Journal of Pharmaceutical and Biomedical Analysis 53 (2010) 1003–1010

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Effect of physical fitness and endurance exercise on indirect biomarkers of growth hormone and insulin misuse: Immunoassay-based measurement in urine samples

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ARTICLE INFO

Article history:
Received 2 February 2010
Received in revised form 23 June 2010
Accepted 26 June 2010
Available online 7 July 2010

Keywords:
Growth hormone
Insulin
Indirect biomarkers
Doping
Urine

ABSTRACT

Indirect biomarkers of recombinant human growth hormone (rhGH), insulin-like growth factor-I (IGF-I), insulin-like growth factor-II (IGF-II), insulin-like growth factor binding proteins (IGFBP-2 and IGFBP-3) and insulin (C-peptide) were measured together with urinary parameters of renal damage (β_2-microglobulin and proteinuria) by immunoassays, in house validated for the purpose, in 61 subjects (36 elite athletes, 18 recreational athletes and 7 sedentary individuals) with different levels of physical fitness and endurance exercise.

Validation parameters were good for the evaluated assays, excluding a high inter-assay imprecision and inaccuracy of 24 and 26% obtained for GH assay. The range of concentrations found in urine samples under investigation was generally covered by the calibration curves of the studied immunoassays. However, for the samples below or above the calibration curve, opportune dilution or concentration were performed. Particularly, C-peptide samples had to be diluted 1:5 and β_2-microglobulin ones assayed using a triple sample volume, to fall within the calibration range.

Urinary C-peptide was the only biomarker statistically higher in samples of elite athletes when compared to recreational athletes and sedentary individuals.

Among elite athletes, taekwondo athletes showed the highest IGF-II basal values while weightlifting athletes showed the lower IGF-I and IGFBP-3 basal values. The trend observed in weightlifters basal samples was confirmed in their training samples: IGF-I, IGF-II, IGFBP-3 and β_2-microglobulin were lower in with respect to those from synchronised swimming.

Over the training season, within athlete variability was observed for IGFBP-3 for weightlifting athletes.

In the studied subjects, no direct associations were found between biomarkers of GH or insulin misuse and urinary parameters of renal damage, eventually due to high-workload endurance training.

The variations observed in different biomarkers should be taken in consideration in the hypothesis of setting reference concentration ranges for doping detection.

1. Introduction

The International Olympic Committee (IOC), World Anti-Doping Agency (WADA) and most sports authorities have banned the use of recombinant human growth hormone (rhGH) and insulin by athletes [1]. However, misuse of these two substances appears to have increased dramatically recently as a result of the lack of a reliable and time-lasting detection window test to identify exogenously administered compounds from endogenous ones.

Insulin-like growth factors-I and -II (IGF-I and IGF-II), insulin-like growth factor binding proteins-2 and -3 (IGFBP-2 and IGFPB-3), are within a group of GH-related compounds that have been proposed as indirect biomarkers of the rhGH misuse in sport, after

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doi:10.1016/j.jpba.2010.06.032

rhGH administration protocols in a group of trained adult males [2–9].

Measurement of C-peptide in serum can reflect the effectiveness of the conversion of proinsulin to insulin in the β cells since its only source is from endogenous pancreatic β cells. Thus, the amount of C-peptide in serum, together with that of insulin has been suggested to distinguish endogenous from exogenous insulin sources [10].

In order to verify if physiological variables such as physical fitness and endurance exercise could affect concentration ranges of these rhGH and insulin dependent substances, we recently evaluated all the above-reported biomarkers in serum samples of elite athletes of various sport disciplines at different moments of the training season, in recreational athletes at baseline conditions and in sedentary individuals [11,12].

The obtained results demonstrated that:

- baseline values of serum insulin and IGF-II from sedentary individuals were significantly higher and lower, respectively, than those of recreational and elite athletes;
- baseline concentrations of serum IGFBP-2 and IGFBP-3 from elite athletes were statistically, different from those of sedentary individuals and recreational athletes. Furthermore, among elite athletes, the specific sport affected serum IGFBP-3, insulin and C-peptide.

Even if variations in serum values of different biomarkers were within normal athletes population ranges and in case of IGF-II, IGFBP-2 and IGFBP-3 they neither get near nor overlap values observed after rhGH administration [2,3,6], we concluded that these variations should be taken into consideration in the hypothesis of setting athlete's reference concentration ranges for doping detection.

In the context of the above-reported studies, we also collected urine samples from athletes and as final part of our study, we sought to establish whether the physiological variations observed in serum samples could be also observed in urine samples, and if eventual variations could be associated to a transient decline in renal function, due to high-workload endurance training.

Indeed, even though the effect of rhGH and insulin administration on urinary concentration of above-reported biomarkers has not studied yet, some preliminary observations supported our study.

Firstly, an inverse relationship between serum increased and urinary decreased IGF-I concentrations following low dose rhGH administration has been shown, suggesting IGF-I accumulation within the kidney [13].

Secondly, a significant increase in urinary IGFBP-3 and rhGH was demonstrated, after 6-day subcutaneous rhGH administration, which lasted 36 h after the last injection [14].

Finally, in the above-reported study the authors also pointed out that a rise in urinary rhGH could not only be produced by hormone administration, but could also be due to temporary decline in renal function (expressed as a transient increased glomerular permeability to macromolecules) as a consequence of endurance exercise [14,15].

In this paper, we present the values of urinary GH, IGF-I, IGF-II, IGFBP-2, IGFBP-3, insulin, C-peptide together with total proteinuria and β_2-microglobulin as markers of a tubular damage in elite athletes of various sport disciplines at different moments of the training season; in recreational athletes at baseline conditions and sedentary individuals. Urinary biomarkers were measured by immunoassays in house validated for the purpose.

2. Materials and methods

Validation of the assays was performed in the laboratories of Drug Abuse and Doping Unit of the Department of Therapeutic Research and Medicines Evaluation, Istituto Superiore di Sanità, Rome, Italy.

2.1. Biomarkers assays

The immunoassay used for the GH measurement was the Human GH-ELISA kit from Biosource (Nivelles, Belgium). Calibration samples were analysed in quintuplicate at the following concentrations: 0, 0.54, 2.84, 7.30, 27.5 and 70.0 μIU/ml. These samples were calibrated by the manufacturer using the international standard WHO 2nd IS 98/574 (with 1 μIU corresponding to 0.33 ng). The quality control (QC) samples 1 and 2 were prepared using the GH calibration sample at 7.20 ± 1.85 μIU/ml and at 17.80 ± 3.60 μIU/ml. As reported by the manufacturer, the cross-reactions with human chorionic gonadotropin, human placental lactogen hormone and prolactin were insignificant.

Both the IGF-I and IGF-II assays correctly measured total (free and bound) IGF-I and IGF-II. Conversely conventional assays, in which IGFBPs are not removed, result in incorrect IGF-I and IGF-II values which reflect more the present amount of IGFBPs rather than the exact IGF-I and IGF-II concentration.

The immunoassay for total IGF-I measurement was the radioimmunoassay Human IGF-I RIA from Mediagnost (distributed by Pantec Torino, Italy). Calibration samples were analysed in quintuplicate at the following concentrations: 0, 0.156, 0.313, 0.625, 1.25, 2.5, 5.0 and 10.0 ng/ml. These samples were calibrated by the manufacturer using the WHO international reference IGF-I standard code 87/518. QC sample was supplied by the manufacturer (QC 3) at a concentration of 2.30 ± 0.20 ng/ml. Due to the high specificity of the assay, no cross-reactivity with IGF-II, IGFBP-2 and IGFBP-3 was reported.

The immunoassay for total I IGF-II measurement was the radioimmunoassay Human IGF-II RIA from Mediagnost (distributed by Pantec Torino, Italy). Calibration samples were analysed in quintuplicate at the following concentrations: 0.0, 0.4, 0.9, 2.4, 5.0, 10.0, 22.5 and 50.0 ng/ml. No information on international reference IGF-II standard used to calibrate curve samples was provided by the manufacturer. Two QC samples were supplied by the manufacturer (QC 4, 2.94 ± 0.59 ng/ml and QC 5, 14.30 ± 2.88 ng/ml). Cross-reactivity with IGF-I was reported as less than 0.05%.

The immunoassay for the IGFBP-2 measurement was the Human IGFBP-2 ELISA kit from GenWayBiotech, Inc. (distributed by Prodotti Gianni, Milano, Italy). Calibration samples were analysed in quintuplicate at the following concentrations: 0, 1, 10, 20, 40 and 80 ng/ml. No information on international reference IGFBP-2 standard used to calibrate curve samples was provided by the manufacturer. The QC samples 6 and 7 at a concentration of 15.0 ± 2.6 and 30.0 ± 4.3 ng/ml were prepared using the highest IGFBP-2 calibration sample. Nor IGF-I, or IGF-II and IGFBP-3 were reported to show any cross-reaction in the assay.

The immunoassay for IGFBP-3 measurement was the Human IGFBP-3 ELISA from GenWayBiotech, Inc. (distributed by Prodotti Gianni, Milano, Italy). Calibration samples were analysed in quintuplicate at the following concentrations: 0, 315, 1025, 2470, 3970 and 9000 ng/ml. These samples were standardized by the manufacturer against the NIBSC/WHO recombinant IGFBP-3 reference reagent coded 93/560. The QC samples were supplied by the manufacturer at the following concentrations: QC 8, 425 ± 89 ng/ml and QC 9, 1866 ± 392 ng/ml. Assay cross-reactivity with IGF-I, IGF-II and IGFBP-2 was reported as less than 0.001%.

The immunoassay used for the insulin measurement was the Human Insulin-Enzyme Amplified Sensitivity Immunoassay

(EASIA) kit from GenWay Biotech, Inc. (distributed by Prodotti Gianni, Milano, Italy). Calibration samples were analysed in quintuplicate at the following concentrations: 0, 4.6, 12.3, 43.3, 120 and 307 μIU/ml. These samples were calibrated by the manufacturer using the 2nd IRP 66/304 insulin reference standard. Quality control samples (QC) supplied by the manufacturer were QC 10, 16.5 ± 4.1 μIU/ml and QC 11, 37.4 ± 4.6 μIU/ml. The assay cross-reacted with animal insulin, but not with human and animal proinsulin.

The immunoassay for the C-peptide measurement was the C-PEP-EASIA kit from Biosource (Nivelles, Belgium). Calibration samples were analysed always in quintuplicate at the following concentrations: 0, 0.07, 0.16, 0.52, 1.80 and 5.50 pmol/ml. These samples were standardized by the manufacturer using the international NIBSC 84/510 C-peptide reference standard. Quality control samples (QC) were supplied by the manufacturer (QC 12, 0.32 ± 0.08 pmol/ml and QC 13, 0.87 ± 0.22 pmol/ml). Assay cross-reactivity with human and animal proinsulin, insulin and glucagon was negligible.

The immunoassay for the β_2-microglobulin was the Human β_2-microglobulin RIA kit from Mediagnost. Calibration samples were analysed always in quintuplicate at the following concentrations: 0, 0.146, 0.29, 0.76, 1.90 and 7.60, 28.50 ng/ml. These samples were calibrated by the manufacturer using the international standard WHO 1st IS 1985 (with 1 IU corresponding to 14 ng). Quality control sample (QC) was supplied by the manufacturer (QC 14, 2.37 ± 0.6 ng/ml). No cross-reactivity with human IgG was detected in the assay.

A microplate reader Novapath TM microplate Reader (Biorad, Milan, Italy) was used for the ELISA and EASIA immunoassays and a Wizard 3-1480 automatic gamma counter reader (Wallac, Milan, Italy).

The colorimetric (pyrogallol red) assay applied for proteinuria measurement was the Protein in U&CSF from Spinreact (Girona, Spain). Using this assay, proteins reacted in acid solution with pyrogallol red and molybdate to form a coloured complex. The intensity of the colour formed was proportional to the protein concentration in the sample. The calibrator was an albumin/globulin aqueous primary standard 1000 mg/l ready to use. Quality control sample (QC 15) was prepared using the proteinuria calibration sample at 200 mg/dl.

Blank samples for each test kits were the ones provided by the manufacturer.

2.2. Validation protocol

The validation of the techniques applied to measure above-reported urinary biomarkers and β_2-microglobulin and total proteinuria consisted on the following studies.

Intra-laboratory validation protocol consisted in four assays. They were performed on four different days. For each assay, the parameters for the best fit between signal and concentration were calculated according to the mathematical model proposed by the manufacturers. As a measure of the goodness of fit, the error (%) in the retro-calculation of the assigned concentration of the calibration samples was monitored. Five replicates of two QC samples were analysed for the determination of intra-assay precision and accuracy, while the inter-day precision and accuracy were determined for all values obtained along three independent experimental assays of the aforementioned QC samples. Precision was expressed as the relative standard deviation (RSD%) of the performed measurements. Accuracy is expressed as the relative error (%) of the value obtained with respect to the assigned value for the QC samples.

To calculate the limits of detection (LOD) and quantification (LOQ), the blank calibration sample was analysed 5 times in the same run. The standard deviation of the values obtained was taken as the measure of the noise. LOD and LOQ were defined as the mean value obtained for the blank sample plus (or less, depending on the sign of the slope of the calibration curve) 3 and 10 times the estimated value of the noise, respectively.

Once the validation parameters were established, a single immunoassay run per biomarker was run to verify if real samples fell into the calibration curve ranges. On the basis of preliminary results, it was decided to analyse samples for C-peptide diluted 1:5 and samples from β_2-microglobulin assayed using a triple sample volume. For samples below or above the calibration curve points of the other assays, opportune dilutions or concentration (e.g. doubling the sample volume) were performed. In those cases, precision and accuracy tests were repeated diluting or concentrating the corresponding QC samples to verify that in these conditions the two validation parameters still satisfied the international established criteria [16,17]. The diluted and concentrated QC samples were also used for linearity tests, comparing theoretical and measured concentration in all cases.

Stability studies were carried out using QC samples supplied by the manufacturers, as well as real samples. The stability in freeze/thaw cycles of QC samples was assessed by comparing the results of the QC samples analysed immediately after their preparation (F/T0) with those obtained after one (F/T1) or two (F/T2) cycles of freezing at −80 °C for 30 min and thawing at room temperature for 30 min. The stability after freeze/thaw cycles was also evaluated in three study urine samples by comparing the results obtained after one (F/T1), two (F/T2) and three (F/T3) freeze/thaw cycles. Stability in storage/transportation conditions was evaluated in QC and three study samples prepared in laboratory, left in dry ice for a time interval of approximately 48 h, mimicking courier transportation, and then thawing at room temperature and analysed. Finally, mid-term stability test was performed for the three study samples stored at −20 °C. Three replicates of the samples were analysed once a month during a six months period. The stability was expressed as a percentage of the initial concentration (first analysed batch) of the biomarkers both in QC and real samples.

2.3. Subjects and population study design

A total of 61 healthy Caucasian subjects (28 males and 33 females) participated in the study. All participants completed a detailed questionnaire assessing physical activity, sport practice, weekly training workload, smoking, use of drugs or dietary supplementation, and underwent a complete medical revision. Subjects were informed and gave written consent to participate in the study, which was approved by the Instituto Municipal de Asistencia Sanitaria Ethic Committee of Clinical Research (CEIC/IMAS no. 2000/1145/I) and was conducted in accordance with the Helsinki Declaration. Subjects were divided in three different main groups: elite athletes (members of sport federations, national and international sporting squads), recreational athletes (subjects regularly practicing low intensity sport in the last 5 years and not belonging to any sports organisation) and sedentary persons (subjects no practising physical training). Elite athletes participated in different sports (synchronised swimming, taekwondo and weightlifting). A detailed description of the subjects is given in Table 1. To study the effect of specific type of exercise and different training workloads in elite athletes, urine samples from athletes of different sports were collected in three different occasions of the training season: at the beginning of the season when training workload was minimal (baseline), in the middle of training preparation when training workload was at a mean level (training), and immediately after the first competition when training workload was maximum (competition). Spot urine samples were collected and stored at −80 °C

Table 1
Anthropometric and physiological characteristics of the studied subjects (mean ± S.D.).

	Number (♂/♀)	Age (years)	Height (cm)	BMI	Training (h/week)	Sample collection**
Sedentary individuals	2/5	28 ± 9	170 ± 12	21.1 ± 2.8	–	Basal*
Recreational athletes	9/9	23 ± 5	171 ± 10	22.4 ± 2.3	3–9	Basal*
Elite athletes	17/19	21 ± 3	174 ± 9	23.3 ± 4.0	8–20	Basal* Training* Competition*
Sports						
Synchronised swimming	–/14	23 ± 3	170 ± 6	20.6 ± 1.0	Up to 25	Basal* Training*
Taekwondo	5/4	21 ± 3	176 ± 11	21.0 ± 1.1	Up to 20	Basal*
Weightlifting	12/1	19 ± 4	173 ± 8	25.6 ± 4.9	Up to 11	Basal* Training* Competition*

Significantly different ($p < 0.05$) from: 1 sedentary; 2 recreational athletes; 3 elite athletes; 4 Synchronised swimming; 5 Taekwondo; 6 Weightlifting.
* Body mass index.
** Basal: at the beginning of the season when training workload was minimal; Training: in the middle of training preparation when training workload was at a mean level. Competition: immediately after the first competition when training workload was maximum.

until analysis. Urine samples from study subjects were examined in triplicates.

2.4. Calculations and statistical analysis

Mathematical models and transformations suggested by the manufacturers were used for fitting the signal with the concentration of analyte. Mean, standard deviation, and coefficient of variation (defined herein as the measure of the "within-athlete" variability of the marker in different conditions) were calculated for each biomarker.

Spearman's rank correlation coefficient was used to study the association between values of β_2-microglobulin and proteinuria, and each of the four urinary biomarkers: IGF-I, IGF-II, IGFBP-2, and IGFBP-3. For these computations, basal data of all individuals were used. The choice between non-parametric and parametric tests for the following comparisons, applied to all biomarkers, depended on the number of available data. Wilcoxon tests for paired data, the Kruskal–Wallis test, pairwise Wilcoxon tests were applied. Concerning parametric procedures, one-way ANOVA was applied and the Tukey's test for multiple posthoc comparisons was used in case of significant overall differences. Finally, linear mixed models were chosen to compare data over the training season: whenever the overall comparison showed significant results, multiple posthoc comparisons were carried out using the Tukey's test within the framework of the chosen model. The statistical package SPSS 2001 for Windows, version 12 (SPSS Inc., Chicago, IL, USA), and R., version 2.9.2 (The R Foundation for Statistical Computing) were used. p-Values < 0.05 were considered to be statistically significant.

3. Results

3.1. Evaluation of assays

Validation parameters for the investigated immunoassays are shown in Table 2. The errors between the assigned concentration of the calibration samples and the re-calculated values obtained with the equations were almost always lower than 20%. Mean correlation coefficients (r^2) obtained for the five replicates of calibration curves were the following: $r^2 = 0.959 \pm 0.033$ for GH, $r^2 = 0.981 \pm 0.036$ for IGF-I, $r^2 = 0.991 \pm 0.006$ for IGF-II, $r^2 = 0.992 \pm 0.025$ for IGFBP-2, $r^2 = 0.994 \pm 0.030$ for IGFBP-3, $r^2 = 0.990 \pm 0.006$ for insulin, $r^2 = 0.996 \pm 0.024$ for C-peptide and $r^2 = 0.982 \pm 0.004$ for β_2-microglobulin.

The performance of the assays, measured in terms of precision and accuracy when measuring QC samples, as provided by manufacturer and diluted or concentrated, was within the international established criteria [16,17], apart from GH assay, where the values of inter-assay precision and accuracy for QC 2 (concentration between the third and the fourth calibration samples) were out of the acceptable range and IGF-II assay, where the value of inter-assay precision for QC 6 (concentration between the fourth and the fifth calibration samples) was out of the acceptable range, too. With respect to linearity tests for diluted and concentrated QC of different biomarkers, measured concentrations always fell within ±20% theoretical concentrations using the previously established calibration curves.

3.2. Stability studies

For the freeze/thaw stability assays for all QC and real urine samples, no relevant degradation was observed after one and two freeze/thaw cycles, with differences from initial concentration lower than 17%. Similar results were obtained for stability experiments in storage/transportation conditions and in case of mid-term stability test, (differences to the initial concentration always lower than 20%) assuring the validity of stored samples analysis.

3.3. Biomarkers values in selected population of athletes

A preliminary investigation was performed to verify a possible effect of gender and age in urine concentrations of the biomarkers analysed in the selected groups. Analysis of variance showed that neither gender nor age influenced the analysed biomarkers. However, the variables were not taken into consideration as covariates for further statistical analysis of data.

3.3.1. Anthropometrical and physiological data

Anthropometrical and physiological data of sedentary and both recreational and elite athletes are summarized in Table 1. Significant differences ($p < 0.001$) were observed in age between elite athletes and sedentary persons. Among elite athletes, synchronised swimming athletes were the ones with lowest body mass index while weightlifting athletes were the ones with the highest.

3.3.2. Factors affecting biomarkers concentrations

Physical fitness (athletes *vs.* sedentary persons) did not influence both direct (urinary GH) and indirect biomarkers (urinary IGF-I, IGF-II, IGFBP-2 and IGFBP-3) of rGH misuse (Table 3 and Fig. 1), nor it was associated with selected biomarkers of transient renal damage: β_2-microglobulin and total proteinuria. Regarding this latter parameter, no proteinuria was observed in any of the sedentary persons. Conversely, although also the majority of ath-

Table 2
Validation parameters for GH, IGF-I, IGF-II, IGFBP-2, IGFBP-3, insulin, C-peptide, β_2-microglobulin and total proteinuria.

	LOD	LOQ	Calibration range	QC		Intra-assay		Inter-assay	
						Precision (%)[a]	Accuracy (%)[b]	Precision (%)[a]	Accuracy (%)[b]
GH (μIU/ml)	0.20	0.50	0.54–70						
				1	7.20 ± 1.85	0.13	6.42	4.64	11.60
				2	17.80 ± 3.60	3.08	3.97	24.22	26.57
IGF-I (ng/ml)	0.10	0.50	0.156–10						
				3	2.30 ± 0.20	1.04	16.30	6.39	11.30
IGF-II (ng/ml)	0.10	0.50	0.40–50						
				4	2.94 ± 0.59	14.60	1.00	14.00	16.90
				5	14.30 ± 2.88	15.30	6.08	18.00	14.80
IGFBP-2 (ng/ml)	0.06	0.21	1.00–80.00						
				6	15.0 ± 2.6	3.34	7.84	5.47	7.22
				7	30.0 ± 4.3	4.87	4.27	7.72	10.02
IGFBP-3 (ng/ml)	10.50	135	315–9000						
				8	425 ± 89	1.56	14.52	1.81	15.58
				9	1866 ± 392	4.83	5.80	5.58	4.74
Insulin (μIU/ml)	0.15	0.50	4.6–324						
				10	16.5 ± 4.1	3.60	12.40	5.40	11.20
				11	37.4 ± 4.6	5.30	14.60	9.70	13.70
C-peptide (pmol/ml)	0.01	0.06	0.07–5.50						
				12	0.32 ± 0.08	6.90	11.40	10.00	8.00
				13	0.87 ± 0.22	8.10	14.90	16.00	12.70
β_2-microglobulin (ng/ml)	0.01	0.145	0.146–28.50						
				14	2.37 ± 0.60	8.00	1.00	7.50	16.90
Total proteinuria (mg/dl)	4	10							
				15	200	9.60	14.50	6.70	15.00

[a] Measured as relative standard deviation (RSD).
[b] Measured as relative error.

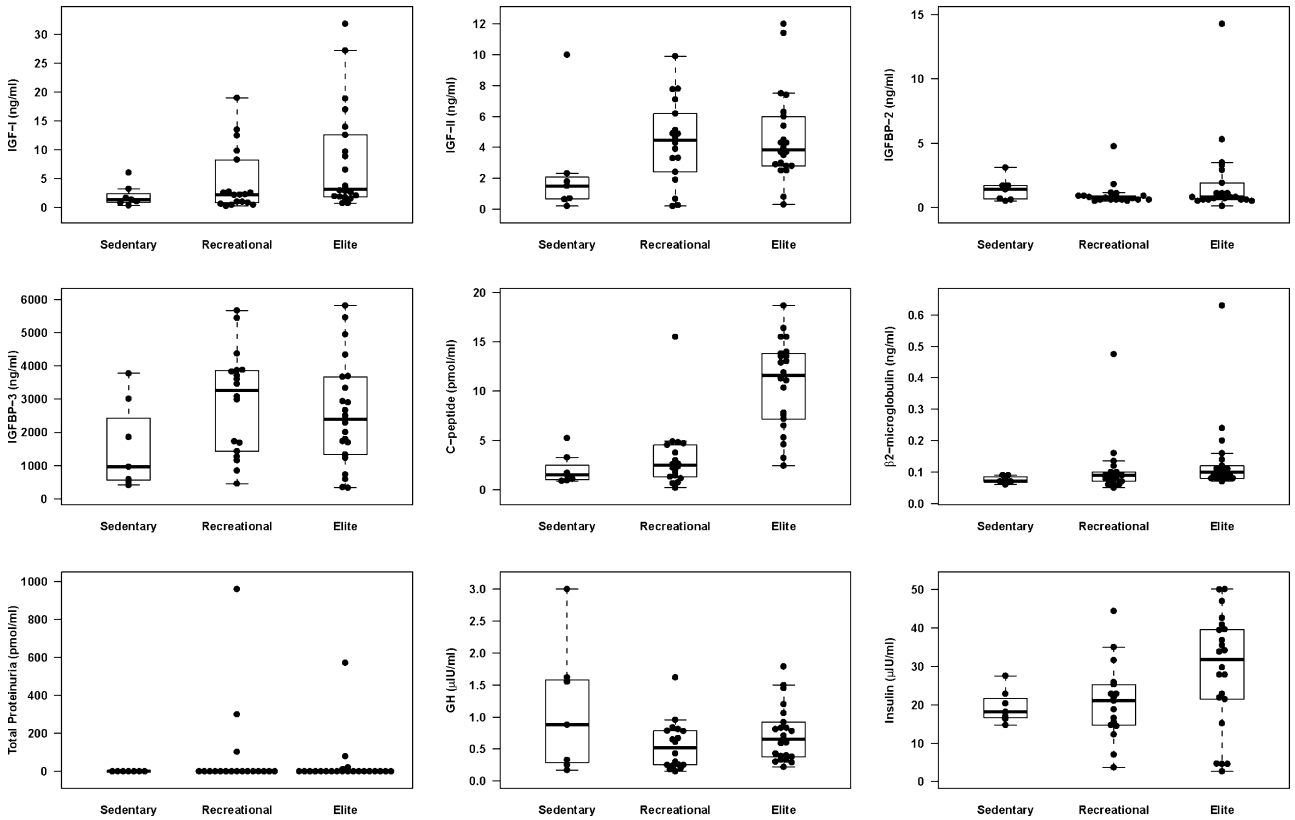

Fig. 1. Box-plots represent concentrations in basal conditions of GH, IGF-I, IGF-II, IGFBP-2, IGFBP-3 insulin, C-peptide, β_2-microglobulin and proteinuria urine concentrations in: (1) sedentary persons; (2) recreational athletes; (3) elite athletes. The median (solid line) and the interquartile range are shown as well as the minimum and the maximum. The black circles represent all the values.

letes did not show any proteinuria, the few recreational ($n = 3$) and elite athletes ($n = 2$) showing aberrant great values of urinary proteins, altered the values of mean and standard deviation of this parameter.

In case of insulin biomarkers, whereas urinary insulin concentration showed only an increasing trend associated with physical training, C-peptide concentrations were significantly higher in elite athletes with respect to both sedentary persons and recreational athletes ($p < 0.001$) (Table 3 and Fig. 1).

Among elite athletes, the specific practiced sport affected baseline urinary IGF-I, IGF-II and IGFBP-3 concentrations with weightlifters showing the lowest IGF-I, IGF-II, and IGFBP-3 values. When comparing biomarkers values during training in different sports, significant lower values of IGF-I, IGF-II, IGFBP-3 and β_2-microglobulin were observed for weightlifting *vs.* synchronised swimming (Table 3 and Fig. 2). These results are in agreement with what observed at the baseline for weightlifting athletes.

Within the same sport, significant increase in urinary IGFBP-3 along the sport season was observed for weightlifting athletes. In addition, when considering the "within-athlete" coefficient of variation of different biomarkers along the training season for weightlifters, values between 50 and 90% were observed. In the studied subjects, no direct associations were found between biomarkers of GH or insulin misuse and urinary parameters of renal damage, eventually due to high-workload endurance training. However, athletes presenting positive values of proteinuria showed also high concentration of biomarkers under investigation.

4. Discussion

4.1. Immunoassays evaluation

As already stated in previous studies, before proposing eventual indirect biomarkers of proteins misuse in athletes, the existence of reliable assays for their measurement is crucial to assure that both in healthy volunteers and athletes in different conditions, the concentrations are independent of the method used and can be reproduced in different laboratories obtaining the same results [11,12].

When analysing urine samples, the only biological fluid accepted in doping control of all sport disciplines (blood and serum are considered only in few disciplines and for selected doping agents or procedures), the problem of assay reliability is still more crucial due to the fact that proteins concentration in urine is generally low and that the majority of immunoassays for the reported biomarkers are not specifically manufactured for this biological fluid.

Indeed, the only immunoassay specifically validated by the manufacturer for urine samples as well as for serum and plasma was the β_2-microglobulin ELISA assay. Concerning the others, the ones showing the highest sensitivity and the lowest LOQ and LOD in the manufacturer instructions were purchased and subsequently in house validated. Nonetheless, the response obtained in some validation parameters was not optimal (as it can be seen in Table 2) and because of values outside of the calibration range, it was been necessary to dilute or to concentrate (in terms of using 2 or 3 times the fluid volume indicated in the assay) the urine samples. In any case, the stability demonstrated for all the biomarkers during storage and transportation tests was important to ensure the reproducibility of results obtained if performed in the same or in different laboratories.

Anyway, the reliability obtained in the intra-laboratory validation parameters made the subsequent analysis in athletes' population of a non-negligible importance.

Table 3
Seasonal changes in serum insulin and C-peptide in sedentary, recreational and elite athletes. Values are mean ± S.D.

	Time	GH (μIU/ml)	IGF-I (ng/ml)	IGF-II (ng/ml)	IGFBP-2 (ng/ml)	IGFBP-3 (ng/ml)	Insulin (μIU/ml)	C-peptide (pmol/ml)	β_2-microglobulin (ng/ml)	Proteinuria (mg/dl)
Sedentary individuals	B	1.1 ± 1.0	2.1 ± 1.9	2.4 ± 3.4	29.4 ± 18.9	1587.7 ± 1342.4	19.6 ± 4.4	2.1 ± 1.6[c]	0.15 ± 0.02	0.00 ± 0.00
Recreational athletes	B	0.6 ± 0.4	4.6 ± 5.5	4.3 ± 2.7	21.0 ± 18.9	2915.9 ± 1562.6	20.8 ± 10.0	3.3 ± 3.4[c]	0.22 ± 0.20	75.72 ± 232.55
Elite athletes	B	0.7 ± 0.4	7.9 ± 8.9	4.6 ± 2.9	39.9 ± 63.0	2562.3 ± 1604.6	31.2 ± 13.4	10.7 ± 4.5[a,b]	0.28 ± 0.22	28.76 ± 124.66
Sport										
Synchronised swimming	B	0.8 ± 0.4	9.8 ± 9.6[f]	3.1 ± 2.2[e]	35.7 ± 27.3	3556.5 ± 1203.6[f]	28.2 ± 16.8	9.4 ± 5.7	0.19 ± 0.04	9.91 ± 28.04
	T	0.6 ± 0.1	4.6 ± 3.8[f]	6.7 ± 3.0[f]	66.5 ± 96.6	3243.0 ± 1362.2[f]	18.0 ± 9.9	6.6 ± 4.5	0.36 ± 0.18[f]	2.73 ± 4.52
Taekwondo	B	0.5 ± 0.4	9.8 ± 9.7[f]	6.8 ± 2.9d[,f]	48.3 ± 94.5	2218.6 ± 1861.4	27.8 ± 15.4	11.5 ± 2.9	0.36 ± 0.35	67.09 ± 189.63
Weightlifting	B	0.9 ± 0.5	1.5 ± 0.5[d,e]	3.1 ± 0.6[e]	35.7 ± 42.0	1590.0 ± 792.8[d]	31.5 ± 14.0	11.4 ± 5.2	0.19 ± 0.05	0.00 ± 0.00
	T	0.8 ± 0.6	1.2 ± 0.6[d]	1.8 ± 1.4[d]	23.1 ± 16.8	1607.9 ± 866.8[d,g]	28.6 ± 7.7	6.1 ± 5.6	0.20 ± 0.08[d]	0.00 ± 0.00
	C	0.8 ± 0.3	2.2 ± 1.7	2.7 ± 1.5	35.7 ± 27.3	3013.1 ± 1675.3[g]	35.3 ± 25.8	9.3 ± 7.4	0.31 ± 0.30	4.26 ± 12.06

Values are mean ± S.D. Abbreviations: B, baseline; T, training; C, competition.
[a] Significantly different ($p < 0.05$) from sedentary individuals.
[b] Significantly different ($p < 0.05$) from recreational athletes.
[c] Significantly different ($p < 0.05$) from elite athletes.
[d] Significantly different ($p < 0.05$) from synchronised swimming.
[e] Significantly different ($p < 0.05$) from taekwondo.
[f] Significantly different ($p < 0.05$) from weightlifting.
[g] Baseline concentrations.

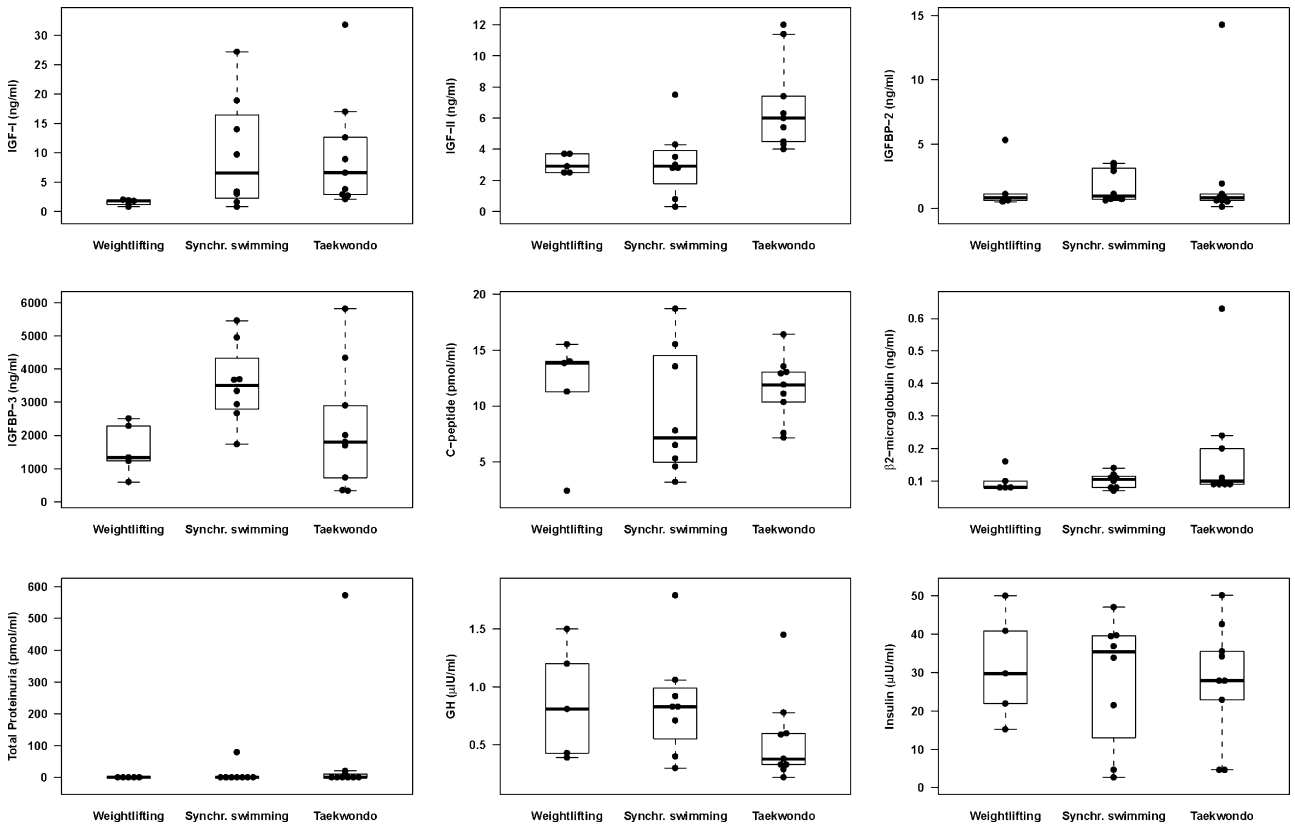

Fig. 2. Box-plots represent concentrations in basal conditions of GH, IGF-I, IGF-II, IGFBP-2, IGFBP-3, insulin, C-peptide, β_2-microglobulin and proteinuria urine concentrations in described sports. The median (solid line) and the interquartile range are shown as well as the minimum and the maximum. The black circles represent all the values.

In particular, we sought to investigate the physiological fluctuations of urinary concentration of these biomarkers according to different physical fitness and different sport discipline and related training workloads. Moreover, since the presence of urinary proteins can be associated to renal damage, we measured two parameters (β_2-microglobulin and proteinuria) indicative of this eventual occurrence.

4.2. Biomarkers values in selected population of athletes

An important result, observed for the first time in this study, is that elite athletes showed their own baseline concentrations of urine C-peptide, different from those of sedentary individuals and recreational athletes.

Furthermore, it has to be noticed that C-peptide values in elite athletes were very high: 50% of subjects had values above the highest point of the calibration curve. Interestingly, insulin showed increasing values in elite athletes compared to the recreational ones and sedentary persons, although not statistically significant.

The second evidence is that different training workload at baseline and during sport season influences serum IGF-I, IGF-II and IGFBP-3 concentrations in elite athletes. In particular, weightlifters showed the lowest IGF-I, IGF-II, and IGFBP-3 values, with taekwondo athletes presenting the highest IGF-II values and synchronised swimmers the highest IGFBP-3. Of note, the weightlifters were the athletes which trained for the lowest number of weekly hours and that at baseline did not have any positive value of proteinuria, while one-third of the swimming and taekwondo athletes showed measurable proteinuria at baseline Moreover, in case of this latter sport disciplines, athletes with proteinuria also presented high values of β_2-microglobulin. So, we can hypothesize that the specific exercises of this combat sport discipline could generate transient microtraumas in renal parenchyma that could modify glomerular filtration, in accordance with previous observations [14]. As matter of fact, even if weightlifters maintained the values of IGF-I, IGF-II, IGFBP-3 and β_2-microglobulin lower than those of swimmers during the training season (training values were available only for these two sports) during competition when exercise workload was maximum, levels of these biomarkers increased (IGFBP-3 significantly) also in weightlifters with respect to training time, Accordingly, for the first time, 40% weightlifting athletes presented urinary proteinuria.

This study evidences differences in both rhGH and insulin indirect biomarkers as a function of physical fitness and training workloads. However, these variations were statistically significant only in some cases, while in the others only trends towards changes during sport season in different disciplines or in athletes as compared to sedentary persons were observed. This latter occurrence could be due to the few number of athletes recruited for different sport disciplines.

Indeed, an important limitation of this study is that number of samples was quite low and obtained results have to be considered only preliminary evidence. Indeed, at moment no comparison with observations made in other populations can be done, since these compounds were for the first time measured in athletes' urine and normal population ranges have not been established.

In addition to that, it has to be recognized that specific sensitive immunoassays targeted to the measurement of compounds under the study do not exist, at moment, for urinary matrix. Nonetheless, these first observations may serve as an eye opener to demonstrate that different sport discipline and training workloads can be relevant when trying to interpret concentrations due to physiological variabilities in sport men from those coming from exogenous insulin and rGH administration. In this latter concern, information is quite scanty: only two studies investigated changes in urinary concentration of two indirect biomarkers of rhGH exogenous

administration. The first study showed that after one subcutaneous injection of 12 IU rhGH every day for 6 days in one healthy individual, IGFBP-3 urinary concentrations increased up to 5 times the baseline concentration in the last treatment day and returned to pre-treatment value 36 h after the last injection [14]. The second study, performed in six normal individuals subcutaneously injected with 2 IU rhGH twice a day for two subsequent days, demonstrated a significant decrease (−25%) of 24 h urinary IGF-I during administration period, followed by a significant increase (+30%) 2 days after the end of the treatment, and a return to baseline value the subsequent day [13]. Indeed, if comparing the results from these two studies with the seasonal changes observed for IGF-I and IGFBP-3 in our athletes, measured values neither get near nor overlap the variations observed after rhGH administration in the reported studies.

5. Conclusions

We measured indirect urinary biomarkers of eventual rhGH and insulin in athletes as a final part of a project aimed to study physiological variations of these biomarkers in elite and recreational athletes *vs.* sedentary people to support the fact that trained individuals have their own serum and urinary ranges of biomarkers. Although the immunoassays used in the present study for measuring urinary concentration of IGF-I and IGF-II, IGFBP-2 and IGFBP-3, insulin and C-peptide showed acceptable performance, in several cases they cannot attain the sensitivity required to detect the low concentrations of investigated substances in urinary matrix. In this concern, it has been proposed to start a new study project including recently developed nanoparticle technology for amplifying the effective sensitivity of biomarker detection and creating a urine test suitable for rhGH, insulin and related compounds of protein nature [18,19].

Nonetheless, in our opinion the observed variations in urinary concentration of C-peptide of elite athletes *vs.* recreational ones and sedentary individuals and the fluctuation in the different biomarkers among elite athletes from different sport disciplines have to be considered, among others factors, in future studies and decisions regarding the problem of disclosing GH and insulin intake misuse in sports. Indeed, notwithstanding several proposals for direct and indirect detection of GH and insulin misuse in sport, this problem is, at moment, far from any definitive solution [20–22] and all the contributions from different studies should be welcome.

Acknowledgments

This study has been funded by “Commissione per la Vigilanza ed il Controllo sul doping e per la tutela della salute nelle attività sportive” from the Italian Ministry of Health.

The authors thank Dr. Montse Bellver and Dr. Ramon Olive from the Centre d’Alt Rendiment, Sant Cugat, Barcelona, Spain for providing urine samples.

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