Immunoassays for the measurement of IGF-II, IGFBP-2 and -3, and ICTP as indirect biomarkers of recombinant human growth hormone misuse in sport Values in selected population of athletes

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ABSTRACT

Insulin-like growth factor-II (IGF-II), insulin-like growth factor binding proteins (IGFBPs) -2 and -3 and C-terminal telopeptide of type I collagen (ICTP) have been proposed, among others, as indirect biomarkers of the recombinant human growth hormone misuse in sport.

An extended intra- and inter-laboratory validation of commercially available immunoassays for biomarkers detection was performed. ELISA assays for total IGF-II, IGFBP-2 and IGFBP-3 (IGF-II/ELISA1: DSLabs, IGFBP-2/ELISA2: Biosource, and IGFBP-3/ELISA3: BioSource) and an EIA assay for ICTP (ICTP/EIA: Orion Diagnostica) were evaluated. The inter- and intra-laboratory precision values were acceptable for all evaluated assays (maximum imprecision of 30% and 66% were found only for the lowest quality control samples of IGF-II and IGFBP-3). Correct accuracy was obtained for all inter-laboratory immunoassays and for IGFBP-2 intra-laboratory immunoassay. The range of concentrations found in serum samples under investigation was always covered by the calibration curves of the studied immunoassays. However, 11% and 15% of the samples felt below the estimated LOQ for IGF-II and ICTP, respectively, in the zone where lower precision was obtained. Although the majority of evaluated assays showed an overall reliability not always suitable for antidoping control analysis, relatively high concordances between laboratory results were obtained for all assays. Evaluated immunoassays were used to measure serum concentrations of IGF-II, IGFBP-2 and -3 and ICTP in elite athletes of various sport disciplines at different moments of the training season; in recreational athletes at baseline conditions and finally in sedentary individuals. Serum IGF-II was statistically higher both in recreational and elite athletes compared to sedentary individuals. Elite athletes showed lower IGFBP-2 and higher IGFBP-3 concentration with respect to recreational athletes and sedentary people. Among elite athletes, serum IGFBP-3 (synchronized swimming), and ICTP (rhythmic gymnastics) concentrations were sport-dependent. Over the training season, within athlete variability was observed for IGFBP-2 in case of taekwondo and IGFBP-2 and -3 in case of weightlifting.

Variations due to those aspects should be taken in careful consideration in the hypothesis of setting reference concentration ranges for doping detection.

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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) by athletes [1]. However, misuse of rhGH appears to have increased dramatically recently as a result of its increased availability and the lack of a reliable and time-lasting detection window test to identify its abuse due to its biological particularities. Exogenously administered rhGH is virtually indistinguishable from the predominant naturally occurring isoform (both substances have identical amino acid sequences) and is cleared from the body within 24 h. A method

for detection of rhGH based on ratios between the predominant and other minor isoforms has been recently reported, but its time-window is rather limited, about 24 h after the last exogenous administration [2].

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), procollagen type III peptide (P-III-P) and C-terminal cross-linked telopeptide of type I collagen (ICTP) serum concentrations have been proposed as indirect biomarkers of the rhGH misuse in sport [3–11].

IGF-I and -II are polypeptide hormones produced by many tissues and are thought to mediate some of the actions of GH [12]. Circulating IGF-I and -II exist as a ternary complexes with IGF-binding proteins (IGFBPs) and the acid-labile subunit (ALS) [13,14].

ICTP, the carboxyterminal telopeptide region of type I collagen, liberated during the degradation of mature type I collagen and P-III-P, the extension fragment of procollagen III, are indexes of bone and collagen turnover [15,16].

It has been shown that acute endurance-type exercise transiently increased serum total IGF-I, IGFBP-3, acid-labile subunit (ALS), ICTP and P-III-P whereas rhGH treatment significantly potentiated exercise-induced changes in serum biomarkers [3,4,17].

The first step for the use of these substances as indirect rhGH misuse biomarkers is the existence of reliable assays for their measurement to assure that both in healthy volunteers and athletes in different conditions, the concentrations measured are independent of the method used and can be reproduced in different laboratories. This is especially important if mathematical models or cut-off values based in these biomarkers are going to be established for the detection of GH misuse.

We recently evaluated the immunoassays for the measurement of IGF-I and P-III-P and showed the need for harmonization of immunoassay parameters to improve the reproducibility and comparability of results between different laboratories [8]. With evaluated immunoassays we found statistically higher values of serum P-III-P in elite athletes, and a trend towards higher IGF-I concentration in both recreational and elite athletes compared to sedentary population. These findings were partly attributed to elite athletes' younger age and, in the case of P-III-P serum levels, also to physical fitness [9].

In this paper, we report an extended validation of commercially available immunoassays (one for each studied biomarker) for IGF-II, IGFBP-2, IGFBP-3 and ICTP, a comparison of results from two different laboratories and finally the measurement of these biomarkers in elite athletes of various sport disciplines at different moments of the training season; in recreational athletes at baseline conditions and sedentary individuals.

2. Materials and methods

Validation of the assays was carried out in two independent laboratories: Drug abuse and Doping unit, Istituto Superiore di Sanità, Rome, Italy (Laboratory 1) and Bioanalysis and Analytical Services Research Group, Institut Municipal d'Investigació Mèdica, Barcelona, Spain (Laboratory 2).

2.1. Biomarkers assays

The immunoassay for the IGF-II measurement was the Non-Extraction IGF-II ELISA from Diagnostics Systems Laboratories, Inc. (Texas, USA). Calibration samples were analysed in quintuplicate at the following concentrations: 0, 70, 200, 500, 1000, 2000 and 3000 ng/ml. Quality control (QC) samples were supplied by the manufacturer (QC 1, acceptance range 140–260 ng/ml; and QC

2, acceptance range 700–1300 ng/ml). Standards for calibration and QC samples used were calibrated against a preparation of recombinant DNA-derived human IGF-II, originally quantified by amino-acid analysis.

The immunoassay for the IGFBP-2 measurement was the RayBio Human IGFBP-2 ELISA Kit from RayBiotech, Inc. Calibration samples were analysed in quintuplicate at the following concentrations: 0, 21, 210, 420, 840 and 1680 ng/ml. The QC sample 3 was supplied by the manufacturer (QC 3, acceptance range 311.7–421.7 ng/ml). The QC sample 4 was prepared using the IGFBP-2 calibration sample at 840 ng/ml (QC 4). No information about standards calibration was available.

The immunoassay for IGFBP-3 measurement was the IGFBP-3 ELISA from BioSource (Nivelles, Belgium). Calibration samples were analysed in quintuplicate at the following concentrations: 0, 340, 880, 2400, 4000 and 8000 ng/ml. The QC samples were supplied by the manufacturer (QC 5, acceptance range 324–601 ng/ml; and QC 6, acceptance range 1286–1970 ng/ml). The standards used were calibrated against recombinant IGFBP-3.

For ICTP, the UniQ ICTP EIA kit (Orion Diagnostica, Espoo, Finland) based on the competitive immunoassay technique was evaluated. Calibration samples were analysed in quintuplicate at the following concentrations: 0, 1, 2.5, 5, 10, 25 and 50 ng/ml. The QC samples were supplied by the manufacturer (QC 7, acceptance range 5.8–8.2 ng/ml; and QC 8, acceptance range 18.9–28.3 ng/ml).

A microplate reader Novapath TM microplate Reader (Bio-rad. Milan, Italy) in Laboratory 1 and a Labsystems Multiskan MS (Vantaa, Finland) in Laboratory 2 were used for the selected immunoassays.

2.2. Validation protocol

The validation of the techniques applied to measure IGF-II, IGBP-2 and -3, and ICTP consisted on the following studies.

2.2.1. Intra-laboratory validation

Intra-laboratory validation protocol consisted in four assays. They were performed by Laboratory 1 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. In the case of QC samples 1 and 2 (IGF-II), 3 (IGFBP-2), 5 and 6 (IGFBP-3), and 7 and 8 (ICPT), an acceptable concentration range was defined by the manufacturer instead of an assigned value; in those cases the accuracy was evaluated as "correct" or "incorrect" if the concentration obtained was inside or outside the acceptance range. Only for QC sample 4, which had an assigned value, accuracy was expressed as the relative error (%) of the value obtained with respect to the assigned value for the QC sample.

To calculate the limits of detection (LOD) and quantification (LOQ), the blank calibration sample was analysed five 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.

2.2.2. Inter-laboratory validation

Inter-laboratory validation was carried out for each technique, by analysing selected serum samples from the studied populations in two different laboratories. The intraclass correlation coefficient (ICC) and dispersion of results obtained between different laboratories were calculated.

2.3. Stability studies

Stability studies were carried out using QC samples supplied by the manufacturers, as well as human serum 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 $-80\,^\circ\mathrm{C}$ for 30 min and thawing at room temperature for 30 min. The stability after freeze/thaw cycles was also evaluated in three human serum 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 samples prepared in laboratory 1 and sent to laboratory 2 using a regular courier system, i.e., packed in dry ice and arriving at the destination within a time interval of approximately 48 h. The stability was evaluated by monitoring the percentage degradation of the analyte in each storage condition.

2.4. Subjects and population study design

A total of 221 healthy Caucasian subjects (134 males and 87 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 five years and not belonging to any sports organisation) and sedentary persons (subjects no practicing physical training). Elite athletes participated in different sports (swimming, synchronized swimming, taekwondo, rhythmic gymnastics, triathlon, 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, blood 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).

Blood samples were collected at 9:00 h in fasting conditions. Venous blood samples were obtained from the antecubital vein and, after a 20 min interval for coagulation, they were centrifuged. Serum samples were stored at $-80\,^\circ\mathrm{C}$ until analysis.

Serum samples from study subjects were examined in triplicates.

2.5. Calculations and statistical analysis

Mathematical models and transformations suggested by the manufacturers were used for fitting the signal with the concentration of analyte.

Concerning inter-laboratory validation, the intraclass correlation coefficient was calculated using the random effects mode to evaluate the agreement of results between laboratories [18]. To evaluate the dispersion of the results obtained between different laboratories, a modification of Bland–Altman plots was used [19]. The mean values of concentrations were represented in front of the relative differences between concentrations. The 95% limits of agreement (95%LA) were calculated according to the following expression: relative difference mean $\pm\,1.96\,\times$ standard deviation of relative differences.

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.

Multifactor analysis of variance (ANOVA) using the Tukey multiple-comparison, repeated ANOVA measures and post-hoc Student test were carried out using the statistical package SPSS 2001 for Windows, version 12 (SPSS Inc., Chicago, IL, USA). p-values <0.05 were considered to be statistically significant.

Several mathematical equations were tested to relate the concentrations of the markers to the age of the subjects. Best fitting was obtained for the linear regression of the logarithm of concentration and the reciprocal of the age. Upper and lower predicted limits of confidence for a 95% range were determined.

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 always lower than 20%. Mean correlation coefficients (r^2) obtained for the five replicates of calibration curves were the following: $r^2 = 0.9907 \pm 0.006$ for IGF-II, $r^2 = 0.9922 \pm 0.014$ for IGFBP-2, $r^2 = 0.9938 \pm 0.032$ for IGFBP-3 and $r^2 = 0.9904 \pm 0.004$ for ICTP.

For IGF-II assay, the values of precision for QC 2 (concentration around the fourth calibration sample were acceptable while accuracy was incorrect in intra-assay). Differently, QC 1, containing a concentration below the calculated LOQ, showed poorer performance in terms of R. Interestingly, the calculated LOQ was higher than the second point of calibration curve.

The performance of the IGFBP-2 assay for both QC samples (the lowest-QC 3- containing concentrations of analyte between the second and third calibration sample) was good, with RSD% and relative errors lower than 8% in all cases. However, estimated LOQ was slightly higher than the first calibration sample for IGFBP-2 immunoassay.

IGFBP-3 assay showed an acceptable performance for the highest QC sample (QC 6 concentration between the second and third calibration sample) with precision and accuracy lower than 8%. Conversely, although the estimated LOQ was lower than the first calibration sample, QC 5 (with concentrations of analyte between the first and second calibration sample) showed poor performance in terms of intra-assay precision and accuracy and inter-assay precision.

For ICTP assay, precision was always better than 15% (QC 7 with concentrations of analyte between the third and fourth calibration sample, and QC 8 between the fourth and fifth calibration sample),

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

	Number (♂/♀)	Age (years)	Height (cm)	BMI[a]	Training (h/week)	Sample collection[b]
Sedentary individuals	38/26	30 ± 4	170 ± 9	21.4 ± 2.0	–	• Basal
Recreational athletes	55/25	30 ± 4	173 ± 8	21.2 ± 2.2	5–10	• Basal
Elite athletes						• Training • Competition
Swimming	3/9	18 ± 2[5,8]	175 ± 7[6]	20.8 ± 1.3[7,8,9]	Up to 35	• Basal • Training
Synchronized swimming	–/14	23 ± 3[4,7]	170 ± 6	20.6 ± 1.0[7,8,9]	Up to 25	• Basal • Training
Taekwondo	10/6	21 ± 4[8]	175 ± 10[7]	21.6 ± 1.9[7,9]	Up to 20	• Basal • Training • Competition
Rhythmic gymnastic	–/6	16 ± 4[5,8]	162 ± 5[4,6,8,9]	16.4 ± 1.0 [4,5,6,8,9]	Up to 35	• Basal • Training
Triathlon	16/–	27 ± 6[4,6,7,9]	177 ± 4[7]	23.5 ± 1.3 [4,5,7]	Up to 25	• Basal
Weightlifting	12/1	22 ± 5[8]	173 ± 8[7]	25.6 ± 4.9[4,5,6,7]	Up to 14	• Basal • Training • Competition

Significantly different ($p < 0.05$) from: [1]sedentary; [2]recreational athletes; [3]elite athletes; [4]swimming; [5]synchronized swimming; [6]taekwondo; [7]rhythmic gymnastics; [8]triathlon; [9]weightlifting.

[a] Body mass index

[b] 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.

but accuracy was generally incorrect. Estimated LOQ was higher than the first calibration sample.

The results of inter-laboratory validation for ELISA assays are presented in Fig. 1. Best agreement between results was obtained for IGFBP-2 ($ICC_{IGFBP-2} = 0.772$) followed by IGFBP-3 ($ICC_{IGFBP-3} = 0.692$), IGF-II ($ICC_{IGF-II} = 0.689$) and ICTP ($ICC_{ICTP} = 0.584$). However, the spread of obtained results was always very high: IGF-II (95%LA = −88.9–28.8%), IGFBP-2 (95%LA = −87.7–48.9%), IGFBP-3 (95%LA = −65.9–68.9%) and ICTP (95%LA = −69.3–153.2%).

Table 2
Validation parameters for IGF-II, IGFBP-2 IGFBP-3, and ICTP obtained in Laboratory 1

QC	ng/ml	Intra-assay		Inter-assay	
		Precision (%)[a]	Accuracy	Precision (%)[a]	Accuracy
IGF-II					
1	140–260[b]	7.4–30.1	Incorrect	18.6	Correct
2	700–1300[b]	0.8–15.1	Incorrect	11.7	Correct
LOD (ng/ml)			94.3		
LOQ (ng/ml)			311.2		
Calibration range[b] (ng/ml)			70–3000		
IGFBP-2					
3	311.7–421.7[b]	3.3–3.5	Correct	3.8	Correct
4	840	2.3–7.1	1.9[c]	7.2	5.0[c]
LOD (ng/ml)			9.5		
LOQ (ng/ml)			31.5		
Calibration range[d] (ng/ml)			21–1680		
IGFBP-3					
5	324–601[b]	5.3–66.0	Incorrect	43.7	Correct
6	1286–1970[b]	1.7–3.8	Correct	7.4	Correct
LOD (ng/ml)			64.5		
LOQ (ng/ml)			212.9		
Calibration range[d] (ng/ml)			340–8000		
ICTP					
7	5.8–8.2[b]	4.1–13.3	Incorrect	14.9	Correct
8	18.9–28.3[b]	4.7–13.6	Incorrect	8.0	Correct
LOD (ng/ml)			1.4		
LOQ (ng/ml)			4.6		
Calibration range[d] (ng/ml)			1–50		

[a] Measured as relative standard deviation (RSD). Minimum and maximum RSD obtained are shown.

[b] Acceptance concentration range according to the manufacturers.

[c] Measured as the relative error respect the assigned QC sample value.

[d] Calibration range provided by the manufacturer.
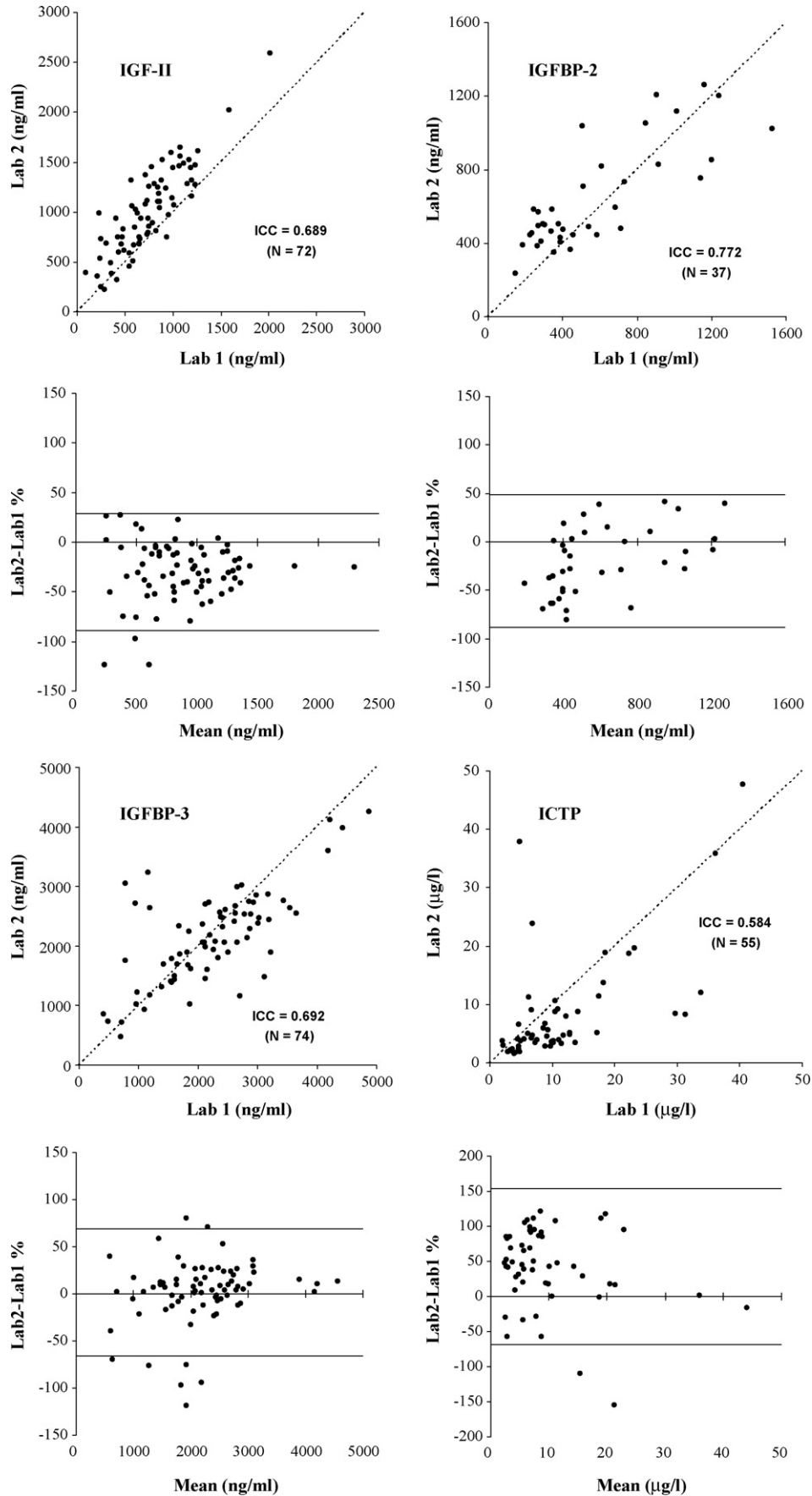
Fig. 1. Inter-laboratory comparison of IGF-II, IGFBP-2, IGFBP-3, and ICTP assays. Top, graphical comparison between laboratories and intraclass correlation coefficient (ICC), dotted lines represent total concordance. Bottom, modified Bland–Altman plots (see text), dotted lines represent the 95% limits of agreement.

Table 3
Seasonal changes in serum growth hormone markers in studied population

Group	Time	IGF-II (ng/ml)	IGFBP-2 (ng/ml)	IGFBP-3 (ng/ml)	ICTP (µg/l)
Sedentary individuals	B	747 ± 376.7 [2,3]	742.0 ± 544.5	2234 ± 955.5	17.1 ± 20.0
Recreational athletes	B	1151.8 ± 453.4	576.8 ± 562.8	2597.7 ± 747.2	14.9 ± 12.1
Elite athletes	B	1433.3 ± 276.3	259.8 ± 121.3 [1,2]	4336.3 ± 621.5 [1,2]	12.8 ± 10.7
Taekwondo	B	1428.7 ± 312.6	316.1 ± 144.8	4655.8 ± 889.1 [7]	7.77 ± 4.56 [8]
	T	1323.5 ± 232.6	391.6 ± 137.3 * [7]	3996.9 ± 498.7 *	10.90 ± 5.00 * [8]
	C	1392.1 ± 293.4	462.0 ± 249.4 *	3972.2 ± 531.1 *	6.87 ± 2.64 [5,6]
Weightlifting	B	1351.5 ± 195.5	202.5 ± 91.8	4654.8 ± 469.6 [7]	15.90 ± 12.96 [8]
	T	1202.9 ± 235.9 [7]	236.9 ± 67.5 *	4531.0 ± 567.8	10.88 ± 8.83 [8]
	C	1378.5 ± 187.3	192.2 ± 90.3	4898.2 ± 410.7 *	10.49 ± 7.32 [4]
Swimming	B	1333.3 ± 264.6	208.6 ± 84.4	4080.3 ± 332.9	14.82 ± 5.71 [8]
	C	1468.2 ± 272.5	247.9 ± 68.2	3946.1 ± 312.8	9.90 ± 2.64 [4]
Synchronized swimming	B	1428.7 ± 323.6	218.2 ± 111.1	3895.7 ± 530.2 [4,5,8]	8.12 ± 2.60 [8]
	T	1570.2 ± 358.9 [5]	184.2 ± 120.2 [4,8]	4011.2 ± 861.9	9.02 ± 4.64 [8]
Rhythmic gymnastics	B	1518.1 ± 153.1	301.4 ± 87.0	4758.2 ± 236.7 [7]	35.70 ± 18.27 [4,5,6,7,9]
	T	1595.5 ± 100.7	348.5 ± 70.2 [7]	4486.5 ± 263.5	32.96 ± 23.31 [4,5,7]
Triathlon	B	1512.1 ± 283.1	294.6 ± 125.6	4245.0 ± 353.5	10.18 ± 9.88 [8]

Values are mean ± S.D. Abbreviators: B, baseline; T, training; C, competition. Significantly different ($p < 0.05$) from: [1]sedentary individuals; [2]recreational athletes; [3]elite athletes; [4]taekwondo; [5]weightlifting; [6]swimming; [7]synchronized Swimming; [8]rhythmic gymnastic; [9]triathlon; *baseline concentrations.

3.2. Stability studies

For the freeze/thaw stability assays for all QC and real samples, no relevant degradation was observed after one and two freeze/thaw cycles, with differences from initial concentration lower than 15%. Similar results were obtained for stability experiments in storage/transportation conditions.

3.3. Biomarkers values in selected population of athletes

A preliminary investigation was performed to verify a possible effect of gender and age in serum concentrations of IGF-II, IGFBP-2, IGFBP-3 and ICTP in the analysed study groups. Analysis of variance showed that gender affected IGF-II and IGFBP-3 values, with lower IGF-II and IGFBP-3 plasma levels in males when compared with females. Age affected IGF-II, IGFBP-2 and ICTP values with higher ones in younger people. For this reason, statistical analysis of data was always adjusted for gender and age of considered individuals.

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 or recreational athletes. Considering the different sports, the elite athletes of rhythmic gymnastics group were the youngest and triathlon ones were the oldest. These differences were significant ($p < 0.001$). Moreover, rhythmic gymnastic 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

After adjusting for age and gender, significant lowest baseline serum IGF-II concentrations were observed in sedentary individuals vs both recreational and elite athletes ($p < 0.001$) while these latter athletes showed significant baseline lowest IGFBP-2 and highest IGFBP-3 serum concentration ($p = 0.011$ and $p < 0.001$, respectively). Differently, ICTP baseline values were similar in the three study groups. (Table 3, Fig. 2).

Among elite athletes, the specific practiced sport affected baseline serum IGFBP-3 (lowest values in synchronized swimming vs taekwondo, weightlifting and rhythmic gymnastics) and ICTP (highest values in rhythmic gymnastics vs all the other disciplines) even after adjusting for age and gender. When comparing values during training in different sports, significant higher values of IGF-II were observed for synchronized swimming than for weightlifting ($p = 0.002$) and statistical lower IGFBP-2 values for synchronized swimming vs taekwondo or rhythmic gymnastics ($p < 0.001$) (Table 3, Fig. 3).

Over the training season, a within athlete variability was observed for IGFBP-2 in case of taekwondo and IGFBP-2 and -3 in case of weightlifting.

Within the same sport, changes in some biomarkers along the sport season were observed for taekwondo and weightlifting athletes. However, these variations did not show the same trend. Serum IGFBP-2 increased and serum IGFBP-3 decreased along the sport season in taekwondo athletes while ICPT only increased in training conditions. For weightlifters, serum IGFBP-2 and IGFBP-3 only increased in training conditions and in competitions, respectively.

Taking all the athletes as a whole, the "within-athlete" coefficient of variation of biomarkers concentration along the sport season was quite low for IGF-II (9.3%) and IGFBP-3 (9.8%), and higher, although not clinically relevant, for IGFBP-2 (24.7%) and ICTP (28.6%). as compared with baseline situations.

4. Discussion

4.1. Immunoassays evaluation

As already stated in case of IGF-I and P-III-P [9], before proposing eventual indirect biomarkers of rhGH misuse in athletes, it is necessary to investigate the physiological fluctuations of serum concentration of these biomarkers due to different physical fitness and different sport discipline and related training workloads. The aim is the differentiation, with a margin of minimum error, between fluctuations associated with exposure to such influences and the atypical variations caused by rhGH and other stimulants of rhGh axis [3,4].

Immunoassays evaluated in the present study were chosen from the few (in many cases no more than three were on the market) commercially available and considering the manual techniques most frequently used.
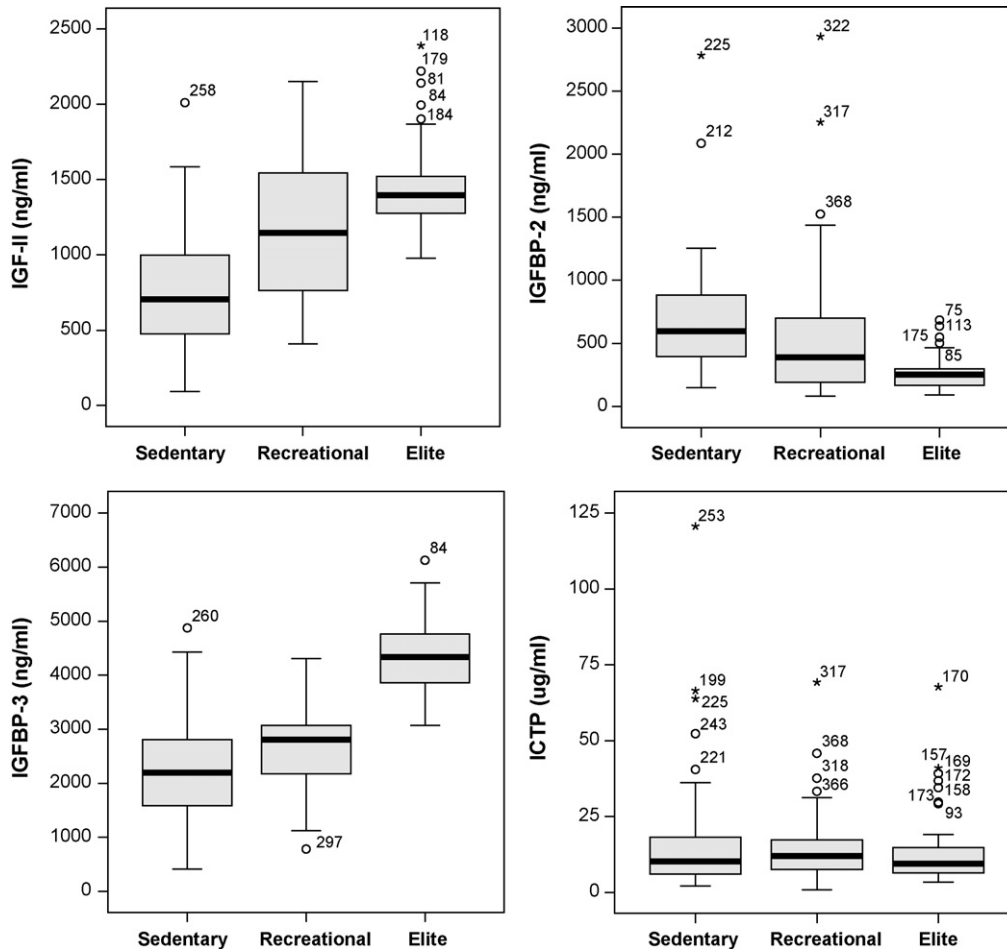
Fig. 2. Box-plots represent concentrations in basal conditions of IGF-II, IGFBP-1, IGFBP-2 and ICTP serum concentrations in: (1) sedentary persons; (2) recreational athletes; (3) elite athletes. The mean (solid line) and the 95% intervals of confidence (dashed lines) are shown. Empty circles represent outlier values; stars represent extreme values.

Taking into account the calibration range of the assays (Table 1), biomarkers concentration found in serum samples under investigation was always covered by the calibration curves of the studied immunoassays. However, 11% and 15% of the samples felt below the estimated LOQ for IGF-II and ICTP, respectively, in the zone where lower precision was obtained (Fig. 1). Conversely, in case of IGFBP-2 and IGFBP-3, values in serum samples were considerably above the estimated LOQ, and in the range of QC samples concentration where values of precision and accuracy resulted adequate. In particular, concerning these latter parameters, although in some intra-assay test imprecision was high and accuracy not correct since one or two of the five QC replicates fell outside the acceptable range, in the inter-assay calculations where replicates were 20, precision was better than 12% apart for the low QC sample of IGFBP-3 and accuracy was always acceptable.

Although the majority of evaluated assays showed an overall reliability not always suitable for antidoping control analysis [20], relatively good concordances between results were obtained for ELISA assays in the two laboratories. The UniQ ICTP EIA showed the lowest inter-laboratory data correlation, but the cause of the discrepancies found in inter-laboratory results was unknown.

Generally speaking, biomarkers values found for both sedentary people and athletes always fell in a part of calibration curve where precision and accuracy were both acceptable. This fact was indeed confirmed by the good inter-laboratory values concordance. Thus, although recognizing the limitations of performed assays, the subsequent analysis in athlete's population was considered of importance. As shown in the following section, in those cases where significant variations were found between different physical fitness or sport disciplines, statistical difference in biomarkers was so clear-cut that it cannot be attributed to variations in precision and accuracy.

The stability demonstrated for all the biomarkers during storage and transportation is important in ensuring the reproducibility of results obtained in the same or in different laboratories and supported the concordance of inter-laboratory validation.

4.2. Biomarkers values in selected population of athletes

The first important result of our study is that elite athletes showed their own baseline concentrations of serum IGF-II, IGFBP-2 and IGFBP-3, different from those of sedentary individuals and recreational athletes (although in case of IGF-II, difference between elite and recreational athletes is not significant). Moreover, also among different sport disciplines, biomarkers such as IGFBP-3 and ICPT were different at baseline. Specifically, syncronized swimming and rhythmic gymnastics athletes were the ones with different IGFBP-3 and ICTP baseline concentrations, as compared to athletes from other sports. It has to be observed that in both cases, athletes are only females and that the rhythmic gymnastics group was the one formed by the youngest subjects and age could be the main reason for the difference, as already shown especially in case of collagen turnover biomarkers [7,9].
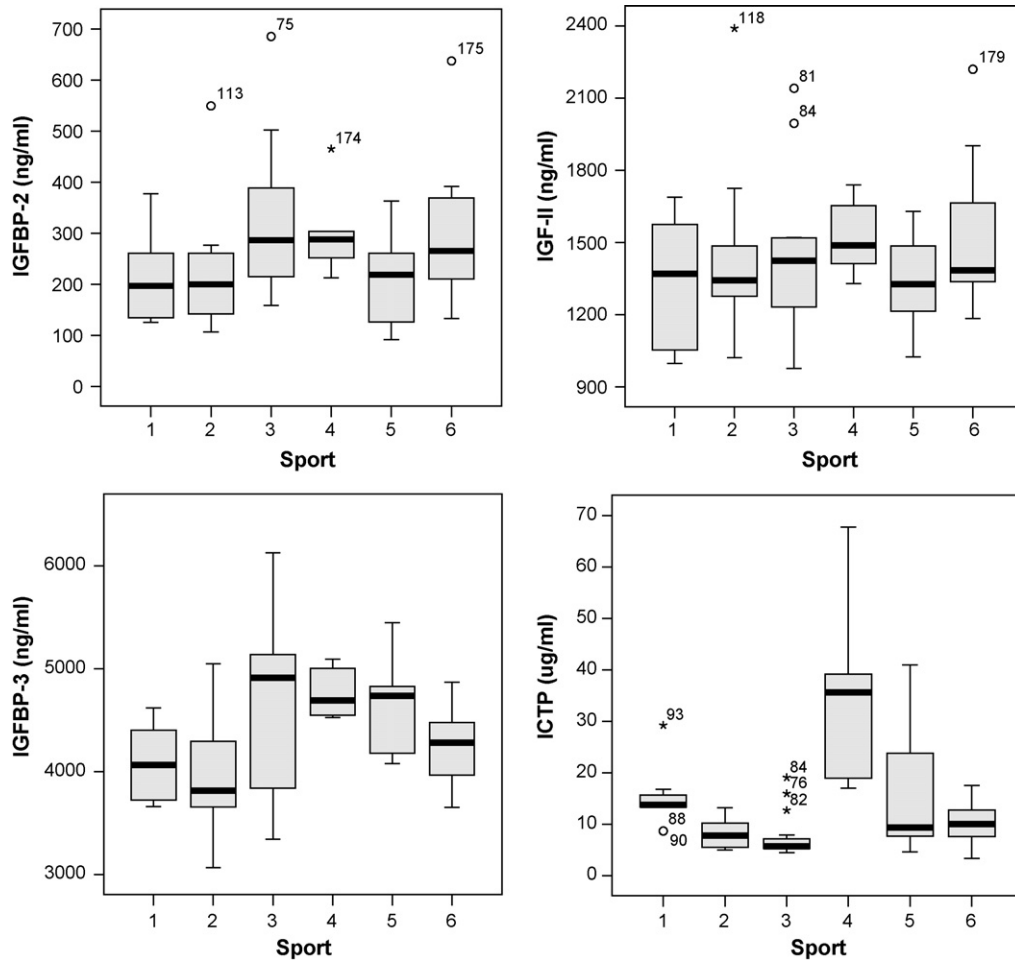
Fig. 3. Box-plots represent concentrations in basal conditions of IGF-II, IGFBP-1, IGFBP-2 and ICTP serum concentrations in: (1) swimming; (2) synchronised swimming; (3) taekwondo (4) rhythmic gymnastic (5) weightlifting (6) triathlon at basal conditions. The mean (solid line) and the 95% intervals of confidence (dashed lines) are shown. Empty circles represent outlier values; stars represent extreme values.

The second evidence is that different training workload during sport season influences serum IGFBP-2, IGFBP-3 and ICPT concentrations in elite athletes. Nonetheless, these variations are statistically significant only in case of taekwondo athletes and weightlifters, while in the other cases only trends towards changes during sport season were observed. This latter result could be due to the few number of athletes in different sport disciplines.

In details, IGFBP-3 serum concentration was significantly lower in taekwondo athletes. Taking into account that IGF-I is predominantly bound to this binding protein, this result is in accordance with the decrease in IGF-I, previously observed in these athletes during training season [9]. We can hypothesize that the specific and prolonged training workload of this combat sport discipline decreases IGFBP-3 reservoirs and consequently IGF-I serum circulation. This fact could also explain the increment of IGFBP-2 serum levels in taekwondo athletes. Probably, IGFBP-2, as secondary alternative carrier for the IGFs, increases in response to IGFBP-3 decrease.

Finally, ICTP transiently increased during training in taekwondo athletes as compared to the other sporting groups, as it was after acute exercise [4,7], probably reflecting increased bone and connective turnover in response to minor injuries caused by this particular sport discipline [7].

In summary, the data here presented indicate that physical fitness, and specific training workload in different sports can affect IGF-II, IGFBP-2, IGFBP-3, and ICTP serum values, but these varia-tions, although statistically significant in many cases, are within normal athlete population ranges [3,4,7]. Indeed, the observed changes neither get near nor overlap values observed after rhGH administration, for serum IGFBP-2, IGFBP-3, and ICTP [3,4]. Nevertheless, the results presented here can only be initial observations applicable in the context of the studied Caucasian athlete's population.

5. Conclusion

We evaluated four immunoassays for measuring serum levels of IGF-II, IGFBP-2 and-3, and ICTP. Although the majority of evaluated assays showed acceptable performance, the overall reliability appeared not always suitable for antidoping control analysis. An harmonization of some analytical parameters (e.g. precision and accuracy and an estimated LOQ below the first point of the calibration curve) and immunoassay performance, that may affect the results is needed with the aim of improving reproducibility and comparability between different laboratories and in different studies to define mathematical models for detection of rhGH exogenous administration.

The observed variations in serum concentration of different biomarkers in elite athletes *vs* recreational athletes or sedentary individuals and between different sport disciplines and training season in elite athletes should be taken in careful consideration together with age and gender in the hypothesis of

setting athlete's reference concentration ranges for doping detection.

Acknowledgments

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