. Also the total iron binding capacity (TIBC) was estimated by applying the formula [TIBC (μg/dL) = transferrin (g/L) x 140] derived from the stoichiometric relationship between divalent transferrin and iron.

Statistical analysis

All data were analyzed for normality by Shapiro-Wilk test. Since the majority of the variables were not normally distributed, non-parametric tests were adopted. The effect of training and detraining (sampling time: January, March, May and July) on the parameters tested was analyzed with the Friedman's test (χ^2) and paired comparisons were performed with the Wilcoxon's test (z). The Spearman's coefficient (ρ) was used to explore the correlation between adropin and apelin levels as well as with the other parameters determined. The statistical analyses were performed using SPSS, version 21 (IBM Corporation, Armonk, NY, USA). The results were considered statistically significant at $p \leq 0.05$. Data were expressed as median (10^{th}-90^{th} percentile).

Results

The aerobic endurance, assessed by post-exercise La^- levels in Mognoni's and HIT tests changed at the end part of the competitive season and by the detraining period [Mognoni $\chi^2(3)=19.53$, $p<0.001$; HIT $\chi^2(3)=15.53$, $p=0.001$; see **Figure 1**]. In the Mognoni's test, La^- levels in January [3.50(2.55-6.82) mM] were higher than in March [2.85(2.02-5.33) mM, z=-2.552, p=0.011] and in May [3.02(2.08-4.66) mM, z=-2.601, p=0.009]. In the same line, La^- levels in the HIT test in March [2.47(1.37-6.21) mM] were lower than in January [4.40(1.65-8.35) mM, z=-2.045, p=0.041] and May [3.65(1.52-6.07) mM, z=-2.090, p=0.002]. This data would indicate a progressive adaptation with training. However, the July La^- values were higher in both tests (see **Figure 1**) which would reflect the detraining occurring during the vacation period between the end of the season and the beginning of the next pre-season. Accordingly, the cardiovascular implication was higher after the detraining period in both Mognoni's [$\chi^2(3)=13.00$, $p=0.005$] and HIT [$\chi^2(3)=11.44$, $p=0.010$] tests (see **Figure 1**). Nevertheless, we failed to find any significant effect of training and detraining on either jump height [$\chi^2(3)=5.23$, $p=0.156$] or PPO [$\chi^2(3)=1.46$, $p=0.692$] in the CMJ test.

Figure 2 shows a significant increase in the apelin concentration from 341.8(283.0-444.8) ng/mL in January to 433.3(373.4-677.5) ng/mL in March. Nonetheless, no statistically significant changes were found in either May or July compared to the previous time points. No significant changes were observed in adropin at any time.

A full blood cell panel along with several biochemical parameters was performed in January, March and July sampling times, but it could not be performed in May. These data are displayed on **Table 1**. The detraining period induced some alterations in erythrocyte indices. The mean corpuscular volume decreased while mean corpuscular

hemoglobin increased in July in comparison with the previous sampling points (**Table 1**). The red blood distribution width was slighter lower in July compared with January levels (z=-2.284, p=0.022, **Table 1**). HsCRP, an inflammatory marker, was found to be lower in July compared to January (z=-2.528, p=0.011). After detraining, levels of both biomarkers of muscle damage CK and LDH, were significantly lower compared with the previous time points (see **Table 1**).

Players' weight [January 77.0 (69.8-82.3) kg, March 77.0(69.0-83.5) kg, May 76.5(68.0-84.8) kg and July 77.6(70.1-86.9) kg)] and percentage of body fat [January 8.4(5.8-13.1) %, March 8.1(6.5-12.0) %, May 8.2(5.5-12.0) %, July (9.2(7.0-12-4) %] did not significantly change at the end of the competitive season or after the detraining period [$\chi^2(3)=6.126$, p=0.106; $\chi^2(3)=4.576$, p=0.206; respectively].

Finally, no correlation was found between adropin or apelin concentrations and performance parameters at all time points measured (**Supplementary Table 1**). On the other hand, significant correlations were found between adropin and apelin levels and other hematological and biochemical parameters measured (**Supplementary Table 2**), although those correlations did not provide additional insights.

Discussion

No significant changes were observed in adropin levels, while apelin exhibited variations that seem not to be related with performance. On the other hand, the patterns of changes in the biomarkers of fatigue and inflammation, i.e., HsCRP, CK and LDH reflected the effects of the training throughout the season.

While apelin levels showed a significant increase only in the first time point, possibly linked to an increased effort, fluctuations in adropin levels did not reach statistical

220 significance. In both cases, however, the distribution widths within the study cohort
221 were large: this was particularly true in the case of adropin but was also evident for
222 apelin at the end of the season and after the rest period. It thus seems that the use of
223 these two markers is not useful to assist in the performance assessment of professional
224 soccer players. It should be also mentioned that, while other myokines seem to be
225 relatively stable within person [13-16] thereby allowing longitudinal analyses, there is
226 paucity of such data on apelin and adropin.

227 The main limitation of our study is the low number of subjects included on each
228 experimental group that can decrease the power of the statistical analyses performed.
229 Since season openings in Europe are regularly at mid/late August, we did not have a
230 first season's start sample to compare. In addition, the specific design of this study with
231 only professional athletes recruited limits the generalizability of its results in less
232 trained individuals. Moreover, due to a problem of samples conservation, the
233 hematological and biochemical panel could not be determined at May. Nevertheless,
234 this study needs to be evaluated in both larger and different cohorts before they can be
235 translated into clinical practice.

236 In conclusion, a one-season follow-up of professional soccer player's training did not
237 lead to observe changes in circulating apelin and adropin levels. Accordingly, neither
238 apelin nor adropin were associated with changes in performance level.

Competing financial interests

The authors declare no competing financial interests.

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299

Table and Figures Legends

Table 1. Players' hematological and biochemical parameters in January, March, and July (i.e., at the beginning of the next pre-season).

Figure 1. Blood lactate (La^-) and heart rate (HR) in the Mognoni and HIT protocols, and height jump and peak power output in the counter-movement jump test (CMJ) during the competitive season (January, March and May) and in the next pre-season stage (July). During the Mognoni's Test, La^- concentration was determined immediately after the test, while the mean HR of the test's last minute of running was considered. During the HIT protocol La- concentration was determined just after the test and the mean HR of 5-min run was considered. CMJ height and peak power output was averaged from the 3 best of 6 jump repetitions. Data represented as median (horizontal line), 1^{st} to 3^{rd} quartile (box) and 10^{th} to 90^{th} percentile (whiskers). Significant comparisons are indicated.

Figure 2. Apelin and adropin concentrations during the competitive season (January, March and May) and in the next pre-season stage (July). Data represented as median (horizontal line), 1^{st} to 3^{rd} quartile (box) and 10^{th} to 90^{th} percentile (whiskers). Significant comparisons are indicated.

Table 1.

	In-season January	In-season March	Pre-season July	Friedman's p-value
RBC ($x10^6/\mu L$)	5.03(4.60-5.73)	5.12(4.78-5.45)	5.11(4.72-5.87)	0.247
Hemoglobin (g/dL)	15.1(13.8-16.0)	15.3(14.3-16.2)	15.1(13.7-16.7)	0.302
Hematocrit (%)	44.0(41.0-47.3)	44.5(42.0-46.8)	44.9(40.1-47.7)	0.591
MCV (fL)	87.2(81.9-89.6)***	85.9(83.0-91.2)***	84.7(80.9-88.4)	<0.001
MCH (pg)	30.0(27.9-31.2)*	30.1(27.9-31.1)**,#	29.5(27.8-30.9)	<0.001
MCHC (g/dL)	34.3(33.1-35.5)	34.5(33.2-35.9)	34.5(33.2-36.6)	0.581
RDW (%)	13.0(12.5-13.5)*	12.9(12.4-13.5)	12.8(12.3-13.6)	0.011
Reticulocytes (%)	0.67(0.51-1.02)**	0.66(0.43-0.88)**	0.82(0.57-1.33)	0.005
IRF (%)	3.2(1.44-5.98)	2.8(0.94-6.28)	3.2(1.26-5.78)	0.721
Fe (µg/dL)	82(60-117)	96(58-131)	84(67-145)	0.627
Ferritin (ng/mL)	125.7(69.1-246.7)	130.2(48.7-244.7)	113.4(51.6-217.6)	0.070
Transferrin (mg/dL)	249(213-273)	242(211-290)	259(207-297)	0.085
TIBC (µg/dL)	349(299-383)	339(296-405)	363(290-416)	0.085
Transferrin saturation (%)	25(18-32)	26(18-41)	26(18-39)	0.349
sTfR (mg/L)	1.30(0.84-1.63)	1.15(0.90-1.49)	1.19(0.83-1.62)	0.591
WBC ($x10^3/\mu L$)	4.97(4.01-6.52)	4.70(3.89-7.5)	5.01(4.25-7.76)	0.516
Lymphocytes ($x10^3/\mu L$)	2.2(1.4-3.3)	2.1(1.5-2.9)	2.3(1.6-3.0)	0.272
Neutrophils ($x10^3/\mu L$)	2.3(1.4-2.9)	2.4(1.4-4.1)	2.3(1.5-3.9)	0.179
Monocytes ($x10^3/\mu L$)	0.4(0.3-0.6)	0.4(0.3-0.6)	0.4(0.3-0.6)	0.971
Basophils ($x10^3/\mu L$)	0.02(0.01-0.03)	0.02(0.01-0.04)	0.02(0.01-0.03)	0.928
Eosinophils ($x10^3/\mu L$)	0.1(0.1-0.4)**	0.1(0.1-0.5)	0.2(0.1-0.5)	0.009
hsCRP (mg/L)	0.97(0.40-8.22)*	0.71(0.16-2.25)	0.30(0.13-2.49)	0.022
Creatine kinase (U/L)	392(240-2053)**	382(168-713)*	294(125-925)	<0.001
Lactate dehydrogenase (U/L)	205(73-271)*	190(148-231)*,#	158(127-217)	<0.001
Cortisol (ng/mL)	238.0(190.3-277.4)	214.9(183.9-271.7)	214.1(135.2-260.1)	0.165
Testosterone (ng/mL)	7.21(4.15-9.57)	7.61(5.39-9.34)	7.16(5.39-9.95)	0.766
FTCR	0.74(0.47-0.95)	0.82(0.58-0.99)	0.91(0.61-1.57)	0.241

RBC: red blood cell. MCV: mean corpuscular volume. MCH: mean corpuscular hemoglobin. MCHC: mean corpuscular hemoglobin concentration. RDW: red blood cell distribution width. IRF: non-mature reticulocyte fraction. sTfR: soluble transferrin receptor. TIBC: total iron-binding capacity. WBC: white blood cell. hsCRP: high-sensitivity C-Reactive protein. FTCR: free-testosterone to cortisol ratio. Data as median (10^{th}-90^{th} percentile).
*p<0.05, **p<0.01 and ***p<0.001 *vs* July. #p<0.05, ##p<0.01 and ###p<0.01 *vs* January.

Highlights

- Myokines are involved in metabolic adaptive changes induced by regular exercise.
- We investigated the association of two myokines (adropin and apelin) with physical performance.
- No significant changes were observed in adropin.
- Apelin exhibited variations that seem not to be related with performance.
- Apelin and adropin levels are not related to performance in professional soccer players.