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Development of a test for recombinant human growth hormone

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Background

Abuse of recombinant human growth hormone (rhGH) in the field of sport only appeared with the ready availability of recombinant drug. Before this the source was from pituitaries of deceased individuals, which carried risks often associated with use of human derived products. hGH was claimed to have performance benefits centred on increases in lean body mass, increased muscle mass, support of bone and connective tissue and improved recovery from injury. However it also was found to have adverse consequences which may include metabolic disturbances, altered glucose tolerance, immune response to repeated injection with damage to the endogenous hGH axis and exposure to counterfeits and poorly made preparations. There also appears to be no objective evidence for efficacy in fit, healthy adults except in respect of recovery from injury. This paper, presented at the Cologne Workshop aims to provide an overview of current status of efforts in development of a test for hGH doping.

Strategies for Detection of rhGH Abuse:

1. Isoform Patterns of Human Growth Hormone

There is currently no direct test for rhGH abuse because the amino acid sequences of rhGH and endogenous 22 kDa hGH are identical. There is no practical means of distinguishing the source of 22 kDa hGH in circulation. Concentrations in serum are extremely variable and pulsatile with changes more than 50 fold occurring several times during a 24 hour period. Recombinant and endogenous hGH do differ in that rhGH is provided only as the 22 kDa form, and endogenous hGH possesses a number of isoforms resulting from alternate splicing

of the GH-N gene, and post-translational modification of the 22 kDa product. Assays are in development which exploit this difference.

Basis for a Test for rhGH Doping - Heterogeneity of Endogenous hGH

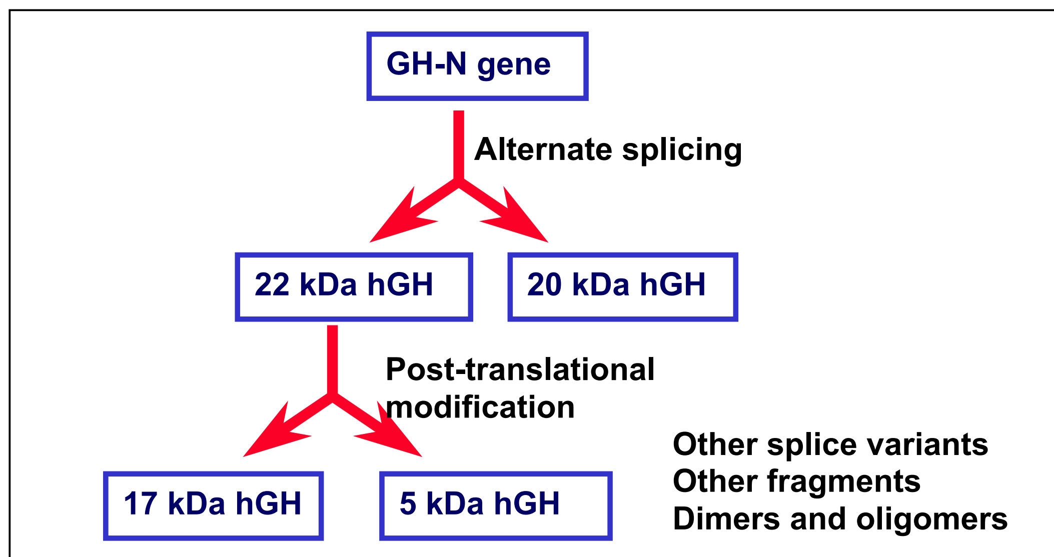


Figure 1 Candidate hGH Isoforms as Markers of hGH Doping

Specific Growth Hormone Isoform Immunoassays

20 kDa hGH is produced by alternative splicing of the GH-N gene, and corresponds to 22 kDahGH which is missing amino acids 32-46 (Baumann, 1991). It is produced by the pituitary in parallel with 22 kDa after stimulation or suppression of hGH production and is increased in acromegaly and reduced in growth hormone deficient patients. It is suppressed by rhGH treatment. A specific two-site immunoassay (ELISA) developed by Mitsui in Japan (Tsushima, 1999) is being further developed by the Japanese doping control laboratory at Mitsubishi Kagaku Bio-Clinical Laboratories.

17 kDa hGH corresponds to amino acids 44-191 in the 22 kDa hGH sequence and is produced by the pituitary in parallel with 22 kDa hGH after stimulation or suppression of hGH production. It is increased in acromegaly and reduced in growth hormone deficiency. It does not rise after 22kDa rhGH treatment (Howe, *et al., 1999)*. A specific immunoassay for its detection is still in development. A reciprocal 5 kDa peptide, consisting of the amino acid sequence 1 to 43 from the complete 22kDa molecule, also appears but is poorly understood (Singh, *et al.*, 1983; Baumann, 1991).

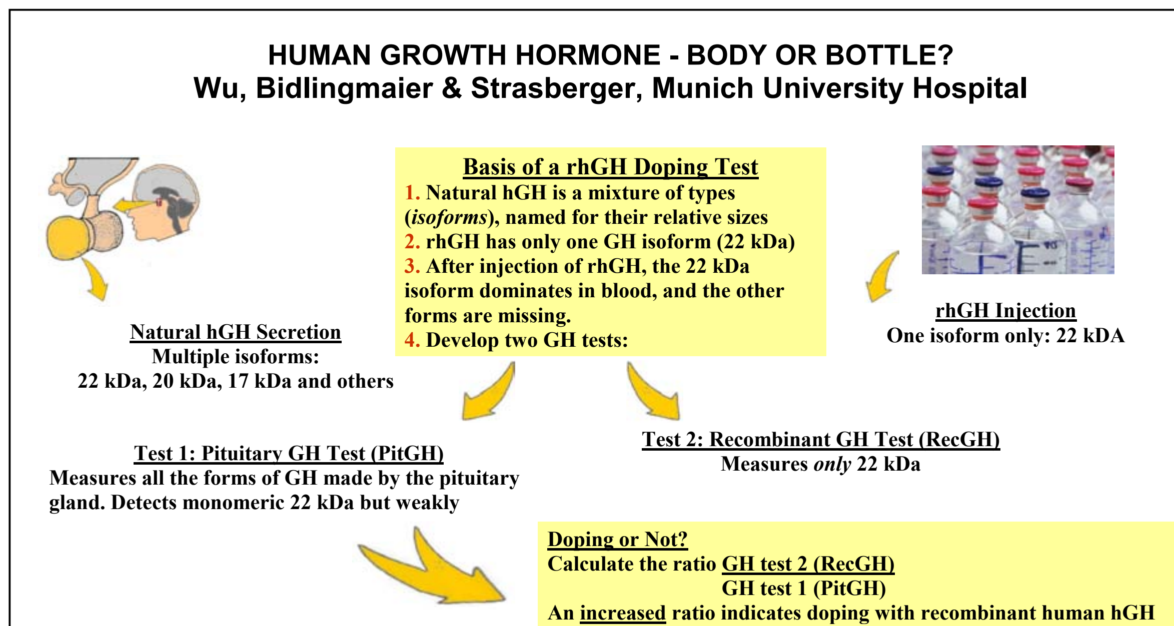


Figure 2 Development of isoform testing protocol which was distributed to a few WADA accredited laboratories in 2004.

The principle of the ELISA assay developed by Strasberger (Wu, *et al.*, 1999) is shown in Figure 2. The method uses two ELISAs with differing specificities for hGH isoforms. This is achieved by using two different capture antibodies. As well, a second, similar set of ELISAs has been developed as a confirmation procedure, using a different pair of capture antibodies but working under the same principle. The second antibody and reporter system is the same in all four assays. All antibodies are monoclonal. For the screening procedure a "recombinant" (REC) assay uses a 22 kDa hGH-specific capture antibody 5D7, and a second "pituitary" (PIT) hGH assay, which uses a capture antibody (1B3), which detects a broad group of isoforms but has relatively poor affinity for 22 kDA GH. The ratio REC/PIT, as in Figure 2, is calculated. If the value of this ratio is found above a value of 1.2 (on current data) then a confirmation procedure is carried out using different capture antibodies in both the REC assay (Ab 8B11) and the PIT assay (Ab 8A9) to confirm an excessive ratio of REC/PIT.

The logical steps for deciding whether a sample is positive for rhGH using this assay can be seen in Figure 3. The performance characteristics of the assays require that the hGH concentration must be >0.5ng/mL in both of the REC assays in order to return a meaningful result. Using these four assays the distribution of the ratios we have found for a number of baseline samples is shown in Figure 4. This clearly shows that values above a ratio of 1.2 for positive samples define rhGH use.

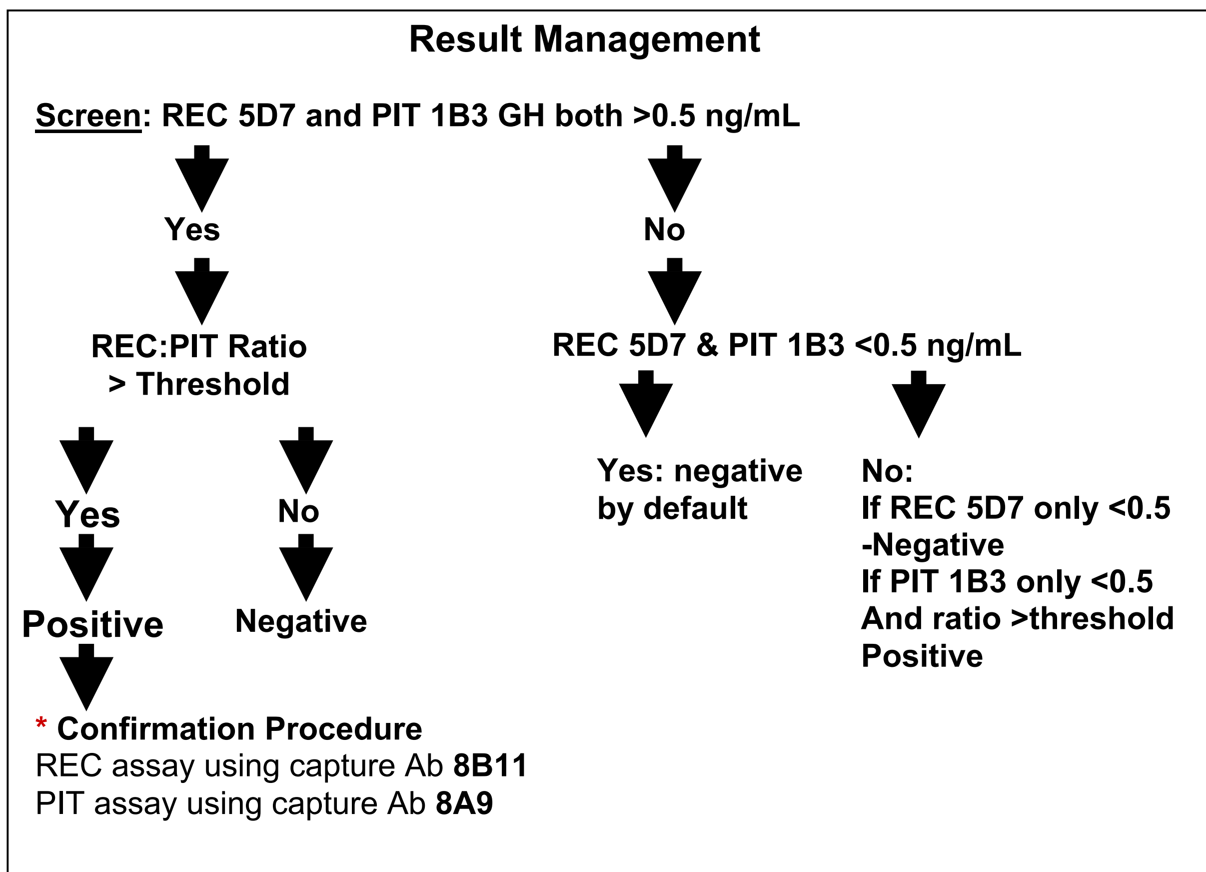
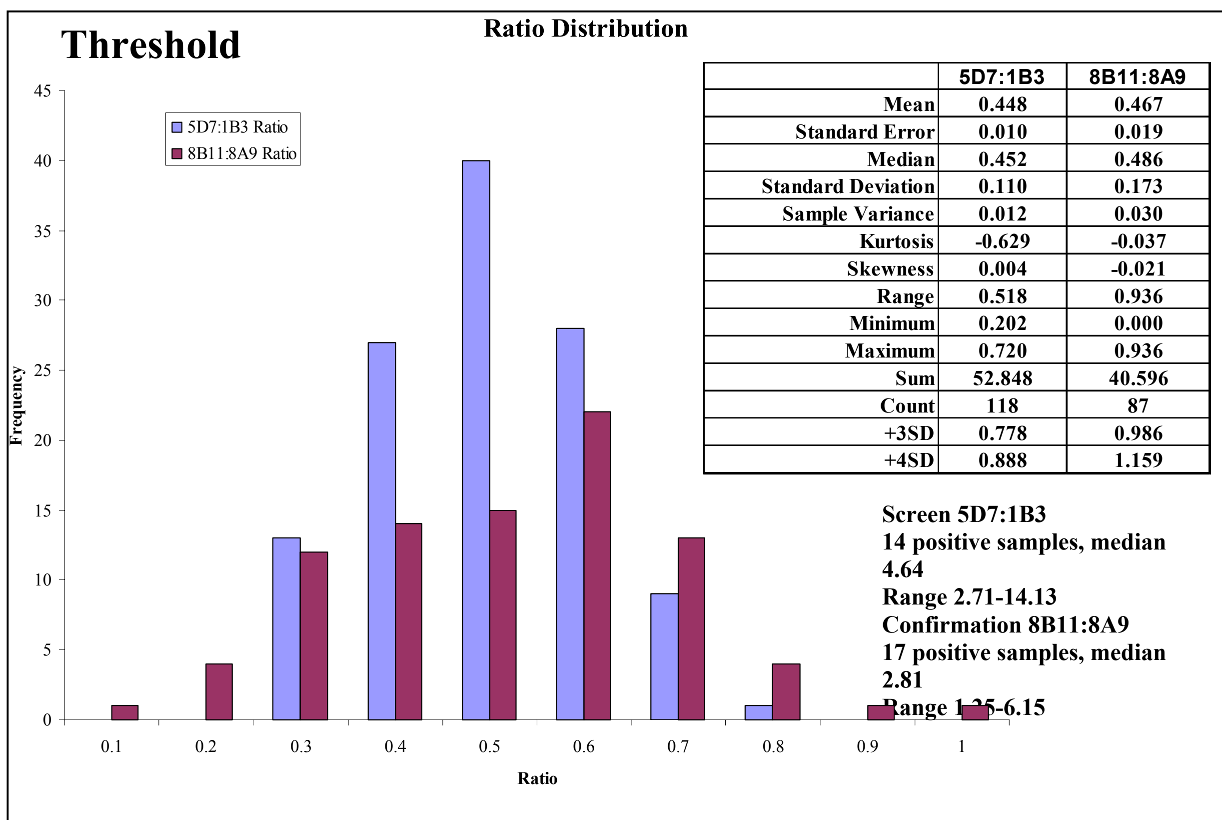


Figure 3 The logical steps for the isoform test.



	5D7:1B3	8B11:8A9
Mean	0.448	0.467
Standard Error	0.010	0.019
Median	0.452	0.486
Standard Deviation	0.110	0.173
Sample Variance	0.012	0.030
Kurtosis	-0.629	-0.037
Skewness	0.004	-0.021
Range	0.518	0.936
Minimum	0.202	0.000
Maximum	0.720	0.936
Sum	52.848	40.596
Count	118	87
+3SD	0.778	0.986
+4SD	0.888	1.159

Figure 4. The distribution of ratios for the isoform assay in normal subjects.

Strategies for Detection of rhGH Abuse:
2. Human Growth Hormone Responsive Markers

The disturbances in isoform composition caused by the use of rhGH are short-lived, with the window of testing probably restricted to about 24 hours after a single injection. Another approach uses hGH-responsive markers to infer rhGH use. The GH2000 project showed that the effects of rhGH treatment persist for 3-4 weeks (Wallace, *et al.*, 1999; Wallace, *et al., 2000*). The rhGH responsive markers can be divided into two groups

- the IGF-I axis markers:-
    - IGF-I; Insulin-like growth factor I is mostly produced by liver and act as the main driver of somatic growth;
    - IGFBP-3; IGF binding protein 3 which carries IGF-I in circulation and controls delivery of IGF-I to tissue; and
    - ALS; Acid labile subunit which forms a complex in circulation with IGF-I and IGFBP-3
- Bone and connective tissue turnover markers:
    - PINP; N-terminal propeptide of type I procollagen which is released from new type I collagen when bone is forming
    - ICTP; C-terminal telopeptide of type I collagen which is a collagen breakdown product produced during bone removal or remodelling
    - PIIINP; N-terminal propeptide of type III procollagen which is released from new type III collagen during formation of cartilage or tendons

Studies into variation across a number of ethnic groups have been carried out (Howe, *et al.*, 2004). These used the EPO2000 sample set which was collected as part of the validation study for EPO (Sharpe, *et al, 2002*). More than 3000 samples from over 1100 elite athletes were obtained. Within this group of elite athletes there were three blood samples collected over a two week period and all the sera were maintained at -80°C with minimum freeze thaw cycles. The parameters IGF-I, IGFBP-3, ALS, PINP, ICTP and PIIINP were measured by radioimmunoassay (RIA) and, with demographic information such as age, sex, Body Mass Index (BMI) and ethnicity were subjected to statistical analysis by random effects ANOVA and multiple regression analysis.

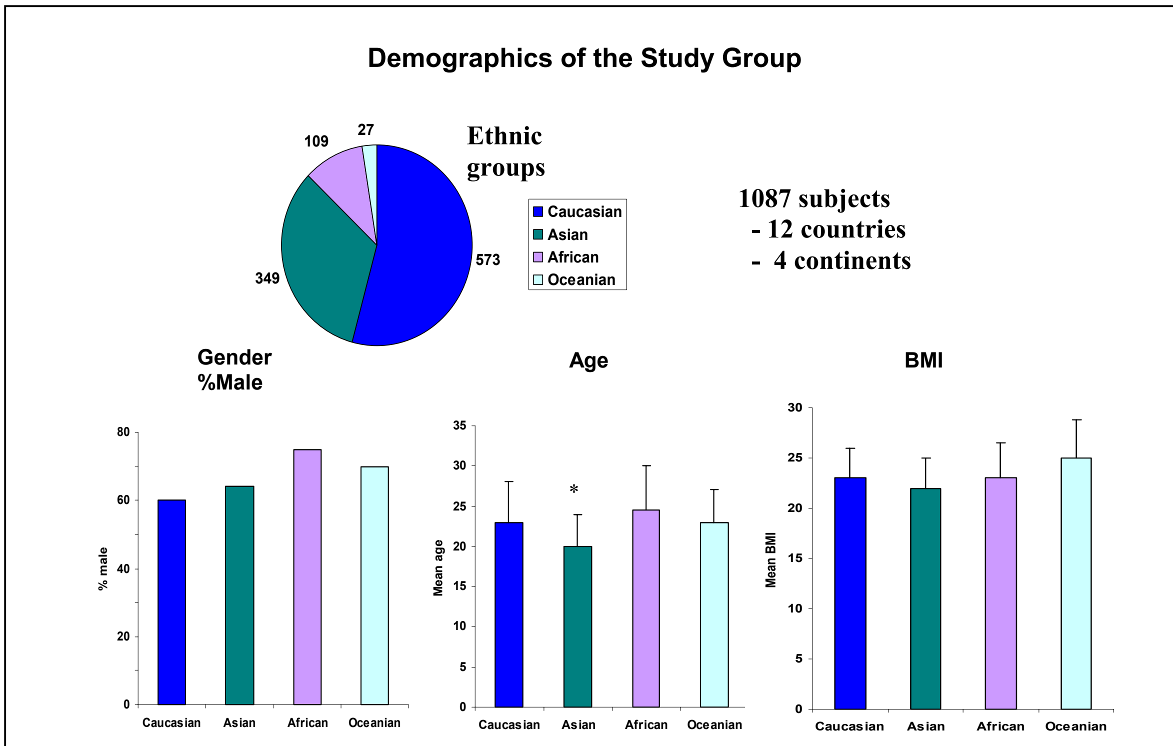


Figure 5 The demographics of the study group for the markers.

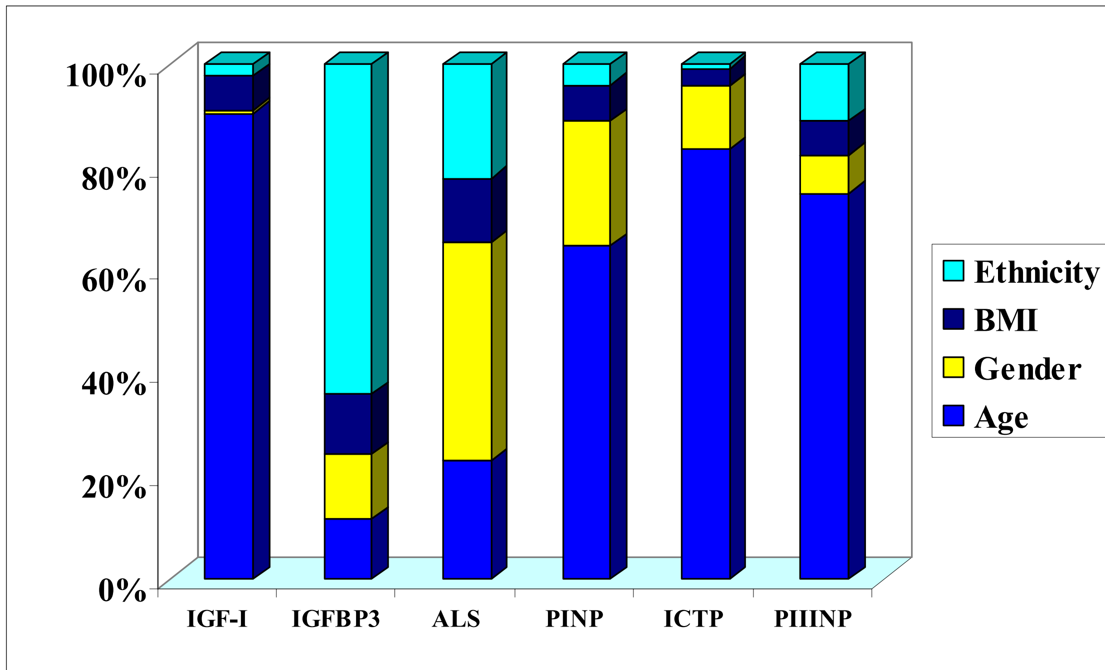


Figure 6 Percentage of total explained variability attributable to the four demographic markers of ethnicity, BMI, gender and age for each of the measured markers.

The demographic distribution of the parameters for the athletes that were used in these studies are shown in Figure 5. While the largest group was Caucasian, the Asian and African ethnic groups were well represented. The group from the Oceanian region was small but significant. The effect of the specified demographic factors upon the explained variability in each of the

hGH-dependent markers is shown in Figure 6. This shows the main effect on IGF-1 was age; IGFBP3 was affected mainly by ethnicity; ALS had a large gender effect but was also well affected by age and ethnicity; PINP had significant age and gender effects; ICTP was mainly affected by age; and PIIINP was mainly affected by age. However, these factors only explain between 21% (IGF-I) and 48% (ICTP) of the total variation in the hGH-dependent markers.

More analysis of available data will clarify other factors affecting the serum concentrations of these markers. Complete interpretation of testing based on markers of hGH action will require more demographic information on the athlete. Currently only sex and sport are provided to the laboratory on the athlete declaration form. Data such as age and ethnicity are not recorded. Effective use of these markers for rhGH use will require more extensive communication between the laboratory and the anti-doping agency. Future and past athlete profiles will also assist in interpretation of changes that may suddenly occur and which may be due to rhGH use.

Current Directions

It will be necessary to continue the analysis of factors affecting variation in markers of hGH action in elite athletes. A complete population study of 20 kDa and 22 kDa hGH levels and a study of the Munich isoform assays in the elite athlete population will need to be integrated with marker studies. The measurement of isoforms and their ratios is dependant on a steady supply of reagents and antibodies. This may be available mid to late 2005, at which time laboratories may commence offering a service assay.

A study investigating the interaction of rhGH and Testosterone treatment in recreational athletes is being undertaken by a multicentre consortium, funded by WADA, with project management based at the Garvan Institute of Medical Research in Sydney. This will use 100 recreational athletes, 60 male and 40 female. The volunteers are subjected to medical examination and baseline studies, randomised into placebo or rhGH treatment (females), and placebo, rhGH or rhGH plus testosterone treatment (males only). This is a double blind controlled study with eight weeks of treatment followed by a six week washout period. The clinical phase of the study is projected to reach completion at the end of 2005.

The work is being performed by several groups:-

- IGF-I, IGFBP-3, ALS measurements by the Kolling Institute in Sydney;
- PINP, ICTP, PIIINP measurements by the ANZAC Institute Bone and Mineral Group in Sydney;
- Haematology and serum testosterone, LH, FSH, E2, EPO and sTfR measured by NMI in Sydney;
- DHT measured by the ANZAC Institute in Sydney;
- General biochemistry at St Vincents Hospital in Sydney
- hGH Isoforms will be measured using the following assays;
    - Munich Assays – NMI,
    - 20/22 kDa assays - Anti-Doping Center, Mitsubishi Kagaku Bio-Clinical Laboratories, Inc.;
- Novel markers by SELDI-TOF MS, studied at the Kolling Institute in Sydney;
- Performance enhancement studies - Garvan Institute, with the NSW Institute of Sport and UNSW UniGym;
- Statistical analysis - Garvan Institute.

Acknowledgements

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