Naud J[1], Julien A[2], Ayotte C[1]

Statistical analysis of the hGH recombinant and pituitary ratios obtained from routine testing of athletes samples with the CMZ isoform kits

Doping Control Laboratory, INRS-Institut Armand-Frappier, Laval, Canada[1]; Faculté des sciences et de génie-Service de consultation statistique, Université Laval, Québec, Canada[2]

Abstract

Circulating hGH consists of several isoforms, dimers and oligomers. In comparison, recombinant hGH which can be abused for performance enhancement, is a monomeric 22 kDa species. A commercial immunoassay was developed by Bidlingmaier and Strasburger and commercialized to WADA accredited laboratories to detect hGH administration in athletes' serum samples. Two kits, both of which must be used for confirmation, are composed of one recombinant (rec) and one pituitary (pit) assay. The ratio of rec to pit is calculated and has to exceed the decision limits of the guidelines first published by WADA in 2010 to be reported as an adverse analytical finding. Close to 20 samples were reported positive in the following two years by several laboratories. However, in March 2013, all testing was halted following a decision rendered by CAS in the case of Veerpalu vs. FIS (CAS 2011/A/2566). The main reason to grant the appeal was related to the determination of the test's decision limits, which had not been not published in peer-reviewed literature.In that context, we undertook in 2013 a biostatistical analysis of our data collected from the analysis of more than 3000 male and female samples received for routine testing since 2010. Decision limits were established in these groups with a parametric bootstrap method and were found to support those recommended by WADA in 2010.The details of the biostatistical analysis, number of tests, statistical approach, distribution of the populations and the calculated decision limits are presented.

Introduction

HGH is a hormone necessary for the skeletal growth that is synthesized and secreted by cells located in the pituitary gland at the base of the brain. It is naturally produced in humans. Recombinant hGH is also available artificially and is believed to be abused by athletes in order to increase performance [1,2]. Circulating hGH consists of several isoforms, the 22 kDa being most abundant, followed by the 20 kDa [3,4]. The administration of exogenous hGH changes the proportion of various hGH isoforms in blood by increasing the proportional share of the 22 kDa isoform compared to others [5]. Even though the concentration of total hGH will vary substantially, it is assumed that the ratio between the relevant types of hGH isoforms measured by the test remains relatively stable.
This test was first utilized during the 2004 Summer Olympic Games in Athens. Following production and validation of the commercial kits on an improved technical platform, tests were conducted during the 2008 UEFA European Football Championship and the Summer Olympic Games in Beijing. In 2010, having evaluated the distribution of thousands of values obtained internationally, WADA determined the decision limits, and published a Guidelines [6].
The present statistical study was initiated when the test was halted to verify whether the decision limits adopted by WADA were supported by the results obtained in the laboratory from 2010 until spring 2013. The distribution of the populations and the 99.9 percentile are presented and compared to the decision limits contained in the 2010 guidelines for hGH testing.

Experimental

Reagents
Preparation of the quality controls (QCs), human pituitary growth hormone and somatrophin were purchased from NIBSC (Potters Bar, Hertfordshire, UK) (code 80/505 and 98/574, respectively), goat serum and BSA from Sigma-Aldrich (St Louis,

 ISBN 978-3-86884-040-7

MO, USA). hGH LIA kit1 and kit2 are produced by CMZ (CMZ-Assay GmbH, Berlin, Germany).

Samples
Blood samples collected in BD SST II tubes were obtained from athletes as part of routine testing programs. After their reception, the tubes were centrifuged to obtain serum. Only samples that had a concentration over the LOQ of 0.02 ng/mL (determined during validation, vide infra) were conserved for the statistical analysis.

Assay Method
Sera were analysed with hGH LIA kit1 or kit2 following the protocol provided by the manufacturer. This hGH isoform differential test is based on a dual-antibody sandwich-type immunoassays with one antibody labelled with acridinium ester to produce a luminescent signal detected on a Berthold luminometer [5,6]. The assay is controlled by in-house QCs that are prepared by spiking goat serum with pituitary human growth hormone or somatrophin at a final concentration of 2 ng/mL. The result of the test is expressed as a “rec/pit” ratio calculated from the concentrations measured with each kit.

Assay limit of quantification
Limit of quantification was defined as the lowest concentration measured with a laboratory repeatability (s_r) below 15% and an intermediate precision (s_w) under 20%. The intra-assay CV and Inter-assay CVs were determined by the measurement of 5 samples at pit concentrations ranging from 0.1 ng/mL to 10 ng/mL and at rec concentrations ranging from 0.25 ng/mL to 25 ng/mL. Samples were analysed in 5 independent assays by 2 operators on 5 days. All samples were serially diluted with sheep serum. Somatrophin was added to each sample to obtain a ratio close to the DL (as recommended in the 2010 Guidelines).

Statistical Analysis
Only blood samples that had a rec or pit assay concentration over the limit of quantification were used. They were regrouped into different categories, i.e. male or female and analysed on kit 1 or kit 2. To calculate the distribution of the population for each group, a parametric bootstrap method was utilised. To that end, the data for each group was analysed to find the distribution F(a,b) that fitted best and the parameters were noted. The 99.9 theoretical percentile of each distribution F(a,b) for the different categories was also calculated. For each distribution F(a,b), a bootstrap sample containing the same number of observations was created. This step was repeated randomly to generate 1000 samples. The parameters of the new distributions F(a*,b*) were again noted and the 99.9 theoretical percentile for each new distribution was calculated. Finally, the 0.5 and 99.5 percentiles of the 1000 percentiles obtained correspond to the limits of the confidence interval at 99%. The extent of the confidence interval is inversely related to the size of the original sample.

Results and Discussion

Preliminary Analysis of data from initial test:
Over the period of 2010 to 2013, 3924 blood samples were received in the laboratory for hGH testing (Table 1). Of those, 3511 (89.5%) had a rec or pit concentration over the LOQ of 0.02 ng/L and were utilised for the statistical analysis. Among these, 334 sera (9.5%) were from female athletes and were analysed on kit1 (248 samples, 7.1%) or kit2 (86 samples, 2.5%). The remaining 3177 sera were from male athletes and were also analysed on kit1 (2612 samples, 74.4%) or kit2 (565 samples, 16.1%).

Group		N	Distribution	Parameter 1	Parameter 2	99.9 Percentile	Confidence Intervals at 99%		Outliers
Gender	Kit						Lower Limit	Higher Limit	
F	1	249	Log Normal	-0.7519	0.4043	1.6444	1.4070	1.9104	0
F	2	88	Gamma	5.3359	0.1131	1.7366	1.3654	2.0713	0
M	1	2605	Gamma	5.6779	0.100	1.5920	1.5327	1.6499	3
M	2	564	Normal	0.6275	0.2428	1.3779	1.3148	1.4443	7

Table 1: Data sorted according to the gender and kit used during screening procedure. The parameters and the related distribution retained for each group are indicated. The statistical limits at 99.9% and their corresponding confidence intervals are also noted.

ISBN 978-3-86884-040-7

As observed recently [7], the kit- and gender-specific distribution of rec and pit concentrations have longer right tails. To obtain a better representation of the distribution, we display the concentrations as boxplots with a Log scale (Figure 1).
For both kit1 and kit2, the proportion of low hGH values is higher for male than female athletes. For kit1, the median of rec and pit values observed for male and female athletes are 0.067 and 0.140 ng/mL, and 0.515 and 1.239 ng/mL, respectively. For kit2, the median of rec and pit concentrations for males are 0.217 and 0.427 ng/mL, whereas for females, the median of rec and pit values are 0.981 and 1.843 ng/mL. However, the medians of the kit- and gender-specific ratios are closer with values of 0.550 and 0.481 for male and female on kit1 and 0.630 and 0.563 for male and female on kit2.

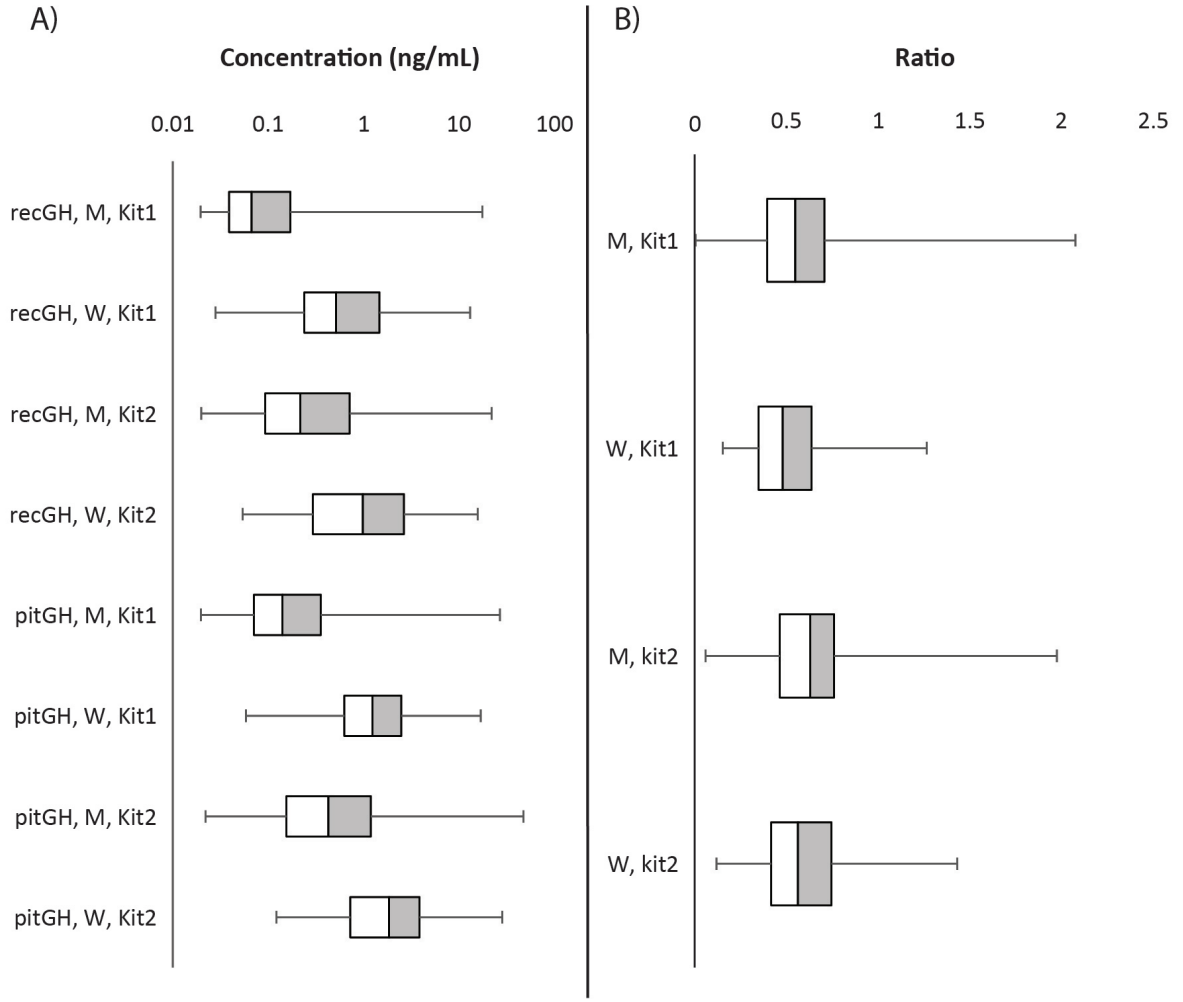

Figure 1: Data dispersion from routine testing samples for the kit- and gender-specific distribution of rec and pit concentrations and their ratio. The left and right boundaries of each boxplot are the 25th and 75th centiles, and the band inside the box indicates the median. The whiskers extend to the minimum and the maximum values observed.

Distribution of ratios:

All ratios with concentrations over the LOQ are displayed in Figure 2 and 3. As expected, a wider dispersion is observed when the concentrations of rec and pit are lower than the Laboratory LOQ (Figure 2 B and D). The distribution is narrower for samples with concentrations over 0.02 ng/mL, even for samples with a rec concentration below 0.100 ng/mL (data not shown). It must be noted that 52% of all the sera tested would have been considered automatically negative according to the WADA 2010 Guidelines, since their rec value was lower than 0.100 ng/mL. In the new Guidelines published in June 2014, this “limit” was increased to 0.150 ng/mL [8]. Based on our results, there is no evidence supporting the exclusion of test results when they are measured over the laboratory LOQ and it is unclear why WADA maintained that requirement.

 ISBN 978-3-86884-040-7

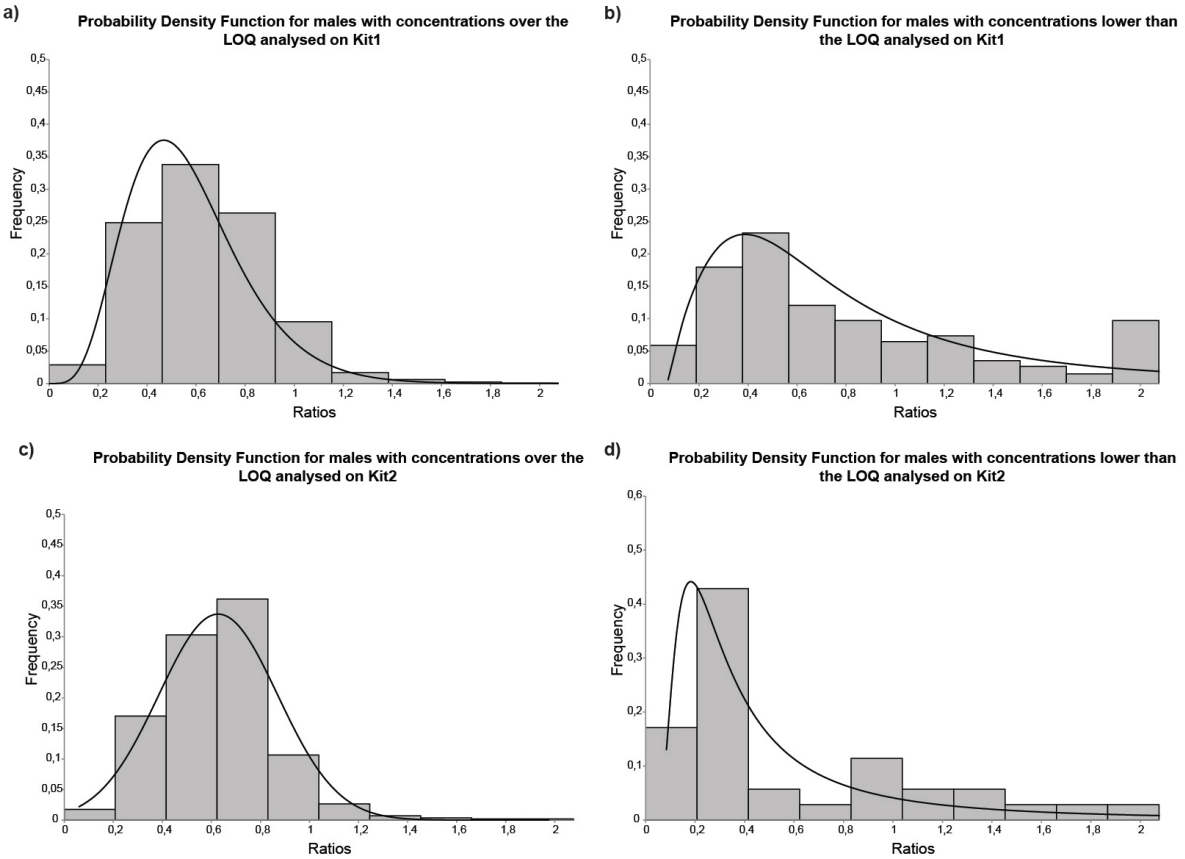

Figure 2: Probability density function for ratios observed on kit 1 or kit2 for male athletes. All ratios were sorted according to the kit used for values over or lower than the LOQ. Their relative frequencies were calculated and plotted as histogram. The distribution retained for each group was added to each graph. The distributions for samples with concentrations below LOQ are shown to indicate the difference of the population observed but they were not included in the calculation of the statistical limits.

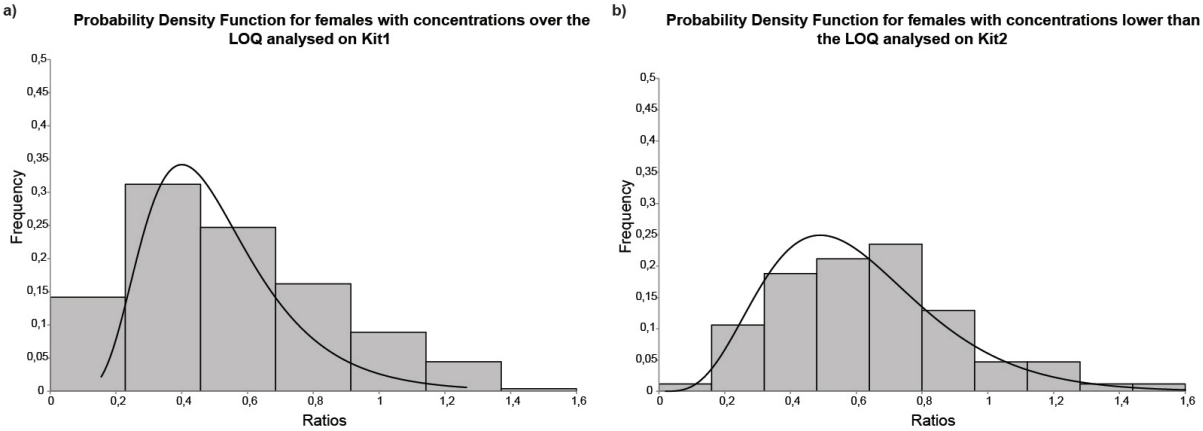

Figure 3: Probability density function for ratios observed on kit 1 or kit2 for female athletes. All ratios were sorted according to the kit used for values over the LOQ. Their relative frequencies were calculated and plotted as histogram. The distribution retained for each group was added to each graph.

 ISBN 978-3-86884-040-7

"Decision Limits":
For the modeling, all ratios obtained in the laboratory were considered as negative and values obtained on kit 1 or kit 2 were considered as independent. We were unable to find a single distribution model that could fit the datasets obtained for the 4 different sub-categories. The best fit distribution for each group is displayed in Table 2. The distribution model for each group is subject to change with a larger set of results, particularly for the female athletes group which is quite small.
For all groups, 10 outliers were found based on the statistical limits calculated (data not shown). On these, 3 were found for kit 1 and 7 for kit 2. All abnormal observations were considered as negative and kept for the determination of the distribution for each group. Even if we used the superior limit of the confidence intervals, many of these observations stay abnormal. However, many of the outliers (56%) have a concentration in recGH that is lower than 0.100 ng/mL, and by default, considered automatically negative based on the Guidelines although it is impossible to know if these samples were really negative. The decision limits were determined from all the results, including the outliers, as long as the concentrations were above the LOQ. For female athletes the limits for kit 1 and 2 are 1.64 and 1.74, respectively. However, the extent of the confidence interval is more important because both groups are relatively small. For the male athletes, the calculated decision limits for kit1 and kit2 are 1.59 and 1.38, respectively. For all the groups, these results support the limits published by WADA in 2010 and 2014. Our limits are slightly lower due to i) all tests being done in the same laboratory; ii) the relative homogeneity of the athlete population (although there is no relation); iii) 99.9 theoretical percentile used during our statistical analysis while the limits were calculated by WADA using the 99.99 percentile in 2010. If the 99.99% theoretical percentile is used instead of 99.9%, the limits are respectively for male athletes and for kit 1 and 2, 1.90 and 1.53. For female athletes they are respectively, 2.12 and 2.08. The limits published in 2014, were calculated using a different statistical model where the influence of the rec and pit concentrations on the ratios were taken into account.

Conclusions

The biostatistical analysis presented here supports the previous and current decision limits published by WADA. Based on our results, there is no evidence suggesting that samples should be reported automatically negative when the rec concentration is below 0.150 ng/mL as long as over the laboratory LOQ. Even if we do not see a clear difference between ratios obtained with low or high concentrations of rec and pit, ongoing analysis is made to test this new statistical model with our data.

References

1. Nelson, A.E. and K.K. Ho, Abuse of growth hormone by athletes. Nat Clin Pract Endocrinol Metab, 2007. 3(3): p. 198-9.
2. Rigamonti, A.E., et al., Growth hormone abuse: methods of detection. Trends Endocrinol Metab, 2005. 16(4): p. 160-6.
3. Baumann, G., Growth hormone heterogeneity in human pituitary and plasma. Horm Res, 1999. 51 Suppl 1: p. 2-6.
4. De Palo, E.F., et al., Growth hormone isoforms and segments/fragments: molecular structure and laboratory measurement. Clin Chim Acta, 2006. 364(1-2): p. 67-76.
5. Bidlingmaier, M., et al., High-sensitivity chemiluminescence immunoassays for detection of growth hormone doping in sports. Clin Chem, 2009. 55(3): p. 445-53.
6. WADA-AMA, hGH isoform differential immunoessays for anti-doping analyses. 2010 (Version 1.0): p. 1-13.
7. Hanley, J.A., et al., hGH isoform differential immunoassays applied to blood samples from athletes: Decision limits for anti-doping testing. Growth Horm IGF Res, 2014.
8. WADA-AMA, hGH isoform differential immunoessays for anti-doping analyses 2014 (Version 2.1): p. 1-8.

Acknowledgements

We would like to thank the “Service de consultation statistique” of Université Laval who completed the statistical analysis.