

Working Towards The Development Of A Physiological Reference Range

For Growth Hormone-Dependent Markers In Injured Athletes.

(ABBREVIATED & ANONYMISED FOR CONFIDENTIALITY)

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Contents Page:

1/ Abstract	(Page 7)
2/ Acknowledgements	(Page 9)
3/ Introduction	(Page 10)
○ 3.1- <i>A history of doping and drug testing</i>	
○ 3.2- <i>The WADA Prohibited List</i>	
○ 3.3- <i>Growth hormone abuse in sport</i>	
○ 3.4- <i>Cadaveric and recombinant GH</i>	
4/ Basic Science	(Page 15)
○ 4.1- <i>GH physiology</i>	
○ 4.2- <i>GH secretion</i>	
○ 4.3- <i>The ‘Somatopause’</i>	
○ 4.4- <i>rhGH</i>	
5/ Literature Review	(Page 19)
○ 5.1- <i>GH studies</i>	
○ 5.2- <i>GH / IGF-I axis</i>	
○ 5.3- <i>The Somatomedin Hypothesis</i>	
○ 5.4- <i>IGF-I as a ‘marker’, not a second messenger</i>	
○ 5.5- <i>Manipulation of the GH-IGF Axis</i>	
○ 5.6- <i>Detecting abuse with GH</i>	
○ 5.7- <i>How were these markers initially identified?</i>	
○ 5.8- <i>IGF-I as a marker</i>	
○ 5.9- <i>P-III-P as a marker</i>	
○ 5.10- <i>The stability of the markers</i>	
○ 5.11- <i>Using both markers in combination</i>	
6/ Materials & Methods	(Page 29)
○ 6.1- <i>The GH-2004 Injury Study</i>	
○ 6.2- <i>Why was the OMI chosen?</i>	

- 6.3- *My involvement in the Injury Study*
- 6.4- *Ethical approval*
- 6.5- *Subject recruitment*
- 6.6- *Inclusion criteria*
- 6.7- *Exclusion criteria*
- 6.8- *Study Protocol*
- 6.9- *Blood samples*
- 6.10- *Saliva samples*
- 6.11- *Subsequent visits*
- 6.12- *Laboratory measurements*
- 6.13- *IGF-I Immunoassay Kit*
- 6.14- *P-III-P Radioimmunoassay (RIA) Kit*

7/ Results (Page 38)

- 7.1- *Demographics*
- 7.2- *Follow-up away from the OMI*
- 7.3- *Analysis of samples*
- 7.4- *Data analysis*
- 7.5- *Serum IGF-I & P-III-P results*

8/ Discussion (Page 47)

- 8.1- *Differences in mean serum IGF-I and P-III-P levels for male and female participants*
- 8.2- *The expected rise in GH-dependent markers after injury*
- 8.3- *Difficulties in following up participants*
- 8.4- *Difficulties in recruiting participants of certain ethnic groups*
- 8.5- *Challenges associated with the detection of GH Abuse*
- 8.6- *Factors to consider when establishing a reference range*
- 8.7- *Statistical & Discriminant Function Analysis*
- 8.8- *Superimposing the data: A useful focus for further discussion*

9/ Conclusions (Page 58)

- 9.1- *What are the reasons for athletes doping with GH?*

- 9.2- *The Injury Study at the OMI*
- 9.3- *Introducing a 'marker' test for doping: the challenges ahead*

10/ References (Page 62)

- *Journal articles*
- *Textbooks*
- *Books, Press Reports & Websites*

11/ Bibliography..... (Page 71)

12/ Appendices (Page 72)

- *Appendix A – Introductory letters to Physicians and Physiotherapists at the Olympic Medical Institute*
- *Appendix B – Letter to elite participant's own Sports Physician or General Practitioner*
- *Appendix C – GH-2004 Injury Study Subject Information Sheet*
- *Appendix D – GH-2004 Injury Study Consent Form*
- *Appendix E – GH-2004 Injury Study History Sheet*
- *Appendix F – GH-2004 Injury Study Generic Participant List*
- *Appendix G – P-III-P radioimmunoassay kit insert*
- *Appendix H – IGF-I radioimmunoassay kit insert*
- *Appendix I – MSNBC.com Article: Athletes were tested for hGH in Athens*
- *Appendix J – UOS GH-2004 Injury Study data for serum IGF-I*
- *Appendix K – UOS GH-2004 Injury Study data for serum P-III-P*

1/ Abstract:

Growth hormone (GH) is a drug abused in elite sport and a banned substance prohibited by the World Anti-Doping Association (WADA).

The detection of illicit usage of GH poses many challenges. Until very recently, there were no tests available to detect GH abuse in sport.

Unlike many other drugs of abuse which are produced artificially and can be detected as exogenous substances, GH is a naturally occurring substance and can be produced endogenously by the human body. Detection is further hampered by the fact that endogenous and exogenous GH have matching amino acid sequences.

Traditional drug testing in sport has involved urinary sampling, but this is not viable for GH detection because GH is not secreted into the urine in sufficient or reliable quantities. Consequently, blood sampling is necessary for the detection of GH abuse.

2 main approaches have now been pioneered to detect GH abuse. The first method relies on the detection of different pituitary GH isoforms (the 'isoform assay method'), whereas the second method depends on the measurement of GH-dependent proteins (the 'marker method'). Both methods utilise immunological assays.

The GH-2004 Project is a multi-centre research group based primarily at the department of Endocrinology and Metabolism within the University of Southampton. It is funded by research grants from WADA and the US Anti-Doping Agency (USADA) and has several key objectives, all aimed at developing a reliable test for GH abuse using the marker method.

In collaboration with the Olympic Medical Institute in London, one of its main objectives is to evaluate the effects of injury on plasma concentrations of GH-

dependent markers. This particular branch of the project is identified as the 'GH-2004 Injury Study'.

The markers of interest are Insulin-like Growth Factor-I (IGF-I) and N-terminal extension peptide of Procollagen type III (P-III-P), both of which are generated by the anabolic actions of GH. The background of doping, GH and how these 2 markers came to be selected will be discussed within this dissertation.

It is hoped that a reference range for IGF-I and P-III-P in injured athletes can be established at the end of the project. This will be used in the detection of GH abuse using the 'marker method' to enable clear differentiation between athletes who choose to abuse GH and those who choose otherwise.

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3/ Introduction:

3.1- A history of doping and drug testing

The term *doping* is thought to originate from the Dutch word *dop*. This was a grape-skin derived alcoholic beverage drunk by Zulu warriors before battle to enhance their prowess in war. As far back as ancient times, Greek athletes were known to have used stimulating potions and special diets for fortification. By the 1920's, it was evident that restrictions regarding the use of drugs in sport were required.

In 1928, the International Amateur Athletic Association (IAAF) became the first international sport federation to ban the use of stimulating substances. Many other federations followed suit but restrictions were ineffective as no tests were made to detect these illegal substances.

It was only after the amphetamine-related death of a Danish Cyclist (Knud Enemark Jensen) in competition at the Rome Olympic Games in 1960 that pressure mounted for sports authorities worldwide to introduce drug tests.

In 1966, UCI (Cycling) and FIFA (Football) were amongst the first international federations to introduce doping tests at their respective World Championships.

In 1967, the International Olympic Committee (IOC) instituted its own Medical Commission and set up the first IOC list of prohibited drug substances. The first IOC drug tests were at the Olympic Winter Games in Grenoble in March 1968 and the Olympic Summer Games in Mexico in October 1968.

The IOC subsequently led the development of doping tests and would go on to test at every major Games after that.

The 1972 Olympic Games in Munich saw the first systematic testing commence with the analysis of over 2000 urine samples by gas chromatography (GC) with nitrogen-selective detection for stimulants.

Systematic urinary screening was initiated in 1983 at the Pan American Games, and subsequently blood testing in 1994 for the first time at the Lillehammer XVII Olympic Winter Games in an attempt to detect blood doping (Bowers, 1997).

Over the years, there has been variable success on several fronts. Steroid drug tests were developed in the mid-1970's and anabolic steroids are now easily detected in urine, as are stimulants such as amphetamine. Paradoxically, success on these fronts has led to the development of other methods such as blood doping and the use of erythropoietin (EPO) injections and gradually resulted in the anti-doping war being shifted to different arenas.

A major re-appraisal of anti-doping legislation and the role played by public authorities came about as a result of a huge doping scandal at the 1998 Tour de France competition. A large number of prohibited medical substances, including growth hormone, were found by police during a raid. Up to this point, anti-doping had been debated in several discrete forums with different definitions, policies and sanctions being applied. A result of this perplexity was that doping sanctions were often disputed and occasionally overruled in civil courts.

There was also concern about possible conflict of interests as the IOC ran the Olympic Games and also established the prohibited substances list. Along with ever-increasing public and media pressure, this scandal led to the IOC formally recognising the need for an independent international agency to set unified standards for anti-doping work and co-ordinate the efforts of sports organisations and public authorities.

A World Conference on Doping in Sport was convened by the IOC in Lausanne in February 1999. Following on from that, the World Anti-Doping

Agency (WADA) was established in November 1999 to concentrate efforts on combating the ever-present and possibly growing threat of doping. (WADA website)

3.2- The WADA Prohibited List

WADA has produced a list of prohibited substances that have been classified according to their mode of action. This was initially based on the IOC prohibited list but modified and revised annually in accordance with new evidence and scientific advancements. (WADA; The 2005 Prohibited List: International Standard). They have also produced a list of methods by which prohibited substances can be detected.

The criteria for a doping offence are as follows:

- (1) the prohibited substance is discovered in the athlete's body fluids;
- (2) if the athlete attempts to use a prohibited substance or method;
- (3) the athlete fails to submit a sample once requested, or fails to avail themselves for out-of-competition testing, unless the athlete can demonstrate that the occurrence of the substance is as a result of a physiological or pathological condition.

3.3- Growth hormone abuse in sport

GH has been a drug of abuse in sport since the early 1980s. Recent press reports indicate that growth hormone (GH) has now become a significant player on the doping scene in elite sport. (Mackay, 2004)

The IOC was quick to ban GH but until recently, no tests were available to detect their exploitation in sport. Unlike many other drugs of abuse, GH is a naturally occurring substance. The demonstration of exogenous GH

administration must therefore rely on detecting concentrations in excess of an established reference interval.

Anecdotal evidence suggested that large numbers of elite athletes had been abusing GH for its ergogenic effects for many years and indeed, several subsequently confessed to doing so. The Canadian Sprinter Ben Johnson was probably the most infamous of these athletes. He and his coach admitted, at a government inquiry, to knowingly using GH repeatedly in conjunction with other prohibited substances. (Goodbody, 1989; TIME magazine, 1989)

Following the loss of his Olympic Gold Medal in the 100m sprint after testing positive for anabolic steroids at the Seoul Olympic games, Johnson admitted during subsequent inquiries that in addition to anabolic steroid abuse, he had also been using GH illegally over many years.

Much more recently, Tim Montgomery, another elite sprinter and former 100m world record holder, admitted to having received a 2 month supply of GH and a steroid compound known as “the clear” (Fainaru-Wada & Williams, 2004).

Over the years, supplies of growth hormone have been found in the personal possession of a Chinese swimmer in the World Championship in Perth, Australia; in the personal baggage of a national team trainer entering Australia for the Sydney 2000 Olympic Games and in a team car during the Tour de France competition.

Circumstantial evidence for GH abuse also comes from the losses of recombinant human GH (rhGH) from the production line, the distribution networks, wholesale and retail outlets. Several months before the Sydney Olympic Games, there was a carefully targeted burglary on a wholesale pharmacy in Sydney. rhGH was stolen in large quantities but rather tellingly, nothing else was taken. None of these drugs appear to ever have been recovered as described in the following article.....

Olympic Jitters at Power Drug Theft by Deborah Cameron

“The theft of a huge quantity of an undetectable bodybuilding drug from a Sydney importer has raised serious concerns among sports officials and doctors about whether Australia’s elite athletes are ‘clean’. With just six months to go until the Olympics, and as some sports prepare for selection trials, the timing of the theft of 1575 (multidose) vials of human GH (hGH) is seen as highly significant”. (16 February 2000)

3.4- Cadaveric and recombinant GH

Initially, GH was initially extracted from cadaver pituitary glands until the advent of recombinant DNA technology which enabled recombinant human GH (rhGH) to be produced. The medical use of cadaveric GH ceased in 1985.

Concerns in recent years have highlighted the potential for contamination of cadaveric GH with Creutzfeld–Jacob prion (CJD). Since then, a small number of recipients of contaminated cadaveric GH have gone on to develop Bovine Spongiform Encephalopathy (BSE) and some have died from complications of the disease. (Belay & Schonberger, 2005; Cordery et al, 2003; Swerdlow et al, 2003)

Worryingly, supplies of pituitary-derived GH are still in circulation, probably as a result of high demand. These are virtually indistinguishable from rhGH. It is highly likely that some of these batches will be contaminated with CJD. It is also likely that athletes who purchase these black-market goods may not appreciate the significant risk to their health in the long term.

4/ Basic Science:

4.1- GH physiology

GH (also known as Somatotrophin) is a peptide hormone which is secreted in a pulsatile fashion by the acidophilic cells of the anterior pituitary gland (Adenohypophysis). Structurally, it comprises a single chain polypeptide containing 191 amino acids, 2 disulfide bridges and 4 helical structures.

It was extracted from the pituitary gland for the first time in 1957 (Li & Papkoff, 1956) and by the 1980s, its anabolic actions for use in elite sport had been described. *The Underground Steroid Handbook* is thought to be the first publication to bring GH to the attention of the sports world as a potent performance-enhancing anabolic agent (Duchaine, 1983).

There are several plasma GH variants with different molecular weights. The predominant GH variant has a molecular weight of 22 kDa with a short half-life in the plasma of between 15 and 20 min after secretion or intravenous injection. The 22kDa isoform represents approximately 75% of circulating GH, with the non-22kDa isoforms making up the rest.

As a protein hormone, GH has to be administered by injection otherwise it undergoes complete digestion in the gastrointestinal system if taken orally. After subcutaneous or intramuscular injection, blood concentrations of GH reach a peak between 1 and 3 hours after and fall to undetectable levels after a day.

Circulating GH is cleared from the blood stream via receptor-mediated degradation and completely degraded to its basic amino acids by the kidney and liver. Only trace quantities of GH (between 0.001- 0.01% of pituitary GH) secretion subsequently emerge in the urine (Albini et al, 1988; Saugy et al, 1996). This pattern of urinary excretion has been shown to be too small and variable to be useful in designing a test to detect GH abuse.

4.2- GH secretion

GH is secreted in discrete pulses (between 6 to 12) every 24 hours. The major stimuli to GH secretion in man are sleep, exercise and stress (Muller et al, 2003).

The phase of deep slow-wave sleep (commonly occurring during the early hours of sleep) correlates most consistently with the sleep-associated circadian burst of GH secretion. Disturbing a person's sleep pattern results in impaired GH secretion. Hypnotic medications that reduce the period of slow-wave sleep decrease GH secretion; whereas drugs that enhance slow-wave sleep increase GH secretion (Van Cauter & Copinschi, 2000).

Exercise-induced growth hormone response (EIGR) is a well recognised physiological response. Although the exact mechanisms is not known, several factors such as neural input, direct catecholamine stimulation, lactate, nitric oxide, and changes in acid-base balance are thought to play a role. Resistance training also results in a significant EIGR (Godfrey et al, 2003).

GH secretion reaches its maximum around late adolescence and falls progressively thereafter (Weltmann et al. 1994). The other stimulatory (and inhibitory) factors are shown below (see *Figure A*).

(Figure A)

Factors affecting Growth Hormone Secretion:

(Neal, 2000; Nussey & Whitehead, 2001)

<u>Stimulates GH secretion:</u>	<u>Inhibits GH secretion:</u>
*Exercise	**Insulin-like growth factors (IGFs)
*Stress	**Hyperglycaemia
*Sleep	Somatostatin
Hypoglycaemia	Senescence
GHRH	Excess GH
Decreased free fatty acids	Elevated free fatty acids
Increased amino acids	Glucocorticoids
Oestrogens	Progesterone
Serotonin	Serotonin antagonists
Alpha-adrenergic agonists	Beta-adrenergic agonists
Dopamine agonists (e.g. L-Dopa, Bromocriptine)	Dopamine antagonists (e.g. Phenothiazines)
L-Arginine	
Starvation	
Puberty	
Clonidine	
Ghrelin	
Androgens	

**Major stimuli for GH secretion*

***Major inhibitors for GH secretion*

4.3- The 'Somatopause'

The total amount of GH secreted over 24 hours in normal adults over the age of 65 closely overlaps with sufferers of organic GH-deficiency (GHD) secondary to pituitary pathology or its treatment (Toogood et al. 1996).

Sarcopenia, the loss of muscle mass, is one of the main problems associated with ageing (Goldspink & Harridge, 2004).

From a physiological point of view, most middle-aged and elderly subjects can be considered incompletely GHD. This is evidence for the so-called 'Somatopause' (by analogy with the menopause).

4.4- rhGH

Since the early 1980s, rhGH has been used as a treatment for patients with GH deficiency. Commonly, this tends to be in children although GHD adults are sometimes treated as well. If given in adults, higher doses are generally needed in women.

It is a single daily subcutaneous injection, usually given at night. There are several different manufacturers who produce it and trade names for it include *Genotropin, Humatrope, Norditropin, Saizen* and *Zomacton*. (BNF version 49)

Side effects of GH excess include insulin resistance, peripheral oedema and arthralgia (Mastaglia et al, 1970). rhGH administration can also result in benign intracranial hypertension and a theoretical slight increased risk of developing malignancy.

5/ Literature Review:

5.1- GH studies

The first scientific study demonstrating a clear regulatory role for GH in adults was only published in peer-reviewed medical literature in 1989 (Jorgensen et al, 1989; Salomon et al, 1989). For obvious ethical reasons, there are no proper scientific studies proving GH to be performance-enhancing in normal human subjects. Few, however, would doubt this capability (Dean, 2002).

GH plays an essential role in regulating body composition both in adult humans and in other species. Administration of exogenous GH to pigs, cattle and sheep results in high birth weights, increased rates of protein synthesis and increased muscle mass. GH is used as a 'partitioning agent' in cattle, specifically diverting ingested calories away from fat synthesis and towards protein synthesis.

In animal experiments, animals made transgenic for GH have been observed to have greatly increased lean tissue and decreased fat. Similar changes in body composition are noted in humans with acromegaly, a condition of excess GH secretion from the pituitary gland. Conversely, GHD adults have reduced lean body mass and increased fat mass, particularly central abdominal fat mass.

GH also induces lipolysis in adipose tissue and leads to a reduction in fat mass. The use of rhGH given in physiological 'replacement' doses to adults with GHD results in the following: a 5 kg increase in lean body mass within the first month and a comparable loss of 5 kg of fat, particularly from the intra-abdominal region where fat accumulates in the GHD state, on average (Salomon et al. 1989).

In addition, subnormal exercise performance and muscle strength of GHD adults are returned to normal (Cuneo et al. 1991a,b).

5.2- GH / IGF-I axis

GH stimulates many metabolic processes in all cells. These functions include promoting linear growth, increasing protein synthesis, amino acid uptake, free fatty acid release and reparative functions. (Nussey & Whitehead, 2001). Perhaps its best-known actions are the generation of Insulin-like Growth Factor-I and its binding proteins (IGF-I and IGFBPs), mainly from the liver.

IGF-I is so named because of its similarity in anabolic activity to Insulin. It is also known as Somatomedin-C (Neal, 2000). The absence of IGF-I was thought to produce dwarfism, even if GH levels were normal (Laron type dwarfism). Pygmies, who lack IGF-I receptors, are noted to have short stature.

Apart from the liver, most other tissues in the body also express IGF-I. There are local (autocrine and paracrine) as well as systemic forms of IGF-I which have different functions.

95% of circulating IGF-I is bound to binding proteins (IGFBP-1 to IGFBP-6), which modulate its actions and bioavailability (Clemmons et al 1989; Wilson et al, 1998).

IGF-I to IGFBP3 in particular form very stable complexes and this provides a circulating reservoir of these growth factors. (Nussey & Whitehead, 2001). Therefore, unlike the rapid fluctuations seen in circulating GH concentration, IGF-I concentrations are relatively stable and their half-life much longer.

5.3- The Somatomedin Hypothesis

It was previously believed that IGF-I produced by the liver was responsible for many of GH's in-vivo actions (Le Roith et al, 2001), such as increases in total body protein turnover and muscle synthesis as noted in GHD adults and endurance-trained athletes (Fryburg et al, 1991; Healy et al, 2003). IGF-I can

also inhibit GH secretion by a negative feedback mechanism (Berelowitz et al. 1981).

It is now clear that hepatic IGF-1 production is regulated by other factors in addition to GH (e.g. nutritional status, Insulin). It has also been discovered that at least 2 different kinds of IGF-I that are expressed by skeletal muscle are derived from the IGF-I gene by alternative splicing. One is expressed in response to physical activity and has now been termed 'mechano-growth factor' (MGF). MGF has a different peptide sequence which is responsible for replenishing the satellite (stem) cells in skeletal muscle. (Goldspink & Harridge, 2004)

More recent insights have also revealed that there is also a darker side to the GH/IGF-I signalling system. Both proteins have been implicated as potential contributing factors in certain human cancers, and normal activity through this signalling pathway has been linked to diminished lifespan in experimental animals. (Woelfle et al, 2005)

Clearly, more work is needed in the future to understand the exact roles played by GH and IGF-I within the human body.

5.4- IGF-I as a 'marker', not a second messenger

'Circulating IGF-I should.... be considered more as a 'marker' of GH action on the liver than as the mechanism by which GH exerts its effects' (Sonksen, 2001).

GH receptors are present on all cells in the body. One GH molecule binds to two receptors and causes dimerisation of the receptors, which subsequently triggers intracellular signalling. It would appear that GH exerts effects on most of these cells.

There are hundreds of GH-dependent 'markers' produced under the influence of GH. IGF-I just happens to be the best known of these with the majority of

circulating IGF-I coming from the liver. Experiments involving IGF-I liver-specific mice indicate that despite near zero circulating levels of IGF-I, these mice are still able to thrive (Sjogren et al, 2002; Wallerius et al, 2001; Isaksson et al, 2001).

Given the recent evidence, we should perhaps now consider IGF-I more as a marker of GH action on the liver rather than the 'second messenger' of GH action.

5.5- Manipulation of the GH-IGF Axis

Novel technologies and ways of manipulating the GH-IGF axis are also starting to surface. GH secretagogues, rhIGF-I, and rhIGF-I/recombinant human IGF-I-binding protein 3 (rhIGFBP-3) complexes are already in circulation.

As a consequence of the Human Genome Project and the unravelling of human DNA, gene doping is likely to become a reality soon. Certain genotypes confer athletic advantages, and the transmission of a genetic code with or without the aid of a vector would allow incorporation of the DNA into target tissues where expression of that gene can lead to enhanced local production of an anabolic substance such as IGF-I. This would confer target tissue specificity without altering systemic concentrations of the product and would therefore not be detectable by urine or blood testing (McHugh et al, 2005).

Proof-of-concept experiments have already been undertaken. Laboratory mice injected with a recombinant adeno-associated virus genetically manipulated to induce myocyte overexpression of IGF-I induced a 15% increase in muscle mass and a 14% increase in muscle strength without inducing a systemic increase in IGF-I (Barton-Davis et al, 1998). This is further evidence against the 'hormonal' action of IGF-I and in favour of autocrine and paracrine actions.

5.6- Detecting abuse with GH

As a substance secreted in bursts, GH poses unique difficulties in developing satisfactory methods of detection (Rigamonti et al, 2005). Unlike most synthetic anabolic steroids, GH is also an 'endogenous substance' and indistinguishable from the naturally occurring hormone. This makes it particularly difficult to detect when used as a drug of abuse.

As mentioned previously, the predominant isoform of GH secreted by the anterior pituitary gland is the 22 kDa isomer. There are, however, some other isomers. The most prominent of these is a 20 kDa isomer that is normally present in the circulation at concentrations averaging around 10% of the 22 kDa isomer.

2 promising avenues have now been taken to detect GH abuse. At present, these techniques for identifying GH doping abuse have yet to be sufficiently well validated for introduction into the arena of competitive sport.

(1) The first approach is based on assessing of the effect of exogenous GH on pituitary GH isoforms and utilises immunoassays that can distinguish the isomers of pituitary-derived GH from the monomer of recombinant human GH (Strasburger et al, 1996; Wu et al. 1999). This method was originally termed the "direct method", but it is now more accurately referred to as the "**isoform assay method**".

It is now possible to distinguish between endogenous GH secretion and exogenous rhGH (but not pituitary-derived GH) administration. Only the 22-kDa isoform is contained in rhGH and exogenous rhGH administration leads to a marked decreased in the endogenous pituitary-derived non-22- kDa isoforms by negative feedback mechanisms.

Utilising these facts has led to a high ratio of 22- to non-22-kDa being proposed as a means of detecting exogenous GH usage. By using assays based on a combination of relatively non-specific polyclonal antibodies and

specific assays using two monoclonal antibodies (each of which has been designed to recognise specific epitopes on the 22 kDa and 20 kDa molecules), Strasburger and colleagues have developed a promising method for detecting rhGH abuse. (Wu et al. 1999).

Age, gender and pathological state are not thought to affect the relative proportions of GH isoforms. It is uncertain, however, whether ethnic background affects the isoform ratio (Wilson et al, 1998).

This approach has a limited window of opportunity lasting less than 24 hours after an injection (Wu et al, 1999) as GH secretion soon returns to baseline values after the last dose of GH treatment (Wu et al, 1990).

Limitations of this method include an increase in the 22-kDa isoform brought about by exercise, possibly lowering the test's sensitivity (Banfi et al, 1994). In addition, the isoform method is unable to detect pituitary-derived GH doping or the abuse of GH secretagogues. This method might also be confounded by the use of a balanced mixture of 22kDa and 20kDa rhGH, a possibility so far not reported but might become a reality in future (Rigamonti et al, 2005).

It would seem that this method may be best suited for 'out of competition' testing, when an unannounced blood sample may well be taken within 24 h of the last rhGH injection. More work is needed to validate this approach however.

Richard Pound, head of WADA, has in fact confirmed that the testing for GH was carried out for the very 1st time at a major sporting event recently. Approximately 300 blood samples were screened for GH at the Athens Olympic Games in 2004 using the Isoform Assay Method. None of these tested positive for GH. (MSNBC.com AP article, 2004)

(2) The second method of detecting GH abuse was previously called the "indirect method" but should be more accurately known as the "**marker method**". It is based on measurement of markers of GH action and considers

circulating concentrations of several GH-sensitive substances that exceed normal physiological variability. This was the main outcome of the international research collaboration, the GH-2000 Project and subsequent to that, the GH-2004 Project.

Two groups of potential markers were established by the GH-2000 research team: one group included members of the IGF-IGFBP axis, the other included markers of bone and collagen turnover and mineralisation (Wallace et al, 2000).

The measurement of multiple markers in conjunction with specific equations, known as “discriminant functions”, can be utilised to detect GH abuse with enhanced sensitivity and specificity compared with single-marker analysis. (McHugh et al, 2005). This will be discussed in more detail later in this report.

It works on the principle that when given in supra-physiological doses, GH over-stimulates a number of processes normally regulated by GH. It should, therefore, be possible to detect GH abuse if these GH-dependent markers are present in amounts that greatly exceed the normal physiological range.

This approach is not able to distinguish the athlete with a GH-secreting pituitary tumour but it should be able to detect normal subjects administered supra-physiological amounts of GH.

This method has good sensitivity for up to a fortnight after the last injection of GH and is not influenced by extreme exercise. It is also suitable for post-competition samples and has a greater sensitivity in men than in women.

‘A test based on the results of the GH-2000 project has a sensitivity better than 90% of picking up a man taking GH with a probability of less than 1: 10,000 of being wrong’ (Sonksen, 2001).

5.7- How were these markers initially identified?

Exercise is known to be a powerful stimulus to GH secretion. A pilot ‘washout’ study was designed which investigated the effects of 1 week’s rhGH administration and acute exercise on a wide range of substances influenced by GH. The intention was to identify a subgroup of GH-dependent ‘markers’ that were sensitive to rhGH administration but comparatively insensitive to the acute effects of exercise (Wallace et al, 1999, Longobardi et al, 2000).

The results of that pilot study suggested 8 possible candidates that were suitable to take forward into other studies: 4 markers were produced by the liver and four produced from collagen and bone (see *Figure B*).

(Figure B)

Markers identified by pilot study as being potentially useful in developing a test for GH abuse

<u>Liver produced GH-dependent markers</u>	<u>Bone and Collagen produced GH-dependent markers</u>
*IGF-I	*P-III-P (Procollagen III terminal peptide)
IGFBP2 (IGF-binding protein-2)	PICP (Type 1 collagen telopeptide)
IGFBP3 (IGF-binding protein-3)	ICTP (C-terminal propeptide of type I collagen)
ALS (Acid labile subunit)	Osteocalcin

A ‘double-blind, placebo-controlled’ study of 1 month’s rhGH administration at 2 doses to more than 100 healthy volunteers was carried out in 4 countries. (Wallace et al. 2000). All 8 markers were measured on the blood samples taken before, during and for 3 months after the rhGH or placebo administration. *Analysis subsequently indicated that the best discrimination between active treatment and placebo was obtained using two of the markers – **IGF-I** and **P-III-P**.

5.8- IGF-I as a marker

IGF-I is an excellent candidate marker for the following reasons:

- (1) it has little diurnal or day-to-day variation (Wilson et al, 1998);
- (2) It has low basal scatter (Dall et al, 2000) and minimal changes with exercise.
- (3) It increases 1.3- to 2.3-fold in a uniform dose-dependent fashion after GH administration (Kniess et al, 2003);

Total IGF-I is used because free IGF-I is less responsive to GH (Longobardi et al, 2000). IGF-I is bound in the circulation to IGF-binding proteins (IGFBP), Of these, it binds mostly to IGFBP-3. IGFBP-3 increases with GH administration but has a less uniform and dose-dependent curve than IGF-I (Kniess et al, 2003), hence the poorer acceptability of IGFBP-3 for use as a marker.

IGF-I does have limitations though. It has been shown that high dose oestrogens, when administered orally or transdermally, can suppress circulating IGF-I levels (Kam et al, 2000).

5.9- P-III-P as a marker

Procollagen III terminal peptide (P-III-P) is a marker of type 3 collagen formation which is mainly found in soft tissues. It, too, is considered an ideal marker for the following reasons:

- (1) It exhibits little diurnal variation;
- (2) It exhibits little day-to-day variation;
- (3) It exhibits little gender variation;
- (4) It does not rise with exercise;
- (5) It increases in a dose-dependent fashion after GH administration (Wallace et al, 2000).

However, as a soft tissue marker, P-III-P secretion may increase after certain injuries or with certain pathological conditions such as lung or liver fibrosis or alcoholic cirrhosis (Kurdy et al, 1998).

5.10- The stability of the markers

A subset of those who volunteered for the GH2000 cross-sectional study used to construct the reference range volunteered to provide samples on other occasions during the year. Many of them also agreed to undertake a simulated extreme exercise event under 'laboratory' conditions.

The results from this longitudinal study, when considered with the information obtained from the placebo-treated group during the double-blind study, showed remarkable stability in the blood concentration of the markers over time.

This would suggest that the blood concentration of the markers is most probably genetically determined and relatively unaffected by day-to-day variation of environmental conditions.

5.11- Using both markers in combination

By using IGF-I and P-III-P in combination, statistical evaluation indicated that improved specificity and sensitivity could be generated by combining both markers in the analysis (Powrie et al, 2005).

As with GH levels in urine (Krogsgaard et al), analysis of the GH-dependent markers in urine samples showed much poorer discriminating power in separating active treatment from placebo.

6/ Materials & Methods:

6.1- The GH-2004 Injury Study

The GH-2004 Project leads on directly from the GH-2000 Project. The previous research project showed that developing a test for GH using GH-dependent markers was indeed feasible. There are 4 branches to the GH-2004 Project: (i) the Longitudinal Study; (ii) the Double-Blind Study; (iii) the Cross-Sectional Study and (iv) the Injury Study.

The Injury Study had been running for several months at the University of Southampton (UOS). The aim of this particular study was to collect blood and saliva samples from people involved in sports who had recently become injured to determine what happens to the body's endogenous growth hormone-dependent markers and how this might have an impact on testing regimes. The information gathered will act as a guide to determine the reference range to be used in anti-doping tests.

The target numbers were 50 participants from each of the 4 main ethnic groups – Caucasian, Afro-Caribbean, Oriental and Indo-Asian. Another objective of the Injury Study was to determine if a different reference range for injured athletes would be required for subjects from different ethnic groups.

The subjects recruited at UOS were predominantly amateur athletes who had recently suffered a musculoskeletal injury. It was decided that collaboration with the Olympic Medical Institute (OMI) to recruit elite athletes to the study would be useful when considering marker concentrations in injured elite athletes as well as helping to increase participant numbers.

6.2- Why was the OMI chosen?

The OMI is a medical treatment and support centre for elite British athletes and was formed as the result of a partnership between the British Olympic Association (BOA) and the English Institute of Sport (EIS).

In addition, it has residential rehabilitation services which enable injured athletes to have multi-disciplinary input, allowing career-threatening injuries to be dealt with comprehensively. The multi-disciplinary team includes sports physicians, sports physiologists, nutritionists, psychologists, sports and rehabilitation physiotherapists.

An evolution of the British Olympic Medical Centre, the OMI is housed within the grounds of Northwick Park Hospital in northwest London. Its proximity to the hospital ensures that athletes who require blood or radiological investigations rapidly are able to gain access to it. It also ensures swift specialist input from the hospital's consultants when that is requested.

Elite athletes from different types of sports are referred from all over the United Kingdom, ensuring a constant supply of potential participants of all different ethnicities for the study.

6.3- My involvement in the Injury Study

A researcher was needed to collect information and samples from participants at the OMI site. I met the GH-2004 team in Southampton in February 2005 and proceeded to begin the OMI branch of the Injury Study in March 2005. It was agreed that I would have 2 supervisors- 1 based at UOS, 1 based at the OMI. Regular contact was maintained between the OMI and UOS site by means of telephone calls and e-mail communications. Where possible, I attended GH-2004 team meetings in Southampton.

The Injury Study data collected at the OMI for this report is for the 4 ½ months between the middle of March 2005 to end of July 2005.

6.4- Ethical approval

The ethical aspects had previously been reviewed at the start of the original study and granted by the Southampton and South West Local Research Ethics Committee and WADA independently. Amendments were sent in

regarding the inclusion of the OMI site for collecting samples from injured participants.

6.5- Subject recruitment

All Sports Physicians and physiotherapists at the OMI were informed about the study verbally and by means of 2 letters and asked to refer patients if they thought they satisfied the criteria. (*Appendix A*)

Patients who attended the Sports Medicine clinics at the OMI and satisfied the inclusion and exclusion criteria were invited to participate in the study. Similarly, rehabilitation in-patients who satisfied the above criteria were also invited to participate in the study.

The objectives of the study were explained to the potential participants and a written Subject Information Sheet (*Appendix C*) was given to them to read. The issues of ethical research, patient confidentiality and possible side effects (if any) were discussed. They were also given time to ask any questions and have any uncertainties addressed. Participants who were agreeable to providing samples were given a consent form to sign before being included in the study (*Appendix D*).

6.6- Inclusion criteria

- Any participant involved in sport who had suffered a musculoskeletal injury within the last 84 days (3 months).
- Male or female.
- Any age range.

6.7- Exclusion criteria

- Alcoholism.
- Hepatic Cirrhosis.

- Diseases resulting in abnormally high growth hormone levels (e.g. Acromegaly, Pituitary secreting tumour).
- Diseases resulting in abnormally low growth hormone levels (e.g. established GHD).
- Patients on rhGH injections for medical indications.

6.8- Study Protocol

Each participant was allocated an individual Identity (ID) Number (e.g. SO 1001, SO 1002.....). Using a standardised history sheet (*Appendix E*), the following items of information were obtained from the participants:

- Name
- Age
- Gender
- Address
- Phone Number
- E-mail Address
- Participants Ethnicity: *Caucasian/ Afro-Caribbean/ Indo-Asian/ Oriental/ Pacific Islander/ Mixed Race/ Other*
- Family Ethnicity: *Caucasian/ Afro-Caribbean/ Indo-Asian/ Oriental/ Pacific Islander/ Mixed Race/ Other*
 - o Mother
 - o Father
 - o Maternal Grandmother
 - o Maternal Grandfather
 - o Paternal Grandmother
 - o Paternal Grandfather
- Height
- Weight
- Nature of injury
- Number of days since injury had occurred
- Past Medical History
- Medications (Prescribed and Over-the-counter)

- Sporting Event
- Level: *Olympic/ International/ National/ Regional/ County/ Club*
- Sports Physician or General Practitioner's details (if applicable)

The following samples were then taken from the participant:

6.9- Blood samples:

5mls of venous blood was taken using a sterile needle and vacutainer. It was collected and allowed to clot in a disposable SST II Advance Yellow top vacutainer bottle.

This sample was subsequently centrifuged at 3000 rpm for 15 minutes in a Denley BR401 Refrigerated Centrifuge Machine.

Using a disposable pipette to ensure cross-contamination of samples was kept to a minimum, serum was equally distributed into 4 unused plastic aliquots. Blue lids were used to seal the sample. The aliquots were labelled and subsequently placed in a freezer to be frozen at -4 °C.

The used pipette, vacutainer and remaining organic material were then discarded in a safe fashion.

(Blood samples which could not be centrifuged immediately were stored upright in the fridge up to the permitted maximum duration of 12 hours prior to spinning. Any sample that was kept longer than 12 hours in the fridge was discarded.)

6.10- Saliva samples:

1ml of saliva was collected in a single unused plastic aliquot. This was sealed with a white lid, labelled and stored immediately in the freezer.

The different coloured aliquot lids and labels ensured that there was no confusion about what each aliquot contained and whom the sample belonged to, when it was tested in the laboratory.

6.11- Subsequent visits

Where possible, participants had repeat blood and saliva samples taken on a weekly basis or longer until their injury exceeded the 84 day mark (see figure C).

In several instances, the idea of having subsequent blood and saliva samples taken by the athlete’s own sports physician or general practitioner (GP) was discussed (*Appendix B*).

(Figure C)

Day of Injury (cross out and write in exact day if appropriate, e.g. day 25)	Blood	Saliva	State of injury	Any new injury since commenced study (if yes, provide date)
Day 1				
Day 7				
Day 14				
Day 21				
Day 28				
Day 42				
Day 84				

6.12- Laboratory measurements:

Once an adequate number of samples had been collected, they were couriered to UOS to be logged, catalogued and stored again in a freezer.

They were subsequently transferred to the Drug Control Centre at King's College London (KCL) where they were thawed and tested.

Immunoassays to measure the serum and saliva concentrations of IGF-I and P-III-P were carried out at KCL.

6.13- IGF-I Immunoassay Kit

IGF-I levels are quantified in the extracted samples using a highly sensitive and specific immunoradiometric assay: The DSL-5600 (Diagnostic Systems Laboratories (DSL) 5600 Active® IGF-I Coated Tube IRMA, Sinsheim, Germany) immunoassay. It utilises a modified version of the standard hydrochloric acid–ethanol extraction procedure (*Appendix H*).

Assay-specific references need to be used whenever IGF-I levels are used for diagnostic purposes (Ranke et al, 2003). Each individual laboratory should aim to establish its own values. The following tables in *Figures D & E* describe serum IGF-I levels measured in 3 separate studies using the DSL-5600 Kit (*Appendix H*). All values are expressed in **ng/ml**.

(Figure D)

Adult Male IGF-I Normal Range

<u>AGE IN YEARS</u>	<u>MEAN</u>	<u>SD (STANDARD DEVIATION)</u>	<u>MEDIAN</u>	<u>ABSOLUTE RANGE</u>
18-20	489.0	206.7	475.0	197.0 - 956.0
20-23	420.1	114.7	413.0	215.0 - 628.0
23-25	320.7	106.3	304.0	169.0 - 591.0
25-30	236.7	81.2	241.0	119.0 - 476.0
30-40	211.9	102.5	188.0	100.0 - 494.0

(Figure E)

Adult Female IGF-I Normal Range

<u>AGE IN YEARS</u>	<u>MEAN</u>	<u>SD (STANDARD DEVIATION)</u>	<u>MEDIAN</u>	<u>ABSOLUTE RANGE</u>
18-20	367.9	106.1	341.0	193.0 – 575.0
20-23	288.9	109.8	279.0	110.0 – 521.0
23-25	274.9	93.1	273.0	129.0 – 480.0
25-30	253.5	106.6	236.0	96.0 – 502.0
30-40	217.7	76.2	209.0	130.0 – 354.0

6.14- P-III-P Radioimmunoassay (RIA) Kit

The serum concentration of P-III-P was determined by RIA (RIA-gnost® PIIP, International CIS, Gif sur Yvette, France).

In children and adolescents, an age-dependence for P-III-P levels is detectable. The highest values are observed in newborn infants. P-III-P levels fall with increasing age, reaching the given normal range at the age of 20. Raised values are also observed in pregnant women but they return to normal within 8 weeks post partum.

Pathological conditions affecting the liver, which are associated with active proliferation of connective tissue, give rise to raised P-III-P values. According to the degree of severity of the disease, P-III-P levels in serum are raised in chronic active hepatitis (CAH), fibrosis and liver cirrhosis. There is a good correlation with histological findings in fibrosis and cirrhosis.

There are also other conditions in which P-III-P levels are raised without a detectable change in the liver (e.g. pulmonary fibrosis, rheumatic disorders, myocardial infarction, acromegaly, multiple trauma).

The lower detection limit is about 0.1 units of PIIP/ml, the upper detection limit is 14 units of PIIP/ml. The normal range for RIA-gnost® P-III-P has been determined using serum samples from 451 healthy men and women Kit (*Appendix G*). By calculating the 5th and 95th percentile, a normal (i.e. non-pathological) range of **0.3-0.8 U/ml** has been established.

7/ Results:

7.1- Demographics

Between mid-March 2005 to end of July 2005, 18 elite athletes were recruited to the study from the OMI. There were 12 male and 6 female participants.

1 athlete (SO 1006, SO 1011) was recruited to the study twice as there was a considerable amount of time between resolution of his 1st injury and occurrence of his 2nd injury. He fulfilled the inclusion criteria on both occasions,

The mean age for all the OMI participants was 23 \pm 1.1 years. The age ranges were 17 to 35 years for the male participants; 19 to 27 years for the female participants.

The mean height for all the participants was 177.5 \pm 3.1 cm. The range of heights were 170 to 194 cm for the male participants; 154 to 181 cm for the female participants.

The mean weight for all the participants was 78.6 \pm 3.6 kg. The range of weights were 63 to 102 kg for the male participants; 56.5 to 73 kg for the female participants.

11 competitive sports were represented in total. These were canoeing, sculling, rowing, snowboarding, alpine skiing, bobsleighting, softball, judo, taekwando, athletics and triathlon.

There were 7 Olympic-level participants and 11 international-level participants.

Of the 18 participants, 14 were classed as Caucasian and 4 were classed as Afro-Caribbean. None of the participants were of Oriental or Indo-Asian origin.

The injuries were all musculo-skeletal in nature. This could be further subdivided into the following classification:

- Bony injuries (e.g. definite fractures): 2 participants
- Soft tissue injuries (e.g. ligamentous ruptures and tears, tendinopathies, enthesopathies and muscle tears): 12 participants
- Other musculo-skeletal injuries (e.g. dislocations, muscle spasms, presumed sprains): 5 participants.

The demographic data is expressed in the following tables (see *Figures F & G*).

(Figure F)

**OMI Injury Study Participants –
Classification by chosen sport, level and ethnicity**

<u>Study No.</u>	<u>Sport</u>	<u>Level</u>	<u>Ethnicity</u>	<u>Serum Samples - (Days After Injury)</u>	<u>Saliva Samples - (Days After Injury)</u>
SO 395	Alpine Skiing	Olympic	Caucasian	8, 33	33
SO 1001	Alpine Skiing	International	Caucasian	91	91
SO 1002	Alpine Skiing	International	Caucasian	89	89
SO 1003	Canoeing	International	Caucasian	33	33
SO 1004	Sculling	International	Caucasian	16, 23	16, 23
SO 1005	Snowboarding	Olympic	Caucasian	3	3
SO 1006	Bobsleighbing	Olympic	Caucasian	70, 92	70
SO 1007	Softball	Olympic	Caucasian	150	150
SO 1008	Judo	Olympic	Caucasian	60	60
SO 1009	Alpine Skiing	International	Caucasian	69	69
SO 1010	Triathlon	International	Caucasian	21, 35	21, 35
SO 1011	Bobsleighbing	Olympic	Caucasian	13	13
SO 1012	Rowing	International	Caucasian	42, 49	42, 49
SO 1013	Bobsleighbing	Olympic	Caucasian	75	75
SO 1014	Bobsleighbing	Olympic	Caucasian	28	28
SO 1015	Athletics	International	Afrocaribbean	Declined	42
SO 1016	Taekwando	International	Afrocaribbean	28	28
SO 1017	Athletics	International	Afrocaribbean	45	45
SO 1018	Athletics	International	Afrocaribbean	3	3

(Figure G)

OMI Injury Study Participants –
Classification by entry date, gender, age and injury diagnosis

<u>OMI Injury Study No.</u>	<u>Date entered study</u>	<u>Gender (M/F)</u>	<u>Age</u>	<u>Injury Diagnosis (where possible)</u>
SO 395	14/03/2005	X	X	Fracture To Left Tibial Plateau
SO 1001	17/03/2005	X	X	Ruptured Left Anterior Cruciate Ligament And Left Medial Collateral Ligament
SO 1002	17/03/2005	X	X	Ruptured Right Anterior Cruciate Ligament
SO 1003	21/03/2005	X	X	Anterior Dislocation Of Left Shoulder
SO 1004	04/04/2005	X	X	Annular Tear To Intervertebral Disc
SO 1005	07/04/2005	X	X	Fracture To Left Navicular & Cuneiform Bones
SO 1006	11/04/2005	X	X	Left Tibialis Posterior Tendinopathy
SO 1007	13/04/2005	X	X	Ruptured Right Lateral Meniscus
SO 1008	20/04/2005	X	X	Epicondylitis- Right Elbow
SO 1009	10/06/2005	X	X	Ruptured L Anterior Cruciate Ligament
SO 1010	21/06/2005	X	X	Low Back And Right Shoulder Pain
SO 1011	21/06/2005	X	X	Acute Lumbar Annular Tear
SO 1012	04/07/2005	X	X	Acute Lumbar Annular Tear
SO 1013	04/07/2005	X	X	L Patellar Enthesopathy
SO 1014	04/07/2005	X	X	Lumbar Muscle Spasm
SO 1015	11/07/2005	X	X	Left Sacroiliac Joint Dysfunction
SO 1016	11/07/2005	X	X	Right Hand Sprain
SO 1017	31/07/2005	X	X	Right Hamstring Muscle- Partial Rupture
SO 1018	31/07/2005	X	X	Left 1st Metatarsophalangeal- likely Cartilage Tear

7.2- Follow-up away from the OMI

Of the 18 participants, only 1 participant consented to have his blood and saliva checked regularly away from the OMI. He initially consented to us contacting his team doctor (by post) to help with taking his blood and saliva samples fortnightly and sending us the samples via pre-printed and pre-paid envelopes. He subsequently changed his mind and declined further follow-up.

7.3- Analysis of samples

It was decided that due to severe time constraints, only the serum samples would be analysed. The saliva samples will be analysed in due course, before the completion of the GH-2004 Project. The measurement of serum IGF-I and serum P-III-P were carried out using the DSL-5600 Active® IGF-I and RIA-gnost® PIIIP radioimmunoassay respectively.

7.4- Data Analysis

Statistical analysis was carried out using Microsoft Excel 2002 software program.

7.5- Serum IGF-I & P-III-P Results

2 samples (from participants SO 1001, SO 1002) were not tested due to problems with storage and subsequent freezing in the early stages of the study. 1 athlete (SO 1015) declined to donate any blood samples and contributed saliva samples only. 20 serum samples from 16 participants (11 males and 5 females) at the OMI were tested for IGF-I & P-III-P levels. The results are as follows: *(see Figure H)*

(Figure H)

<u>Study No.</u>	<u>Gender</u>	<u>Day after injury</u>	<u>IGF-I Levels (ng/ml)</u>	<u>Serum P-III-P levels (U/ml)</u>
SO 395	X	33	719.90	1.102
SO 1003	X	33	985.46	0.983
SO 1004	X	16	648.36	0.447
SO 1004	X	23	540.34	0.459
SO 1005	X	3	829.78	0.456
SO 1006	X	70	516.78	0.493
SO 1006	X	92	570.94	0.672
SO 1007	X	150	578.88	0.520
SO 1008	X	60	675.76	0.335
SO 1009	X	69	627.76	0.616
SO 1010	X	21	651.90	0.807
SO 1010	X	35	850.10	0.727
SO 1011	X	13	585.62	0.677
SO 1012	X	42	751.16	0.335
SO 1012	X	49	765.48	0.441
SO 1013	X	75	502.98	0.466
SO 1014	X	28	744.20	0.362
SO 1016	X	28	650.30	0.383
SO 1017	X	45	464.88	0.625
SO 1018	X	3	895.08	0.235

Mean serum IGF-I level for all OMI participants

= 677.78 +/- 31.17 ng/ml (where n = 20)

Mean serum IGF-I level for male OMI participants only

= 664.59 +/- 41.93 ng/ml (where n = 14)

Mean serum IGF-I level for female OMI participants only

= 708.56 +/- 36.99 ng/ml (where n = 6)

Mean serum P-III-P level for all OMI participants

= 0.56 +/- 0.05 U/ml (where n = 20)

Mean serum P-III-P level for male OMI participants only

= 0.62 +/- 0.06 U/ml (where n = 14)

Mean serum P-III-P level for female OMI participants only

= 0.41 +/- 0.03 U/ml (where n = 6)

In order to see how injury might alter one's interpretation of IGF-I and P-III-P values for injured male and female athletes, larger numbers of participants were needed than those accrued over the 4 ½ month period at the OMI. For

this purpose, some data taken from the Injury Study participants at the UOS site has been included (*Appendices J & K*). The following graphical displays for injured males and females athletes have been designed by combining the 2 sets of data (*See Figures I, J, K & L*):

(Figure I)

(Figure J)

(Figure K)

(Figure L)

8/ Discussion:

8.1- Differences in mean serum IGF-I and P-III-P levels for male and female participants

The mean serum IGF-I level for male OMI participants was 664.59 +/- 41.93 ng/ml (where n = 14); the mean serum IGF-I level for female OMI participants was unexpectedly higher at 708.56 +/- 36.99 ng/ml (where n = 6). By applying a t-Test (assuming equal variances for the 2 samples), $p > 0.05$. This shows that the differences between mean serum IGF-I levels for male and female participants were not significant.

The mean serum P-III-P level for male OMI participants was 0.62 +/- 0.06 U/ml (where n = 14); the mean serum P-III-P level for female OMI participants was 0.41 +/- 0.03 U/ml (where n = 6). By applying a t-Test (assuming equal variances for the 2 samples), $p > 0.05$. This shows that the differences

between mean serum P-III-P levels for male and female participants were not significant.

In view of these findings, serum IGF-I (X-axis) was plotted against serum P-III-P (Y-axis) for all OMI participants (male and female participants combined).
(See *Figure M*)

(Figure M)

8.2- The expected rise in GH-dependent markers after injury

One would expect rising levels of serum IGF-I and P-III-P soon after injury, rising to a peak and then gradually decreasing again. Based on the current data, plotting regression lines does not support this theory in 3 out of 4 of the graphs (*Figures I, J & L*).

Several serum IGF-I sample readings were noticeably higher than the rest. Analysing the data more closely did not produce an identifiable pattern of bony or soft tissue injury which might explain this. The same findings applied to serum P-III-P sample readings.

More data will be needed before firm conclusions can be drawn about the natural time-course and levels of these markers after injury.

8.3- Difficulties in following up participants

Although most participants were willing to contribute blood and saliva samples while they were at the OMI, most were not interested in regular follow-up with their own general Practitioners or Sports Physicians. This was usually due to reasons of time constraints or dislike of venepuncture.

Many of the participants did not live locally and attended as out-patients with appointments several weeks to months apart. Some came as in-patients for short-term rehabilitation. Although that was preferable in terms of follow-up, often the potential participant did not fulfil the inclusion criteria.

8.4- Difficulties in recruiting participants of certain ethnic groups

Throughout the study, there have been difficulties recruiting athletes of Oriental and Indo-Asian origin. This may reflect the paucity of elite level athletes from those ethnic backgrounds in the United Kingdom.

There may be several reasons for this- perhaps participation and the sacrifice necessary to get to the upper echelons of sport are not as highly encouraged in these cultures. It may also reflect the types of sport a person from such a background may be interested in (as a sport which is not classed as an Olympic Sport is far less likely to have one of its members attending the OMI).

The difficulties experienced are similar to those experienced by the GH-2000 Project Study Group. At present, the GH-2004 Group is collaborating with clinicians in Singapore to address these problems.

8.5- Challenges associated with the detection of GH Abuse

Certain factors can cause difficulty when trying to distinguish between those who have taken exogenous GH and those who have not. These are:

- (1) the heterogeneous nature of GH;
- (2) the short half-life of GH in circulation ;
- (3) the presence of GH-binding proteins in plasma;
- (4) the potential cross-reactivity with homologous polypeptide hormones such as prolactin;
- (5) the heterogeneous immuno-reactivity of monoclonal antibodies used in commercial immunoassays
- (6) the amino acid sequence identity between the main fraction of pituitary-derived GH and recombinant GH.

(McHugh et al, 2005)

8.6- Factors to consider when establishing a reference range

1) The effects of exercise: As mentioned previously, exercise is a potent stimulus for GH secretion (Godfrey et al, 2003). Both acute and chronic physical activity increase the concentrations of some of the GH markers. This effect is augmented by GH administration, but the general pattern of the increase remains the same. Total but not free IGF-I also increases after acute exercise (Wallace et al, 1999).)

2) Aging and adolescence: As with GH, there is also a decline in the secretion of IGF-I with aging (Corpass et al, 1993, Weltman et al. 1994; Keenan et al. 1999; Harridge, 2003). In fact, all the potential GH-dependent markers showed a very clear age-related fall. It also shows that even elite athletes who exercise at rates much higher than the normal population still show a decline in GH production as they age.

In adolescents, it should be noted that stages of puberty as opposed to chronological age determines the level of GH secretion.

3) Elite versus amateur athletes: Circulating levels of GH markers in elite athletes after an event are different from the values found in standard reference intervals (Healy et al, 2005).

4) Gender: Women have lower GH peaks and higher troughs and are relatively more GH resistant (Wilson et al, 1998; Nussey & Whitehead, 2001; Powrie et al, 2005).

5) Ethnicity: It is thought that ethnic differences play a role in differing levels of GH-dependent markers. The extent of these differences is uncertain at present.

6) Injury: The GH/ IGF-I axis is thought to be involved in the regulation of fracture and wound healing (Bail et al, 2001; Weiss et al, 2002). As traumatic injury is a frequent occurrence in sport, the potential impact of injury, particularly on bone markers has to be carefully evaluated.

8.7- Statistical & Discriminant Function Analysis

Single-marker analysis lacks sufficient specificity to detect exogenous GH abuse. A combination of markers is necessary in conjunction with discriminant function analysis to improve sensitivity and specificity. To generate these mathematical equations, a training set of data is applied to create the

discriminant model, and a confirmatory set of data then similarly applied to ensure that the model is applicable to the population in general (not just the sample set). The time course disparity of the markers can be exploited by mathematical modelling to further enhance the sensitivity of the test to detect GH abuse.

It was initially proposed that IGF-I to IGFBP-2 and IGFBP-3 to IGFBP-2 ratios could be used (Kicman et al). However, of the various potential markers considered, the GH-2000 team found that IGF-I and P-III-P were the simplest and most effective combination providing the best sensitivity and specificity during rhGH therapy (Dall et al, 2000). Kniess et al subsequently reported significant rises in the concentrations of the products (IGF-I _ P-III-P) and (IGF-I _ IGFBP-3) in a GH administration study, independently confirming the GH-2000 study results.

A recent paper by Powrie et al (submitted for publication) looked at the overall results of the GH-2000 study, particularly the double-blind placebo controlled study of GH administration to 102 trained but non-elite subjects. They also obtained blood from 813 elite athletes across 15 sporting disciplines drawn within 2 hours of a major national or international event to establish reference ranges for this group. They report the following:

Construction of a doping test

The data from the double blind study of GH administration was used to construct a formula that allowed best discrimination between those on active treatment or placebo. The data was used to optimize the discrimination while subjects were actively administering GH rather than in the washout period. The data from the cross sectional study of elite athletes was used to assess the physiological differences between the elite and non-elite athletes and to allow calibration of the doping test for elite athletes. It was also used to assess and take account of the effects of age.

All markers measured in the double blind study were assessed in order to determine which combinations would provide optimal discrimination. The

patterns of marker levels changed over time. Most markers were elevated while the drug was still being administered but the rate at which the markers declined over the washout period varied. Men and women were considered separately due to their differing sensitivities to the effects of GH.

The combination of markers IGF-I and P-III-P provided the best and most consistent discrimination between subjects in the placebo and active drug groups in the double-blind trial during both the treatment and early washout period. **Figure 3** shows that this combination results in complete separation of active and placebo groups even in those treated with low dose GH. The use of either individual marker would result in overlap between the groups. IGF-I declined more rapidly following cessation of GH administration than did P-III-P.

Figure 3: Individual datapoint plot of P-III-P against IGF-I levels in male subjects after 3 weeks treatment with low dose (€) or high dose (▲) GH or placebo (◆).

Using discriminant analysis and correcting for age (with age expressed for both men and women on a reciprocal scale) the following proved to be the best formulae:

$$\text{Men} = -6.586 + 2.905 * p3p + 2.100 * igf - 101.737 / \text{age}$$

$$\text{Women} = -8.459 + 2.454 * p3p + 2.195 * igf - 73.666 / \text{age}$$

where marker levels are recorded on a logarithmic scale writing $igf = \log(\text{IGF-I})$ and $p3p = \log(\text{P-III-P})$, for convenience. The larger the value found, the more likely it would be that the individual was receiving the active drug.

These age-adjusted scores have mean 0 and standard deviation 1 over the samples of elite athletes, for males and females separately. This age

adjustment has only been validated over age range 16-50. Once the adjustment was made to take account of the dependence of IGF-I and P-III-P on age there were no significant differences in derived scores between different sporting disciplines.

*After omission of one large negative value for the male sample, the statistical distributions of both age-adjusted derived scores were tested for Normality (with a mean of zero and a standard deviation of one) with non-significant results ($p > 0.1$ for each sample). The 99% reference ranges can therefore be taken as (-2.576, +2.576). For males, this range covered 98.9% of the available sample; for females it covered 99.5%. A cut-off level of derived marker score can be chosen which will reduce the false positive rate to an acceptable level, which could be set at whatever is deemed to be reasonable. A threshold value of 3.7 will be exceeded by chance in only around 1 observation in 10,000. **Table 1** shows the sensitivity of the test using this cut-off value when applied to our study of GH administration. On this basis this test can detect up to 86% of men and 60% of women actively taking GH and we consider that this provides the basis for a robust test of GH doping.*

Table 1: Sensitivity of the GH doping test both during and after the administration of low and high dose GH to men and women. The table shows the number of subjects from the trial testing positive on various days during and after the administration of GH using the formulae with a cut-off value for the test of 3.7 (specificity of 1:10,000). GH was administered on days 0-28 (shaded area).

(Extract taken from Powrie et al, 2005)

8.8- Superimposing the data: A useful focus for further discussion

By superimposing the data from the OMI Injury Study on to Powrie et al's datapoint plot of serum P-III-P against IGF-I (with permission from the GH-2000 Study Group), the following graph was obtained. (See *Figure N*)

(Figure N)

Several factors need to be borne in mind when considering the 2 sets of data.

Firstly, the serum concentration of IGF-I in Powrie et al's data was determined by a different hydrochloric acid–ethanol extraction radioimmunoassay (RIA) (Nichols Institute Diagnostics, San Juan Capistrano, CA, USA) whereas the samples from the OMI were tested using the DSL-5600 Active® IGF-I Coated Tube IRMA. This decision was prompted by the Food and Drug Administration's (FDA) decision to withdraw that particular Nichols IGF-I assay from the market. The DSL assay is known to have a higher 'normal' reference range. This may partly explain why the OMI data is skewed to the right.

Secondly, Powrie et al's data in this graph is only considering male participants whereas the OMI data considers both males and females.

Thirdly, Powrie et al's data is looking at participants in a double-blind study, whereas the OMI data considers injured participants.

Placing these limitations to one side for a moment, it is possible to see how the OMI data supports the use of a 'marker' test coupled with discriminant function analysis to increase its sensitivity and specificity. The serum concentration of P-III-P in both sets of data was determined by the same type of assay (RIA-gnost® PIIP) which may explain why the OMI data remains under the cut-off line (i.e. 'normal' values) if positive skewing is ignored.

However, both sets of IGF-I data were assayed against the same International Reference Preparation of IGF-I and would be expected to be closer. This suggests that there are problems with assay standardisation which will need to be solved before working out the true injury 'effect' on setting a reference range and subsequent cut-off level.

For a better comparison to occur, either the samples have to be aligned by re-assaying some of the GH-2000 samples with the DSL assay or a 'correction factor' will need to be devised to link findings from both IGF-I assays.

9/ Conclusions

9.1- What are the reasons for athletes doping with GH?

There are perhaps 4 main motives for this:

(1) As a drug of abuse, GH has many features that would make it attractive to the would-be 'doper'. It is a powerful anabolic agent, readily available in large quantities and undetectable by current anti-doping methods in place. It is also relatively safe in the short term as risks associated with taking it are minimal.

(2) It is well documented from surveys of elite athletes that they would be willing to take high risks in order to win medals. A survey carried out in 1995 by a prominent sporting magazine in the USA polled a series of elite athletes on several questions (Bamberger & Yaeger, 1997).

One of these questions was: 'You are offered a banned performance-enhancing substance with two guarantees :....(1) you will not be caught; (2) you will win....Would you take the substance?'

98% of respondents answered 'Yes'!

Another question posed was: 'You are offered a performance-enhancing substance that comes with two guarantees:.....(1)you will not be caught; (2)you will win every competition you enter for the next 5 years and then you will die from the side-effects of the substance.....Would you take it?'

More than 50% of the respondents answered 'Yes'.

Although this may not be a truly representative survey, it does highlight the fact that the motivation to win is indeed very strong in the elite athlete population and a reason why drug abuse in sport may be prevalent. The potential financial rewards of winning that elusive gold medal are immense.

(3) Injuries are common in most sports as a result of overuse and overtraining. Some athletes believe that the prevention of such injuries (e.g. tendinopathies) can be reduced or even eliminated completely. There is also the view that GH may prevent stress fractures and speeds the healing process. Indeed, research has shown that GH stimulates proliferation of cartilage in the growing epiphyseal plate, stimulates linear growth, and increases bone mass, mineral content, and the number of bone modelling units (Nussey & Whitehead, 2001).

(4) GH and testosterone act via separate mechanisms but have synergistic effects on anabolic metabolism. This advantage has not been lost on athletes, who use cocktails of anabolic agents to gain the maximal effect, as illustrated by the recent Montgomery case.

9.2- The Injury Study at the OMI

By comparing data ranges in 'normal' subjects (provided by the IGF-I and P-III-P kit inserts) and injured athletes, it would appear that serum IGF-I and P-III-P levels do increase quite considerably after injury. This builds on the work that was done by the GH-2000 Project and strongly suggests that developing reference ranges for GH-dependent markers with regards to GH doping is indeed possible. Due to various constraints of time, participant ethnicity and participant numbers however, the data obtained for this study is preliminary and no firm conclusions can therefore be drawn at this stage.

The scarcity of Oriental and Indo-Asian elite athletes was noted and this is being dealt with by further collaborations in countries where such potential participants can be found in greater abundance.

Participants should ideally be followed up and have several serum and saliva samples taken chronologically over a period of weeks to months after injury to monitor how IGF-I and P-III-P levels change.. This was often not possible for various reasons.

As with GH, the data does suggest that increasing age appears to blunt the serum IGF-I and P-III-P response, irrespective of type of injury.

Clearly, more data is required before age-, gender- or ethnicity-specific reference ranges can be firmly established for a 'marker' test. As such, performing detailed statistical analysis on the current data (beyond descriptive data analysis) will only result in speculation and inaccurate conclusions.

9.3- Introducing a 'marker' test for doping: the challenges ahead

With regards to introducing a 'marker' test for GH doping, recent research has shown that this is feasible (Powrie et al, 2005; McHugh et al, 2005; Rigamonti et al, 2005). However, certain issues will need to be ratified before introduction of such a test.

There are numerous IGF-I and P-III-P assays available commercially. These assays are important in detecting increased secretion of GH-dependent markers in athletes who dope but each assay has its own 'normal' reference range. Calibrants and assay reagents will therefore need to be standardised for comparison.

IGFBPs have similar affinities and compete with conventional antibodies for IGF-I. IGFBPs should be dissociated and separated from the IGF-I before assay, e.g. by acidification (Blum & Breier, 1994). Acid ethanol extraction followed by cryoprecipitation has a significantly improved IGF-I recovery of 90%– 95%. High affinity antibodies should be used wherever possible to improve the accuracy of the IGF-I assays.

At present, the exact levels of specificity for a 'marker' test for GH have yet to be decided. This depends largely on the level of specificity that a court of law would demand to uphold a prosecution of drug doping. Courts of law do not currently deal in terms of scientific probabilities.

Current medical practice accepts as 'normal' any value that lies within 2 SD from the mean on a calibration curve (i.e. 95% of the population is within the 'normal' range). By this definition, 5% of the population lie outside the 'normal range'. This would create an unacceptably high false-positive rate if applied to athletes.

Clearly, a compromise between what is ideal and what is achievable will be necessary if further progress is to be made on catching those who abuse GH.

Finally, there are the ethical issues to consider. Any testing for GH doping must be fair to the athlete, consistent in the process by which testing is carried out and accountable to the public.

Developing a definitive reference range for IGF-I and P-III-P levels in injured athletes will require more work, but as the preliminary results of this study show, that is certainly a realistic and achievable aim.

(Total Word Count = 9587 words)

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(by alphabetical order)

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12/ Appendices:

Appendix A – Introductory letters to Physicians and Physiotherapists at the Olympic Medical Institute

Appendix B – Letter to elite participant's own Sports Physician or General Practitioner

Appendix C – GH-2004 Injury Study Subject Information Sheet

Appendix D – GH-2004 Injury Study Consent Form

Appendix E – GH-2004 Injury Study History Sheet

Appendix F – GH-2004 Injury Study Generic Participant List

Appendix G – P-III-P radioimmunoassay kit insert

Appendix H – IGF-I radioimmunoassay kit insert

Appendix I – MSNBC.com Article: Athletes were tested for hGH in Athens

Appendix J – UOS GH-2004 Injury Study Data for serum IGF-I

Appendix K – UOS GH-2004 Injury Study Data for serum P-III-P