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Genetic and protein biomarkers in blood for the improved detection of GH abuse

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Graphical abstract

Increase of *FN1* and *RAB31* expression in PBL and FN1 concentrations in serum samples after treatment with recombinant Growth Hormone

Highlights

- Expression of *FN1* and *RAB31* genes in lymphocytes appear as sensitive markers of rhGH administration.
- FN1 protein in serum also appears as a sensitive marker already 24h after the first rhGH dose.
- FN1 gene and protein levels in serum are not influenced by gender, age, sport and other doping substances.
- Serum and/or plasma can be used for FN1 concentrations measurements.

Abstract

Human Growth Hormone (hGH, somatotropin) is one of the relevant forbidden substances to be detected in sport drug testing. Since the appearance of recombinant hGH (rhGH) in the 80's, its expansion and availability through the black market has increased, so the detection of its abuse continues to be a challenge at present. New techniques or biomarkers that are robust, reliable, sensitive and allowing a large detection time window are welcome. rhGH produces an increase of insulin-like growth factor 1 (IGF-1). *FN1* (fibronectin 1) and *RAB31* (member of RAS oncogene family) genes have been suggested as two potential biomarkers for IGF-1 abuse. Following this line, in the present study some genetic and proteomic approaches have been performed with fourteen healthy male subjects treated with rhGH (which produces increase of IGF-1 concentrations) to study *FN1 gene*, FN1 protein, *RAB31 gene* and RAB31 protein as potential biomarkers for rhGH abuse. The results showed that both, *RAB31* and *FN1* genes and FN1 protein could be potential biomarkers for rhGH administration. Preliminary assessments of gender, age, acute sport activities and GHRP-2 (pralmorelin, a rhGH releasing peptide) influence suggest they are not relevant confounding factors. Thus, the selected markers present high sensitivity and a larger detection window for rhGH detection than IGF-1 itself.

Key words: Growth hormone, FN1, Fibronectin, RAB31, doping substances, doping abuse

1. Introduction

Lipolytic and anabolic activities of human Growth Hormone (hGH) render GH an ergogenic and performance-enhancing substance approached by some professional athletes and adolescents participating in sports [1, 2]. Since the appearance in 1981 of rhGH for the treatment of children and adults with growth disorders or GH-deficient diseases, its expansion and availability through the black market has increased [3] and currently is classified as a prohibited substance on the World Anti-Doping Agency (WADA) Prohibited list [4].

Currently, there are two different strategies to detect rhGH abuse in sport: a) direct strategy which involves methods based on the relationship between variants of hGH, and b) indirect strategy, based on variations in surrogate parameters resulting from rhGH administration, also known as the biomarkers method. The direct strategy is based on the ratios of various isoforms or hGH variants, which remain rather constant under physiological conditions, but are altered after the exogenous administration of rhGH [5-9]. This fact significantly alters the physiological or natural isoforms ratios [5, 8, 10] which can be detected by specific immunoassays but only for a short period of time. Indirect strategy allows a larger detection time window to suspect the rhGH abuse [11]. The approach, studied and developed by several research groups [12-18], measures the two markers IGF-1 and the N-terminal pro-peptide of type III collagen (P-III-NP), which were selected among several biomarkers studied, and has longer retrospectivity

than the direct strategy [17]. However, the indirect approach is not without limitations. Among them we can mention its dependence on the gender and age of the athletes [12, 18-21], the fact that only serum (and not plasma) can be used [22] and also the more rapid elimination of IGF-1 [18] as compared with P-III-NP, which reduces the retrospectivity of the unified algorithm for both markers used in the approach. Therefore, the search for new biomarkers or the development of new techniques which are reliable, robust and sensitive, and allowing a large detection window, is still appropriate.

Studies to find genetic biomarkers to detect the use of performance-enhancing drugs have been performed by some authors. These approaches have supported the suggestion of new gene related biomarkers with promising sensitivity in peripheral blood lymphocytes (PBL) either after *in vitro* PBL incubation with IGF-1 [23] and dihydrotestosterone (DHT) [23], or after administration of erythropoietin (EPO) [24] or autologous blood transfusion [25]. Specifically, based on the results obtained by Mancini et al [23], *FN1* and *RAB31* genes were suggested as potential biomarker of IGF-1 abuse. *FN1* is a gene which presents several alternative splicing and encodes a glycoprotein of 220 kDa (FN1) that can be found in serum and plasma as a dimer. Due to the presence of multifunctional domains in its structure, FN1 interacts with diverse components of the coagulation and fibrinolysis [26]. This protein contains binding sites for collagen, heparin and fibrin and is a specific ligand for several integrin adhesion cell receptors regulating processes of importance in haemostasis as platelet adhesion and aggregation, tissue remodelling during wound healing, and activation of fibrinolysis by the plasminogen activators [26, 27]. Largely responsible for the presence of FN1 in plasma is its synthesis by hepatocytes although there is a smaller contribution by macrophages, lymphocytes, platelets and endothelial cells [28, 29]. Altered levels of

FN1 gene and protein have been associated with some pathologies [30-37], although with expected low prevalence in elite athletes [23]. They include mammary and renal cancer, gestational pathologies as preeclampsia, myocardial infarction or Duchenne muscular dystrophy, among others; and in the treatment with carbon ion irradiation and X-irradiation treatment in the prostate and colon cancer [30-37]. However, no environmental or physiological factors have been described as agents which cause changes in the normal FN1 concentrations. On the other hand, *RAB31* is a gene which encodes a small GTP-binding protein of 22 kDa (RAB31) that plays a crucial role in vesicle and granule targeting, and displays low serum concentrations. This protein is required for the integrity and normal function of the Golgi apparatus and the trans-Golgi network [38, 39] and it has been associated with the development and progression of different cancers [40, 41].

Because of the treatment with rhGH causes an increase of IGF-1 concentrations [12, 18], it would be expected that *FN1* and *RAB31* also show an increase in their mRNA and/or corresponding protein levels and could be used as potential biomarkers of rhGH abuse. In this context, a clinical trial with fourteen healthy men volunteers treated with a low dose (0.026 mg⁻¹ kg⁻¹ day⁻¹ person⁻¹ s.c.) of rhGH for three consecutive days was carried out and blood samples (serum, plasma and PBL) were collected for ten days after the first dose. In these samples *IGF-1*, *FN1* and *RAB31* genes and corresponding protein levels were evaluated by quantitative real-time PCR (RTqPCR) and proteomic (immunoassays) approaches respectively. Also to assess the potential influence of gender, age, acute sport activities (cycling, tennis, swimming, triathlon) and other doping substances related to hGH such as GHRP-2 (pralmorelin, a rhGH releasing peptide), preliminary investigations were carried out on the expression of *FN1* gene and the concentrations of protein, suggesting no influence of these potential confounding

factors. Overall, two potential genetic biomarkers and one potential protein biomarker with high robustness, sensitivity and wide detection time window are proposed for the detection of rhGH abuse in cheating athletes.

2. Methods and materials

2.1 GH and GHRP-2 treatment in human subjects: study design

Two clinical trials were performed in the clinical trials unit at the Hospital del Mar for Medical Research Institute (IMIM, Barcelona), one with rhGH and other with GHRP-2. For rhGH, the trial was carried-out with fourteen caucasian healthy men volunteers in a randomised and controlled fashion. Recombinant 22 kDa hGH (Genotonorm®, Pfizer, New York, USA) was administered subcutaneously to 10 volunteers, while 4 other volunteers were used as controls without drug treatment (blank subjects). The volunteers were recruited applying a number of selection criteria that included a history of normal growth and development, normal analytical values as well as medical history and physical examination performed prior to enrolling in the trial. They were from 20 to 30 years old, mean weight 72.9 ± 8.7 and mean body mass index (BMI) of 22.5 ± 3.5 . The protocol was approved by the Ethical Committee of Clinical Research (CEIC) from Parc Salut Mar (n° 2013/5037)), and the volunteers were administered daily for 3 days with $0.026 \text{ mg}^{-1} \text{ kg}^{-1} \text{ day}^{-1} \text{ person}^{-1}$ rhGH s.c. after overnight fasting. Since the first administration of rhGH, blood (EDTA and serum gel separator tubes) samples were collected at different time points along 10 days (0h, 8h, 24h, 48h, 72h, 96h, 168h and 216h) and processed for obtaining serum, plasma and PBL.

For GHRP-2 (pralmorelin; a rhGH secretagogue), a clinical trial was carried with 5 caucasian healthy men volunteers in an obert and controlled fashion.

Pralmorelin-GHRP-2 KAKEN100® (KaKen Pharmaceuticals Co., Ltd., Tokyo, Japan) was administered intravenously as a bolus to 3 volunteers, 2 other volunteers were used as controls without drug treatment (blank subjects). The volunteers were recruited applying a number of selection criteria as described above for the rhGH trial. The protocol was approved by the Ethical Committee of Clinical Research (CEIC) from Parc Salut Mar (n° 2014/5760) and the volunteers were administered intravenously with a single dose of 100 µg GHRP-2 after overnight fasting. Since the first administration of GHRP-2, blood samples were collected at different time points along 10 days (0h, 8h, 24h, 48h, 72h, 96h, 168h and 216h) and processed for obtaining serum.

2.2 Serum, plasma and PBL

Blood samples were withdrawn from the antecubital vein. Serum was obtained from blood after clotting and it was stored at -20°C. Plasma and PBL were obtained from blood tubes containing tri-potassium ethylenediaminetetraacetic acid (EDTA-K₃). After centrifuging the samples for 10 min at 250 g, plasma was aliquoted and stored at -20 °C until use and PBL were purified, immediately after blood collection, with Histopaque-1077 (Sigma-Aldrich, Spain) following the manufacturer's instructions. The final pellet of PBL was stored at -80 °C until RNA extraction.

2.3 RNA isolation, cDNA synthesis and duplex qRTPCR

Total RNA was extracted from samples of PBL using TRIzol reagent (Invitrogen, Spain) following the manufacturer's instructions. An aliquot containing 0.5 µg of total RNA was reverse transcribed using M-MLV reverse transcriptase (Invitrogen, Spain), 5 µM random hexamers and 2 mM dideoxynucleotides (Roche, Spain) in a total reaction volume of 20 µl.

Levels of human genes *IGF-1*, *FN1* and *RAB31* relative to *TBP* (tata box binding protein gene, house keeping gene) were determined by multiplex (duplex) qRT-PCR experiments using Taqman type probes with HEX and FAM as fluorochromes, BHQ1 as quencher and ROX for background normalization. Probes and oligonucleotide primers were designed (Primer Express 2.0.0 (ABI PRISM, Applied Biosystems) and synthesized by Sigma (Spain), being the selected sequences: a) for *TBP*, forward primer 5'-ccgaaacgccgaatataatcc-3', reverse primer 5'-ccgtggttcgtggtctct-3' and probe 5'-[FAM]cggtttgctcgcggaatcatgaggat[BHQ1]-3'; b) for *IGF-1*, forward primer 5'-gctgcttttgattcttgaaggt-3', reverse primer 5'-tggtgaaggtgagcaggca-3' and probe 5'-[HEX]tgtcctctcgcctctcttctacctggc[BHQ1]-3'; c) for *FN1* forward primer 5'-ggaaggaaaaagacagacgagc-3', reverse primer 5'-cgaaaggggtctttgaactgt-3' and probe 5'-[HEX]gtaaccctccacacccaatcttcat[BHQ1]-3'; and d) for *RAB31* forward primer 5'-aggatcactttgaccacaacatca -3', reverse primer 5'-ggagccaatgaatgaaaccg-3' and probe 5'-[HEX]aagttctcatctgggacactgctggta[BHQ1]-3'. Triplicates of duplex qRT-PCR mixtures contained 5 µl of Taqman Gene Expression Master Mix (Applied Biosystems, Spain), 0.2 µM of each primer, 0.2 µM of *IGF-1*, *FN1* or *RAB31* probe, 0.25 µM of *TBP* probe, 1 µl containing cDNA resulting of retrotranscription from 25 ng of total RNA and RNase-free water to a final volume of 10 µl. The amplification program to quantify each gene was carried out in a PCR LightCycler 480 II thermocycler (Roche), as follows: 1 cycle at 50 °C for 10 min; 60 cycles with steps at 95 °C for 15 s and at 60 °C for 1 min with simultaneous detection of FAM, HEX, and ROX. Gene expression was normalized to *TBP* using the mathematic model $2^{-\Delta\Delta CT}$ described by Livak y Schmittgen [42], where $\Delta\Delta CT = (C_{T,Target} - C_{T,TBP})_{TIME X} - (C_{T,Target} - C_{T,TBP})_{Time 0}$. The RTqPCR assay data are reported as mean $\pm$ standard deviation (SD) and were analyzed with the student's t-test. Differences were considered significant at a p-value less than 0.05.

2.4 FN1, RAB31 and PIIINP ELISA

FN1, RAB31 and PIIINP proteins serum concentrations were measured with enzymatic methods using ELISA kits.

FN1 was measured by the recently established two antibodies sandwich fibronectin ELISA kit (kit 1: Abcam, Cambridge, MA, USA). Alternatively (figure 6), fibronectin human ELISA kit (kit 2: Millipore, Billerica, Massachusetts, USA) was also used to compare with basal fibronectin concentrations previously reported with older single anti-fibronectin antibody assays. RAB31 concentrations were measured by ELISA kit (Mybiosource, San Diego, California, USA) and PIIINP concentrations by ELISA kit with paramagnetic particles purchased from Siemens and using an ADVIA Centaur CP system (Siemens Healthcare Laboratory Diagnostics, Camberley, UK). In all cases, samples were processed following the manufacturer's instructions. The results are reported as mean $\pm$ SD. Differences (student's t-test) were considered significant at a p-value less than 0.05.

2.5 IGF-1 Radioimmunoassay

IGF-1 serum concentrations were measured with an enzymatic method using commercial radioimmunoassay kits based on a sandwich ELISA (IRMA) purchased from Beckman Coulter (California, USA) and using a 1470 Wizard gamma scintillation counter (PerkinElmer, Waltham, Massachusetts, USA). Samples were processed following the manufacturer's instructions and the assay data are reported as mean $\pm$ SD. Differences (student's t-test) were considered significant at a p-value less than 0.05.

2.6 Subjects for the preliminary study of gender, age and exercise

To assess the potential influence of gender and age, plasma and PBL samples from 13 sedentary healthy women and 14 sedentary healthy men aged between 21 and 30 years were collected and stored at -20 °C until analysis. On the other hand, 62 elite athlete samples from acute sport activities were enrolled in the study: 16 cyclists, 32 tennis players, 8 swimmers and 6 triathlon athletes to assess the potential influence of acute sport exercise. Serums for these athletes obtained after competition and having been authorized by each subject for their use for research, were shared by the Barcelona Antidoping Laboratory, belonging to the same institution IMIM-Hospital del Mar Medical Research Institute (Barcelona, Spain).

3. Results

Based on the results obtained by Mancini et al [23] in which *FNI* and *RAB31* in PBL were suggested as long effective potential genetic biomarkers of IGF-1 exposure and because of rhGH abuse produces an increase of IGF-1 concentrations, genetic and protein approaches have been applied to a clinical trial in which 10 male healthy subjects were treated for three days with a low dose of rhGH. After obtaining peripheral blood (PBL, serum and plasma) samples, mRNA and protein levels were measured with techniques based on qRT-PCR and radioactivity or colorimetric immunoassays, respectively.

3.1 Quantitative expression of *IGF-1*, *FNI* and *RAB31* genes in PBL after rhGH treatment

From previous results [23], primer and taqman probes were designed for *IGF-1*, *FNI* and *RAB31* detection by RT-qPCR assays. Then relative mRNA levels expression of *IGF-1*, *FNI* and *RAB31* were evaluated in subjects after treatment of rhGH and

compared with untreated control subjects. As displayed in figure 1, changes in relative mRNA levels of *FN1* and *RAB31* but no differences in mRNA levels of *IGF-1* were observed in PBL. A significant increase of PBL mRNA level of *FN1* and *RAB31* were produced at 24 and 48 hours respectively after starting treatment. The maximum expression level of *FN1* was reached at 72h with 8.91 ± 3.71 fold increase, though high expression levels were found at all times studied. For *RAB31* the maximum level was reached at 48h with 3.82 ± 1.53 fold increase, though high significant expression levels were found until 168h. From this time *RAB31* mRNA levels were declining until being close to the basal state at 216h when compared with untreated control subjects.

3.2 IGF-1, PIIINP, FN1 and RAB31 proteins serum concentrations after rhGH treatment

To determine whether the potential identified genetic biomarkers showed alterations in their corresponding protein serum concentrations, ELISAs to quantify PIIINP, FN1 and RAB31, and radioimmunoassay to quantify IGF-1 were performed. Significant increase in serum concentrations for IGF-1, PIIINP and FN1 but not for RAB31, were found, when compared treated and untreated control subjects, as can be seen in figure 2. In treated subjects maximum IGF-1, PIIINP and FN1 serum concentrations were reached respectively at 72h with 441.7 ± 91.5 ng/ml, at 168h with 11.6 ± 4.11 ng/ml and at 72h with 229.5 ± 41.7 µg/ml, while the baseline concentrations in untreated control subjects were found around 200 ng/ml for IGF-1, 7 ng/ml for PIIINP and 105 µg/ml for FN1. The IGF-1 serum concentrations decayed quickly beyond 72h and reached the baseline concentrations at 168h, whilst PIIINP and FN1 showed higher concentrations at all times studied when compared with untreated control subjects. Statistically significant differences were high at all times for FN1 but low (and only at 48h, 72h, 96h and 216h) for PIIINP.

3.3 IGF-1 and FN1 serum concentrations after GHRP-2 treatment

To assess if subjects treated with another substance related to GH release could show changes in FN1 concentrations, serum samples from three caucasian subjects treated intravenously with 100 µg of GHRP-2 (pralmorelin dihydrochloride) were collected and compared with two untreated control subjects. IGF-1 and FN-1 concentrations were assessed by radioimmunoassay and ELISA respectively, and the results were compared between both groups. As displayed in figure 3, the results showed that the concentrations detected for both proteins, IGF-1 and FN1, were similar between treated and untreated control subjects, with no statistically significant differences found due to treatment with GHRP-2.

3.4 Biological matrix effect for FN1 measurements

Based on the FN1 ELISA kit instructions used in this study (kit 1) which indicate no differences in the FN1 concentrations in plasma or serum, samples for both biological matrices obtained from the rhGH clinical trial were compared in order to evaluate the potential matrix effect for FN1 protein measurement, due to the different composition of the plasma as compared with serum. Similar concentrations were found in both matrices and a positive Pearson correlation coefficient of 0.923 ($p \leq 0.001$) with a regression line $y = 0.85x + 10$ (x being the serum concentration and y the plasma concentration) was verified (interval 220-565 µg/ml).

3.5 Age and gender effect in plasma and PBL samples

To preliminary investigate the effect of age and gender, plasma and PBL samples were obtained from male and female untreated subjects.

In the PBL samples, as can be observed in figure 4, no differences were observed as mean values of 1 ± 0.08 (men, $n=12$) and 1.08 ± 0.5 (women, $n=13$) fold mRNA expression were obtained for gender effect, respectively; and homogeneous unified mean values of 1.01 ± 0.20 ($n=25$) fold mRNA expression were obtained for age effect (from 21 to 30 years, mean 25 years).

In the plasma samples, no differences were observed as mean values of 124.75 ± 17.07 (men, $n=12$) and 118.61 ± 29.19 (women, $n=13$) $\mu\text{g/ml}$ were obtained for gender effect, respectively; and unified mean values of 123.50 ± 20.09 ($n=25$) $\mu\text{g/ml}$ were obtained for age effects (from 21 to 30 years, mean 25 years), as displayed in figure 4.

3.6 Exercise effect in FN1 concentrations

To assess the exercise effects on FN1 serum concentrations, four different acute sport activities were evaluated (figure 5). No differences were observed as mean values of 110.3 ± 20.2 ($n=16$), 113.7 ± 12.5 ($n=32$), 112.7 ± 16.5 ($n=8$), 107.7 ± 13.6 ($n=6$) and 119.3 ± 21.9 $\mu\text{g/ml}$ ($n=28$) were obtained for cycling, tennis, swimming, triathlon and control groups, respectively.

3.7 Assessment of FN1 serum concentrations with single anti-FN1 antibody ELISA assay

To compare with basal concentrations reported in some previous studies for the quantification of FN1 in serum, a single anti fibronectin antibody ELISA commercial kit from Millipore was assessed. In line with studies using a similar analytical approach, higher FN1 serum concentrations were found both for treated and untreated control subjects as compared with the sandwich assay used in our study, as can be seen in figure 6. In treated subjects maximum FN1 serum concentrations were 487.37 ± 39.65 $\mu\text{g/ml}$,

while the baseline concentrations in untreated control subjects were found around 250 µg/ml. FN1 concentrations were highly statistically significant different at all times studied when both groups were compared..

4. Discussion

The search for biomarkers is a comprehensive strategy used by researchers to early indicate diagnostic/prognostic signs of a disease or other biological evolving process. The use of the approach for antidoping purposes can also be very useful to improve or complement methods which are currently used in the detection of abuse by cheating athletes. In particular from *in vitro* studies, some genetic biomarkers were suggested as potential tools to detect IGF-1 abuse, such as *FN1* and *RAB31* genes expression in PBL [23]. Also interestingly, previous results obtained by different researchers in the study of various diseases related to kidney [43], bone [44], hypertension [45] or embryological processes [46] performed on different cell types and tissues have shown, after treatment with IGF-1, an increase in the *FN1* expression levels for both, mRNA and protein.

As it is well known that rhGH administration produces an increase in the concentrations of IGF-1 [18], it could be speculated that *FN1* (and *RAB31*) could also be good biomarkers to detect rhGH abuse by athletes. This approach prompted our development of a clinical trial with volunteers treated with rhGH. In this trial, an increase in *FN1* mRNA levels in PBL after administration of rhGH was observed, which was in agreement with an increase of IGF-1 concentrations in serum already at 8 hours after starting the treatment. However, although IGF-1 protein levels were increased along the rhGH treatment returning to baseline after 168h, *IGF-I* mRNA expression in PBL did not differ when compared with untreated control subjects. This

fact is not so surprising as the increase in IGF-1 blood levels should be due to the action of rhGH in other tissues different from lymphocytes such as the liver, which is the largest producer of IGF-1 [47]. Therefore, either *IGF-1* mRNA levels in PBL are very low because of B lymphocytes do not secrete IGF-1 or the enhanced expression is confined to macrophages in the lymph nodes [48-50].

For *RAB31*, a member of RAS oncogene family, despite being related to different types of cancers [40, 41], no study on their expression in cells or tissues treated with IGF-1 has been performed. In our study, its mRNA levels in PBL, like those of *FNI*, are increased following treatment with rhGH. However, significant differences between treated and untreated control subjects were not shown until 48h after first administration of rhGH and disappeared at 168h, so it seems to be a less attractive genetic biomarker than *FNI*, which maintained high levels of expression even until 216h after the treatment. Regarding the RAB31 protein serum levels, they did not statistically differ between treated and untreated control subjects at any of the tested times, which is detrimental to its potential biomarker's capabilities.

By contrast, FN1 concentrations in serum samples showed a similar profile to that obtained for *FNI* mRNA expression with a slight increase already at 24h after start of rhGH administration maintained at least until 216h. Thus it seems that both, *FNI* genetic and FN1 protein levels, could be used as a potential biomarker of rhGH abuse. In fact, when this biomarker was compared with the current biomarkers approved by WADA a wider detection windows and more sensitivity was observed. For IGF-1, a rapid stimulation is produced after rhGH administration but these IGF-1 levels decreases quickly returning to baseline after 168h. For PIIINP, high basal variability and detectability with low statistical significance was found.

After ascertaining that FN1 could be such a potential biomarker, it was relevant to assess its robustness and specificity through some experimental studies. In this regards, samples from clinical trials with other doping substances different from rhGH but prompting the release of hGH (growth hormone secretagogues), and samples of different age, gender or acute sport activities were used to obtain preliminary data. In the case of hGH secretagogues, samples from a clinical trial with GHRP-2 were studied but no difference was observed between treated and untreated subjects in IGF-1 and FN1 serum concentrations. In spite of the effects of GHRPs in increasing hGH, the lack of increase of IGF-1 (and subsequently of FN1) were not surprising according to the previous data published by Okano et al., which showed that GHRP-2 treatment produces an increase of hGH but not of IGF-1 serum concentrations [51]. Therefore, it seems that the administration of hGH secretagogues will not confound FN1 biomarker results as compared with rhGH administration.

On the other hand, results obtained to test the influence of gender, age and acute sport activities suggested that none of these three variables produced differences when 13 women and 12 men between 21 and 30 years and 62 elite athletes practicing four different kinds of sports were evaluated, yielding values very close to the control group. Interestingly, some previous studies to assess the FN1 concentrations in plasma samples showed age influence in healthy subjects between 0 and 82 years [52, 53]. However, these studies were performed in age subgroups that included a subgroup between 15 and 39 years of age, which showed homogeneous FN1 concentrations, as observed in our experiences. Despite these promising results a wider investigation with more volunteers including female and a wide range of age which would strengthen the results is needed.

In the human blood circulation the most common concentration range reported using immunoassays for FN1 is approximately 250–300 µg/ml [54]. However, the basal

common concentrations established by the manufacturer of the recent Abcam commercial ELISA kit (kit 1) used in this study is around 100 $\mu\text{g/ml}$, which was corroborated by the analysis of samples from the control group in our study. The Abcam ELISA kit used is based on the recognition of the FN1 in solution by two antibodies (capture and detector antibody) and the specific epitope for any of both is not known (manufacturer's information). When an antibody recognizes a glomerular protein (the form of FN1 in solution), the epitope may be composed of amino acids from different parts of the polypeptide chain that have been brought together by protein folding. Antigenic determinants of this kind are known as conformational or discontinuous epitopes which are brought together in the three-dimensional structure [55]. Accordingly, it is known that different antibodies with different epitope recognition give different results for FN1 in serum [52]. Further analyses with another commercial kit (kit 2) which use a single antibody for the FN1 detection were carried out to compare with the FN1 reference ranges described in some previous studies.. After testing all treated and untreated control subjects with kit 2, FN1 basal level around 250 $\mu\text{g/ml}$ was observed, as expected, while concentrations in rhGH treated subjects went up close to twice this value, in agreement with the observation made with kit 1. For any future inter-laboratory comparison, a standardization of the method used for FN1 quantification must be taken into account. However, it is interesting to note the kit 1 is faster to develop and has a simpler protocol for easy implementation in any laboratory. Alternatively, approaches based on mass spectrometry for the quantification of such an abundant protein could be developed without much high difficulty.

5. Conclusion

FN1 and *RAB31* gene expression in PBL could be used as potential genetic biomarkers with high retrospectivity to support current detection methods used for rhGH abuse. FN1 protein also can be used as a biomarker, showing high concentrations in serum and plasma samples after treatment with rhGH, even after a low dosage. The detection time window is larger than for IGF-1 and has more sensitivity and lower variability than PIIINP, the biomarkers currently used by WADA for the detection of rhGH doping.

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Figure 1. Time-courses of mRNA level expression of *IGF-1*, *FN1* and *RAB31* after rhGH treatment in treated (■) and untreated control (♦) subjects. Mean values of every time-point are represented. Bars show standard deviations. Levels of statistical significance compared with control subjects at the same corresponding time: * $0.01 < p \leq 0.05$, ** $0.001 < p \leq 0.01$, and *** $p \leq 0.001$.

Figure 2. Time-courses of serum concentrations of FN1 (kit 1), RAB31, IGF-1 and PIIINP after rhGH treatment in treated (■) and untreated control (♦) subjects. Mean values of every time-point are represented. Bars show standard deviations. Levels of statistical significance compared with control subjects at the same corresponding time: * $0.01 < p \leq 0.05$, ** $0.001 < p \leq 0.01$, and *** $p \leq 0.001$.

Figure 3. Time-courses of serum concentrations of IGF-1 and FN1 (kit 1) after GHRP-2 treatment in treated (■) and untreated control (♦) subjects. Mean values of every time-point are represented. Bars show standard deviations.

Figure 4. Age and gender effect on *FN1* gene expression and FN1 protein concentrations (kit 1). **Left.** PBL and plasma samples versus age. **Right.** PBL and plasma samples versus gender. Box plot compares median of *FN1* gene expression and FN1 concentrations in women (n=13) and men (N=12). Box plot explanation: upper horizontal line of box, 75th percentile; lower horizontal line of box, 25th percentile; horizontal bar within box, median; upper horizontal bar outside box, 90th percentile; lower horizontal bar outside box, 10th percentile.

Figure 5. Exercise effect on FN1 serum concentrations (kit 1). Box plot compares median of FN1 concentrations in serum samples from each acute sport activities and the control sedentary group. Box plot explanation: upper horizontal line of box, 75th percentile; lower horizontal line of box, 25th percentile; horizontal bar within box, median; upper horizontal bar outside box, 90th percentile; lower horizontal bar outside box, 10th percentile.

Figure 6. Time-courses of serum concentrations of FN1 (kit 2) after rhGH treatment in treated (■) and untreated control (◆) subjects. Mean values of every time-point are represented. Bars show standard deviations. Levels of statistical significance compared with control subjects at the same corresponding time: * $0.01 < p \leq 0.05$, ** $0.001 < p \leq 0.01$, and *** $p \leq 0.001$.

AGE

GENDER

FN1