

Yvette Dehnes<sup>1</sup>, Mads Drange<sup>2</sup>, Stian B. Ottersen<sup>3</sup>, Peter Hemmersbach<sup>1,3</sup>

## **Longitudinal profiles of indirect markers for GH misuse**

Norwegian Doping Control Laboratory, Oslo University Hospital, Oslo, Norway<sup>1</sup>; Anti-Doping Norway, Oslo, Norway<sup>2</sup>; School of Pharmacy, Oslo University, Oslo, Norway<sup>3</sup>

### **Abstract**

An indirect detection method for rhGH misuse using GH dependent markers has been under development for well over a decade, and measurement of the GH markers insulin-like growth factor-1 (IGF-1) and N-terminal peptide of type III procollagen (PIIINP) in combination with discriminant function analysis can differentiate between doped and not doped [1]. Final validations and establishment of population based thresholds were recently established [2]. However, methods based on population based threshold will always suffer from limited sensitivity due to the inter-individual variability these values must reflect. We wanted to see if we could increase the sensitivity of the method by establishing individual profiles of the markers and started up a research project in 2008 aimed at establishing individual profiles for IGF-1 and PIIINP. Volunteers belonging to top national power sport athletes, top national endurance sport athletes and bodybuilders (using rhGH), gave about 5 blood samples each over a period of 2 years. Promising results with regard to low individual variability in the markers for the power and endurance sport athletes, prompted Anti-Doping Norway to set up a monitoring program in order to create individual biomarker profiles of a limited number of athletes from their national registered testing pool. The rationale for this decision was to use the individual profile data to identify athletes for targeted testing with the hGH isoform method, and to gain experience regarding the prevalence of GH-doping amongst Norwegian athletes.

### **Introduction**

Testing for growth hormone has been a major issue for most anti-doping organizations for several years. How to approach the testing for this prohibited substance has also been a matter of discussion in Norway. First of all there has been a discussion about use: to what extent, in which sports, at which levels and for what purpose is it being used? Secondly there has been a discussion about how to test, to what extent and by which method.

In the fall of 2008, Norwegian Doping Control Laboratory, Anti-Doping Norway and the Norwegian School of Sports Sciences started up a research project aimed at establishing individual GH biomarker profiles and to determine within-subject variability. The rationale behind this was an assumption that rhGH testing would benefit greatly from combining the direct method (isoform assay) [3] with longitudinal profiles of the indirect method (GH-sensitive markers IGF-1 and PIIINP). A critical parameter for increased sensitivity with longitudinal profiles is significantly lower within-subject than between-subject variability. To determine this, a group of national level athletes were followed over a period of 2 years. Further, we wanted to see how GH-doping would alter the longitudinal biomarker profiles, but a long-term GH-application study would never be approved by Norwegian ethical committees. We therefore recruited bodybuilders who had a private consume of GH to an uncontrolled study. Promising results prompted Anti-Doping Norway to establish biomarker profiles for a limited number of athletes in their ordinary national test program in 2011.

### **Experimental**

#### Volunteers:

9 power- and weightlifters (average age 24.7 years (range 19-36)) and 6 endurance athletes (average age 26.7 years (21-32)), all male, were recruited through another research project by the Norwegian School of Sports Sciences. 5 GH-users (average age 33.7 years (24-46)), all male, were recruited through a personal trainer at a gym. The numbers of volunteers

reflect those that completed the study, as some decided to quit before completion. All volunteers gave written informed consent. One of the hGH-users (ID 24) stopped doping in the middle of the project, but continued to give samples to the study.

#### Sample collection:

The subjects were called in at irregular intervals for testing. They had to sit for 10 minutes prior to venipuncture. After mixing of the SST tubes, the blood was allowed to clot before centrifugation and separation of serum. Sera were aliquoted and kept frozen (-30 °C) until analysis. The athletes were asked to fill out a form describing any injury and training intensity for the previous 8 weeks. The GH-users volunteered to give detailed information about their use of doping agents in the months prior to each test. They were tested whether or not they were using GH at the time of each sample collection. 5 samples from each volunteer were collected over a period of 2-2.5 years.

#### Assays:

IGF-1 was measured with the Immulite 2000 assay (Siemens Medical Solutions, UK) and PIIINP was measured with UniQ PIIINP RIA (Orion Diagnostica, Finland), both assays conducted according to the manufactures descriptions. rhGH was measured with the hGH isoform differential immunoassays (CMZ-Assay, Germany). During validation, the intra-assay CV for IGF-1 was found to be 5% for 5-65 nmol/L; inter-assay CV for 7, 20 and 30 nmol/L IGF-1 was 8, 7 and 6%, respectively. Intra-assay CV for 2.5, 5.2 and 10 µg/L PIIINP was 6.4, 5.8 and 2.5%, respectively, and inter-assay CV for 2.5 and 10 µg/L PIIINP was 6.9 and 3.0%, respectively. Assays were run during the entire collection period, and hence the five samples from each athlete have been analyzed with different lots of assay kits. This was done in order to mimic the situation in routine analysis.

#### Athletes:

A total number of 39 athletes were included in the program, 26 male and 13 female athletes. 27 athletes came from power sports and 12 from endurance sports. They were all athletes competing at a high international level in their respectful sports. Sample collection was distributed over the year, and adapted to the competition schedule of each athlete. The samples were stored at -30 °C (never more than three months) and analyzed in bulks. As the biomarker method is not an implemented method, only one SST tube was collected. Sample collection and transportation followed the WADA ABP guideline with some exemptions: Altitude training, hypoxic devices and blood transfusions/withdrawal were not registered. Samples were transported under cool conditions (around 4°C), but without temperature logger. The maximum time from sample collection to analysis or freezing was 72 hours.

## Results and Discussion

#### Results:

The normal levels for each marker varied greatly between the athletes; however, the intra-individual variability was significantly lower than the total variability of both groups of athletes, while the hGH-users displayed large intra-individual variability (Figure 1, Table 1).

	Inter-individual CV			Intra-individual CV		
	IGF-1	PIIINP	GH-2000 score	IGF-1	PIIINP	GH-2000 score
<b>Athletes</b>	36 %	30 %	25 %	<b>13 %</b>	<b>14 %</b>	<b>10 %</b>
<b>hGH-users</b>	40 %	35 %	27 %	37 %	35 %	27 %
<b>Elite athletes</b>	36 %	29 %	27 %	<b>12 %</b>	<b>14 %</b>	<b>12 %</b>

Table 1: Estimates for inter-individual and intra-individual variability for the GH-dependent biomarkers and the GH-2000 score.

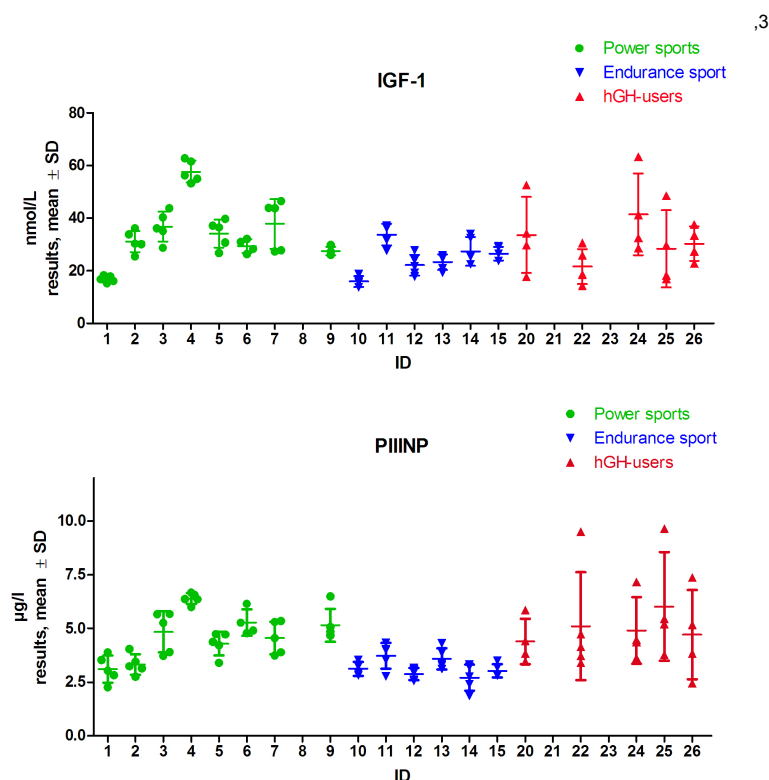


Figure 1: Individual measurements of IGF-1 and PIIINP for each subject.

The GH-2000 scores [4] varied amongst the different athletes (ID 1-15), especially in the group of power athletes (ID 1-9), but the within-subject variability was fairly low (Figure 2). The variability in the scores for the hGH-users (ID 20-26) was significantly higher, with a mean intra-individual CV of 27%, almost threefold higher than that of the athletes (10%). Samples from the hGH-users were collected in periods both on or off hGH, and on or off anabolic steroids, and this obviously increases the variability. Samples collected at times with at least 4 weeks off hGH have been marked with a green circle in Figure 2.

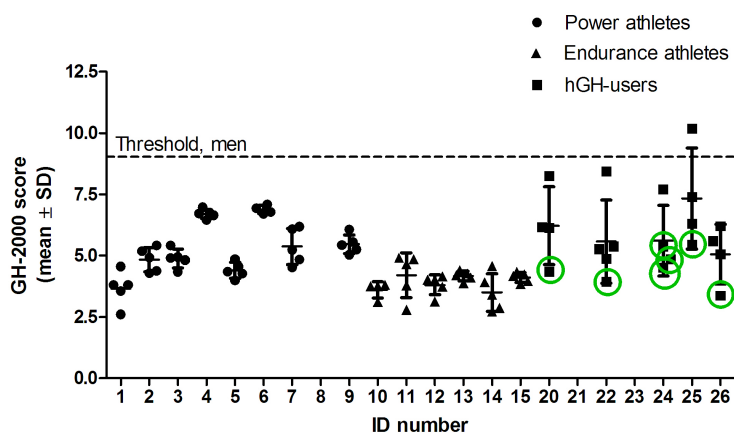


Figure 2: The distribution of GH-2000 scores for collected samples of power athletes (ID 1-9), endurance athletes (ID 10-15) and hGH-users (ID 20-26). Green circles: samples collected when users were off hGH.

The longitudinal score profiles are plotted in Figure 3. The highly irregular score sequences seen for some of the hGH-users, are quite striking when compared to the score sequences for the athletes.

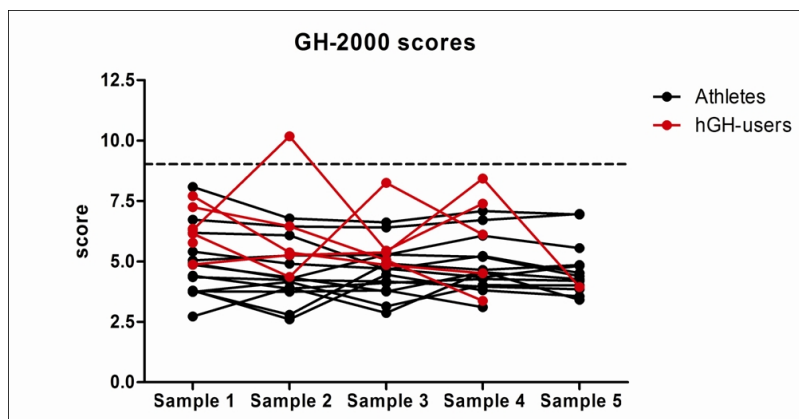


Figure 3: Longitudinal profiles of the GH-2000 scores for power and endurance sports athletes (in black) and the hGH-users (in red).

The within-subject variability for IGF-1 and PIIINP was significantly lower than between-subject variability for the elite athletes (Table 1). In fact, the variability results for the elite athletes were very similar to what we found for the lower level athletes part of the research project, though the means for both markers were slightly higher for the elite athletes. The calculated GH-2000 scores for both male and female elite athletes are shown in Figure 4. Our results correlate well with the scores for elite athletes recently published by Eritokritou-Mulligan and colleagues [2].

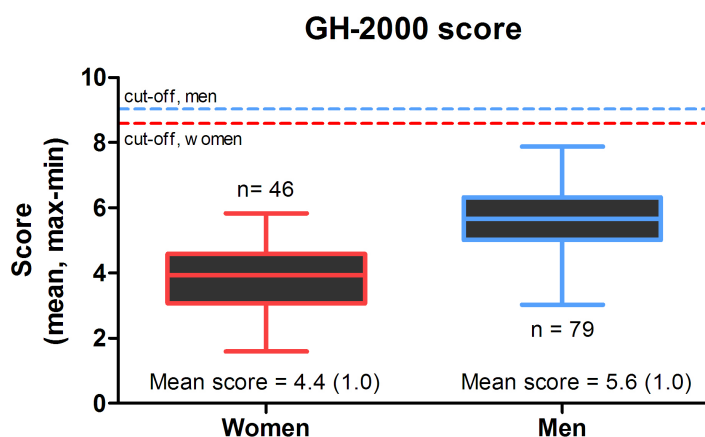


Figure 4: The calculated GH-2000 score for male and female elite athletes.

The individual distribution of scores for some athletes is plotted in Figure 5, as it nicely illustrates the low variability in scores seen with most. The scores for the hGH-users from the research project have been added for comparison (in red).

#### Discussion:

Considering the long collection period, the irregular collection intervals and the fact that the samples have been measured with different batches of assay kits, the found low within-subject variability is encouraging. Our results are also comparable to recently published results for a larger number of elite athletes where samples were collected for a shorter period of time (up to 1 year) [5,6].

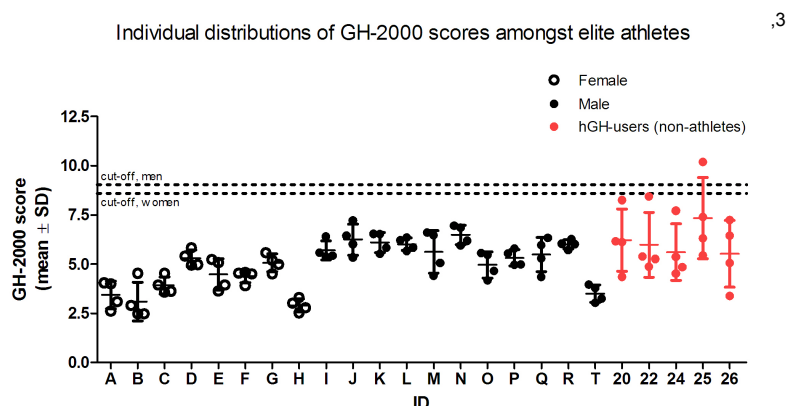


Figure 5: The distribution of GH-2000 scores for elite athletes (ID A-T, black), with the scores for hGH-users (ID 20-26, red) for comparison.

Only one of the samples from the hGH-users exceeded the suggested threshold which was recently published by the GH-2004 group [2]; this sample was one of six samples positive for rhGH when analysed with the isoform immunoassay. The other 5 positive for rhGH had scores between 7.7 and 8.4. Several samples collected during periods of hGH-use were increased for both markers (figure 1) and scores (figure 2), but they were all below threshold and were dispersed at levels corresponding to the normal levels for some of the athletes.

The reason for the apparently low scores in this study for samples collected during hGH-use is presumably related to the doses of hGH used by our volunteers. The average doses they used were 4-5 U/day, often divided into a morning and an evening dose. This is similar to the doses the volunteers in the Kreisch study received [7]. In comparison, Powrie and colleagues administered approximately two and four times these doses of GH to their 'low dose' and 'high dose' groups, respectively [4].

Another issue is the co-use of testosterone and hGH, which is the case for several samples including the sample with score > 10. It has been shown that the addition of testosterone to GH can amplify the response of PIIINP 1.5 fold [8]. Hence, it may be the combined effect of testosterone and rhGH on PIIINP that pushed the score of this sample above the threshold. This underscores the need for longitudinal profiling to detect GH-misuse, as population-based thresholds are unlikely to have the desired sensitivity. Further, the longitudinal profiles are an excellent tool for selecting samples for targeted testing (e.g. direct hGH-analysis) even if thresholds are not exceeded.

The test results from the samples collected as part of the national test program in 2011 were slightly higher than those for the lower level athletes in the research project, as expected. However, both the low within-subject variability and the high between-subject variability were almost identical for the elite and lower level athletes, for both the markers and the GH-2000 score. The low intra individual variability in the samples collected as part of the ordinary test program are important because they show that pre-analytical factors such as transportation time, temperature, time of day, training and other factors not controlled for in this study, have had little impact on the test results. This indicates a robustness which is important for a possible implementation of the method. The results also indicate a low prevalence of rGH doping in the 39 athletes at the time they were tested.

## Conclusions

There is high between-subject variability, but fairly low within-subject variability for both biomarkers (IGF-1 and PIIINP) for athletes not doping with hGH, an important criterion for the desired increased sensitivity of longitudinal profiles.

The isoform assay (direct method) identified 6 out of 12 samples collected within 24 hours of the last rhGH-injection (according to the subjects), and while the GH-2000 score (indirect method) only identified 1 out of 15 samples collected while the subjects claimed to be using hGH, the longitudinal biomarker method identified abnormal profiles for all the GH-users followed over time. The isoform ratio and the biomarker method have different and complementary qualities, and we believe the fight against GH-misuse would benefit from using both methods.

## References

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- [1] J. K. Powrie, E. E. Bassett, T. Rosen, J. O. Jørgensen, R. Napoli, L. Sacca, J. S. Christiansen, B. A. Bengtsson, P. H. Sonksen, *Growth Hormone & IGF Research* 2007, 17, 220.
- [2] A. Sartorio, M. Jubeau, F. Agosti, N. Marazzi, A. Rigamonti, E. E. Müller, N. A. Maffiuletti, *Journal of Endocrinological Investigation* 2006, 29, 237.
- [3] I. Erotokritou-Mulligan, E. Eryl Bassett, D. A. Cowan, C. Bartlett, P. Milward, A. Sartorio, P. H. Sönksen, R. I. G. Holt, *Clinical Endocrinology* 2010, 72, 520.
- [4] I. Erotokritou-Mulligan, N. Guha, M. Stow, E. E. Bassett, C. Bartlett, D. A. Cowan, P. H. Sonksen, R. I. G. Holt, *Growth Hormone & IGF Research* 2012, 22, 53.
- [5] I. Erotokritou-Mulligan, E. E. Bassett, A. Kniess, P. H. Sönksen, R. I. G. Holt, *Growth Hormone & IGF Research* 2007, 17, 416.
- [6] A. E. Nelson, U. Meinhardt, J. L. Hansen, I. H. Walker, G. Stone, C. J. Howe, K.-c. Leung, M. J. Seibel, R. C. Baxter, D. J. Handelsman, R. Kazlauskas, K. K. Ho, *Journal of Clinical Endocrinology & Metabolism* 2008, 93, 2213.

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