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Evaluation of immunoassays for the measurement of insulin and C-peptide as indirect biomarkers of insulin misuse in sport: Values in selected population of athletes

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ABSTRACT

Insulin and C-peptide have been proposed as possible biomarkers of human insulin hormone misuse in sport. An extended intra- and inter-laboratory validation of commercially available immunoassays was performed.

Enzyme Amplified Sensitivity Immunoassay (EASIA) assays (Human Insulin-EASIA and C-peptide EASIA kits from BioSource) were evaluated for insulin and C-peptide in serum.

The intra- and inter-laboratory precision and accuracy values were good for the evaluated assays with maximum imprecision and inaccuracy of 16% and 23%, respectively, obtained just for one day C-peptide assay evaluation. The range of concentrations found in serum samples under investigation was always covered by the calibration curves of the studied immunoassays. However, a 19.7% of the samples felt below the estimated insulin limit of quantification.

High concordance between laboratory results was obtained for insulin assay (intraclass correlation coefficient –ICC = 0.857), whereas that for C-peptide was lower (ICC = 0.539).

Evaluated immunoassays were used to measure serum concentrations of insulin and C-peptide in elite athletes of various sport disciplines at different moment of training season, in recreational athletes at baseline conditions and finally in sedentary individuals. Serum insulin was statistically lower both in recreational and elite athletes when compared to sedentary individuals. Among elite athletes, the specific sport affected serum insulin (e.g., weightlifting) and C-peptide (e.g., triathlon) concentrations. Over the training season, a within athletes variability was observed for taekwondo, swimming and weightlifting athletes. Variations due to those aspects should be taken in careful consideration in the hypothesis of setting reference concentration ranges for doping detection.

1. Introduction

Insulin is among the proteic compounds reported to be abused in sport, particularly in body building [1–3]. Several facts support the hypothesis that insulin administration positively influences performance, e.g. by increasing muscle glycogen utilizing hyperinsulinaemic clamps prior to sports events or during recovery phases. Muscle glycogen stores are the primary source of carbohydrate during exercise. Thus, the greater the muscle glycogen stores, the longer the exercise time to exhaustion. Moreover, insulin has been shown to work in synergy with steroids. Steroids spawn new muscle whereas insulin inhibits catabolism in muscle and liver by increasing the synthesis of glycogen and proteins and promoting the entry of glycogen and amino acids into muscle cells before an event, thereby improving endurance [4]. Indeed, recent studies have indicated that use of insulin increases muscle size by its action in inhibiting protein breakdown [5–8].

Since 1999, insulin has been included in the list of prohibited substances of the International Olympic Committee (IOC) and the World Anti-Doping Agency (WADA), with the exemption for those

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athletes demonstrably suffering from diabetes [9,10] and in several recent WADA hearings insulin misuse has been frequently mentioned in terms of doping offences.

As insulin has a half life of 4 min in the human body, it vanishes rapidly and would be very difficult to detect [2]. Indeed, despite the recent establishment of efficient methods to determine recombinant modified insulins, the issue of detecting the misuse of recombinant human insulin has not been solved yet [11].

Regarding insulin biosynthesis, modifications liberate the centre portion of the molecule, or C-peptide (Connecting peptide, half life ranging between 24.9 and 45.3 min), from the C- and N-terminal ends of the proinsulin. Most proinsulin (~85%) is converted to insulin and C-peptide, which are then secreted in equimolar amounts into serum. Measurement of C-peptide in serum can reflect the effectiveness of the conversion of proinsulin to insulin in the beta cells since its only source is from endogenous pancreatic beta cells. Thus, the amount of C-peptide in serum can help in distinguishing endogenous from exogenous insulin sources [12]. It has been demonstrated that endurance training and dietary variation in elite athletes affects serum concentration of both insulin and C-peptide [13,14].

In the eventuality of proposing the use of serum insulin and C-peptide as indirect biomarkers of insulin abuse in sport, the existence of reliable assays for their measurement should be needed to assure that both in healthy volunteers and athletes in different situations, the concentrations measured are independent of the used method.

In addition, the variations of serum insulin and C-peptide concentrations due to different physical performance, different sport discipline and training workloads can be relevant when trying to interpret concentrations due to physiological variabilities in sportsmen from those coming from exogenous insulin administration.

To our knowledge, none of these two aspects has been investigated up to now.

In this paper, an extended validation of commercially available immunoassays for insulin and C-peptide (one for each studied biomarker) is reported together with 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 performed in two independent laboratories: Drug Research and Control Department, 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 evaluated for the insulin measurement was the Human Insulin- Enzyme Amplified Sensitivity Immunoassay (EASIA) kit from BioSource (Nivelles, Belgium). Calibration samples were analysed always in quintuplicate at the following concentrations: 0, 5.1, 13.8, 44.4, 128, 324 μIU/ml. Quality control samples (QC) were supplied by the manufacturer (QC1, acceptance range 12.4–20.6 μIU/ml and QC2, acceptance range 39.7–66.1 μIU/ml).

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, 1.8, 5.5 pmol/ml. Calibration samples 3 and 5, supplied by the manufacturer, were used as control samples (QC3, acceptance value 0.16 pmol/ml and QC4, acceptance value 1.80 pmol/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 insulin and C-peptide consisted of the following studies.

2.2.1. Intra-laboratory validation

Intra-laboratory validation protocol consisted in four assays. They were performed by Laboratory 1 along 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 manufacturer. 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-assay 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 (R.S.D.%) of the performed measurements. In the case of QC samples 1 and 2 (insulin), 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. For QC sample 3 and 4 (C-peptide), 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 samples.

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) three and ten 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 human serum samples 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 analyzed 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 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.

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

	Number (♂/♀)	Age (years)	Height (cm)	Training (h/week)	Sample collection[a]
Sedentary	38/26	30 ± 4	170 ± 9	–	9.00 h
Recreational athletes	55/25	30 ± 4	173 ± 8	5–10	Basal
Elite athletes	41/36	22 ± 5	173 ± 8	7–35	Basal Training Competition
Swimming	3/9	18 ± 2	175 ± 7	up to 35	Basal Training
Synchronized swimming	–/14	23 ± 3	170 ± 6	up to 25	Basal Training
Taekwondo	10/6	21 ± 4	175 ± 10	up to 20	Basal Training Competition
Rhythmic gymnastic	–/6	16 ± 4	162 ± 5	up to 35	Basal Training
Triathlon	16/–	27 ± 6	177 ± 4	up to 25	Basal
Weightlifting	12/1	22 ± 5	173 ± 8	up to 14	Basal Training Competition

[a] 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.

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 0900 hours 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 °C until analysis. Serum samples from study subjects, apart those used for inter-laboratory validation tests, were examined in triplicate by Laboratory 1.

2.5. Calculations and statistical analysis

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

Concerning inter-laboratory validation, the intraclass correlation coefficient (ICC) was calculated using the random effects mode to evaluate the agreement of results between laboratories [15]. To evaluate the dispersion of the results obtained between different laboratories, a modification of Bland–Altman plots was used [16]. 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 ± 1.96 × 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.

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: $r^2 = 0.9902 \pm 0.002$ for insulin, $r^2 = 0.9922 \pm 0.014$ for C-peptide.

The performance of the insulin assay, for both QC samples (QC1 containing concentrations of analyte between the second and third calibration samples and QC2 containing concentrations of analyte between the third and the fourth calibration sample) was good in terms of precision (R.S.D.% always lower than 10%) and accuracy. However, estimated LOQ was higher than the first calibration point of calibration curve.

The performance of the C-peptide assay for QC samples (QC3, being the third calibration sample and QC4 being fifth calibration sample) was still within the international established criteria [17–19], with maximum imprecision and inaccuracy of 16 and 23%

Table 2
Validation parameters for insulin and C-peptide obtained in Laboratory 1.

Insulin	Intra-assay		Inter-assay		
QC	μIu/Ml	Precision (%[a])	Accuracy	Precision (%[a])	Accuracy
1	12.4–20.6[b]	1.6–5.0	Correct	5.4	Correct
2	39.7–66.1[b]	4.3–5.3	Correct	9.7	Correct
LOD (μUI/ml)		4.9			
LOQ (μUI/ml)		9.0			
Calibration range (μUI/ml)		5.1–324			
C-peptide	Intra-assay		Inter-assay		
QC	pmol/ml	Precision (%[a])	Accuracy[c]	Precision (%[a])	Accuracy[c]
3	0.16	3.7–6.9	4.3–11.4	10.0	8.0
4	1.80	2.9–8.1	5.3–23.7	16.0	12.7
LOD (pmol/ml)		0.04			
LOQ (pmol/ml)		0.06			
Calibration range (pmol/ml)		0.07–5.50			

[a] Measured as relative standard deviation (R.S.D.).
[b] Acceptance concentration range according to the manufacturers.
[c] Measured as the relative error respect the assigned QC sample value.

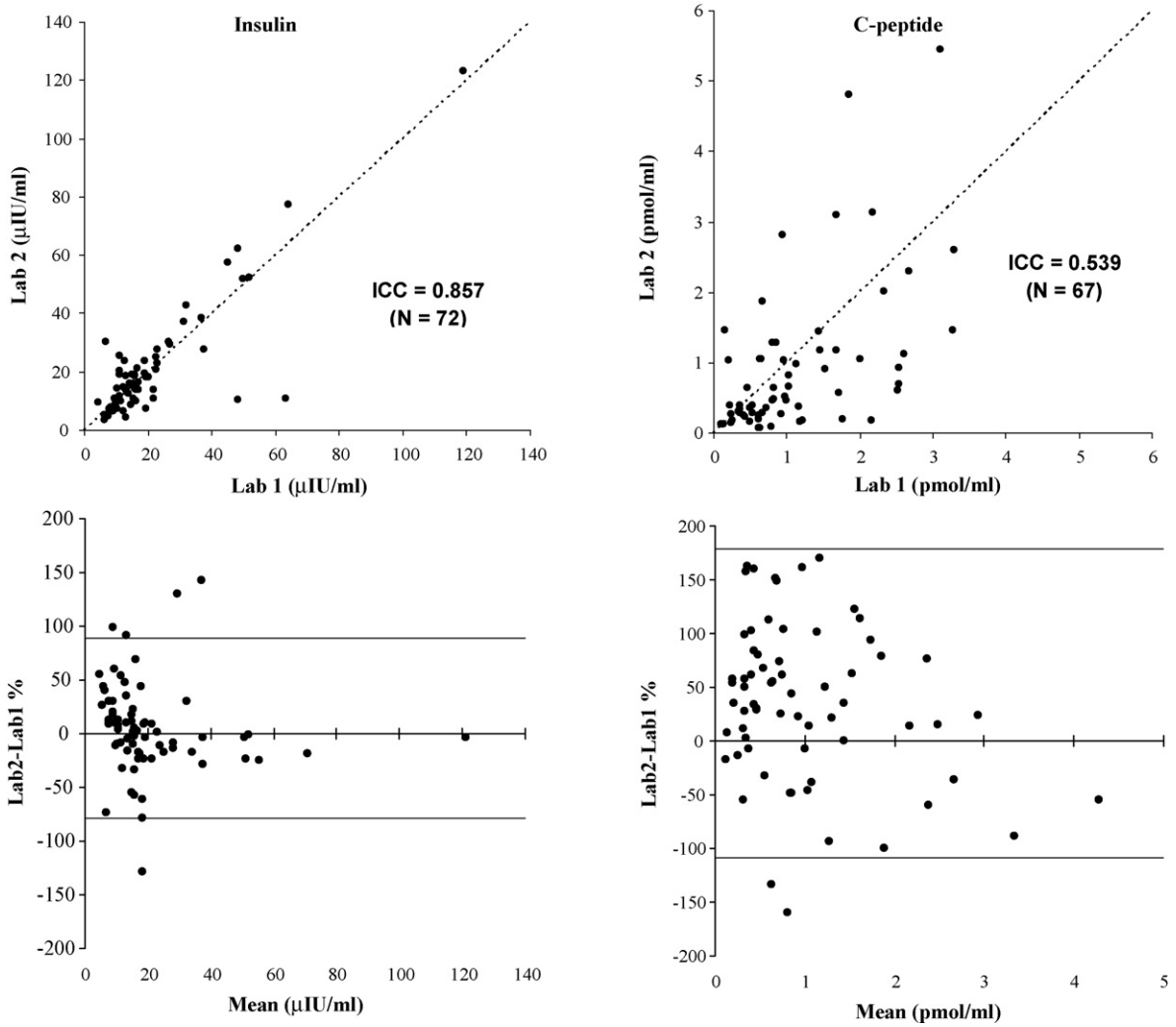

Fig. 1. Inter-laboratory comparison of insulin and C-peptide assays. Top: Graphical comparison between laboratories and intraclass correlation coefficient (ICC), dotted lines represent total concordance. Bottom: Modified Bland–Altman plots (see text), dotted lines represent the 95% limits of agreement.

obtained just for a single C- peptide one-day assay evaluation. Furthermore, estimated LOQ was lower than the first calibration sample.

The results of inter-laboratory validation for insulin and C-peptide EASIA assays are presented in Fig. 1. Better agreement between results was obtained for Insulin ($ICC_{INS} = 0.857$) than for C-peptide assay ($ICC_{C\text{-}PEP} = 0.539$). However, the spread of obtained results was always very high: insulin (95% LA = −78.8–88.5%) and C-peptide (95% LA = −109.2–178.8%).

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 insulin and C-peptide in the analysed recreational athletes group. Analysis of variance showed that neither gender nor age influenced the analysed biomarkers. However, these two variables were not taken into consideration as covariates for further statistical analysis of data.

3.3.1. Factors affecting biomarker concentrations

Significantly higher baseline serum insulin concentrations were observed in sedentary individuals vs both recreational and elite athletes ($p < 0.001$) Differently, C-peptide baseline values were similar in the three study groups (Table 3, Fig. 2).

Among elite athletes, the specific practiced sport affected baseline serum insulin (highest values in Weightlifting vs all the other studied disciplines) and C-peptide (highest values in Triathlon vs all the other disciplines) (Table 3, Fig. 3).

When comparing values during training in different sports, significant higher values of insulin and C-peptide were observed for Weightlifting than for Synchronised Swimming ($p = 0.005$) and statistical higher C-Peptide values for Weightlifting vs Taekwondo ($p < 0.001$) (Table 3, Fig. 3). Within the same sport, changes in insulin and C-peptide along the sport season were observed for Taekwondo, Swimming and Weightlifting athletes. However, these variations did not show the same trend. Serum insulin and C-peptide increased along the sport season in Taekwondo and Swimming, while insulin decreased in Weightlifting. Taking all the athletes as a whole, the "within-athlete" coefficients of variation of insulin and C-peptide concentration along the sport season were 30.1% and 47.2%, respectively.

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

Group	Time	Insulin (μIU/ml)	C-peptide (pmol/ml)
Sedentary	B	26.33 ± 23.05[2,3]	0.76 ± 0.61
Recreational athletes	B	15.94 ± 11.16[1]	1.10 ± 0.84
Elite athletes	B	13.29 ± 9.86[1]	0.96 ± 0.87
Taekwondo	B	11.10 ± 2.56[5]	0.57 ± 0.25[9]
	T	11.84 ± 1.73	0.57 ± 0.12[5]
	C	16.13 ± 2.14 *	1.16 ± 0.15 *
Weightlifting	B	20.39 ± 5.04[4,6,7,8,9]	1.22 ± 0.30[9]
	T	14.99 ± 3.55[7,*]	1.17 ± 0.25[4,7]
	C	16.93 ± 4.34	1.37 ± 0.30
Swimming	B	9.36 ± 3.33[5]	0.38 ± 0.26[9]
	C	14.58 ± 2.80*	0.60 ± 0.20*
Synchronized Swimming	B	11.08 ± 2.51[5]	0.63 ± 0.20[9]
	T	9.42 ± 1.93[5]	0.42 ± 0.14[5]
Rhythmic Gymnastics	B	9.15 ± 4.18[5]	0.58 ± 0.33[9]
	T	10.08 ± 2.99	0.56 ± 0.21
Triathlon	B	15.73 ± 2.72[5]	1.97 ± 0.22[4,5,6,7,8]

Abbreviators: B, baseline; T, training; C, competition.
Significantly different ($p < 0.05$) from: [1]Sedentary; [2]Recreational athletes; [3]elite athletes; [4]Taekwondo; [5]Weightlifting; [6]Swimming; [7]Synchronized Swimming; [8]Rhytmic gymnastic [9]Triathlon; * Baseline concentrations.

4. Discussion

4.1. Immunoassays evaluation

As already stated, before proposing eventual indirect biomarkers of insulin 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.

Results obtained in this study show that tested assays were reliable and suitable for measurement of biomarkers concentrations in

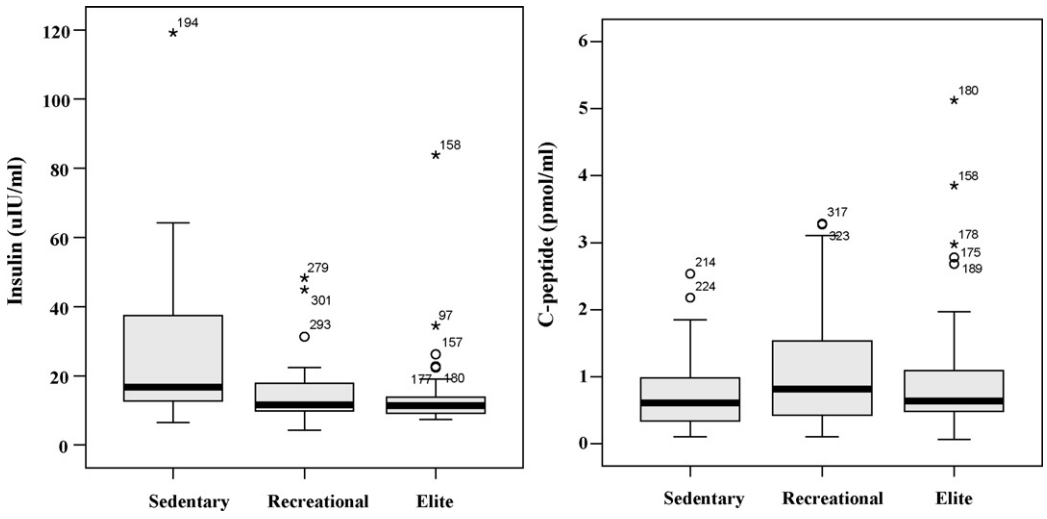

Fig. 2. Box-plots represent change of insulin and C-peptide serum concentrations in: (1) sedentary persons; (2) recreational athletes; (3) elite athletes at basal conditions. The mean (solid line) and the 95% intervals of confidence (dashed lines) are shown. Empty circles represent outlier values; stars represent extreme values.

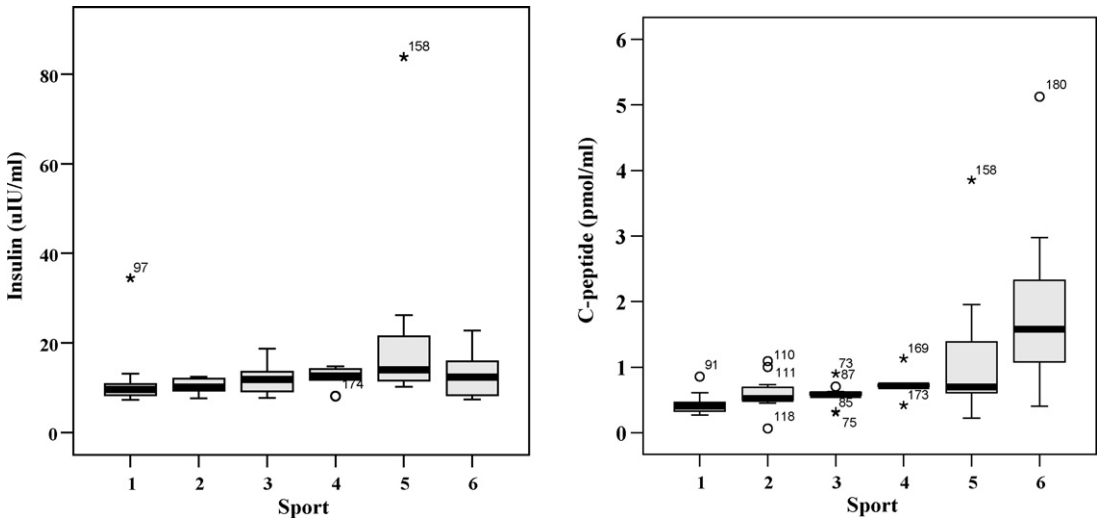

Fig. 3. Box-plots represent change of insulin and C-peptide serum concentrations in: (1) swimming; (2) synchronised swimming; (3) taekwondo (4) rhythmic gymnastic (5) weightlifting (6) triathlon at basal conditions. The mean (solid line) and the 95% intervals of confidence (dashed lines) are shown. Empty circles represent outlier values; stars represent extreme values.

investigated subjects. Indeed, insulin and C-peptide values found in serum samples under investigation were always within by the calibration curves of the studied immunoassays and fell in a part of curves where precision and accuracy were both acceptable. However, a 19.7% and 1.2% of the samples felt below the estimated LOQ for insulin and C-peptide, respectively (Fig. 1). The stability demonstrated for all the biomarkers during storage and transportation is important to ensure the reproducibility of results obtained in the same or in different laboratories.

In spite of that, whereas good concordances between results in the two laboratories were obtained for insulin assay, this was not true in case of C-peptide assay and due to poor concordance, dispersion of the results was very high. Inter-laboratory validation is important in sports drug testing, taking into consideration that a result should be reproducible in different laboratories located in different parts of the world, specially if cut-off values based in these biomarkers are going to be established for insulin misuse in sport.

Although it can be recognized that other assays could have been evaluated in order to obtain the best possible performance parameters, immunoassays evaluated in the present study were chosen from the more commonly used and commercially available as a first attempt in the evaluation of immunoassays for the measurement of insulin and C-peptide as indirect biomarkers of insulin misuse in sport applying specific requirements for doping purposes [17].

Furthermore, the good reliability obtained in the intra-laboratory validation parameters made the subsequent analysis in athletes population of importance.

4.2. Biomarkers values in selected population of athletes

Before proposing eventual indirect indicators of insulin (and eventually rGH) misuse in athletes, it is necessary to investigate the physiological fluctuations of serum concentration of those 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 substances misuse [20].

The first important result of our study is that sedentary individuals showed their own baseline concentrations of serum insulin, significantly higher than those of recreational and elite athletes. These results are in accordance with previously observed in ice-hockey players with respect to sedentary persons [13]; indeed, at baseline conditions, these athletes had significantly lower baseline insulin levels compared with the sedentary group. However, in contrast with our results on the C-peptide baseline values similar in the three study groups the ice-hockey players, tended to have baseline C-peptide levels lower than sedentary group. Nevertheless, these players had a higher body mass index as compared with the sedentary group and had carefully designed dietary changes in fat and carbohydrate intake. As a result of that, the decrease in relative fat content and simultaneous increase in carbohydrate content were associated with a decrease in both insulin and C-peptide levels [13].

It has been established that athletes displayed markedly increased insulin sensitivity [21]. The high insulin sensitivity in athletes may be explained by increased sensitivity to insulin in each insulin-responsive cell or increased number of such cells. This high sensitivity was matched by a adaptively reduction in insulin secretion resulting in unchanged disposition index. [21]. It has been also showed that serum insulin concentrations at rest were significantly higher in sedentary people compared with the trained individuals [14].

Among different sport disciplines, insulin and C-peptide serum levels were different at baseline. In particular, weightlifting and triathlon athletes were the ones with different insulin and C-peptide baseline concentrations respectively, when compared to athletes from other sports (Table 3). It can be observed that in both cases, athletes were only males (apart for one female in weightlifting athletes), and with the highest body mass index. Moreover, triathlon athletes were also the oldest (Table 1).

There is some evidence that insulin clearance rates in healthy adult subjects differ between the sexes and decline with age [22–24] and are influenced by factors such as sedentariry, the degree of obesity [25] and circulating GH concentrations [26]. Moreover, serum insulin and C-peptide concentrations increase with aging and are lower in trained than in sedentary individuals, so that exercise training seems to prevent the aging-associated increase in insulin resistance reflected in elevated insulin and C-peptide concentrations [14].

The second evidence is that different training workload during sport season influences serum insulin and C-peptide. Nonetheless, these variations are statistically significant only in case of taekwondo, swimming and weightlifting athletes.

It is well established that serum insulin concentrations decrease during physical exercise. This has been noticed during graded as well as prolonged exercise [27]. It has been also shown that the

degree of reduction in serum insulin concentrations with physical training is dependent on variations in body fat, glucose tolerance, and route of glucose administration [28–30]. Insulin serum concentration variations in weightlifting athletes are in agreement with these last observations, while the increase in serum insulin during training season in taekwondo and swimming athletes is are not.

These two latter sport disciplines presented a similar increase in serum C-peptide during training season.

Concerning the use of C-peptide as a marker to detect insulin misuse in sport, we observed a correlation ($r^2 = 0.640$) between serum insulin and C-peptide in those athletes who showed significant variations of these parameters during the sport season. Since C-peptide is uncorrelated with synthetic insulin, a comparison between the two values could indicate a possible abuse of insulin for doping purposes.

In summary, the data here presented indicate that physical fitness and specific training workload in different sport disciplines can affect insulin and C-peptide serum concentrations. These preliminary results should be confirmed in a higher number of athletes. At moment, given high variability among individuals from different sports during different periods of training, these measurements cannot stand alone as a reason to disqualify an athlete. Nonetheless, they demonstrate that although these statistically significant variations are within normal population values, specific concentration ranges for elite athletes can be hypothesized to distinguish between normal physiological fluctuation of these biomarkers and those caused by exogenous intake of insulin (and eventually rGH).

5. Conclusions

Two immunoassays for measuring serum levels of insulin and C-peptide have been evaluated as indirect biomarkers of insulin misuse in sport. Although the evaluated assays showed acceptable performance and reliability suitable for antidoping control analysis, a harmonization of some analytical parameters that may affect the inter-laboratory concordance of results is needed to improve reproducibility and comparability between different laboratories and in different studies to define mathematical models for detection of insulin exogenous administration.

The observed variations in serum concentration of different biomarkers in elite athletes vs recreational athletes or sedentary individuals and among 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.

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