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The detection of growth hormone and insulin-like growth factor-I misuse in athletes

by

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ABSTRACT

FACULTY OF MEDICINE

Doctor of Philosophy

THE DETECTION OF GROWTH HORMONE AND INSULIN-LIKE GROWTH FACTOR-I MISUSE IN ATHLETES

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There is widespread evidence that growth hormone (GH) has been misused by athletes for many years because of its anabolic and lipolytic properties. The anabolic effects of insulin-like growth factor-I (IGF-I) also make this an attractive performance-enhancing agent although there is no published evidence to suggest that IGF-I has a beneficial effect on athletic performance.

The GH-2000 and GH-2004 research groups devised a method for detecting GH misuse based on serum concentrations of GH-dependent markers. Currently, no test is available to detect IGF-I misuse and the first aim of my research was to investigate the detection of IGF-I misuse in athletes, using the principles of the GH-2000 marker method. A further aim was to investigate the effects of IGF-I administration on glucose homeostasis, body composition and physical fitness in recreational athletes. 56 recreational athletes were recruited to a randomised, double-blind, placebo-controlled recombinant human IGF-I (rhIGF-I)/rhIGF binding protein-3 (rhIGFBP-3) administration study. Serum IGF-I and procollagen type III amino-terminal propeptide (P-III-NP) concentrations increased after rhIGF-I/rhIGFBP-3 administration, though the increase in P-III-NP was substantially less than the rise observed after rhGH administration. rhIGF-I/rhIGFBP-3 administration caused a reduction in insulin secretion and fasting triglycerides. There were also significant improvements in aerobic athletic performance, though no changes in body composition were observed.

The GH-2000 marker method for detecting GH misuse required further validation before it could be implemented by anti-doping organisations. Serum GH-dependent markers from 157 elite adolescent athletes and 498 elite adult athletes were measured using commercial immunoassays and the results were used to devise decision limits for detecting GH misuse in athletes. The stability of GH-dependent markers in serum was investigated using blood samples from 20 healthy volunteers and we showed that storage of serum at -20°C for up to 3 months had no significant effects on analyte results. The GH-2000 test was introduced at the London 2012 Olympic and Paralympic Games and two athletes with positive results for GH misuse have been banned from competition.

It is possible to detect messenger RNA (mRNA) for GH and IGF-I in the peripheral circulation. A final aim of my research was to investigate the use of blood mRNA technology to detect GH and IGF-I misuse. The intra-individual variability of mRNA concentrations for these proteins was investigated, along with the response in circulating mRNA to administration of rhGH and rhIGF-I/rhIGFBP-3. These mRNA species demonstrated high physiological variability in blood and the method did not appear to offer a reliable alternative to serum peptide assays.

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DECLARATION OF AUTHORSHIP

I, **DR NISHAN GUHA**, declare that the thesis entitled:

"The detection of growth hormone and insulin-like growth factor-I misuse in athletes"

and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

1. this work was done wholly or mainly while in candidature for a research degree at this University;
2. where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. where I have consulted the published work of others, this is always clearly attributed;
4. where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. parts of this work have been published as:

Guha N, Erotokritou-Mulligan I, Burford C, Strobridge G, Brigg J, Drake T, Bassett EE, Cowan D, Bartlett C, Sönksen PH, Holt RI. "Serum insulin-like growth factor-I and pro-collagen type III N-terminal peptide in adolescent elite athletes: implications for the detection of growth hormone abuse in sport" ***J Clin Endocrinol Metab*****. 2010 Jun; 95(6): 2969-76.**

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Signed:…….....

Date…….....

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DEFINITIONS AND ABBREVIATIONS

AAS	Anabolic androgenic steroid
ALS	Acid-labile subunit
ANOVA	Analysis of variance
BIA	Bioelectrical impedance analysis
BMI	Body mass index
CV	Coefficient of variation
EPO	Erythropoietin
GH	Growth hormone
GHIS	Growth hormone insensitivity syndrome
GHRH	Growth hormone-releasing hormone
HbA_{1c}	Glycated haemoglobin
IGF-I	Insulin-like growth factor-I
IGFBP	Insulin-like growth factor binding protein
IOC	International Olympic Committee
NEFA	Non-esterified fatty acid
P-III-NP	Procollagen type III amino-terminal propeptide
PCR	Polymerase chain reaction
QC	Quality control
rhIGF-I/rhIGFBP-3	Recombinant human insulin-like growth factor-I/recombinant human insulin-like growth factor binding protein-3
RIA	Radioimmunoassay
USADA	United States Anti-Doping Agency
WADA	World Anti-Doping Agency
WTCRF	Wellcome Trust Clinical Research Facility

CHAPTER 1: INTRODUCTION

1.1 Background

The use of performance-enhancing substances or "doping" has been prevalent in the professional sporting arena for many years and athletes continue to search for artificial ways to gain a competitive advantage (Sjoqvist et al. 2008). It is widely believed that growth hormone (GH) is a popular substance of misuse for athletes because of its lipolytic and anabolic properties (Holt et al. 2008). Over the last 20 years, several high profile athletes have admitted to using GH to enhance their performance despite its inclusion in the World Anti-Doping Agency (WADA) Prohibited Substances List (WADA 2012).

The detection of GH misuse has proven challenging for a number of reasons. Unlike many drugs of misuse, such as synthetic anabolic steroids, GH is a naturally occurring substance. As a result, the accusation of doping with GH must be based on finding abnormally high GH concentrations in the circulation, which cannot be explained by an underlying pathological condition. In addition, GH is secreted in a pulsatile manner and random elevated GH measurements may reflect a spontaneous peak.

Two approaches currently exist for the detection of GH misuse. The first method is based on the measurement of endogenous pituitary GH isoforms in the blood. This method was first established by Professor Christian Strasburger and Dr Martin Bidlingmaier, and was implemented by WADA for the Olympic Games in Athens, 2004 (Bidlingmaier et al. 2003). In November 2009 Terry Newton, a British rugby league player, became the first athlete to test positive for GH, using this test. Newton admitted taking GH and was serving a two-year suspension from the sport when he committed suicide in September 2010.

The second method for detecting doping with GH was developed by the GH-2000 research group. The project proposed an approach based on the measurement of the peptides insulin-like growth factor-I (IGF-I) and procollagen type III amino-terminal propeptide (P-III-NP). IGF-I is released into the circulation from the liver in response to stimulation by GH while P-III-NP is a marker of collagen formation in soft tissues. These markers rise in response to rhGH administration and show little diurnal variation (Wallace et al. 1999; Wallace et al. 2000). Discriminant functions incorporating these two markers and a correction for the effects of age have been

devised and can be used to distinguish individuals treated with GH from those treated with placebo (Powrie et al. 2007). The GH-2000 project reported its results to the International Olympic Committee (IOC) at a workshop in Rome in March 1999. The proposed test was strongly supported but it was felt that a number of issues needed to be resolved before the test could be implemented internationally. As a result, research on this "marker method" has continued over the past 14 years.

In addition to acting as a circulating marker of GH action, IGF-I is thought to mediate many of the anabolic effects of GH (Le Roith et al. 2001). As the tests for GH misuse develop further, it is likely that athletes will turn to IGF-I as an alternative performance-enhancing agent as currently there is no test to detect IGF-I misuse. There is anecdotal evidence that IGF-I misuse has been practised in sport for several years despite the fact that it also appears on the WADA Prohibited Substances List (WADA 2012). Pharmaceutical preparations of IGF-I have been approved by the US Food and Drug Administration for the treatment of children with certain forms of short stature (Kemp et al. 2006). The availability of these preparations will almost certainly result in an increase in the misuse of IGF-I. Furthermore laboratory supplies of IGF-I for non-clinical usage are readily available.

The detection of IGF-I misuse poses many challenges. Most current anti-doping tests use spot urine samples that are relatively easy to collect. IGF-I is excreted in urine at low concentrations and methods for measuring urinary IGF-I are complex and time-consuming (Yokoya et al. 1988). Furthermore, factors other than serum IGF-I concentration contribute to the renal clearance of IGF-I. For example, a significant increase in urinary IGF-I concentration is observed as part of the proteinuria that occurs in response to exercise (De Palo et al. 2003). As a result, blood sample collection rather than urine will be required for IGF-I doping tests.

Recombinant human IGF-I (rhIGF-I) has an identical amino acid sequence to endogenous IGF-I. As a result, techniques such as electrophoresis which rely on electrical charge differences between the endogenous and exogenous forms of prohibited hormones, cannot be used to detect rhIGF-I administration (Kazlauskas et al. 2002). Highly sensitive and specific mass spectrometric methods have been employed successfully to detect anabolic androgenic steroid (AAS) misuse (Saugy et al. 2000). Although several mass spectrometric methods have been developed for measuring IGF-I (Bredehoft et al. 2008; Clemmons 2011) these approaches have not yet been fully validated and current investigations into both GH and IGF-I misuse rely on immunoassay methods using specific antibodies.

1.2 Thesis outline

This thesis begins with a literature review on the history of doping with GH and IGF-I in sport. I have then described the GH-IGF axis, concentrating on the physiology of IGF-I and the potential benefits of exogenous IGF-I to the competitive athlete. I have outlined potential harmful effects and included a description of pharmaceutical preparations of IGF-I that are used clinically. This is followed by a discussion on current methods for detecting GH misuse and their development over the last fifteen years, along with the issues surrounding the use of immunoassays in this field. I have introduced the measurement of circulating nucleic acids in plasma and serum and how this new technology might be applied to the detection of GH and IGF-I misuse.

The rationale and aims of my research are then described followed by the methods employed to address the research objectives. I have presented the data from each of the studies in the subsequent results chapters and discussed the key findings from this research in the final conclusions chapter.

1.3 A brief history of doping in sport

Doping with performance-enhancing substances has been attempted since the ancient Olympic Games. One of the earliest reports of doping was by Charmis, the Spartan winner of the *stade* race (~200 yards) at the Olympic Games of 668 BC, who used a special diet of dried figs (Yesalis et al. 2002). At the modern Olympic Games, one early doping story was that of Thomas Hicks, winner of the Olympic Marathon in 1904, who was given a combination of brandy and strychnine by his support team throughout the race (Rosen 2008). The benefits of stimulants such as amphetamines were soon recognised by athletes and their use began to increase in the 1930s. Amphetamine abuse was particularly prevalent in cycling in the 1960s and the first doping-related death is thought to have occurred at the Rome Olympics in 1960 when a Danish cyclist Knud Jensen collapsed and died during the 100-kilometre team time-trial. It was reported that at autopsy, traces of methamphetamine and another stimulant nicotynal alcohol were found in Jensen's body (Rosen 2008).

The bodybuilding world recognised the potential benefits of testosterone and anabolic steroids on body shape and strength and the abuse of these substances was rife from the 1960s onwards. It was at this time that doping in professional sports became a major problem and indeed state-sponsored doping regimens, for example in the German Democratic Republic, were put in place to maximise the potential for success at world and Olympic level (Ungerleider 2001). The most famous doping scandal at the Olympic Games occurred in Seoul 1988 when the Canadian sprinter Ben Johnson was disqualified after his victory and world record performance in the 100 metres final. Johnson tested positive for the banned anabolic steroid stanozolol and a subsequent inquiry concluded that at least half the athletes competing at the Games were using anabolic steroids to enhance their performances (Dubin 1990). It became apparent that the number of drugs being used by athletes was expanding rapidly; the current list of prohibited substances published by WADA is extensive and includes anabolic steroids, peptide hormones, beta agonists, diuretics and masking agents, stimulants and methods of enhancing oxygen transfer including the use of erythropoietin (EPO) and blood transfusions (WADA 2012).

1.4 The history of growth hormone misuse

Growth hormone was first isolated and purified from human pituitary glands in 1956 (Li et al. 1956) and was initially used to improve growth in children with hypopituitarism (Raben 1958). Cadaveric GH was the only source available until the

mid 1980s when the first reports appeared of a link between pituitary-derived GH and the transmission of Creutzfeldt-Jakob Disease (Koch et al. 1985). Pituitary-derived GH was withdrawn from the market but GH produced by recombinant DNA technology became available in 1987 (Dalboge et al. 1987) and provided a potentially limitless supply of therapy.

Growth hormone was first publicly advocated as a performance-enhancing agent in "The Underground Steroid Handbook" published in 1982, in which the author Dan Duchaine described the potential beneficial effects on athletic performance of anabolic steroids and other substances including GH (Duchaine 1982). It was clear that bodybuilders and other athletes were already misusing GH at this time. In fact, athletes had discovered the performance-enhancing actions of GH by experimenting on themselves, long before scientists designed randomised controlled trials to test the effects of GH in adults with GH deficiency (Jorgensen et al. 1989; Salomon et al. 1989). When Ben Johnson was disqualified from the Seoul Olympic Games, he initially denied the charge of doping but in the subsequent inquiry he admitted to using GH in combination with anabolic steroids over several years to increase muscle strength and to recover more quickly from injuries (Dubin 1990).

The International Olympic Committee (IOC) included GH on its Prohibited Substances List in 1989 although there was not yet a test designed to detect its use and thus it remained an attractive performance-enhancing agent for many athletes. It has proven popular not only for strength disciplines but also in endurance sports: in 1998, large quantities of GH were confiscated from a team car at the Tour de France (Voet 2002). Also in 1998, Yuan Yuan, a Chinese swimmer, was forced to withdraw from the World Swimming Championships after police found vials of GH inside a thermos flask in her luggage (Evans 2003).

In 2006, two reporters for the "San Francisco Chronicle" published the book "Game of Shadows" in which they detailed their two-year investigation into the Bay Area Laboratory Co-Operative (BALCO) (Williams et al. 2006). It was alleged that a number of high profile athletes including US sprinters Tim Montgomery and Marion Jones, Major League Baseball superstar Barry Bonds and National Football League linebacker Bill Romanowski had been supplied with GH along with other performance-enhancing drugs by BALCO owner Victor Conte. BALCO was raided by the US Food and Drug Administration (FDA) in September 2003, when evidence of systematic doping with steroids and GH was discovered and several professional athletes were implicated. Conte later pleaded guilty to illegal steroid distribution and

spent four months in prison. Tim Montgomery and Marion Jones, along with British sprinter Dwain Chambers who was another BALCO client, later admitted to using performance-enhancing drugs including GH.

In response to the BALCO controversy, US Senator George Mitchell was appointed to lead an investigation into performance-enhancing drug use by Major League Baseball players (Mitchell 2007). Mitchell concluded that GH misuse is widespread in baseball and that the use of GH had risen because, unlike steroids, it is largely undetectable. He also reported that players use GH because they believe it assists their recovery from injury and fatigue. A number of players purchased GH through "anti-aging" centres using prescriptions from physicians with whom they had never met.

A recent high profile case involving GH doping was the positive test of British rugby league player Terry Newton in November 2009; he was the first athlete to test positive for the drug since the isoform test was introduced in 2004. Newton admitted to injecting GH in an attempt to recover from injuries and alleged that he knew a number of other rugby league players who were also using GH (BBC 2010). This case ended tragically when Newton committed suicide after serving eight months of his ban from sport. In the past two years, there have been reports in the media of other positive tests for GH including a Canadian college football player, an Estonian cross country skier and a German road cyclist. The anti-doping authorities and international sports federations are currently investigating these cases.

The use of GH is not limited to professional athletes and it is becoming an increasingly popular drug for amateur bodybuilders because of its effects on muscle mass. Cheap supplies of GH are widely available on the internet and an undercover BBC investigation revealed how simple it is to purchase GH for personal use (Pinsent 2009). The actor Sylvester Stallone was arrested in 2007 after 48 vials of GH were found in his luggage at Sydney Airport (Hardy 2007). Stallone admitted taking GH and suggested that everyone over the age of 40 years should try it as it "increases your quality of life". There is evidence that even adolescent athletes have been misusing GH for many years; in a survey of American high school students 5% of male students reported past or present use of GH and 31% of male students knew someone who had taken GH (Rickert et al. 1992).

1.5 Doping with IGF-I

The prevalence of IGF-I misuse is likely to be lower than for GH because there is no natural source available and therefore all IGF-I is produced using recombinant DNA technology. For many years the only available form of IGF-I was produced by biotechnology companies for enhancing cell culture growth and for research into the treatment of conditions such as myotonic dystrophy (Furling et al. 1999).

The recent development of IGF-I preparations for clinical use by two pharmaceutical companies has further increased the availability of this drug. Increlex® (Mecasermin) manufactured by Tercica Inc. (Brisbane, California, USA) contains recombinant human IGF-I (rhIGF-I) alone while iPLEX™ (Mecasermin rinfabate) manufactured by Insmed Inc. (Richmond, Virginia, USA) is a recombinant protein containing rhIGF-I complexed with rhIGF binding protein-3 (rhIGFBP-3) in equimolar proportions. These pharmaceutical preparations are discussed in more detail in section 1.11 below.

Although there are no confirmed cases of IGF-I misuse by athletes, it appears that the drug is already popular among amateur bodybuilders. IGF-I is discussed extensively on Internet bodybuilding forums where its use, both alone and in combination with GH, is described. The list of purported benefits is lengthy and these include increases in muscle size and strength, improvements in energy and endurance, benefits to the immune system and increased bone density. The increasing availability of the drug along with these suggestions of performance-enhancing effects is likely to increase its illicit use by athletes.

1.6 The GH-IGF axis and physiology of IGF-I

1.6.1 *Growth Hormone*

Growth hormone is a peptide hormone synthesised, stored and released by the anterior pituitary gland. The main circulating form of GH consists of 191 amino acids with a molecular weight of 22 kilodaltons (kDa) but there are multiple other isoforms including a 20 kDa form produced by the gene deletion of a region encoding 14 amino acids (Baumann et al. 1986). GH secretion is pulsatile and major stimuli to its secretion include sleep and exercise (Savine et al. 2000). GH secretion is highest during the pubertal growth spurt and falls thereafter (Martha et al. 1989) and it has been shown that GH secretion falls by 14% per decade in adult life (Toogood et al.

1996). The hormonal control of GH release and the GH-IGF axis are illustrated in Figure 1.1.

Growth hormone exerts its actions through binding to specific GH receptors and a major physiological role of GH is to promote linear growth in children by stimulating longitudinal bone growth through actions at the epiphysis (Rogol 2009). GH stimulates skeletal muscle protein synthesis (Fryburg et al. 1991) and also promotes mobilisation of fat by stimulating lipolysis (Hansen 2002).

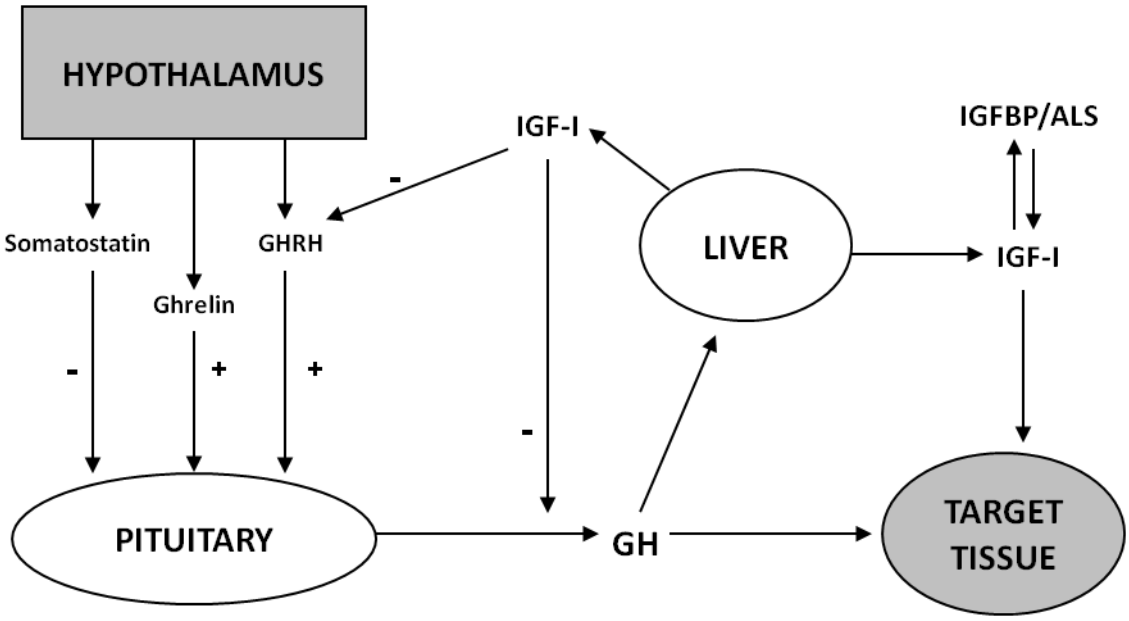

Figure 1.1. The growth hormone (GH)/insulin-like growth factor-I (IGF-I) axis. GH release from the pituitary gland is stimulated by GH-releasing hormone (GHRH) from the hypothalamus and ghrelin from the stomach and hypothalamus. Somatostatin inhibits the release of GH. The actions of GH are mediated in part by the synthesis and release of IGF-I predominantly from the liver. IGF-I binds to the IGF receptor and can act in an autocrine, paracrine or endocrine fashion. IGF-I is bound in the circulation by the IGF binding proteins (IGFBPs) and acid-labile subunit (ALS), which regulate the concentration and function of the IGFs. IGF-I inhibits the release of GHRH and GH by negative feedback on the hypothalamus and pituitary.

1.6.2 *Insulin-like growth factor-I (IGF-I)*

IGF-I is a single chain polypeptide consisting of 70 amino acids, which has a molecular weight of 7.6 kDa and demonstrates structural similarities with the insulin molecule (Blundell et al. 1978; Rinderknecht et al. 1978). GH stimulates the synthesis of IGF-I in most tissues (D'Ercole et al. 1984) and the liver is the major source of circulating IGF-I (Fig. 1.1). Serum IGF-I concentrations are an indicator of GH status; elevated IGF-I levels are found in acromegaly, a condition characterised by sustained hypersecretion of GH. In contrast, IGF-I concentrations are low in patients with GH

deficiency. In addition, insulin and nutritional status are important regulators of IGF-I synthesis. For example, in people with type 1 diabetes, where portal insulin concentrations and hepatic insulin action are reduced, IGF-I concentrations fall despite increased GH secretion suggesting that these individuals develop a state of apparent hepatic GH resistance (Wurzburger et al. 1996). This is because the expression and translation of the IGF-I gene is insulin-dependent (Boni-Schnetzler et al. 1991). Furthermore, while short-term fasting has little impact on IGF-I concentrations, starvation over a period of a few days leads to a reduction in circulating IGF-I (Clemmons et al. 1981) and IGF-I levels are low in people with anorexia nervosa (Gianotti et al. 1998).

1.6.3 *The Somatomedin Hypothesis*

It was previously believed that GH exerted its effects on tissues indirectly by stimulating the production of IGF-I (Daughaday et al. 1972). This theory was based on experiments demonstrating the incorporation of radioactive inorganic sulphate into chondroitin sulphate from rat cartilage. The incorporation of sulphate was reduced in hypophysectomised rats but this could be restored by *in vivo* injections of pituitary extracts and bovine GH. When bovine GH was placed on costal cartilage *in vitro*, however, only minimal effects were observed. This led to the hypothesis that bovine GH was acting through an intermediary substance and further experiments demonstrated that serum from normal rats could stimulate this biological effect. Serum from hypophysectomised rats was ineffective but if these hypophysectomised rats were injected with bovine GH, the serum from these animals was able to stimulate sulphate incorporation. The intermediary substance was initially termed the "sulphation factor" and later known as "somatomedin", which reflected its ability to mediate the effects of GH (also known as "somatotropin") (Daughaday et al. 1972). IGF-I was later shown to be the somatomedin substance that was regulated by circulating GH in rats (Klapper et al. 1983).

This "somatomedin hypothesis" was challenged by work performed on transgenic mice in which the IGF-I gene had been selectively deleted in the liver, resulting in a 75% reduction in serum IGF-I concentration (Sjogren et al. 1999; Yakar et al. 1999). Despite this reduction, these mice demonstrated normal postnatal body growth suggesting that hepatic IGF-I was not crucial in growth regulation. It has been proposed that circulating IGF-I is in fact a marker of GH action on the liver (Sonksen

2001) and that the anabolic actions of GH are thus combined with those of IGF-I acting in an endocrine, paracrine and autocrine fashion (Le Roith et al. 2001).

Mechano growth factor (MGF or IGF-IEc) is derived from alternative splicing of the IGF-I gene and activates skeletal muscle progenitor cells (Goldspink et al. 2008). MGF expression in human skeletal muscle is elevated by resistance exercise and it is believed that MGF has an important role in regulating the muscle hypertrophy response observed after mechanical loading (Hameed et al. 2003; Psilander et al. 2003).

1.6.4 *IGF binding proteins*

IGF-I circulates bound to a family of highly specific IGF binding proteins (IGFBP-1 to -6). At least 80% of circulating IGF-I is bound in a ternary complex comprising IGF-I, IGFBP-3 and an acid-labile subunit (ALS). IGFBP-3 is a 46-53 kDa protein that contains three potential glycosylation sites (Jones et al. 1995). The function of IGFBP-3 is not fully understood but it is known to regulate the actions of IGF-I and may target IGF-I to certain tissues allowing for tissue-specific actions of IGF-I (Rosenfeld et al. 1999). In addition, IGFBP-3 acts to prolong the half-life of IGF-I in the circulation. While unbound or free IGF-I has a half life of only a few minutes, the half-life of the binary complex of IGF-I/IGFBP-3 is 30 minutes and the ternary complex has a half-life of 12 to 15 hours (Guler et al. 1989). As a result, the pulsatility of GH release has little effect on serum IGF-I concentrations, which are more stable than GH concentrations (Jones et al. 1995). IGFBP-3 concentrations in plasma are increased by the administration of rhGH (Wallace et al. 1999).

ALS is an 85 kDa glycoprotein synthesised by the liver. GH is a potent stimulator of ALS production and ALS concentrations are reduced in GH-deficient states (Boisclair et al. 2001). Serum ALS concentrations are also reduced by fasting (Dai et al. 1994), in conditions of acquired GH resistance such as cirrhosis (Holt et al. 1998) and in critical illness (Van den Berghe et al. 2000). As well as extending the half-life of IGF-I, ALS is thought to modulate some of the metabolic effects of the IGFs, for example by preventing severe hypoglycaemia (Zapf 1995). ALS is able to modulate these effects because IGFs in ternary complexes are unable to penetrate capillary endothelia and activate the insulin receptor. Since IGF-I is not stored in any tissue, ALS performs an important physiological role in maintaining a circulating IGF-I reservoir (Domene et al. 2005).

1.6.5 *IGF receptors*

IGF-I may act in an autocrine or paracrine fashion in addition to its endocrine functions (Le Roith et al. 2001). The actions of IGF-I are mediated by the binding of IGF-I to the type 1 IGF receptor (IGF-IR) (Singleton et al. 2001). This is a transmembrane receptor of the tyrosine kinase family that shares structural homology with the insulin receptor (LeRoith et al. 1995) but has a thousand-fold higher affinity for IGF-I than insulin (Nitert et al. 2005). This provides specificity for IGF-I at physiological concentrations. At higher concentrations, IGF-I can also bind to the insulin receptor, although with only 1-5% the affinity of insulin for its own receptor (Guler et al. 1987). Both IGF-IR and the insulin receptor share intracellular signalling mechanisms including the activation of tyrosine phosphorylation (Izumi et al. 1987) and insulin receptor substrate-1 (IRS-1) (Myers et al. 1993) cascades. Hybrid IGF-I/insulin receptors have also been discovered (Federici et al. 1997) in a variety of tissues but their precise role in modulating IGF-I and insulin actions is unclear.

1.7 Effects of IGF-I on intermediate metabolism

IGF-I has both GH-like and insulin-like actions in vivo and the complex interactions between these three peptide hormones allow efficient metabolism of carbohydrate, lipids and protein during fasting and after feeding.

1.7.1 *Carbohydrate metabolism*

IGF-I has insulin-like effects on carbohydrate metabolism and its administration promotes hypoglycaemia along with the suppression of circulating insulin concentrations. The intravenous infusion of IGF-I into rats was shown to provoke hypoglycaemia by stimulating peripheral glucose uptake and glycogen synthesis in a similar manner to insulin (Jacob et al. 1989) although IGF-I did not demonstrate the insulin-like property of suppressing hepatic glucose production in these animals. IGF-I also causes hypoglycemia when administered to human volunteers (Turkalj et al. 1992). Some authors proposed that IGF-I increases insulin sensitivity by suppressing GH secretion and decreasing insulin levels (Zenobi et al. 1992). Other studies have shown that IGF-I administration to humans results in both stimulation of peripheral glucose uptake and suppression of hepatic gluconeogenesis (Boulware et al. 1994; Russell-Jones et al. 1995; Saukkonen et al. 2006).

These hypoglycaemic effects appear to be mediated, at least in part, by the type 1 IGF receptor (IGF-IR); in experimental mice lacking the insulin receptor gene, IGF-I still had a potent glucose-lowering effect (Di Cola et al. 1997). This effect has also been utilised in patients with Rabson-Mendenhall syndrome who suffer from severe insulin resistance secondary to mutations of the insulin receptor. Treatment with rhIGF-I reduces glucose and insulin concentrations in these patients with beneficial effects on glycated haemoglobin (HbA_{1c}) (McDonald et al. 2007), suggesting that IGF-I is acting via its own receptor and bypassing the defective insulin receptor. The suppression of hepatic gluconeogenesis by IGF-I in humans is perhaps surprising as hepatocytes do not appear to express IGF-I binding sites (Caro et al. 1988). It has been postulated that this action of IGF-I is mediated via either the insulin receptor or hybrid IGF-I/insulin receptors (Simpson et al. 1998).

The effects of IGF-I administration on glucose metabolism led to investigations into its potential as a therapeutic agent in patients with diabetes. Several studies have shown that administration of rhIGF-I to adolescents with diabetes results in a reduction in insulin requirements along with improvements in HbA_{1c} (Cheetham et al. 1993; Bach et al. 1994; Cheetham et al. 1995). Similarly, in a study of eight adult patients with type 1 diabetes, rhIGF-I treatment resulted in a decrease in insulin requirements along with decreased mean overnight GH secretion (Carroll et al. 1997). It has been suggested that these reductions in insulin requirements are caused not only by the direct hypoglycemic actions of IGF-I but also by reduced GH secretion (Simpson et al. 1998).

1.7.2 *Protein metabolism*

IGF-I enhances protein anabolism. Early studies showed that rhIGF-I administration caused a reduction in protein degradation but had no effect on protein synthesis (Turkalj et al. 1992; Laager et al. 1993; Hussain et al. 1994). In order to investigate whether the latter effect resulted from low levels of amino acids, Russell-Jones *et al.* administered IGF-I combined with an amino acid infusion to five healthy human volunteers. IGF-I caused a significant increase in protein synthesis but no significant change in the rate of protein degradation (Russell-Jones et al. 1994). In studies looking at the arteriovenous difference of radiolabelled phenylalanine across the forearm, IGF-I infusion for 6 hours caused positive amino acid balance through both the inhibition of protein degradation and the stimulation of protein synthesis (Fryburg 1994). By contrast, *in vivo* studies in humans suggest that insulin inhibits

protein degradation but does not stimulate protein synthesis (Fryburg et al. 1995). Thus, IGF-I appears to combine the insulin-like property of inhibiting proteolysis with the GH-like property of stimulating protein synthesis (Garlick et al. 1998), during periods of adequate substrate (amino acid) availability (Fig. 1.2).

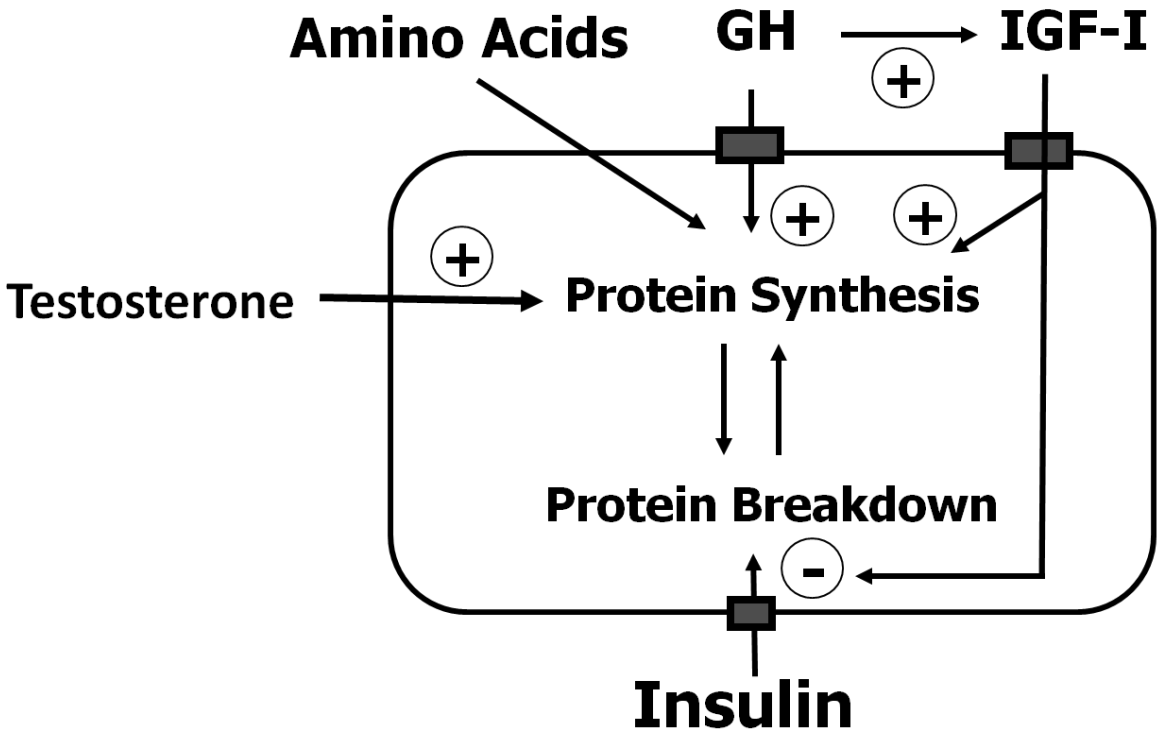

Figure 1.2. The regulation of protein synthesis by IGF-I, GH and insulin. IGF-I combines the insulin-like action of inhibiting proteolysis with the GH-like action of stimulating protein synthesis. GH has direct actions on protein synthesis and also stimulates local production of IGF-I, which then acts in an autocrine/paracrine manner. Figure taken from Guha *et al.* (2009) "IGF-I abuse in sport: current knowledge and future prospects for detection", *Growth Horm IGF Res,* 19(4): 408-11.

1.7.3 *Lipid metabolism*

Adipose tissue produces both IGF-I and IGFBPs (Wabitsch et al. 2000) and it appears that GH is the main regulator of IGF-I production in adipocytes (Vikman et al. 1991). In fact, there is evidence that IGF-I mRNA levels in adipose tissue are as high as those in the liver (Gosteli-Peter et al. 1994). This led to the hypothesis that adipose tissue could be a significant contributor to systemic IGF-I production. It was previously reported that no functional IGF receptors were present in human adipocytes (DiGirolamo et al. 1986). A recent study, however, demonstrated the presence of both insulin receptors and type 1 IGF receptors (IGF-IR) in human mature adipocytes as well as adipocyte precursors (Back et al. 2009) and it has been

proposed that IGF-I plays a critical role in adipocyte differentiation (Peter et al. 1993). This differentiation process is associated with an increase in the ratio of insulin receptors to IGF-IR (Back et al. 2009).

Studies of the effects of rhIGF-I administration on lipolysis and lipid oxidation have yielded conflicting results. Administration of a single subcutaneous dose of rhIGF-I to patients with type 1 diabetes had no effect on the rate of lipolysis (Simpson et al. 2004). Longer-term (eight week) administration of rhIGF-I similarly had no effect on lipolysis and lipid oxidation in GH-deficient adults (Mauras et al. 2000). In GH-deficient patients treated with rhIGF-I for seven days, however, increased rates of lipolysis and lipid oxidation were observed (Hussain et al. 1994).

People with GH insensitivity syndrome (GHIS or "Laron syndrome") have low serum IGF-I concentrations and are treated with rhIGF-I. This results in substantial changes in body composition with a decrease in percentage fat mass and increased lean body mass (Mauras et al. 2000). In addition, increased lipolysis and lipid oxidation rates have been demonstrated in these patients after rhIGF-I treatment (Mauras et al. 2000) although these lipolytic actions were attributed to relative insulin deficiency during treatment. It has been suggested that the potent effects of IGF-I on adipose tissue in Laron syndrome reflect the severity of the IGF-I deficiency in this condition and may not be replicated in healthy participants (Mauras et al. 2005).

The effects of IGF-I on lipolysis are therefore likely to be a result of the complex interaction between IGF-I, GH and insulin concentrations (Yuen et al. 2007). Direct effects of IGF-I on lipolysis cannot be excluded in view of the demonstration of IGF-IR in adipocytes (Back et al. 2009). GH is known to increase the lipolytic activity of adipocytes directly by activating hormone-sensitive lipase (Yip et al. 1999) and also by increasing the sensitivity of these cells to the actions of catecholamines (Marcus et al. 1994). It is possible that GH-stimulated IGF-I release also affects the lipolytic activity of these cells, acting via paracrine/autocrine mechanisms.

1.8 Effects of GH and IGF-I on bone and collagen metabolism

The complex interactions between circulating GH, IGFs, IGFBPs and locally produced IGFs and IGFBPs have a role in the maintenance of normal bone mass (Ueland 2005). Bone remodelling reflects the balance between bone resorption activated by osteoclast cells, and bone formation initiated by osteoblasts (Raisz 1999). GH deficiency is

associated with abnormal bone turnover and GH replacement reverses these abnormalities (Longobardi et al. 1999). Treatment with GH increases biochemical markers of bone resorption in postmenopausal women and these changes are correlated with changes in serum IGF-I (Brixen et al. 1995), suggesting that GH stimulates osteoclast activity through increased systemic and/or local IGF-I production. Administration of rhGH to healthy adults results in increased markers of bone resorption (C-terminal cross-linked telopeptide of collagen type I, (ICTP)) and bone formation (osteocalcin and C-terminal propeptide of type I procollagen (PICP)) with the stimulation of bone formation being more prolonged than bone resorption (Longobardi et al. 2000). Similarly, treatment of normal women with rhIGF-I activates both osteoclasts and osteoblasts with a more prominent effect on bone formation than on resorption (Ebeling et al. 1993). Other studies have shown that low doses of rhIGF-I given to healthy elderly women for 28 days have no effect on bone resorption markers (Ghiron et al. 1995). It has therefore been suggested that both GH and IGF-I activate osteoclasts but that GH has a more potent effect, independent of IGF-I (Ueland 2005).

The GH-IGF axis also affects soft tissue collagen turnover. Type III collagen is found in connective tissue throughout the body and is present in bone post-fracture when it is found in the callus (Kurdy et al. 1998). Procollagen type III amino-terminal propeptide (P-III-NP) is a marker of collagen turnover that has been used to monitor growth (Crofton et al. 1997) and also the progression of hepatic fibrosis (Maurice et al. 2005). GH deficiency in childhood reduces collagen turnover and is associated with a significant reduction in serum P-III-NP concentration (Sartorio et al. 1993). These abnormalities are reversed by long-term GH replacement in association with improvements in bone mineral density (Longobardi et al. 1999). Serum P-III-NP increases after a maximal exercise test and subsequently decreases to baseline levels within two hours (Ehrnborg et al. 2003). In addition, the administration of GH to healthy adults for four weeks causes a significant increase in P-III-NP concentration, which persists for up to eight weeks after cessation of GH treatment (Longobardi et al. 2000).

1.9 The GH-IGF axis and exercise: why might athletes misuse IGF-I?

The relationship between the GH-IGF axis and exercise has been investigated extensively. Exercise provides a potent stimulus for GH release (Sutton et al. 1976)

and this GH response is affected by gender (Wideman et al. 1999; Giannoulis et al. 2005), age, body composition and physical fitness (Holt et al. 2001). The GH response increases with increasing intensity (Pritzlaff et al. 1999) and duration (Wideman et al. 2006) of exercise. It has been suggested that changes in body temperature (Christensen et al. 1984; Wheldon et al. 2006) and blood pH (Elias et al. 1997) are responsible for stimulating GH release during and after exercise although the mechanisms are not fully understood.

It has also been shown that acute exercise causes a small increase in total IGF-I concentration (Cappon et al. 1994; Schwarz et al. 1996) along with increases in IGFBP-3 and ALS concentrations, possibly secondary to mobilisation of preformed intact ternary complexes (Wallace et al. 1999). Similar increases in IGF-I concentration have been observed during continuous moderate-intensity exercise and during high-intensity interval exercise (Copeland et al. 2008). Local increases in IGF-I have been proposed as a mechanism by which collagen synthesis in tendons is enhanced in response to exercise (Olesen et al. 2007). This effect of IGF-I might be tempting to the competing athlete as a method of accelerating recovery from soft tissue injury.

Over the course of prolonged aerobic exercise, the body alters the substrates it utilises for energy (Jeukendrup et al. 1998). In the initial stages of exercise, skeletal muscle uses glucose derived from muscle glycogen stores but as these are depleted, glucose is provided by hepatic glycogenolysis (Petersen et al. 2004). During prolonged exercise, lipolysis is enhanced in adipose tissue in response to a rise in circulating catecholamines, allowing the use of free fatty acids (FFA) as an alternative fuel source (Stich et al. 2000). Endurance-trained athletes demonstrate an increased rate of fat oxidation and slower rate of muscle glycogen breakdown than untrained individuals (Jeukendrup et al. 1997). It has also been shown that administration of supraphysiological doses of rhGH to endurance-trained athletes increases plasma glycerol and FFA concentrations both at rest and during sub-maximal exercise (Healy et al. 2006). These changes were associated with significant increases in serum IGF-I concentration. If the administration of rhIGF-I similarly increases FFA availability during exercise, this could reduce glycogen breakdown and spare glycogen stores, thus enhancing performance and recovery in endurance activities.

The potential effects of IGF-I on muscle protein synthesis are also attractive to the competitive athlete. In a study on hypopituitary males, GH replacement increased serum IGF-I concentrations and also stimulated skeletal muscle gene expression of

IGF-I and collagen (Sjogren et al. 2007). Reduction of protein breakdown and enhanced muscle protein synthesis in competitive athletes may aid power generation in sports such as weightlifting, as well as sprinting. Furthermore, the development of gene therapies in muscle-wasting conditions such as muscular dystrophy has led to the emergence of gene doping as a potential method of performance-enhancement (Unal et al. 2004). The IGF-I gene has been expressed in transgenic mice using viral and plasmid vectors, resulting in skeletal muscle hypertrophy and increased muscle strength (Barton-Davis et al. 1998; Barton 2006). When resistance training is combined with IGF-I overproduction in transgenic mice, there is a significant increase in strength compared with either intervention alone (Lee et al. 2004). These effects, if reproduced in humans, would clearly appeal to competitive athletes not least because local expression of IGF-I may not affect systemic IGF-I concentrations and therefore would be difficult to detect. It must be remembered, however, that research into gene therapy even in clinical practice is at an early stage and the risks of this technique to an athlete's health are unknown.

Several studies have demonstrated a relationship between serum GH and IGF-I concentrations and physical fitness (Poehlman et al. 1990; Eliakim et al. 1996). A study involving 44 healthy volunteers showed a positive correlation between integrated 24-hr serum GH concentrations and VO_2 peak, a measure of maximal oxygen consumption during exercise (Weltman et al. 1994). In addition, one year of exercise training in healthy female volunteers amplified the pulsatile release of GH and this was associated with an increase in fat-free weight along with improvements in VO_2 max (Weltman et al. 1992). Previous studies in adolescent athletes, however, have demonstrated a decrease in serum IGF-I concentration in response to a 5-week period of endurance training (Eliakim et al. 1996; Eliakim et al. 1998). The authors of these studies hypothesized that there may be two phases to the GH-IGF response to training: an initial catabolic response with decreased circulating IGF-I followed by a chronic anabolic adjustment and increased IGF-I concentration.

1.10 rhGH and rhIGF-I administration studies

The benefits of GH administration to the elite athlete have been a source of considerable debate. The potential benefits of exogenous GH are most evident from studies in adults with GH deficiency in whom GH replacement results in improved exercise capacity and maximum oxygen uptake, increased muscle strength and also improved body composition with increased lean body mass (Carroll et al. 1998). All of

these effects have the potential to benefit performance if they were reproduced in healthy athletes. For many years, however, there was no RCT evidence to demonstrate the performance-enhancing effects of GH on healthy individuals (Liu et al. 2008) although there was some evidence that GH alters body composition. Six months of treatment with rhGH in twenty-one healthy elderly men resulted in a significant increase in lean body mass (LBM) with an associated rise in serum IGF-I concentration (Rudman et al. 1990). A study involving administration of growth hormone-releasing hormone (GHRH) to healthy middle-aged and elderly males resulted in significant increases in GH and IGF-I concentrations (Veldhuis et al. 2004). These changes were associated with an increase in free fat mass (FFM) but no significant improvements in skeletal muscle strength were observed.

Several recent studies have shown improvements in physical performance in response to GH administration, and have suggested that benefits are most likely to be seen when GH is combined with other anabolic agents (Giannoulis et al. 2006; Graham et al. 2007; Meinhardt et al. 2010). Graham *et al.* demonstrated an improvement in aerobic performance and respiratory muscle strength in abstinent anabolic androgenic steroid (AAS) users after short-term (six days) administration of rhGH (Graham et al. 2007). In a study of 96 recreational athletes, Meinhardt *et al.* demonstrated enhanced sprint capacity in response to administration of rhGH alone and in combination with testosterone for eight weeks (Meinhardt et al. 2010). They speculated that the observed increase in sprint capacity could translate to an improvement of 0.4 seconds over a 100 meter race.

Administration of rhIGF-I in combination with rhGH to obese postmenopausal women resulted in reductions in fat mass, and these reductions were greater than those achieved by diet and exercise alone (Thompson et al. 1998). Administration of rhIGF-I to eight healthy male volunteers increased cardiac output and ejection fraction but did not affect exercise duration or VO_2 max (Donath et al. 1996). Indeed there is no published RCT evidence to support the belief that rhIGF-I produces performance-enhancing effects in healthy individuals but it is unlikely that this will deter individuals from misusing rhIGF-I. Clinical trials aim to detect large, clinically relevant differences between treatment groups and controls; thus any performance advantages conferred by taking IGF-I may to be too small for detection by standard clinical trials. Benefits may be perceived by individual athletes, however, who are acutely aware of their own training and competitive performances and may be using higher doses of rhIGF-I than have been used in these trials. In addition, the current clinical studies have been designed to test the effects of rhGH or rhIGF-I in isolation.

In reality, it is likely that rhIGF-I is misused as part of a cocktail of performance-enhancing substances along with rhGH and anabolic steroids in the hope of creating additive effects.

1.11 Pharmaceutical preparations of IGF-I

Recombinant human IGF-I has been developed as a potential therapy in a number of conditions including type 1 diabetes, severe insulin resistance and GH insensitivity syndrome (GHIS). The first commercial preparation of rhIGF-I, Increlex® (Mecasermin) was developed by the company Tercica (Brisbane, California, USA). This consists of recombinant human IGF-I that has an identical amino acid sequence to endogenous IGF-I. It is indicated for the treatment of children with growth failure secondary to severe primary IGF-I deficiency or with GH gene deletion who have developed neutralising antibodies to GH. Treatment with rhIGF-I has been shown to increase linear growth in these children with GHIS (Chernausek et al. 2007) although the effects of GH-treatment in GH deficiency are greater, possibly because of the effects of GH on local IGF-I production in bone (Guevara-Aguirre et al. 1997).

Recombinant human IGF-I administration leads to improved insulin sensitivity and decreased HbA_{1c} in patients with both type 1 (Quattrin et al. 2001) and type 2 (Moses et al. 1996) diabetes. A number of worrying side-effects are associated with the use of rhIGF-I alone including hypoglycaemia, seizures, jaw pain, myalgia and fluid retention (Williams et al. 2008). There have also been concerns that increases in serum IGF-I concentration are associated with progression of retinopathy in people with diabetes, as IGF-I concentrations are elevated in the retina of those with retinopathy (Merimee et al. 1983; Chantelau 1998). It has been suggested, however, that IGF-I therapy could result in a short-term deterioration in established diabetic complications before longer-term benefits are observed (Simpson et al. 1998). Such findings would be similar to those of the Diabetes Control and Complications Trial (DCCT 1993) in which intensive insulin treatment was associated with improved glycaemic control but also a transient worsening of established retinopathy over six months. Furthermore, the administration of IGF-I will lead to a reduction in GH secretion, which may lead to a fall in endogenous IGF-I produced locally in tissues that are affected by microvascular complications (Holt et al. 2003).

Recently, Mecasermin Rinfabate (iPLEX™), a recombinant protein complex consisting of rhIGF-I and rhIGFBP-3 in equimolar proportions, has been developed by the company Insmed (Kemp 2007). The drug forms a ternary complex with ALS in the

circulation and therefore prolongs the half-life of the rhIGF-I. It is hoped that the formation of this bound complex will reduce the frequency of significant hypoglycaemia secondary to the insulin-like actions of IGF-I. Once dissociated from the ternary complex, the rhIGF-I should interact with the IGF-IR in an identical fashion to endogenous IGF-I.

The metabolic effects of the rhIGF-I/rhIGFBP-3 complex include suppression of basal endogenous glucose production along with enhancement of insulin-stimulated peripheral glucose uptake (Saukkonen et al. 2006). As a result, this drug has been shown to decrease insulin requirements in adult and adolescent people with type 1 diabetes (Clemmons et al. 2000; Saukkonen et al. 2004) and to improve insulin sensitivity in people with type 2 diabetes (Clemmons et al. 2007). The complex has also been shown to improve glycaemic control in people with severe insulin resistance (SIR) (Regan et al. 2010). The commonest reported adverse reactions to Mecasermin rinfabate are local injection-site erythema and lipohypertrophy although headaches, increased liver and kidney size and alterations in liver function tests have also been observed (Williams et al. 2008). There are few data on the safety profile of long-term use of rhIGF-I/rhIGFBP-3. It is possible, however, that some of the side-effects associated with administration of rhIGF-I alone such as changes in facial features and tonsillar and adenoidal hypertrophy (Chernausek et al. 2007), will occur in the future with the co-administration of rhIGF-I/rhIGFBP-3.

In addition to these preparations used in clinical treatment, rhIGF-I is available from a number of companies for research purposes, for example in the study of cell growth and proliferation.

1.12 Why athletes should not misuse IGF-I

The potential side effects of the current pharmaceutical preparations of rhIGF-I have been outlined above in section 1.11. It is vital that athletes are aware of these and other potential risks of long-term IGF-I misuse. Acromegaly is a disorder characterised by sustained hypersecretion of GH and raised serum IGF-I concentrations. It is possible that misuse of exogenous IGF-I could result in similar adverse effects to those observed in patients with this condition. In particular, longstanding acromegaly is associated with deleterious effects on cardiac muscle structure and cardiac performance (Colao 2008). Chronic GH and IGF-I excess result in concentric biventricular hypertrophy and diastolic dysfunction initially, which can progress to systolic dysfunction at rest if left untreated (Colao et al. 2004). In

addition, there is a high prevalence of cardiac valve dysfunction in patients with active acromegaly (Colao et al. 2003). Maximal oxygen uptake during exercise (VO_2 max) and ventilation threshold (VET), a measure of work rate when breathlessness develops, are reduced in patients with acromegaly compared with age-matched normal individuals (Thomas et al. 2002).

There is controversy over the potential increase in cancer risk associated with the chronic GH and IGF-I excess observed in acromegaly. *In vitro* studies suggest IGF-I stimulates proliferation of transformed neoplastic cell clones and the growth of pre-existing tumour tissues (Wu et al. 2002). Downregulation of the type I IGF receptor (IGF-IR) leads to apoptosis of tumour cells (Baserga et al. 2003). Furthermore, a positive correlation has been observed between circulating IGF-I concentrations and the incidence of prostate, colorectal and breast cancers (Giovannucci et al. 2000; Nam et al. 2005; Key et al. 2010) although the association in these studies does not prove a causal effect.

Epidemiological studies suggesting an increased risk of colorectal cancer in patients with acromegaly have stimulated debate (Colao et al. 2004). Although some studies have suggested an increased incidence of pre-malignant colonic adenomas in acromegaly (Terzolo et al. 1994; Vasen et al. 1994; Delhougne et al. 1995), it has been suggested that if these incidence rates are adjusted for the confounding factors of age and gender and are compared with appropriate control populations, there is no increased adenoma prevalence in people with acromegaly (Renehan et al. 2001). In view of the current conflicting evidence, it is difficult to predict whether long-term IGF-I misuse will result in an increased risk of malignancy but vigilance is required.

1.13 Detection of growth hormone misuse in athletes

1.13.1 *The GH-2000 Project*

The International Olympic Committee (IOC) banned the use of GH by athletes in 1989 but investigation into methods to detect its misuse did not commence until 1991. At this time, the IOC Medical Sub-Commission Doping and Biochemistry in Sport invited Professor Peter Sönksen (now co-principal investigator of the GH-2004 Project research group) to join the Sub-Commission as an advisor on GH. The IOC had very little knowledge at that stage of the issues around doping with GH and little experience in detecting complex peptide and glycopeptide hormones. Indeed there

were several barriers to Professor Sönksen's early suggestions; first, the need to perform blood rather than urine testing caused some concern. In fact blood testing was introduced at the Winter Olympic Games in 1994 for the detection of "blood doping" by blood transfusion and proved to be a convenient method of sample collection, which was even preferred by the athletes (Sönksen 2009). There were also concerns about performing 'invasive' scientific research on elite athletes and it was difficult to obtain funding for this type of research because the IOC did not fund scientific research itself. Eventually, the research arm of the European Union agreed to include anti-doping research in their BIOMED 2 research programme and this provided a valuable source of funding.

Professor Sönksen conceived the 'GH-2000' research project to develop a test for GH misuse. The research team consisted of leading endocrinologists from the UK, Sweden, Denmark and Italy, in partnership with two pharmaceutical companies manufacturing GH (Novo Nordisk, Denmark and Pharmacia, Sweden), along with statisticians from the University of Kent and the IOC Medical Commission. Since GH concentrations fluctuate widely in normal life, it was clear that a simple measurement of serum GH would not be sufficient to detect doping. The GH-2000 team hypothesized that GH administration would lead to the alteration of serum concentrations of biomarker proteins and they investigated proteins in the GH-IGF axis as well as markers of collagen and bone metabolism.

The GH-2000 double-blind GH administration study involved 102 recreational athletes from four countries (Dall et al. 2000; Longobardi et al. 2000). The administration of rhGH resulted in a dose-dependent increase in serum IGF-I as expected, along with increases in its major binding proteins IGFBP-3 and ALS. Furthermore, there were dose-dependent increases in collagen markers procollagen type III N-terminal propeptide (P-III-NP), procollagen type I C-terminal propeptide (PICP), C-terminal cross-linked telopeptide of type I collagen (ICTP), osteocalcin and bone alkaline phosphatase. From these studies, the GH-2000 team proposed a doping test based on IGF-I and P-III-NP (Powrie et al. 2007). These markers were chosen because they provided the best discrimination between athletes receiving rhGH and those receiving placebo. These proteins also exhibit little diurnal or day-to-day variation and are largely unaffected by exercise (Wallace et al. 1999; Wallace et al. 2000). It was necessary to establish reference ranges for IGF-I and P-III-NP in elite athletes as suspicion of GH misuse relied on detecting concentrations that were higher than occurred in normal physiology. 813 elite athletes were recruited and serum levels of IGF-I and P-III-NP were measured in serum samples collected

immediately after a competitive event (Healy et al. 2005). It was shown that sporting discipline and body composition had little effect on serum marker concentrations but that both IGF-I and P-III-NP decline with age, consistent with the known age-related decline in GH secretion.

Although a single marker could be used, it was found that combining these markers in age-adjusted, gender-specific equations (discriminant functions) resulted in improved sensitivity and specificity to detect GH misuse (Powrie et al. 2007). It was possible that incorporating more markers into the discriminant functions would improve the sensitivity of the test but the initial recommendation of the GH-2000 team was to adopt the test using these two markers. It was important that IGF-I and P-III-NP are produced in separate tissues thus reducing the chances of pathological conditions leading to elevated concentrations of both markers and false-positive tests. Standard medical practice accepts values within two standard deviations of the mean as 'normal' values but by definition, 5% of the population lies outside the normal range. If applied to a population of athletes, this would lead to an unacceptably high false-positive rate. The GH-2000 team, working with an IOC lawyer, recommended a false-positive rate of approximately 1 in 10,000. Using this specificity, it was possible to make complete distinction between all men on rhGH and those on placebo on day 21 of treatment. The sensitivity of the test was lower in women than in men probably because women are more resistant to the actions of GH. It is likely, however, that female athletes would need to administer higher doses of rhGH than men to obtain the same performance-enhancing effect. The discrimination of the test was greatest in the period when athletes were administering the drug but it was possible to detect the rhGH group as long as 14 days after the last injection (Powrie et al. 2007). This 'window of opportunity' is an important aspect of any anti-doping test because athletes are aware of testing protocols at major events and can attempt to evade detection by stopping their use of performance-enhancing drugs in the days before competing.

The GH-2000 Project reported these findings to the EU and IOC in January 1999 and an IOC workshop was convened in March of that year to discuss the results. The workshop was supportive of the proposed test though it was recognized that further studies were required to ensure the test was effective in non-White Caucasian ethnic groups and that the results of the test were not affected by injury. Furthermore, since IGF-I and P-III-NP had been measured by commercially available immunoassays for the GH-2000 project, it was suggested that the IOC develop its own immunoassays as it could then control the assay procedures and reagents. The IOC initially agreed

further funding for all of this work but the offer was subsequently withdrawn and this caused major delays in the further development of the GH-2000 'marker method'.

1.13.2 *The isoform or differential immunoassay method*

A second approach to detecting GH misuse was simultaneously being developed by Professor Christian Strasburger and Dr Martin Bidlingmaier in Germany, which relied on the measurement of GH isoforms. As described in section 1.6, GH exists as multiple isoforms and 70% of circulating GH is in the form of a 22kDa polypeptide. Other endogenous isoforms include the 20kDa splice variant as well as dimers, oligomers, acetylated and fragmented forms (Baumann 1999). rhGH contains only the 22kDa isoform and the 'differential immunoassay' or 'isoform' approach to GH misuse detection relies on alterations in the ratio between the 22kDa isoform and other isoforms. When exogenous rhGH is administered, there is suppression of endogenous GH production through negative feedback on the pituitary gland and the ratio between 22kDa GH and non-22kDa GH is increased (Bidlingmaier et al. 2003). The isoform approach relies on the ratio of results between one immunoassay that specifically measures 22kDa GH and another 'permissive' assay that measures all GH isoforms. When this method was applied to a normal population, the ratio between 22kDa and total GH was significantly higher in participants treated with rhGH compared with control participants (Wu et al. 1999). The effect of exercise on the isoform ratio was investigated and it was shown that the proportion of 22kDa GH is decreased after exercise (Wallace et al. 2001) reducing the sensitivity of the test in the immediate post-competition period.

The isoform test has been validated in several WADA-accredited laboratories and has been used at the Olympic Games and by international anti-doping authorities since 2004. Despite a large number of tests being performed, the first positive result did not occur until November 2009 and the previous lack of positive tests is probably as a result of the short 'window of opportunity'. The short half-life and rapid clearance of rhGH means that detection of an altered isoform ratio is most likely during the first 24 hours after the last rhGH injection (Bidlingmaier et al. 2007). Any athlete who stops GH administration several days before a competition will not be detected in the usual post-competition setting. The test is therefore most suitable for an unannounced, out-of-competition scenario as occurred in the case of Terry Newton, the rugby league player with a positive test in November 2009. Furthermore, the isoform method cannot be used to detect doping with cadaveric pituitary-derived GH.

1.13.3 *The GH-2004 Project*

The investigations into the marker method resumed with the establishment of the GH-2004 project supported by the US Anti-Doping Agency (USADA) and WADA. The aims of the project were to address the concerns regarding the method raised at the IOC workshop in 1999, in order to provide further validation of the method and to allow its implementation in WADA-accredited laboratories. The first aim was to develop reference ranges for IGF-I and P-III-NP in elite athletes of differing ethnicity as the original GH-2000 studies included predominantly Caucasian European athletes. 242 male and 62 female elite athletes from different ethnic groups were recruited and blood samples were collected for measurement of IGF-I and P-III-NP and calculation of the GH-2000 discriminant function score (Erotokritou-Mulligan et al. 2008). The study found that there are minor differences in marker concentrations between different ethnicities but that these differences did not affect the performance of the test. The response to rhGH administration in non-White Caucasian, recreational athletes was also investigated and it was shown that there were no significant ethnic effects on maximal change in IGF-I, P-III-NP and GH-2000 score (Holt et al. 2010).

It was vital to exclude the possibility of false-positive results because of injury when using the marker method. The GH-2004 team studied the effects of injury in 127 male and 30 female recreational athletes along with 16 male and 10 female elite athletes (Erotokritou-Mulligan et al. 2008). No changes were observed in IGF-I concentration but there was a significant rise in P-III-NP following both soft tissue and bony injuries. Despite this rise, the GH-2000 score did not increase significantly after injury and it was concluded that injury would not adversely affect the performance of this detection method.

One limitation of anti-doping tests based on measurements at a single time-point is that the concentration of the analytes may vary with time, for example as a result of changes in training intensity during the season. The intra-individual variability of IGF-I, P-III-NP and GH-2000 score was therefore evaluated in four longitudinal studies involving 303 elite and 78 recreational athletes over a period of up to 12 months (Erotokritou-Mulligan et al. 2009). The intra-individual variability for IGF-I ranged between 14 and 16% while variability for P-III-NP was between 7 and 18% and there was no difference in intra-individual variability between elite and recreational athletes. The intra-individual variability estimates incorporated both

biological variation and assay variation. These results suggested that a positive test result for GH misuse would not occur as a result of chance variability within an individual athlete. The low variability of marker levels suggests the test could be employed as part of a profiling or "Athlete's Passport" approach where markers in an individual athlete are monitored over time. Suspicions of doping are raised if increases in marker levels relative to the athlete's baseline, are observed. This approach is already used by WADA to aid the detection of blood doping and testosterone misuse (Sottas et al. 2010).

A further aim of the GH-2004 Project was to determine the stability of IGF-I and P-III-NP concentrations in blood samples exposed to various storage and transportation conditions, in order to minimize pre-analytical variability. Post-competition and out-of-competition drug testing does not usually take place in a clinical setting. Consequently centrifugation and storage of samples in a fridge or freezer may not be possible at the testing site. It was found that P-III-NP concentrations increased by approximately 6-7% per day when stored at room temperature, regardless of whether the blood was mixed with the anticoagulant lithium-heparin or a clotting agent (Holt et al. 2009). This increase did not occur if samples were stored at 4°C and it was therefore suggested that clotted blood samples or serum could be stored at 4°C but not ambient temperature, for up to five days prior to analysis. Furthermore, it was shown that the anti-coagulant ethylenediaminetetraacetic acid (EDTA) inhibited any rise in P-III-NP but also exerted significant matrix effects on the measurement of P-III-NP and was unsuitable as a collection medium.

The GH-2004 research team was also able to validate the marker approach using an independent data set (Erotokritou-Mulligan et al. 2007). A 14-day GH administration study in amateur male athletes had been undertaken at the Institut für Dopinganalytik und Sportbiochemie in Kreischa, Germany. When the male GH-2000 discriminant functions were applied to the IGF-I and P-III-NP results from this study, 90% of the participants who had received GH were correctly identified and no false positive results occurred. This study provided further validation that the marker method could be used to detect individuals receiving exogenous GH.

It is clear that the GH-2000 and GH-2004 Projects provided a wealth of data supporting the effectiveness of the marker method. The progression in development of methods for detecting GH misuse is summarised below in Table 1.1.

Table 1.1. Major steps in developing methods for detecting GH misuse in athletes.

	Year
International Olympic Committee bans use of GH by athletes	1989
Discriminant functions proposed based on serum IGF-I and P-III-NP	1999
Reference ranges developed for IGF-I and P-III-NP in elite athletes	1999
GH isoform method implemented at Athens Olympic Games	2004
Marker method validated in non-Caucasian athletes	2005
Marker method validated in independent GH administration study	2006
Marker method validated in athletes with musculoskeletal injury	2006
Intra-individual variability in markers and GH-2000 score investigated	2007
Pre-analytical conditions investigated to assess stability of markers	2007
First positive test for GH doping using isoform method	2009

There were, however, some remaining issues that needed to be addressed before the test could be implemented and these have provided the basis for part of my research. The issues included the validity of the marker method in elite adolescent athletes as described in section 1.17.3, further investigations into acceptable pre-analytical sample treatment and storage conditions (section 1.17.4) and the use of commercial immunoassays for measuring markers (section 1.14 and 1.17.5).

1.14 The use of immunoassays in anti-doping

An immunoassay is an analytical method that uses antibodies as reagents to quantify specific analytes and this technique is widely used in clinical laboratories. The antibodies are engineered to recognise and bind to a specific site (epitope) on the analyte (antigen) of interest. The strength of binding between antibody and antigen determines the sensitivity of the method. Binding of antibody and antigen is visualised by labelling either the antibody or antigen with a marker that can be detected quantitatively. The radioimmunoassays (RIAs) widely employed in detecting GH misuse, currently rely on antibodies or antigens labelled with a radioactive isotope. Enzyme-linked immunosorbent assays (ELISAs) incorporate an enzyme label that gives a coloured product after antibody has bound to antigen.

A number of immunoassay formats exist (Fig. 1.3). In competitive binding radioimmunoassays, antigen present in the sample competes with a fixed amount of labelled antigen in the presence of a limited quantity of antibody. Free antigen is separated from antibody-bound antigen after the system reaches equilibrium, and the amount of labelled antibody-bound antigen is quantified (e.g. using scintillation counting). This is inversely proportional to the concentration of antigen present in the sample. Standard solutions of known antigen concentrations are used to construct a standard curve and these are subsequently used to calculate antigen concentrations in patient or athlete samples. Competitive radioimmunoassays are precise and cheap as only small amounts of antibody are required. They are, however, difficult to automate and can be time-consuming.

Immunometric assays rely on the binding of antigen in the sample with insoluble or immobilised antibodies. The bound antigen is then detected using a second antibody specific to a different epitope on the antigen. The detecting antibody is labelled to allow quantitation e.g. with radioactive isotope in immunoradiometric assays (IRMAs). These immunometric assays can be very sensitive, are easily automated and cover a wide range of analyte concentrations but require more antibody than competitive assays and can be more expensive.

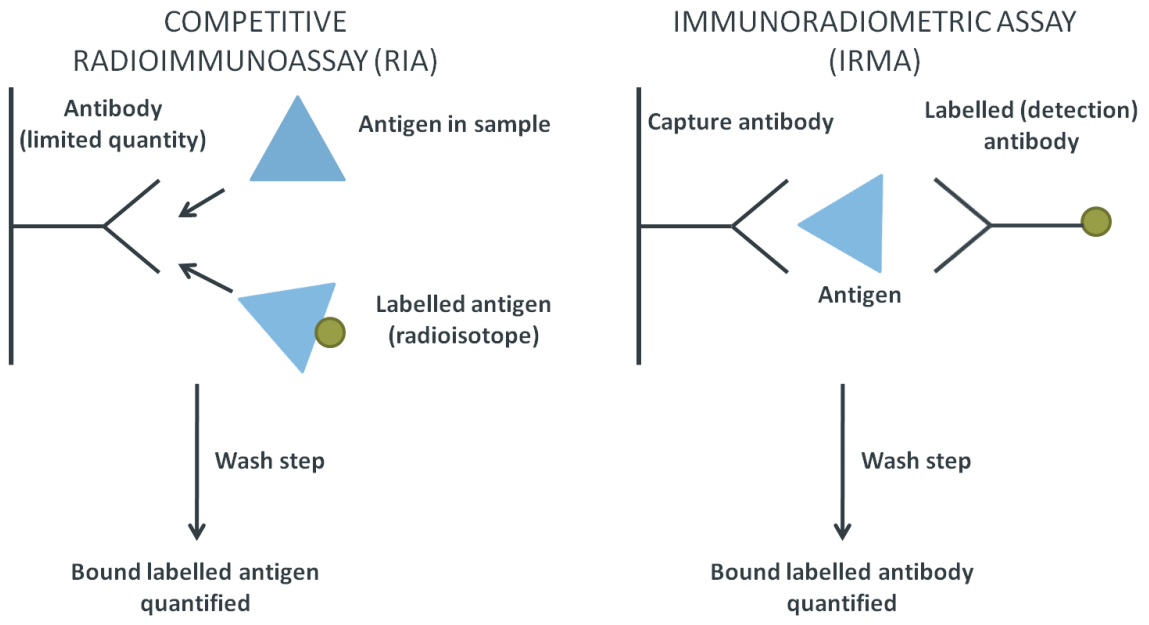

Figure 1.3. The principles of competitive (left) and non-competitive (right) immunoassays. In competitive radioimmunoassays, as the concentration of unlabelled antigen increases, the ability of the labelled antigen to bind to antibody is reduced. There is therefore an inverse relationship between assay signal and unlabelled antigen concentration. In non-competitive radioimmunoassays, there is a positive relationship between assay signal and antigen concentration. Radioisotopes are used to provide the signal in radioimmunoassays but enzymes or fluorescent labels can also be employed.

The use of commercial immunoassays for the detection of GH and IGF-I misuse has created a number of issues as detailed below.

1.14.1 *IGF-I assays*

The initial development of immunoassays for measuring IGF-I in serum relied on competitive RIAs. IGF-I circulates bound to IGFBPs and it was found that these assays suffered from interference from binding proteins, leading to inaccurate and inconsistent results. It was therefore necessary to design techniques to remove the effects of binding protein interference. The definitive method for removal of binding proteins is acid gel filtration chromatography (Daughaday et al. 1982) but this method is labour-intensive and is not used routinely by commercial laboratories. The most common method of removing binding protein interference is by using acid displacement of the IGFBPs, which are then precipitated with ethanol leaving unbound IGF-I in the sample (Daughaday et al. 1982). One problem with this technique is that small binding proteins such as IGFBP-1 and IGFBP-4 that do not bind to ALS are not wholly removed during the precipitation step and remain in the sample (Mesiano et al. 1988). Another problem is co-precipitation of IGF-I and if this loss is not corrected for, there will be an underestimation of IGF-I concentration in the sample (Clemmons 2007). The acid/ethanol precipitation technique can be enhanced by the addition of excess IGF-II to saturate residual binding protein that remains after the precipitation step, since IGF-II has a relatively high affinity for the binding proteins (Blum et al. 1994). This method has become popular with commercial manufacturers over the past twenty years because although it is not as effective as acid gel filtration chromatography, it is much less labour-intensive.

More recent immunoassays for IGF-I rely on the immunometric, non-competitive principles described above as the specificity of the assays for IGF-I is improved by using two antibodies and these assays can be performed rapidly on automated platforms (Khosravi et al. 1996). It is still necessary to use acid/ethanol precipitation and/or addition of excess IGF-II when performing these assays to ensure minimal interference from binding proteins.

There are difficulties in comparing results between commercial IGF-I assays because of a lack of standardization. Until recently, most commercial assays were calibrated against the WHO International Reference Reagent 87/518 (WHO IRR 87/518). It was shown that this reference material is impure, containing approximately 40% IGF-I and therefore its assigned concentration gave rise to inaccuracies in the measurement

of serum IGF-I in clinical samples (Quarmby et al. 1998). Krebs et al. compared five commercial IGF-I assays, using the Nichols Advantage assay (no longer available) as a reference assay (Krebs et al. 2008). They discovered systematic deviations between the assays despite the fact that all assays were calibrated against WHO IRR 87/518. It has been suggested that these differences between IGF-I assays reflects differences in their sensitivity to the effects of IGFBPs (Frystyk et al. 2010). The WHO recognized the need for an International Standard for IGF-I and undertook an international collaborative study to establish the First International Standard for IGF-I, 02/254 (Burns et al. 2009). This recombinant standard was shown to be >97% pure, bioactive and stable. An expert group including members of the Growth Hormone Research Society, the International Society for IGF Research, the Pituitary Society and the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) recommended that assays should be recalibrated with the new standard and assay-specific reference intervals re-established (Clemmons 2011). This should minimise the inter-assay differences that arise from the use of different standards.

When immunoassays are used for doping detection, WADA rules state that two immunoassays are required for each analyte and that the antibodies used in these immunoassays should recognise different epitopes (WADA 2008). It is difficult to satisfy these requirements as commercial manufacturers do not release information about the epitope specificity of their immunoassays. In addition, manufacturers can change their immunoassays and reagents with little notice and indeed some of the IGF-I assays used in the original GH-2000 and GH-2004 studies are no longer available. The GH-2000 team anticipated that commercial assays carry this risk and advised the IOC and subsequently WADA to develop their own assays to ensure that they had control of reagents. The United States Anti-Doping Agency (USADA) started to develop reagents for IGF-I (and P-III-NP) assays 10 years ago but assay development proved to be more difficult than anticipated and the project was not completed. As a result, it has been necessary to develop methods for comparing IGF-I results from current commercial immunoassays with those used in the previous studies and these methods are discussed in section 2.2.1. Furthermore the use of commercial assay reagents has caused some inconsistency in IGF-I results because of changes in the commercial reagents over time ("lot-to-lot variability") (Bidlingmaier 2009). The manufacturers have taken steps to minimise this variability but a rigorous quality control system is required by laboratories using IGF-I assays to allow the detection of changes between different lots of assay reagents (Miller et al. 2011).

1.14.2 *P-III-NP Assays*

For many years, there were only two commercial assays available for measuring P-III-NP: the RIA-gnost® P-III-NP from Cisbio (Gif-sur-Yvette, Cedex, France) and the UniQ™ PIIINP RIA from Orion Diagnostica (Espoo, Finland). Recently, however, several non-radioisotopic assays have been developed and are appearing on the market. P-III-NP is not bound by proteins in the circulation and so there is no need for an extraction step in the assay procedure. When serum is separated by gel filtration, four distinct immunoreactive species of P-III-NP have been described: 1) aggregates of intact P-III-NP and large molecules (lipoproteins and fibrinogen), 2) dimers of P-III-NP, 3) intact monomeric P-III-NP and 4) the Col 1 domain of P-III-NP (Jensen 1997). The Cisbio assay is specific for the Col 1 domain but also detects intact P-III-NP while the Orion assay detects intact P-III-NP and aggregated P-III-NP. The major concern with these P-III-NP assays is that no international standard is available for calibration purposes. The exact nature of standards used for calibration is not disclosed by the commercial manufacturers and it is possible that bovine material is used. A further issue with these assays is that they use different units of measurement: units/millilitre (Cisbio) *vs.* micrograms/litre (Orion) and this can lead to confusion when comparing the results between the two assays. Finally, P-III-NP immunoassay results may also be susceptible to the lot-to-lot reagent variability described above for IGF-I assays.

1.15 The use of blood mRNA technology to detect misuse with GH and IGF-I in athletes

The presence of cell-free DNA in the circulation was first demonstrated by Mandel and Métais in 1948 (Mandel et al. 1948). Evidence accumulated in subsequent years for the spontaneous release of newly synthesized DNA by living cells (Stroun et al. 1977). Interest in this area was further stimulated in the 1960s by the demonstration of increased circulating DNA in people with systemic lupus erythematosus (Tan et al. 1966). This was followed by studies showing increased levels of DNA in blood from people with cancer, which decreased after radiotherapy (Leon et al. 1977). The discovery of fetal DNA in maternal plasma was a major breakthrough and since the 1990s, the study of circulating nucleic acids in plasma and serum (CNAPS) has been a rapidly expanding scientific field.

Circulating DNA and RNA are useful markers in oncogenic disorders. Fragments of circulating DNA can be used as an early diagnostic marker in breast (Deligezer et al.

2008), lung (Board et al. 2008) and gastrointestinal tumours (Kolesnikova et al. 2008). It was shown that measurement of cell surface-bound DNA enabled the identification of people with gastric cancer with a sensitivity and specificity of 75% and 54% respectively (Kolesnikova et al. 2008). Furthermore, it has been suggested that the detection of circulating methylated DNA in people with stage IV melanoma, can predict the response to chemotherapy and therefore disease outcome (Mori et al. 2005).

Circulating nucleic acids have also played an important role in fetal medicine and other areas of research. Analysis of maternal plasma for DNA and RNA has been used in the early identification of a number of maternal complications including pre-eclampsia. Plasma corticotrophin-releasing hormone (CRH) mRNA concentrations were found to be nine-fold higher in women suffering from pre-eclampsia compared with controls matched for gestational age (Ng et al. 2003). The analysis of fetal DNA in maternal plasma has helped in the detection of Rhesus incompatibility (Daniels et al. 2006). Rhodopsin (the visual pigment found in rod cells of the retina) mRNA is detectable in the circulation of healthy individuals and people with diabetes (Butt et al. 2006) and it has been shown that rhodopsin mRNA concentrations increase with increasing severity of diabetic retinopathy (Hamaoui et al. 2004).

The origins of circulating nucleic acids have provoked some debate. Apoptotic and necrotic cells have been proposed as one source of free circulating DNA in serum and plasma (Jahr et al. 2001; Atamaniuk et al. 2006). There is also the possibility that DNA may be released by living cells (Stroun et al. 2001) although the mechanism of this release has not been fully elucidated (van der Vaart et al. 2008). Exogenous sources of circulating DNA have also been described; in people with cervical cancer, human papilloma virus DNA can be detected in 50% of cases (Yang et al. 2004). Another area of uncertainty is the mechanism of clearance of these nucleic acids from the circulation. Studies have suggested that the kidneys may be responsible for the removal of circulating DNA since free DNA can be detected in urine (Su et al. 2004). Binding and uptake of DNA by cells (Chelobanov et al. 2006) and the breakdown of DNA by plasma nucleases (Lo et al. 1999) may also be responsible for DNA clearance.

As the evidence accumulated for the presence of mRNA species in the circulation from a variety of sources, it raised the possibility that circulating mRNA encoding proteins in the GH-IGF axis would also be detectable. This possibility was investigated in a preliminary study at St Thomas' Hospital, London, which examined whether circulating nucleic acids are useful in the detection of endogenous GH production

(Thakkar et al. 2008). Blood samples were collected from 33 healthy volunteers and 13 people with acromegaly and mRNA levels for GH and GHRH were measured using real-time quantitative polymerase chain reaction (RT-PCR). Median mRNA concentrations for GHRH were 30.7 times lower in people with acromegaly than in healthy volunteers. Furthermore, mRNA concentrations for GH were significantly higher in the participants with acromegaly than in controls. In the control participants, mRNA concentrations for both GH and GHRH were significantly lower in older participants compared with younger age groups, consistent with the known age-related decline in GH production.

The preliminary study described above demonstrated the detection and quantitation of mRNA for GH and GHRH in the peripheral circulation and raised the possibility of using this mRNA technology in the detection of GH and IGF-I misuse. Injection of exogenous rhGH, rhIGF-I or rhIGF-I/rhIGFBP-3 complex would act through the negative feedback control mechanisms outlined in section 1.6 and modify serum levels of the endogenous peptides. It is possible that synthesis of mRNA encoding these hormones, and therefore circulating mRNA concentrations, would also be affected.

1.16 Potential advantages of mRNA technology in the detection of GH and IGF-I misuse

Analysis of circulating mRNA concentrations requires collection of whole blood, extraction of RNA from the blood, quantitation of total RNA, reverse transcription of mRNA to complementary DNA (cDNA) and finally real-time quantitative polymerase chain reaction (qPCR). Each of these steps is described in detail in section 2.1.5. qPCR is a technique based on PCR, which is used to amplify a targeted cDNA sequence using specific primers complementary to the sequence of interest (Fig. 1.4).

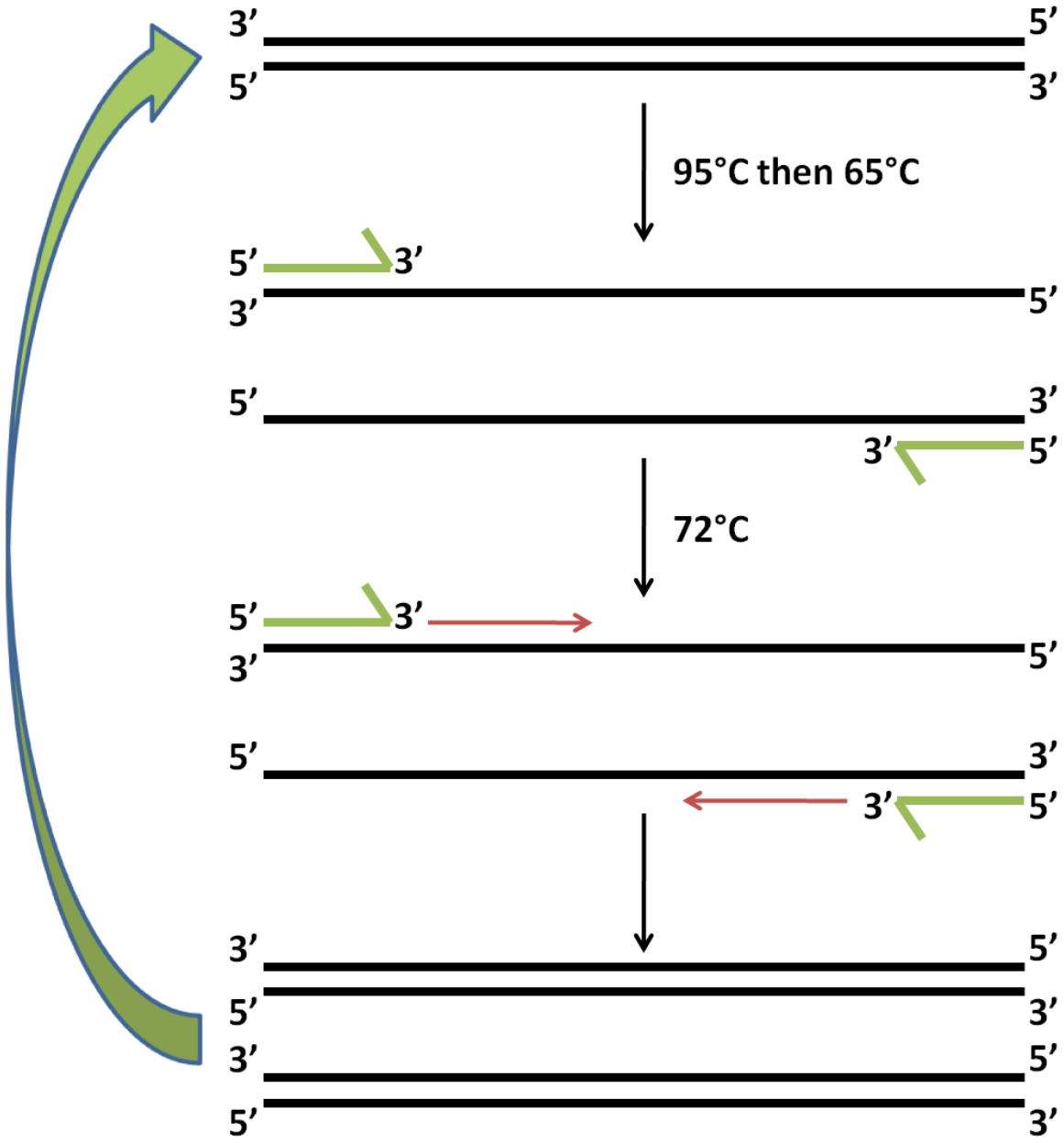

Figure 1.4. The Polymerase Chain Reaction (PCR) cycle. Denaturing of double-stranded DNA occurs at 95°C followed by annealing of specific primers (green lines) at 65°C. DNA polymerase extends the primer by adding complementary nucleotides at 72°C, producing two copies of the template DNA sequence. With repeated cycling of denaturation, annealing and extension steps there is exponential amplification of the target DNA sequence.

In qPCR, the amplified DNA is detected as the reaction progresses in real time, using a fluorescent reporter probe. This probe specifically binds to the target DNA and emits a fluorescent signal as each PCR cycle progresses. Exponential increases of the DNA product targeted by the reporter probe result in an increase in fluorescent signal above background levels and the PCR cycle at which this occurs is known as the threshold cycle, C_t. The threshold cycle number decreases with increasing concentration of cDNA and this concentration is proportional to the mRNA

concentration in the original blood sample. mRNA concentrations are determined using standard curves generated by serial dilution of commercially available cDNA of known concentration.

In order to quantify gene-specific mRNA accurately in circulating blood, the results can be expressed relative to a reference gene product (Holford et al. 2008). This corrects for differences in mRNA concentrations between samples that occur through differences in, for example, RNA extraction procedures or the volume of starting material. Potential methods for correcting mRNA concentrations include expressing results relative to total RNA concentrations or relative to the mRNA concentrations of a reference or "housekeeping" gene. Commonly used housekeeping genes include glyceraldehyde-3-phosphate dehydrogenase (GAPDH), beta-actin and beta-globin. It is vital that the expression of the reference gene is very similar across all samples studied but it has already been shown that GAPDH mRNA concentrations are increased by insulin and noradrenaline (Barroso et al. 1999) and that thyroid status has an effect on levels of beta-actin in whole blood (Holford et al. 2008). As a result, the quantities of total RNA as well as beta-actin and beta-globin mRNA have been measured in the studies described in Chapter 8 and variations in all housekeeping gene quantities have been analysed.

Measurement of circulating mRNA concentrations may help in the detection of GH and IGF-I misuse as this new technology has some advantages over the conventional immunoassay techniques used to measure serum peptide concentrations. Multiple mRNA markers can be measured in a single assay using primers that are specific for different genes and this could reduce both the cost and time required to perform the test. Furthermore, once whole blood has been collected in appropriate tubes, the mRNA is stable at room temperature for up to three days, which would allow for transport of the blood sample to the anti-doping laboratory from the testing site. There is also potential that this technology, if successful in detecting GH and IGF-I misuse, could be applied to other peptide hormones such as insulin, which have also proven difficult to detect using current methods. It is not known, however, whether it is possible to detect and quantify circulating mRNA for IGF-I and IGFBP-3. Furthermore, the physiological variability in circulating mRNA concentrations for these peptides from the GH-IGF axis is unknown and no previous studies have investigated whether administration of exogenous GH or IGF-I affects these mRNA concentrations.

1.17 Aims of my research

The aims of my research were divided into five key areas:

1) To investigate biomarkers of rhIGF-I/rhIGFBP-3 administration in recreational athletes.

2) To investigate the effects of rhIGF-I/rhIGFBP-3 administration on metabolic substrate utilisation, body composition and physical fitness in recreational athletes.

3) To validate the GH-2000 discriminant function method for detecting GH misuse in elite athletes.

4) To determine the effects of pre-analytical storage conditions on the serum concentrations of IGF-I and P-III-NP.

5) To investigate blood mRNA technology as a method for detecting GH and IGF-I misuse in athletes.

To address these aims, six research studies were undertaken as described below.

1.17.1 *Biomarkers of IGF-I misuse in recreational athletes: changes in serum IGF-I, P-III-NP and GH-2000 score*

Our hypothesis was that the administration of exogenous rhIGF-I could be detected by measuring an increase in the concentrations of serum IGF-I and P-III-NP. This was based on the methodology that has previously been successful in detecting exogenous GH administration. The primary aims of the randomised, double-blind, placebo-controlled IGF-I administration study were therefore to:

1) Assess whether the administration of rhIGF-I/rhIGFBP-3 complex induces changes in the GH-dependent markers IGF-I and P-III-NP.

2) Assess whether the formulae derived for the detection of GH misuse are also applicable for the detection of IGF-I misuse.

1.17.2 *The effects of IGF-I on lipid metabolism, carbohydrate metabolism, body composition and physical fitness*

There are limited data on the effects of IGF-I on carbohydrate and lipid metabolism in healthy volunteers. There is currently no evidence to suggest that administering

IGF-I to healthy athletes will improve physical performance or alter body composition. Our hypotheses were that IGF-I administration would result in improved insulin sensitivity, enhanced triglyceride breakdown, increased lean body mass and improved athletic performance. The secondary aims of the randomised, double-blind, placebo-controlled IGF-I administration study were therefore to:

1) Assess the effects of rhIGF-I/rhIGFBP-3 complex on glucose metabolism, insulin sensitivity and substrate utilisation.

2) Assess the effects of rhIGF-I/rhIGFBP-3 complex on body composition.

3) Assess the effects of rhIGF-I/rhIGFBP-3 complex on physical fitness.

1.17.3 *Cross-sectional study of elite adolescent athletes*

During puberty, alterations in the hypothalamic control of the GH-IGF axis and increased gonadal sex steroids lead to a marked increase in GH secretion that peaks during mid to late puberty (Veldhuis et al. 2005). Adolescent athletes compete at national and international events, and it is believed that even high school athletes misuse GH (Rickert et al. 1992). It is therefore vital that any test for GH misuse is validated in adolescent athletes. The original GH-2000 studies included few athletes younger than 18 years and the aim of the current study was to investigate serum IGF-I and P-III-NP in elite adolescent athletes to determine how a test based on measurement of GH-dependent markers could be validated for use in younger athletes.

1.17.4 *The effects of a freeze-thaw cycle and pre-analytical storage temperature on the stability of IGF-I and P-III-NP concentrations*

The stability of IGF-I and P-III-NP concentrations in serum stored at -80ºC and during transport at 4ºC has already been established (Holt et al. 2009). As anti-doping laboratories may not have access to -80ºC storage facilities, one aim of this study was to investigate the stability of these measurements in serum stored at -20ºC. In addition, during major sporting events, many blood samples are analysed without prior freezing and a further aim of the study was to establish the effects of one freeze-thaw cycle on assay results.

1.17.5 *Cross-sectional study of elite athletes and development of decision limits for the implementation of the GH-2000 detection method*

The GH-2000 discriminant formulae were based on measurements from the Nichols Institute Diagnostics IGF-I radioimmunoassay, which is no longer available, and Cisbio P-III-NP assay. WADA rules state that any analyte measured by immunoassay should be measured by two separate assays that recognise different epitopes (WADA 2008). Thus it was necessary to validate two IGF-I assays and a further P-III-NP assay before the test could be introduced. Our hypothesis was that the GH-2000 formulae would function effectively regardless of the assays used to measure the analytes. The aims of this study were therefore to:

1) Measure IGF-I and P-III-NP in a population of elite athletes using two commercial immunoassays for each analyte, and calculate GH-2000 scores.

2) Establish how the assay results from different immunoassays relate to one another.

3) Develop appropriate GH-2000 score decision limits to determine whether an athlete has been misusing GH.

1.17.6 *The use of blood mRNA technology to detect GH and IGF-I misuse in athletes*

1.17.6.1 The intra-individual variability of mRNA concentrations for GH, GHRH, IGF-I and IGFBP-3

The possibility of detecting GH and IGF-I misuse using a new technology based on circulating mRNA concentrations for components of the GH-IGF axis has been proposed (Thakkar et al. 2008). If mRNA concentrations for GH, GHRH, IGF-I and IGFBP-3 are to prove useful in detecting GH and IGF-I misuse, it is important that the physiological variation in these concentrations within and between individuals is established. The aim of this pilot study was to investigate the intra-individual variability of circulating mRNA for GH, GHRH, IGF-I and IGFBP-3 in recreational athletes.

1.17.6.2 Acute changes in blood mRNA concentrations for GH, GHRH, IGF-I and IGFBP-3 in response to rhGH

It is not known if circulating mRNA concentrations for GH, GHRH, IGF-I and IGFBP-3 are altered in response to exogenous GH administration and the aim of this

pilot study was to investigate the acute changes in circulating mRNA for GH, GHRH, IGF-I and IGFBP-3 in response to four daily injections of rhGH.

1.17.6.3 The effects of rhIGF-I/rhIGFBP-3 administration on circulating mRNA for GH, GHRH, IGF-I and IGFBP-3

It is not known whether the administration of IGF-I results in changes in circulating mRNA concentrations for GH, GHRH, IGF-I and IGFBP-3. The aim of this study was therefore to examine changes in mRNA concentrations for these peptides in 30 male recreational athletes participating in the randomised, double-blind, placebo-controlled IGF-I administration study described in section 1.17.1.

CHAPTER 2: METHODS

In this chapter the participants, study design, study protocols and analytical methods employed in each of the research studies are outlined (section 2.1). The determination of sample size numbers for each study and the statistical methods used to analyse the data are also discussed (section 2.2).

2.1 Participants, study design and analytical methods

In all the studies described below, participants provided written informed consent and the study protocols were approved by the Southampton and South West Hampshire Research Ethics Committee. The studies were conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. The studies were regulated by the Research and Development Office of the University Hospital Southampton (UHS) NHS Trust.

2.1.1 *Randomised, double-blind, placebo-controlled IGF-I administration study*

The primary aim of this study was to investigate the effects of IGF-I administration over 28 days on the GH-dependent biomarkers, IGF-I and P-III-NP, in healthy, recreational athletes. The secondary aim of this study was to investigate the effects of IGF-I administration over 28 days on glucose and lipid homeostasis and on the body composition and physical fitness of healthy, recreational athletes.

2.1.1.1 Participants

The study was performed at the Wellcome Trust Clinical Research Facility (WTCRF), Southampton General Hospital. 56 (30 male, 26 female) healthy, recreational athletes aged between 18-30 years were recruited. The athletes engaged in regular physical activity (≥2 sessions/week). Athletes were recruited by poster advertisement in the Faculty of Medicine, University of Southampton and University of Southampton Sports Centre and by approaching University of Southampton Sports Societies.

Participants were ineligible if they were competing at elite level, had a history of using performance-enhancing drugs or were found to be anaemic at screening. Anyone

with previous history of endocrinopathy, diabetes mellitus or neoplastic disease was excluded. Pregnant women were not allowed to participate; pregnancy tests were performed on all female volunteers prior to enrolment and they were advised to use safe contraception for the duration of the study if sexually active. Participants were ineligible if they had participated in other clinical research projects within the previous 12 weeks according to the guidelines set out by the UHS NHS Trust Research and Development office.

2.1.1.2 Study design

The study was a randomised, double-blind, placebo-controlled trial involving three treatment arms. Participants were randomly assigned to receive low dose (30 mg/day) rhIGF-I/rhIGFBP-3 complex, high dose (60mg/day) rhIGF-I/rhIGFBP-3 complex or placebo (Fig. 2.1). Insmed Incorporated (Virginia, USA) provided the rhIGF-I/rhIGFBP-3 complex (Mecasermin Rinfabate, iPLEX™ 60 mg/mL) and matching placebo. Participants and study investigators (including all individuals measuring study outcomes) were blinded to the intervention groups at all times. Insmed Incorporated generated the allocation sequence for IGF-I, prepared the IGF-I and provided placebo in identical packaging labelled with the allocation number. UHS NHS Trust Pharmacy staff were responsible for dispensing the drug kits according to the allocation sequence. Drug vials were stored frozen at -20°C until 30 minutes prior to injection when the required dose was allowed to thaw at room temperature.

I demonstrated the injection technique to each volunteer prior to the first dose. Participants self-administered the drug subcutaneously with their evening meal for 28 consecutive days. All participants were reminded to inject the drug by daily text message. Compliance was assessed by asking the volunteers to complete a treatment diary and by collection of empty drug vials at the end of the treatment period. I assessed adverse effects of the treatment by clinical assessment at weekly intervals during the treatment period.

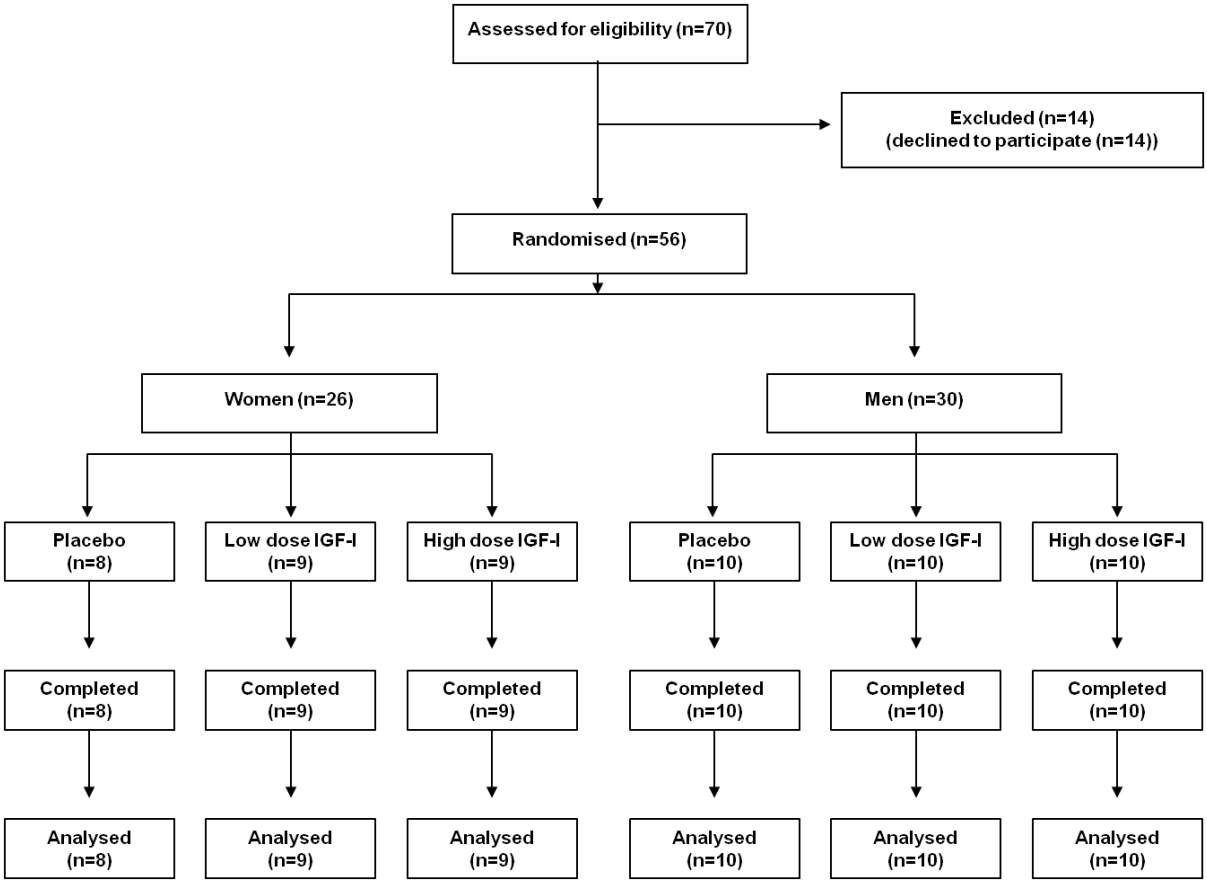

Figure 2.1. Recruitment and randomisation procedures for the rhIGF-I/rhIGFBP-3 administration study

2.1.1.3 Collection of serum GH-dependent markers

Venous blood samples for GH-dependent markers were collected at baseline, at the end of each week during the treatment period (Days 7, 14, and 21) and during the washout period on Days 28, 30, 33, 42 and 84 (Fig. 2.2).

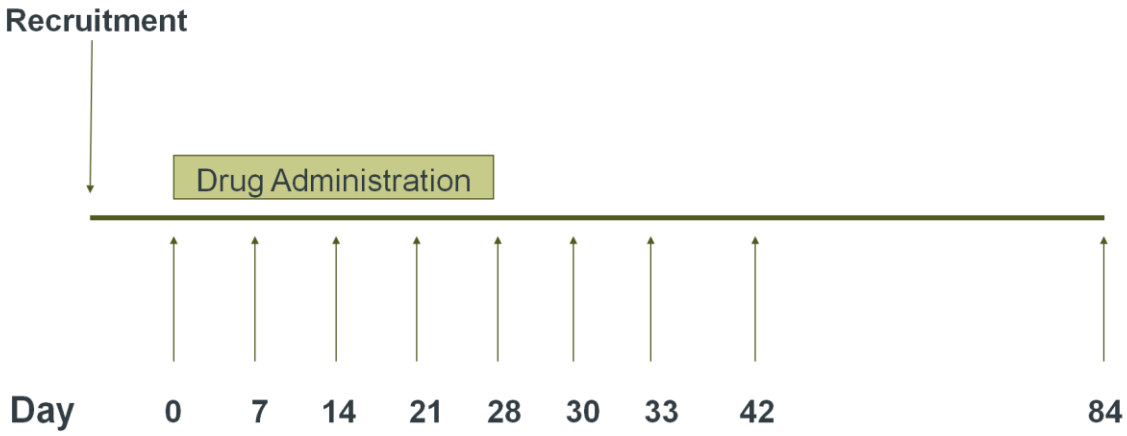

Figure 2.2. rhIGF-I/rhIGFBP-3 administration study protocol. Volunteers were randomised to treatment for 28 days, followed by a 56 day washout period. Serum samples were collected before treatment (Day 0), during treatment (Days 7, 14 and 21) and after treatment (Days 28, 30, 33, 42 and 84).

Blood was collected according to WADA guideline 5.8.2 for blood sample collection (WADA 2008). In brief, 15mL of venous blood was collected from the antecubital fossa into 5mL SSTII *Advance*™ Vacutainers (Becton Dickinson, Oxford, UK) and allowed to clot at room temperature for 15 minutes. Samples were centrifuged for 15 minutes at 1300*g*; serum was separated and stored frozen at -80ºC until analysis. All samples were coded and anonymised before analysis.

2.1.1.4 Analysis of GH-dependent markers (IGF-I and P-III-NP)

IGF-I and P-III-NP assays were performed at the Drug Control Centre, King's College London. All samples from one individual were analysed in the same assay run to minimise the effects of inter-assay variability on the results.

2.1.1.4.1 IGF-I assays

Serum IGF-I was measured using commercial immunoassays. Intra-assay precision was determined at the Drug Control Centre using eight replicates of two quality control (QC) samples. The Siemens Immulite IGF-I assay (Siemens Medical Solutions Diagnostics Limited, Llanberis, UK) is a solid-phase, enzyme-labelled, chemiluminescent, immunometric assay performed on the Immulite 1000 analyser. Excess IGF-II is added to prevent interference from IGF binding proteins (IGFBPs). Intra-assay CV was 3.8% and 4.4% at concentrations of 70 and 226 ng/mL respectively. Inter assay CV (n = 24 independent assays) was 6.2% and 11.3% at 77 and 243 ng/mL respectively (Cowan et al. 2009).

The Immunotech A15729 IGF-I IRMA (Immunotech SAS, Marseille Cedex, France) is a solid-phase, immunoradiometric assay using two monoclonal antibodies prepared against two different antigenic sites of the IGF-I molecule. The first antibody is coated on a solid phase and the second antibody is radiolabelled with ^{125}I. IGF-I is separated from IGF binding proteins (IGFBPs) by acidification and excess IGF-II is added to prevent further interference with the assay from IGFBPs. Intra-assay CV was 1.6% and 2.2% at concentrations of 138 and 455 ng/mL respectively. Inter-assay CV (n = 22 independent assays) was 6.9% at a concentration of 285 ng/mL (Cowan et al. 2009).

The Immulite and Immunotech assays measure IGF-I using the same units (ng/mL) and are calibrated using the WHO IGF-I IRP standard 87/518.

2.1.1.4.2 P-III-NP assays

P-III-NP was measured using commercial immunoassays. The RIA-gnost P-III-NP from CIS Biointernational (Gif-sur-Yvette, Cedex, France) is a two-stage sandwich assay based on the formation of a complex between solid-phase monoclonal anti-P-III-NP antibodies, P-III-NP in the serum samples and ^{125}I-labelled anti-P-III-NP monoclonal antibodies. A volume of 20 μL of serum is used. Intra-assay CV (n = 8 replicate samples) at a concentration of 0.12 and 3.46 U/mL was 10.8% and 18.2% respectively. Inter-assay CV was 9.5% at a concentration of 2.08 U/mL (n = 18 independent assays) (Cowan et al. 2009).

The UniQ™ PIIINP RIA (Orion Diagnostica, Espoo, Finland) is a competitive RIA based on the formation of a complex between solid-phase anti-P-III-NP polyclonal rabbit antibodies and P-III-NP in the serum samples in competition with ^{125}I-labelled P-III-NP. A sample volume of 200 μL is used. Intra-assay CV was 4.1% and 2.4% at a concentration of 4.27 and 56.1 ng/mL respectively. Inter-assay CV was 3.7% at a concentration of 5.31 ng/mL (n = 20 independent assays) (Cowan et al. 2009).

Analyses were performed in duplicate for all assays except the Immulite assay for which analysis was performed in singlicate as instructed by the manufacturer.

2.1.1.5 Anthropometric measurements

Anthropometric measurements were performed before treatment (baseline) and at the end of treatment (Day 28). Height was measured to the nearest millimetre using a wall-mounted Seca 220 stadiometer (Seca, Birmingham, UK). Body weight was measured to the nearest 0.1 kg using Seca 876 electronic scales (Seca, Birmingham, UK) with participants dressed in light clothing. Body mass index (BMI, kg/m^2) was calculated by dividing the participant's weight in kilograms by the square of their height in metres. Hip circumference (measured at the largest posterior extension of the buttocks) and waist circumference (measured at the midpoint between subcostal and suprailiac landmarks) were measured by a single, trained observer. Mid-upper arm circumference was measured at the midpoint between the acromion process of the scapula and the olecranon process of the ulna.

2.1.1.6 Body composition assessment

Body composition was assessed at baseline and at the end of treatment (Day 28) using three methods: 1) Dual-Energy X-ray Absorptiometry (DEXA), 2) Bioelectrical Impedance Analysis and 3) Skinfold thickness.

2.1.1.6.1 Dual Energy X-ray Absorptiometry (DEXA)

DEXA whole-body scanning was performed using the Hologic QDR-4500W DXA Scanner (Hologic, Bedford, USA) according to standardised procedures recommended by the manufacturer. Calibration was performed on the day of each scan. Participants were dressed in light clothing and wore no metal objects. Scan duration was approximately 10 minutes with radiation dose approximately 0.01 millisieverts. Results were analysed using Hologic Discovery software version 13.0. Fat Free Mass (FFM) was calculated using the sum of the estimates of lean tissue mass and bone mineral content for each participant.

2.1.1.6.2 Bioelectrical Impedance Analysis (BIA)

BIA was performed using the Bodystat® 1500 Bio-impedance Analyser (Bodystat Limited, Isle of Man, UK). Participants were dressed in light sportswear and had emptied their bladders. Participants reclined on a flat couch ensuring limbs were not touching the trunk. Electrodes were placed on the right side of the body between the distal prominences of the radius and ulna, the distal end of the third metacarpal, between the medial and lateral malleoli at the ankle and at the distal end of the third metatarsal. Body water percentage, lean mass percentage and body fat percentage were calculated from measurements of electrical resistance made at 50 kHz according to the manufacturer's instructions.

2.1.1.6.3 Skinfold thickness

Skinfold thickness at four sites (triceps, biceps, subscapular and suprailiac) was measured by a single, trained observer using Holtain Tanner/Whitehouse Skinfold Calipers (Holtain Ltd, Crymych, UK). Measurements were taken on the non-dominant side to the nearest 0.1mm. Three measurements were taken at each site and the readings were averaged. Percentage body fat was calculated from the sum of four skinfold measurements using the equations of Durnin and Wommersley (Durnin et al. 1974) and Siri (Siri 1956) below:

Males 17-19 years	Body density = 1.1620 - 0.0630 x Log Σ Skinfolds
Males 20-29 years	Body density = 1.1631 - 0.0632 x Log Σ Skinfolds
Males 30-39 years	Body density = 1.1422 - 0.0544 x Log Σ Skinfolds
Females 16-19 years	Body density = 1.1549 - 0.0678 x Log Σ Skinfolds
Females 20-29 years	Body density = 1.1599 - 0.0717 x Log Σ Skinfolds
Females 30-39 years	Body density = 1.1423 - 0.0632 x Log Σ Skinfolds

% Body fat = (4.95 / Body density - 4.50) x 100

2.1.1.7 Physical fitness assessment

Cardiopulmonary exercise (CPX) testing was performed during recruitment (to allow participants to familiarise themselves with the testing equipment and test protocol), at baseline and at the end of treatment (Day 28). Participants were asked to maintain their normal exercise pattern during the treatment period. Maximal aerobic capacity was measured by incremental treadmill test on a Woodway PPS Med treadmill (Woodway, Waukesha, USA) using the Bruce Protocol (Bruce et al. 1973). The slope of the treadmill was increased by 2% and the speed increased every 3 minutes as shown in Table 2.1. Participants were verbally encouraged to continue until exhaustion. Oxygen consumption (VO_2) was recorded continuously with an on-line gas analyser (Cortex MetaLyser 3B, Cortex Biophysik GmbH, Leipzig, Germany). Breath-by-breath gas exchange values were averaged over 15 second intervals to estimate maximal oxygen consumption (VO_2 max), corrected for total body weight.

Table 2.1. The Bruce Protocol for assessment of maximal oxygen consumption.

Stage	Time (min)	km/hr	Gradient (%)
1	0	2.74	10
2	3	4.02	12
3	6	5.47	14
4	9	6.76	16
5	12	8.05	18
6	15	8.85	20
7	18	9.65	22
8	21	10.46	24
9	24	11.26	26
10	27	12.07	28

2.1.1.8 Serum lipids and glycated haemoglobin

Fasting venous blood samples for triglycerides, total cholesterol, HDL-cholesterol, LDL-cholesterol and glycated haemoglobin (HbA_{1c}) were collected at baseline and at the end of treatment (Day 28). Serum lipids and glycated haemoglobin were measured by the Clinical Biochemistry laboratory of UHS NHS Trust using standard laboratory techniques according to the manufacturers' guidelines.

2.1.1.9 Oral glucose tolerance test

The response to an oral glucose tolerance test (OGTT) was assessed at baseline and at the end of treatment (Day 28). The protocol for these visit days is shown below in Table 2.2.

Table 2.2. Protocol for Baseline and Day 28 visits.

Time (minutes)	Procedures/Investigations
0	Intravenous cannulation Fasting lipids, HbA_{1c}, glucose, insulin, C-peptide, NEFA, glycerol GH-dependent markers (IGF-I and P-III-NP) Indirect calorimetry 75g oral glucose challenge
30	Glucose, insulin, NEFA
60	Glucose, insulin, NEFA, indirect calorimetry
90	Glucose, insulin, NEFA
120	Glucose, insulin, NEFA, indirect calorimetry
180 - 240	Anthropometric measurements DEXA, skinfold thickness, bioelectrical impedance Physical fitness test

Participants attended the WTCRF at Southampton General Hospital at 9am after a 12 hour overnight fast. Participants did not exercise on the morning of the assessment. Fasting venous blood samples were collected for glucose, insulin, C-peptide, non-esterified fatty acids (NEFA) and glycerol analysis. Participants then consumed 113mL Polycal® (Nutricia Clinical, Trowbridge, UK) mixed with water to a total volume of 200mL (equivalent to 75g anhydrous glucose). The participant remained at rest for the duration of the test (120 minutes). Venous blood samples were collected through an indwelling venous catheter at 30 minute intervals for analysis of glucose, insulin and NEFA.

Plasma glucose, serum insulin and serum C-peptide concentrations were measured by the UHS NHS Trust Clinical Biochemistry laboratory and plasma NEFA and glycerol concentrations were measured by the NIHR Biomedical Research Unit for Nutrition

and Lifestyle (University of Southampton and UHS), using standard laboratory techniques according to the manufacturers' guidelines.

2.1.1.10 Indirect calorimetry

Indirect calorimetry (IC) was performed using the Deltatrac II™ metabolic cart (Datex-Engstrom Corp, Helsinki, Finland). IC was performed in the fasting state and at 60 and 120 minutes after the 75g glucose challenge. Participants were asked to lie supine for 20 minutes whilst a canopy was placed over their heads. Ambient air was drawn through the canopy at a constant rate and expired gases were collected in a mixing chamber. The volume of oxygen consumed (VO_2) and volume of carbon dioxide produced (VCO_2) were calculated from the differences between inspired and expired air. Respiratory quotient was calculated as the ratio between VCO_2 and VO_2. Substrate oxidation rates were calculated from the following equations (Frayn 1983):

$CHO_{ox} = (4.55*VCO_2) – (3.21*VO_2) – 2.87*N_u$

$Lipid_{ox} = (1.67*VO_2) – (1.67*VCO_2) – 1.92*N_u$, where N_u is urinary urea nitrogen excretion.

2.1.1.11 Serum growth hormone

The relationship between serum GH concentrations and glucose and lipid metabolism was investigated by analysing venous blood samples for GH at each time-point shown above in Figure 2.2. Serum GH was measured at the Oxford University Hospitals NHS Trust Clinical Biochemistry laboratory using the Siemens Immulite 2000 GH assay (Siemens Medical Solutions Diagnostics Limited, Llanberis, UK), according to the manufacturer's guidelines.

2.1.2 *Cross-sectional study of elite adolescent athletes*

The primary aim of this study was to investigate serum IGF-I and P-III-NP concentrations in elite adolescent athletes and to determine whether the method developed to detect GH misuse in adults is appropriate for use in this population. A secondary aim of the study was to perform method comparisons between two IGF-I and two P-III-NP assays used to measure the adolescent athlete samples.

2.1.2.1 Participants

Sixty-three national and regional sporting organisations were contacted to obtain permission to approach elite athletes (representing county level or above) aged between 12 and 20 years. Permission was obtained to attend training sessions at the following organisations (all UK): Guernsey Amateur Swimming Association, Guernsey Hockey Association, Hampshire Hockey Association, Guernsey Netball Association, Guernsey Cricket Association, Guernsey Squash Racquets Association, Guildford City Swimming Club, City of Southampton Swimming Club, City of Cardiff Swimming Club, Howell's School (Llandaff), Hampshire Athletics Association, Saracens Rugby Football Club (Hertfordshire), Chichester Junior Performance Tennis and Southampton University Sailing Club. Individual participants were also recruited to the study through personal contacts of the GH-2004 research team.

Prior to the event, potential participants were sent a letter or e-mail giving details of the study. A GH-2004 team member then approached the athletes at the event and if the athlete agreed to participate, written informed consent was obtained together with parental consent if the athlete was younger than 18 years. Approximately 185 letters were sent to potential participants of whom 157 were included in the study. Volunteers were excluded if they were suffering from any endocrine pathology or had suffered a recent musculoskeletal injury.

Demographic data on gender, age, ethnic origin, sport, training hours, diet, injuries, medications and menstrual history were recorded. Height was measured to the nearest centimetre using a portable stadiometer (Raven Equipment Limited, Essex) and weight was measured to the nearest 0.1 kg using Seca 876 electronic scales (Seca, Birmingham, UK) with participants dressed in light clothing. Body mass index (BMI, kg/m^2) was calculated by dividing the participant's weight in kilograms by the square of their height in metres. Pubertal staging was not undertaken as this would not be feasible in a real-life anti-doping setting.

2.1.2.2 Study design

Blood samples were collected from adolescent athletes either before or after exercise. Venous blood samples were collected and processed as described in section 2.1.1.3.

2.1.2.3 Analysis of GH-dependent markers (IGF-I and P-III-NP)

All serum samples were analysed in duplicate at the Drug Control Centre, King's College London.

2.1.2.3.1 IGF-I assays

Serum IGF-I was measured using two commercial immunoassays. The Immunotech A15729 IGF-I IRMA is described above in section 2.1.1.4.1. The other IGF-I assay used was the DSL-5600 ACTIVE IGF-I IRMA (Diagnostics Systems Laboratories Inc., Texas, USA). This assay was available from DSL in 2008 when these blood samples were collected. It was replaced with the DSL 10-5600 IGF-I ELISA (described below in section 2.1.4.3) in 2009, though this assay was also subsequently withdrawn from the market and has been replaced at the Drug Control Centre with the Siemens Immulite IGF-I assay described in section 2.1.1.4.1.

The DSL-5600 ACTIVE IGF-I IRMA was a two-site immunoradiometric assay which utilised monoclonal anti-IGF-I antibody-coated tubes along with ^{125}I-labelled goat polyclonal antibodies to IGF-I. Acid-ethanol extraction was used to separate IGF-I from its binding proteins. Intra-assay CV (n = 8 replicates) at concentrations of 74 and 278 ng/mL was 5.5% and 3.9%, respectively. Inter-assay CV (n = 24 independent assays) at concentrations of 89 and 242 ng/mL was 4.3% and 5.9%, respectively (Cowan et al. 2009).

2.1.2.3.2 P-III-NP assays

The P-III-NP assays used in this study are described above in section 2.1.1.4.2.

2.1.3 *The effects of a freeze-thaw cycle and pre-analytical storage temperature on the stability of IGF-I and P-III-NP concentrations*

The aim of this study was to investigate the stability of IGF-I and P-III-NP concentrations in serum stored at -20°C and to establish the effects of one freeze-thaw cycle on assay results.

2.1.3.1 Participants

20 healthy volunteers (12 men, 8 women) aged between 22-34 years were recruited to the study through personal contacts at UK Anti-Doping. Exclusion criteria included

previous history of endocrinopathy and previous use of performance-enhancing drugs. Demographic data on gender, age, ethnic origin, physical activity, diet, injuries, medications, menstrual history, height (self-reported) and weight (self-reported) were recorded.

2.1.3.2 Study design

20mL of whole blood were collected from each volunteer as described in section 2.1.1.3. After centrifugation, the serum was divided into 1mL aliquots. 1 aliquot from 10 volunteers was analysed immediately (there was insufficient time to analyse samples from all 20 volunteers on the day of sample collection). The remaining aliquots and those from the other 10 volunteers were stored overnight at 4°C and then frozen at -20°C. Aliquots from all 20 volunteers were analysed after storage for 1 day at 4°C, 1 day at -20°C, 1 week at -20°C, 5 weeks at -20°C and 3 months at -20°C.

2.1.3.3 Analysis of GH-dependent markers (IGF-I and P-III-NP)

All serum samples were analysed in duplicate at the Drug Control Centre, King's College London. The 10 "fresh" samples assayed immediately after centrifugation were analysed using the DSL 10-5600 IGF-I ELISA (Diagnostics Systems Laboratories Inc., Texas, USA) and the UniQ™ PIIINP RIA (Orion Diagnostica, Espoo, Finland). The Orion assay is described above in section 2.1.1.4.2. The DSL 10-5600 IGF-I ELISA was available in 2009 when these blood samples were collected but this assay has since been withdrawn from the market and has been replaced at the Drug Control Centre with the Siemens Immulite IGF-I assay described in section 2.1.1.4.1.

The DSL 10-5600 IGF-I ELISA was a manual, enzymatically-amplified, one-step sandwich immunoassay. Acid-ethanol extraction was used to separate IGF-I from its binding proteins. Intra-assay CV (n = 8 replicate samples) was 5.2% and 4.3% at concentrations of 125 and 225 ng/mL respectively. Inter-assay CV (n = 6 independent assays) was 10.7% at a concentration of 225 ng/mL. This assay was calibrated with WHO International Reference Reagent WHO IRR 87/518.

Samples from all remaining time-points were analysed using the DSL 10-5600 IGF-I ELISA, the Immunotech A15729 IGF-I IRMA (see section 2.1.1.4.1), the RIA-gnost P-III-NP from CIS Biointernational (see section 2.1.1.4.2) and the Orion UniQ™ PIIINP RIA.

2.1.4 *Cross-sectional study of elite athletes to determine decision limits for the GH-2000 detection method*

The aim of this study was to determine concentrations of IGF-I and P-III-NP in elite athletes using two commercial immunoassays for each analyte, to determine how these assay results relate to one another and to develop appropriate decision limits for detecting GH misuse in elite athletes.

2.1.4.1 Participants

In collaboration with UK Anti-Doping (formerly a part of UK Sport), 24 sporting organisations were contacted to obtain permission to approach elite athletes (representing national level or above). Permission was obtained to attend training sessions at the following organisations (all UK): Gloucester Rugby Football Club, London Irish Rugby Football Club (Middlesex), Saracens Rugby Football Club (Hertfordshire), England Women's Rugby Football Club (Middlesex), Harlequins Rugby League Club (Middlesex), Southampton Football Club, Tottenham Hotspur Football Club (London), Wolverhampton Wanderers Football Club, England Women's Football Club (London), Royal Yachting Association (Hamble), British Triathlon Federation (Loughborough), Great Britain Rowing Team (Caversham), Great Britain Hockey Team (Bisham Abbey) and Great Britain Swimming Team (Loughborough).

I (or another member of the GH Project research group) approached potential volunteers at each training session and the athletes were given a written information sheet. If the athlete agreed to participate, written informed consent was obtained. Participants were required to confirm that they had not taken performance-enhancing drugs prior to taking part. 260 athletes agreed to participate in the study. Demographic data on gender, age, ethnic origin, sport, training hours, diet, injuries, medications, menstrual history, height (self-reported) and weight (self-reported) were recorded.

2.1.4.2 Study design

Venous blood samples for GH-dependent markers were collected either before or after exercise as described in section 2.1.1.3. If an appropriate centrifuge was not available at the collection site, blood samples were transported on ice to the Wellcome Trust CRF, Southampton General Hospital for centrifugation. The large majority of samples were centrifuged within 8 hours from the time of collection and all samples were

centrifuged within 24 hours. WADA guidelines recommend that a temperature-recording device is included with the samples to ensure the samples have been maintained at temperatures between 2ºC and 8ºC prior to centrifugation. A temperature-recording device was used on several occasions to confirm that the temperature of the samples did not rise above 8ºC. Serum was transferred to microcentrifuge tubes after centrifugation and stored frozen at -80 ºC. All samples were coded and anonymised before analysis.

A further 238 samples were collected from elite athletes as part of the UK Anti-Doping Testing Programme. UK Anti-Doping is responsible for collection of anti-doping samples from more than 40 sports along with transportation of these samples to a WADA-accredited laboratory. Testing can occur both in-competition and out-of-competition and any athlete subject to the anti-doping rules of their sport is eligible for testing. UK Anti-Doping employs a group of Blood Collection Officers (BCOs) who are trained phlebotomists and who collect blood samples for testing. The athletes included in this study had provided consent for their samples to be used for research purposes and these samples were also collected according to WADA guidelines for blood sample collection.

2.1.4.3 Analysis of GH-dependent markers (IGF-I and P-III-NP)

All serum samples were analysed at the Drug Control Centre, King's College London. The IGF-I and P-III-NP assays used in this study are described above in section 2.1.1.4. Analyses were performed in duplicate for all assays except the Immulite assay for which analysis was performed in singlicate as instructed by the manufacturer.

2.1.5 *The use of blood mRNA technology to detect GH and IGF-I misuse in athletes*

2.1.5.1 The intra-individual variability of circulating mRNA concentrations for GH, GHRH, IGF-I and IGFBP-3

The aim of this pilot study was to assess the intra-individual variability of circulating mRNA for GH, GHRH, IGF-I and IGFBP-3 over a period of six weeks.

2.1.5.1.1 Participants

10 healthy recreational athletes (8 men, 2 women) aged between 19-29 years were recruited by poster advertisement in the University of Southampton Faculty of Medicine, University of Southampton Sports Centre and by approaching University of Southampton Sports Societies. The athletes engaged in regular physical activity (≥2 sessions/week). Participants were ineligible if they had used performance-enhancing drugs previously or were found to be anaemic at screening. Anyone with previous history of endocrinopathy, diabetes mellitus or neoplastic disease was excluded. Pregnant women were not allowed to participate; pregnancy tests were performed on all female volunteers prior to enrolment and they were advised to use safe contraception for the duration of the study if sexually active.

2.1.5.1.2 Study design

At the beginning of the study (Day 0), demographic data on gender, age, ethnic origin, sport, training hours, diet, injuries, medications and menstrual history were recorded. Height was measured to the nearest centimetre using a portable stadiometer (Raven Equipment Limited, Essex) and weight was measured to the nearest 0.1 kg using Seca 876 electronic scales (Seca, Birmingham, UK) with participants dressed in light clothing. Body mass index (BMI, kg/m^2) was calculated by dividing the participant's weight in kilograms by the square of their height in metres. Body composition was assessed using bioelectrical impedance analysis and skinfold thickness as described in section 2.1.1.6. Physical fitness was assessed by incremental treadmill test as described in section 2.1.1.7.

2.1.5.1.3 Collection and analysis of serum GH-dependent markers

Venous blood samples for GH-dependent markers were collected at baseline (Day 0) and processed as described in section 2.1.1.3. All samples were coded and anonymised before analysis at the Drug Control Centre, King's College London using the IGF-I and P-III-NP assays described in section 2.1.1.4.

2.1.5.1.4 Collection of mRNA blood samples

Blood samples were collected for mRNA analysis at baseline (Day 0) and then at two-weekly intervals over 6 weeks (Fig. 2.3).

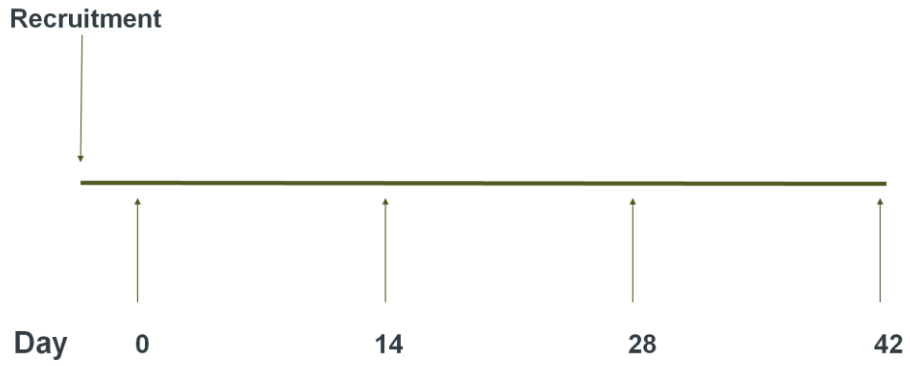

Figure 2.3. mRNA Variability Study Protocol. Venous blood samples were collected at baseline (Day 0) and at two-weekly intervals over 6 weeks (Days 14, 28 and 42).

Venous blood was collected into two 10mL PAXgene™ Blood RNA Tubes (Becton Dickinson, Oxford, UK). Samples were mixed and stored upright at room temperature for a minimum of two hours, then frozen at -20ºC for at least 24 hours before transfer to the -80ºC freezer. Samples were anonymised before analysis and all analyses were performed by Dr Asif Butt at the Department of Chemical Pathology, St Thomas' Hospital, London.

2.1.5.1.5 Extraction of RNA from whole blood

Blood samples were thawed and allowed to equilibrate at room temperature for at least two hours prior to RNA extraction. RNA from whole blood samples was extracted using the PAXgene™ Blood RNA kit (QIAGEN, Crawley, UK) as follows: thawed samples were centrifuged at 4700*g* for 10 minutes at room temperature to form a pellet in the PAXgene™ Blood RNA Tubes. The supernatant was aspirated and the pellet washed by re-suspending it in RNase-Free water. The re-suspended

pellet was centrifuged again at 4700*g* for 10 minutes and 350µL re-suspension buffer (BR1) was added after removal of the RNase-Free water. The washed pellet was re-suspended in 300µL of binding buffer (BR 2) and 40µL Proteinase K (PK). This mixture was incubated in a heating block at 55°C for 10 minutes to enable optimal protein digestion. The lysate was pipetted directly onto a PAXgene™ Shredder Spin column, placed in a 2mL processing tube and the whole assembly centrifuged at 20,000*g* for 3 minutes. The supernatant of the flow-through fraction was transferred to a fresh 1.5mL microfuge tube without disturbing the pellet in the processing tube. This was followed by the addition of 350µL absolute alcohol (Rathburn Chemicals Limited, Walkerburn, UK) before the sample was pipetted into a PAXgene™ spin column, which contains a silica gel-based membrane that selectively binds RNA. The spin column was centrifuged at 15,700*g* and retained contents washed with buffer BR3. On-column DNA digestion was carried out at room temperature by the addition of 80µL RNase-Free DNase I for 15 minutes. DNase I and any contaminants were removed by a second wash step using buffer BR3 followed by two wash steps using buffer BR4. An elution step was then performed using 40µL of elution buffer BR5 and centrifugation at 15,700*g*. This elution step was repeated to give a final volume of 80µL containing extracted RNA which was stored at -70°C until further processing.

2.1.5.1.6 Total RNA quantitation

Extracted RNA was quantified using the Nanodrop™ 2000 spectrophotometer (Thermo Scientific, Wilmington, USA), which uses an algorithm to convert absorbance at 260/280nm to determine the RNA quantity in the sample. The elution buffer for the RNA extraction (section 2.1.5.1.5) was used as a blank sample to "zero" the analyser.

2.1.5.1.7 Reverse transcription

All reagents (Invitrogen Life Technologies, Paisley, UK) and RNA samples were kept on ice prior to the addition of the reverse transcriptase. The order of addition and reagent volumes per reaction are shown in Table 2.3.

Table 2.3. Components for reverse transcription of mRNA.

Reagent	Volume per reaction, µL
RNA Sample	60
10mM dNTP Mix (2'-deoxynucleoside 5'-triphosphate; dATP, dCTP, dGTP, dTTP)	4.5
Oligo$(dT)_{12-18}$ (0.5µg/µL)	2.5
X5 Buffer (250mM Tris-HCl, pH 8.3)	20
0.1M DTT (dithiothreitol)	10
RNaseOUT™ (40U/µL)	1.0
SuperScript II™ (200U/µL)	2
Total reaction volume	**100**

RNA sample, dNTP Mix and Oligo$(dT)_{12-18}$ were added to a 0.2mL PCR tube and heated to 65°C for 5 minutes. The mixture was chilled on ice before the addition of X5 Buffer, DTT and RNaseOUT™. PCR tubes were transferred to a thermal cycler (PTC-100™ Programmable Thermal Cycler, MJ Research Inc., Massachusetts, USA). The thermal cycler was used to incubate the reaction mixture at 42°C for 2 minutes, after which SuperScript II™ reverse transcriptase was added to start the reverse transcription reaction. The reaction mix was incubated for another 50 minutes at the same temperature before inactivating the reaction by heating the mixture at 70°C for 15 minutes. Reverse transcription negative controls were processed simultaneously. These contained all the components of the corresponding reaction but the SuperScript II™ was replaced with water.

2.1.5.1.8 Primer and probe design

Primers and probes for IGF-I, IGFBP-3, GH and GHRH were intron-spanning (exon-exon boundary) and were designed by Dr Asif Butt using Primer Express, version 1.0 (Applied Biosystems Inc., Warrington, UK). The accession numbers, nucleotide sequences and amplicon sizes are shown in Table 2.4. The amplicon sequences were checked by BLAST to confirm mRNA transcript and gene identity.

Housekeeping genes beta-actin and beta-globin were measured using pre-developed Assay Reagents (Applied Biosystems Inc., Warrington, UK).

2.1.5.1.9 Quantitative real-time polymerase chain reaction (qPCR)

Quantitative real-time PCR analysis was performed using an ABI Prism 7000 sequence detection system (Applied Biosystems Inc., Warrington, UK). In-house Taqman assays were used to measure complementary DNA (cDNA) for GH, GHRH, IGF-I and IGFBP-3 by absolute quantification. Standard curves were generated by serial dilution of commercially available cDNA of known concentration (0.5 µg/µL). Liver cDNA (Ambion Inc., Huntingdon, UK) was used to develop standard curves for beta-globin, beta-actin, IGF-I and IGFBP-3, while pituitary cDNA (Takara Bio, Paris, France) was used to develop a standard curve for GH. The working concentration range of the standard curves was 0.005 – 20 ng/mL.

For GH, GHRH, IGF-I and IGFBP-3 measurement, samples (10µL) were amplified in a reaction volume of 25µL containing: 300nM of each amplification primer, 100nM of corresponding probe and X2 Taqman Universal Master Mix (12.5 µL), which contains optimised concentrations of $MgCl_2$, dATP, dCTP, dGTP and dUTP, AmpliTaq® Gold and AmpErase® uracil N-glycosylase. Beta-actin and beta-globin PCR were also analysed in a reaction volume of 25µL comprising 10µL sample, 1.25µL PDAR, 12.5µL X2 Taqman Universal Master Mix and 1.25µL RNase-Free water. In the case of beta-actin and beta-globin, samples were diluted 1:10,000 with RNase-Free water. All reactions were carried out in 96-well optical reaction plates (Applied Biosystems Inc., Warrington, UK). Samples and standards were analysed in duplicate and a calibration curve run in parallel with each analysis. Multiple negative water blanks (no template control) were included in every analysis. Identical thermal profiles were used for all the genes of interest. Thermal cycling was initiated with a two minute incubation period at 50°C to allow the uracil N-glycosylase to act, followed by a first inactivation step of 10 minutes at 95°C and then up to 50 cycles of 95°C for 15 seconds and 60°C for 1 minute.

Table 2.4. Primers and probes for GH, GHRH, IGF-I and IGFBP-3 Taqman qPCR assays.

	GH	GHRH	IGF-I	IGFBP-3
Accession no	NM_000515	NM_021081	NM_001111283	NM_001013398
Forward primer	CTCCGCGCCCATCGT	GCAGGCAGCAGGGAGAGA	AGCGCCACACCGACATG	CCAAGCGGGAGACAGAATATG
Reverse primer	CCTTTGGGATATAGGCTTCTTCAA	ATGCTGTCTACCTGACGACCAA	CTGAGACTTCGTGTTCTTGTTGGT	CATTGAGGAACTTCAGGTGATTCA
Probe	6-FAM-CCAGCTGGCCTTTGACACCTACCAGG-TAMRA	6-FAM-AACCAAGAGCGAGGAGCAAGGGCAC-TAMRA	6-FAM-CCAAGACCCAGAAGTATCAGCCCCCA-TAMRA	6-FAM-TCCCTGCCGTAGAGAAATGGAAGACACA-TAMRA
Exon-Exon	2-3	2-3	3-4	2-3
Amplicon size	73 bp	70 bp	71 bp	75 bp

2.1.5.2 Acute changes in blood mRNA concentrations in response to four injections of rhGH

The aim of this study was to assess changes in mRNA concentrations for GH, GHRH, IGF-I and IGFBP-3 in response to four injections of rhGH (0.1 units/kg) on consecutive days.

2.1.5.2.1 Participants

10 healthy male recreational athletes aged between 20-23 years were recruited. The athletes engaged in regular physical activity (≥2 sessions/week). The recruitment procedures, inclusion and exclusion criteria were identical to those described in section 2.1.5.1.1 above.

2.1.5.2.2 Study design

This study was a non-randomised intervention study. At the beginning of the study (Day 1), demographic data on gender, age, ethnic origin, sport, training hours, diet, injuries, medications and menstrual history were recorded. Height and weight were measured and BMI calculated as described in section 2.1.5.1.2. Body composition was assessed using bioelectrical impedance analysis and skinfold thickness as described in section 2.1.1.6. Physical fitness was assessed by incremental treadmill test as described in section 2.1.1.7.

Participants received a daily injection of rhGH (0.1 units/kg/day) between 8am and 10am. Novo Nordisk Ltd, Crawley, UK supplied rhGH in the form of Somatotropin rDNA 15mg/1.5mL (Norditropin NordiFlex®). Drug vials were stored at 4ºC. I administered rhGH injections to all volunteers.

2.1.5.2.3 Collection and analysis of mRNA blood samples

Blood samples were collected for mRNA analysis at baseline (prior to the first injection of rhGH) and 30 minutes, 1 hour, 2 hours, 4 hours and 8 hours after the first injection. Subsequent blood samples were taken after 1 day (prior to the second injection), 2 days (prior to the third injection), 4 days and 1 week (Fig. 2.4).

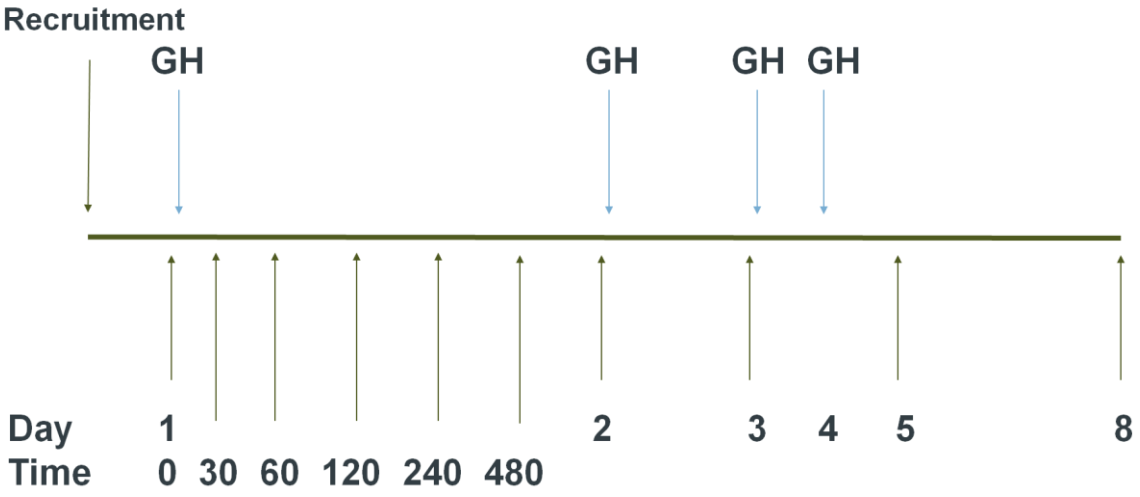

Figure 2.4. mRNA GH Administration Study Protocol. rhGH was administered daily for four days. Venous blood samples were collected at baseline (Day 1, Time 0). Five further blood samples were collected on Day 1 after rhGH administration and subsequent blood samples were collected on Days 2, 3, 5 and 8.

Venous blood was collected and processed as described in section 2.1.5.1.4. Samples were anonymised before analysis and all analyses were performed by Dr Asif Butt at the Department of Chemical Pathology, St Thomas' Hospital, London. mRNA quantitation for GH, GHRH, IGF-I and IGFBP-3 was carried out as described in sections 2.1.5.1.5 to 2.1.5.1.9.

2.1.5.2.4 Collection and analysis of serum GH-dependent markers

Venous blood samples for GH-dependent markers were collected and processed as described in section 2.1.1.3. These samples were collected at all time-points described in section 2.1.5.2.3 above. All samples were anonymised and stored frozen at -80ºC until analysis at the Drug Control Centre, King's College London using the IGF-I and P-III-NP assays described in section 2.1.1.4.

2.1.5.3 The effects of rhIGF-I/rhIGFBP-3 administration on circulating mRNA for GH, GHRH, IGF-I and IGFBP-3

The aim of this study was to assess changes in mRNA concentrations for GH, GHRH, IGF-I and IGFBP-3 in response to the administration of rhIGF-I/rhIGFBP-3 complex to 30 male recreational athletes.

2.1.5.3.1 Participants and study design

This study was undertaken as an extension to the rhIGF-I/rhIGFBP-3 administration study described in section 2.1.1. 30 male recreational athletes were recruited as described in section 2.1.1.1. 10 males were randomly assigned to receive low dose (30 mg/day) rhIGF-I/rhIGFBP-3 complex, 10 males received high dose (60mg/day) rhIGF-I/rhIGFBP-3 complex and 10 males received placebo for 28 days as described in section 2.1.1.2.

Blood samples were collected for mRNA analysis at baseline (Day 0), during the treatment period (Days 7 and 28) and during the washout period on Days 30, 33, 42 and 84 (Fig. 2.5). Venous blood was collected and processed as described in section 2.1.5.1.4. Samples were anonymised before analysis and all analyses were performed by Dr Asif Butt at the Department of Chemical Pathology, St Thomas' Hospital, London. mRNA quantitation for GH, GHRH, IGF-I and IGFBP-3 was carried out as described in sections 2.1.5.1.5 to 2.1.5.1.9.

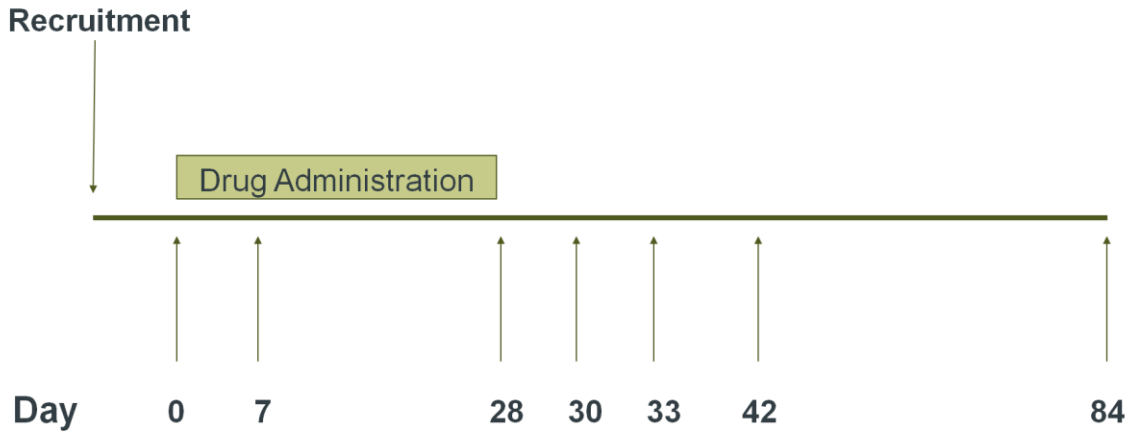

Figure 2.5. mRNA IGF-I Administration Study Protocol. Blood samples were collected before treatment (Day 0), during treatment (Day 7) and after treatment (Days 28, 30, 33, 42 and 84).

2.2 Statistical methods

2.2.1 *Assay comparisons and calculating assay adjustments*

In the original GH-2000 studies, serum IGF-I was analysed using a hydrochloric acid-ethanol extraction radioimmunoassay (Nichols Institute Diagnostics, San Juan Capistrano, USA). Nichols Institute Diagnostics ceased trading in 2005 and it has been necessary to use alternative IGF-I assays since that time. In order to compare results of subsequent studies (Chapters 5 and 6) with the original GH-2000 studies, I have aligned IGF-I assay results from these studies with the original scales used by the GH-2000 group. No significant changes have been made to the Cisbio P-III-NP assay since the GH-2000 studies but in Chapters 5 and 6, I have aligned P-III-NP results from the Orion assay with the Cisbio scale. The relationships between IGF-I assays and between P-III-NP assays have been assessed by performing method comparisons using paired samples, i.e. the same samples were analysed by both assay method; these results are presented in section 5.3.5. Inter-assay agreement was evaluated using simple linear regression and modified Bland-Altman plots.

2.2.2 *GH-2000 detection method*

The previously published GH-2000 discriminant function formulae (Powrie et al. 2007) are as follows ("log" is the natural logarithm):

Male score = -6.586 + 2.905 * log (P-III-NP) + 2.100 * log (IGF-I) - 101.737/age

Female score = -8.459 + 2.454 * log (P-III-NP) + 2.195 * log (IGF-I) - 73.666/age

These discriminant formulae were derived from amateur athletes participating in a rhGH administration study (Dall et al. 2000; Longobardi et al. 2000) and were calibrated against the GH-2000 elite athlete population (Healy et al. 2005). They were defined such that the mean GH-2000 score was 0 and standard deviation was 1 in elite athletes when IGF-I was measured using the Nichols IGF-I assay and P-III-NP was measured using the Cisbio P-III-NP assay. A provisional cut-off point (decision limit) for detecting GH doping using these formulae was set at 3.72. This would equate to a false positive rate of approximately 1 in 10,000 tests, assuming a normal distribution of scores in elite athletes.

The decision limit of the GH-2000 detection method depends on the combination of assays used to measure IGF-I and P-III-NP and on the population of athletes studied

(elite or recreational). I have described the calculation of the decision limits used in each of the following studies below and in the Methods section of the corresponding chapters.

2.2.3 *Randomised, double-blind, placebo-controlled IGF-I administration study*

2.2.3.1 Sample size

A formal power calculation was not possible for this study since this was the first study to investigate markers of IGF-I misuse by administering rhIGF-I/rhIGFBP-3 to healthy volunteers. We were confident that the numbers involved in the study were adequate for purpose for the following reasons: first, in order for an anti-doping test to be used, it is important that there is a very clear difference between doped and clean athletes. Therefore if differences were not seen with small numbers, it is unlikely that the test would be useful in the context of anti-doping. In addition, the numbers involved in this study were similar to the numbers involved in the previous GH-2000 and GH-2004 administration studies as well as other studies assessing the effects of rhGH administration on serum markers (Powrie et al. 2007; Nelson et al. 2008; Holt et al. 2010).

2.2.3.2 GH-dependent markers

The primary aim of this study was to assess whether the administration of rhIGF-I/rhIGFBP-3 complex induces changes in serum concentrations of the GH-dependent markers IGF-I and P-III-NP. The analyses of marker results were performed by Drs Ioulietta Erotokritou-Mulligan and Eryl Bassett, GH Project statisticians, using methods similar to those used in the previous double-blind rhGH administration studies (Bassett et al. 2009). Statistical analyses were performed using SAS® software version 9.0 (SAS Institute Inc., Cary, NC, USA). Differences in baseline characteristics between treatment groups were assessed using ANOVA. As marker concentrations were skewed and their distribution was normalised by log-transformation, all analyses were performed on the log-transformed values of IGF-I and P-III-NP. The concentrations of both markers and the GH-2000 scores on each visit day were assessed against the clean observation values (baseline and placebo-treated samples). Paired *t*-test analysis was used to compare mean IGF-I, P-III-NP and GH-2000 score on each visit day in the study. The analysis was

performed separately for each treatment group (placebo, low dose IGF-I and high dose IGF-I). Maximum IGF-I and P-III-NP concentrations in each participant were calculated and differences in mean maximum concentrations between low dose IGF-I and high dose IGF-I groups were assessed using unpaired *t*-tests. Stepwise discriminant analysis was performed using IGF-I and P-III-NP results from the high dose IGF-I groups. Further pharmacokinetic calculations are described in Results Chapter 3, section 3.2.

2.2.3.3 Comparison between rhIGF-I/rhIGFBP-3 administration and GH-2000 rhGH administration studies

It was possible to compare the effects on serum markers of rhIGF-I/rhIGFBP-3 administration in the current study and rhGH administration in the original GH-2000 study (Dall et al. 2000; Longobardi et al. 2000). The rhGH doses used in the GH-2000 study were 0.1 IU/kg/day (low dose group) and 0.2 IU/kg/day (high dose group) and treatment was administered for 28 days in both studies. The assays used to measure IGF-I and P-III-NP in the two studies were different and therefore assay results from the Immunotech IGF-I and Orion P-III-NP assays used in the current study were aligned with the Nichols IGF-I and Cisbio P-III-NP assay scales, using the methods described in section 2.2.1 and Chapter 5. For each participant in both studies, maximum IGF-I and maximum P-III-NP results were calculated. Maximum percentage increase in each marker was calculated using:

((Maximum marker result – Day 0 marker result) ÷ Day 0 marker result) x 100

The relative increase in each marker was compared between the two studies and between women and men within each study, using unpaired *t*-tests and analysis of covariance (ANCOVA).

2.2.3.4 Body composition, physical fitness and substrate metabolism

Area under the curve (AUC) calculations for glucose, insulin and NEFA were performed using the trapezoidal method. Body composition and physical fitness data from participants in low and high dose treatment groups were analysed separately and combined. Within-group changes after treatment were assessed using paired *t*-tests. The relative effects of rhIGF- I/rhIGFBP-3 administration on male and female treatment groups were compared using unpaired *t*-tests and analysis of covariance

(ANCOVA). Analyses were performed on log-transformed data and $P < 0.05$ was considered statistically significant.

2.2.4 *Cross-sectional study of elite adolescent athletes*

2.2.4.1 Sample size

A minimum sample size of 100 elite adolescent athletes was determined by the GH Project statisticians in collaboration with WADA based on previous experience from the GH-2000 and GH-2004 studies. The sample size was limited by the difficulties in recruiting elite athletes aged less than 18 years within the timeframe of this study.

2.2.4.2 Anthropometric data

Height, weight and BMI measurements for the adolescent study were converted to SD scores (SDS) for chronological age (UK standards 1990) (Freeman et al. 1995). Anthropometric data were compared using unpaired *t*-tests and all statistical comparisons were two-tailed. $P<0.05$ was considered statistically significant. Data from this study were analysed using SPSS (SPSS Inc., Chicago, IL) and SAS (SAS Institute Inc., Cary, NC) software.

2.2.5 *The effects of a freeze-thaw cycle and pre-analytical storage temperature on the stability of IGF-I and P-III-NP concentrations.*

2.2.5.1 Sample size

A sample size of 20 participants was determined by the GH Project statisticians in collaboration with WADA. It was not possible to perform a power calculation for this study as the aim of the study was to investigate the effect of pre-analytical storage conditions on assay results.

2.2.5.2 Estimation of intra-individual variability

Assay results were converted to GH-2000 assay scales as described in section 2.2.1 and then incorporated into the GH-2000 discriminant function formulae as described in section 2.2.2. Analysis of variance (ANOVA) was performed to estimate intra-sample variability for IGF-I and P-III-NP concentrations and for GH-2000 score.

Inter-assay variability was determined by analyzing one QC sample on the same day as the participant samples. Data from this study were analysed using SAS (SAS Institute Inc., Cary, NC) software.

2.2.6 *Cross-sectional study of elite athletes to determine decision limits for the GH-2000 detection method*

2.2.6.1 Sample size

A target sample size of 500 elite athletes was agreed by the GH Project statisticians in collaboration with WADA. This was deemed an appropriate number of participants from which to estimate decision limits for determining GH misuse because of previous experience in the GH-2000 studies in which 813 elite athletes were recruited to study physiological ranges of GH-dependent markers (Healy et al. 2005). In addition, we have proposed a dynamic approach to the use of these decision limits by incorporating data from further elite athletes as the test is used in an anti-doping context, as described in Chapter 7.

2.2.6.2 Calculation of decision limits

GH-2000 scores were calculated as described above in section 2.2.2. Since two IGF-I and two P-III-NP assays were used, scores were calculated using all four combinations of IGF-I and P-III-NP assay results. The aim of the statistical analysis in this study was to determine appropriate decision limits for each IGF-I and P-III-NP assay combination ("assay kit"). All analyses were based on Normal distributions since all empirical distributions were consistent with this. If, for a particular assay combination, GH-2000 scores are Normally distributed then a false positive rate of 1 in 10,000 will be achieved if a decision limit (c) of c = mean + 3.72 * standard deviation, is used. This applies to tests based on each assay pairing separately.

WADA testing requirements state that, for a positive finding to be declared, an athlete's results must exceed the calculated decision limit on both assay pairings. Under these circumstances, the overall chance of a false positive is less than 1 in 10,000 if the multiplier 3.72 is used. To maintain a false positive rate of 1 in 10,000, the standard deviation multiplier needed to be reduced appropriately by considering the correlation between the GH-2000 scores from the two pairings of assays. The

reduced multipliers give decision limits for a "combined" test based on both pairings of assays, with an estimated overall false positive rate of 1 in 10,000 (99.99% specificity).

Each proposed decision limit is based on data from a sample of the elite athlete population and therefore is only an estimate of the "true" limit. Each of the estimated decision limits carries a degree of uncertainty around it. The extent of this uncertainty is inversely proportional to the square root of the total sample size. The larger the sample size used when estimating the decision limits, the smaller the degree of uncertainty will be. This "sample size uncertainty" was assessed using the standard deviation of each decision limit. The sampling distribution of each limit can be approximated by a Normal distribution, which was used to give an upper 95% confidence limit. This upper 95% confidence limit is the proposed new decision limit for each assay pairing.

2.2.7 *The use of blood mRNA technology to detect GH and IGF-I misuse in athletes*

2.2.7.1 The intra-individual variability of circulating mRNA for GH, GHRH, IGF-I and IGFBP-3

2.2.7.1.1 Sample size

This was a pilot study and so formal power calculations were not possible. A sample size of 10 participants was agreed by the GH Project statisticians in collaboration with WADA.

2.2.7.1.2 Estimation of intra-individual variability

mRNA concentrations for target genes were corrected for total RNA concentrations as well as for housekeeping genes beta-actin and beta-globin and the ratios were log-transformed to eliminate skewness. Intra-individual variability in mRNA concentrations was assessed using ANOVA.

2.2.7.2 Acute changes in blood mRNA concentrations in response to four injections of rhGH

2.2.7.2.1 Sample size

This was a pilot study and so formal power calculations were not possible. A sample size of 10 participants was agreed by the GH Project statisticians in collaboration with WADA.

2.2.7.2.2 Assessment of changes in mRNA concentrations

Changes in mRNA concentrations and peptide concentrations between time-points were assessed using paired *t*-tests. The relationship between mRNA concentrations and IGF-I peptide concentrations was assessed using simple linear regression.

2.2.7.3 The effects of rhIGF-I/rhIGFBP-3 administration on circulating mRNA for GH, GHRH, IGF-I and IGFBP-3

2.2.7.3.1 Sample size

This was a pilot study and so formal power calculations were not possible. A sample size of 30 participants was agreed by the GH Project statisticians in collaboration with WADA. A larger number of participants were recruited compared with the studies described above in sections 2.2.7.1 and 2.2.7.2 because the rhIGF-I/rhIGFBP-3 administration study was already taking place and these participants were therefore not exposed to any additional risks.

2.2.7.3.2 Assessment of changes in mRNA concentrations

Changes in mRNA concentrations and peptide concentrations between time-points were assessed using paired *t*-tests. The relationship between mRNA concentrations and IGF-I peptide concentrations was assessed using simple linear regression.

CHAPTER 3: BIOMARKERS OF IGF-I MISUSE IN RECREATIONAL ATHLETES: CHANGES IN SERUM IGF-I, P-III-NP AND GH-2000 SCORE

3.1 Introduction

There are reports that athletes are misusing IGF-I either alone or in combination with GH, despite the presence of both substances on the WADA list of prohibited substances (WADA 2013). At present there is no test to detect IGF-I misuse. The main challenge lies in distinguishing exogenous from endogenous IGF-I, as discussed in Chapter 1. The GH-2000 marker method for detecting misuse with GH is based on the measurement of GH-dependent markers in serum. The administration of recombinant human GH (rhGH) leads to statistically and clinically significant increases in serum GH-sensitive markers (Dall et al. 2000; Longobardi et al. 2000), which can be used to construct formulae that discriminate between those taking GH and those taking placebo. IGF-I and procollagen type III amino-terminal propeptide (P-III-NP) were selected as the best markers to detect GH misuse (Powrie et al. 2007).

If IGF-I is an essential mediator of GH action on target tissues, as proposed by the original somatomedin hypothesis, then IGF-I misuse would be expected to cause similar changes in GH-sensitive serum markers, both in the GH-IGF axis and markers of collagen and bone turnover. It would be ideal if the GH-2000 discriminant functions could be used to detect IGF-I misuse, as well as GH misuse, because this would allow anti-doping organisations to test for both substances without the additional costs of further assays or calculations. The hypothesis tested in this study is that it is possible to detect the administration of exogenous rhIGF-I/rhIGFBP-3 complex by measuring the GH-2000 selected GH-dependent markers in serum. The aims of this study were:

1) To assess whether the administration of rhIGF-I/rhIGFBP-3 complex induces changes in serum concentrations of the GH-dependent markers IGF-I and P-III-NP in healthy, recreational athletes.

2) To assess whether the GH-2000 formulae, previously derived for the detection of GH misuse, are also applicable to the detection of rhIGF-I/rhIGFBP-3 misuse in athletes.

3.2 Methods

56 healthy recreational athletes (30 men, 26 women) aged between 18-30 years were recruited as described in section 2.1.1.1. Participants were randomly assigned to receive low dose (30 mg/day) rhIGF-I/rhIGFBP-3 complex, high dose (60 mg/day) rhIGF-I/rhIGFBP-3 complex or placebo as described in section 2.1.1.2. Participants self-administered the drug with their evening meal as described in section 2.1.1.2. Venous blood samples for GH-dependent markers (IGF-I and P-III-NP) were collected at baseline, during the treatment period on Days 7, 14 and 21 and during the washout period on Days 28, 30, 33, 42 and 84 as described in section 2.1.1.3. IGF-I and P-III-NP were measured by immunoassay at the Drug Control Centre, King's College London as described in section 2.1.1.4.

A formal power calculation was not possible for this study since this was the first study investigating the response of GH-dependent markers to rhIGF-I/rhIGFBP-3 administration in healthy volunteers. The numbers involved in this study were similar to the numbers recruited to the previous GH-2000 and GH-2004 administration studies and other studies assessing the effects of GH on serum biomarkers (Powrie et al. 2007; Nelson et al. 2008; Holt et al. 2010). Differences in baseline characteristics between treatment groups were assessed using ANOVA. As marker concentrations were skewed and their distribution was normalised by log-transformation, all analyses of marker results were performed on log-transformed values of IGF-I and P-III-NP. GH-2000 scores were calculated as described in section 2.2.2. Changes in concentration of both markers and in GH-2000 score on each visit day were assessed using the statistical techniques described in section 2.2.3.2. 99.99% upper threshold levels for this population of recreational athletes were estimated as described in section 2.2.2 and 2.2.3.2. Marker responses in the current study were compared with marker responses in the previous GH-2000 study using the methods described in section 2.2.3.3.

Pharmacokinetic Calculations

During the initial analyses of marker responses, three participants demonstrated a pattern of serum IGF-I changes that was different to all other participants, as discussed further in section 3.4.2 below. I therefore examined the pharmacokinetics of the rhIGF-I/rhIGFBP-3 complex using the simplified approach described below to determine whether differences in absorption and elimination of the drug might explain the differences observed in IGF-I changes.

Pharmacokinetic parameters were derived from measurements of serum IGF-I and P-III-NP according to a single compartment model, using the methods previously described for estimating pharmacokinetic parameters of human insulin and porcine proinsulin (Sonksen et al. 1973). Steady state IGF-I and P-III-NP concentrations for each participant in the low and high dose treatment groups were estimated using mean concentration during the administration period (Days 7, 14, 21 and 28). For the purposes of these estimations, it was assumed that both markers reached steady state concentrations by Day 7. Absorption rates for IGF-I were calculated as follows: 1mg of rhIGF-I/rhIGFBP-3 is equivalent to 0.2mg of rhIGF-I. Absorption rates in the high dose (60 mg/day) and low dose (30 mg/day) groups were therefore 8.33 and 4.17 mcg/min respectively. The metabolic clearance rate (MCR: the volume of blood irreversibly cleared of IGF-I per unit time) was calculated using:

MCR (mL/min) = Absorption rate (mcg/min) ÷ Steady state concentration (mcg/mL)

The elimination rate constant (K_{el}) was estimated graphically from the linear portion of the log concentration *vs.* time curve using:

K_{el} = (LN C_1 - LN C_2) ÷ (t_2 - t_1), where C_1 and C_2 are successive IGF-I concentrations measured at times t_1 and t_2 after the participant had stopped administering rhIGF-I/rhIGFBP-3 and LN is the natural logarithm.

The serum half-life ($t_{1/2}$) was calculated from K_{el} using:

$t_{1/2}$ = 0.693 ÷ K_{el}

The apparent volume of distribution (V_d) was calculated using:

V_d = MCR ÷ K_{el}

Since MCR and V_d are related to body mass, their results are expressed relative to body weight (in kg).

My role in this part of the study was as follows: I recruited all participants to the study using the methods described in section 2.1.1.1. I demonstrated the injection technique to each participant prior to the first dose, delivered the study medication to each participant and was the primary study contact for participants throughout the administration and washout periods. I collected the majority of blood samples for GH-dependent markers and a small number of blood samples were collected by University of Southampton medical students under my supervision. I was also

involved in liaising with the local ethics committee and UHS Research and Development office, and I was responsible for preparing progress reports on the study. All laboratory analyses were performed by Mr Christiaan Bartlett at the Drug Control Centre, King's College London. I performed the pharmacokinetic calculations. Statistical analyses on marker results were performed by Dr Erotokritou-Mulligan, GH-2004 project statistician.

3.3 Results

Table 3.1 shows the baseline characteristics of the groups. The 30 male volunteers comprised 29 white Europeans and 1 Asian. The 26 female volunteers comprised 20 white Europeans, 2 Asians, 1 African and 3 Mixed Race.

In men, there were significant differences between treatment groups at baseline in mean weight. There were no significant differences between treatment groups in men or women in age, height, body mass index, IGF-I or P-III-NP concentrations.

No participants discontinued the study because of adverse effects related to the study medication. Participants in all treatment groups reported local erythema and pain at the site of subcutaneous injections and it is likely that this was a reaction to the solvent used to dissolve the drug and placebo. These symptoms were mild and resolved completely after stopping treatment. Three participants (all in the high dose rhIGF-I/rhIGFBP-3 group) reported increased appetite during treatment.

Table 3.1. Baseline characteristics of 56 recreational athletes. BMI=body mass index, IGF-I=rhIGF-I/rhIGFBP-3 administration. *Significant difference ($P < 0.05$) between placebo, low dose and high dose groups.

Variable	Women			Men		
	Placebo (n=8)	Low dose IGF-I (n=9)	High dose IGF-I (n=9)	Placebo (n=10)	Low dose IGF-I (n=10)	High dose IGF-I (n=10)
Mean age (SD), years	21.9 (2.2)	21.7 (3.4)	21.4 (1.7)	22.0 (2.8)	21.9 (2.7)	23.2 (2.7)
Mean height (SD), cm	167.5 (7.7)	165.2 (2.3)	169.0 (6.6)	185.0 (5.8)	179.2 (10.2)	181.3 (6.2)
Mean weight (SD), kg	61.7 (7.0)	60.2 (4.9)	60.5 (7.4)	92.4 (16.2)*	76.9 (12.0)*	80.7 (12.9)*
Mean BMI (SD), kg/m^2	22.0 (1.6)	22.0 (1.8)	21.2 (2.4)	27.0 (4.3)	23.8 (2.5)	24.6 (3.9)
Mean IGF-I (SD), ng/ml	285 (61)	284 (94)	279 (87)	290 (59)	238 (42)	245 (53)
Mean P-III-NP (SD), ng/ml	3.92 (1.24)	4.25 (0.96)	4.90 (1.36)	4.36 (0.90)	4.11 (0.69)	4.42 (0.83)

3.3.1 *IGF-I concentrations*

Figure 3.1 shows the change in serum IGF-I concentrations after administration of rhIGF-I/rhIGFBP-3 or placebo in recreational athletes. IGF-I concentrations increased in both low and high dose rhIGF-I/rhIGFBP-3 administration groups in both women and men.

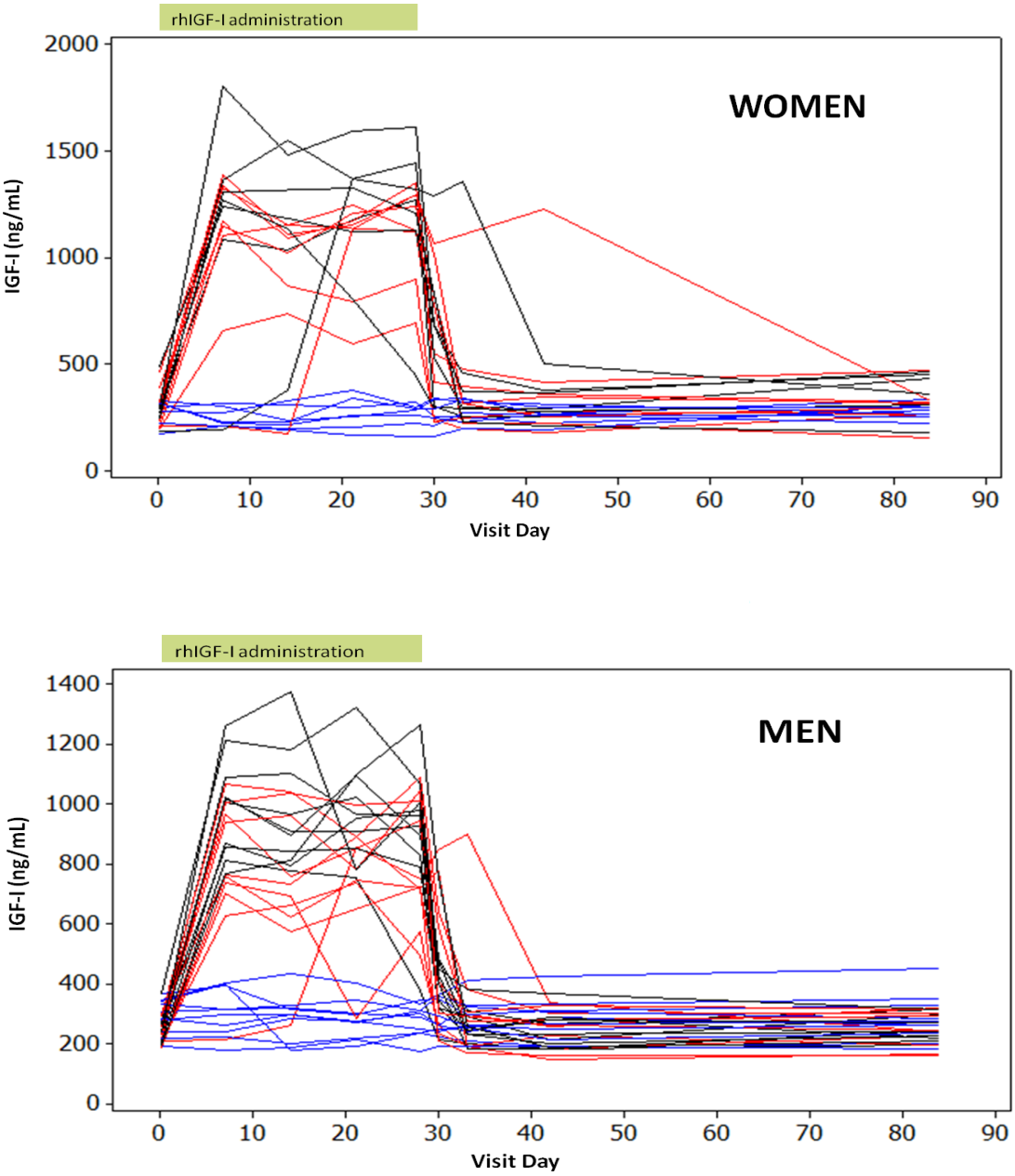

Figure 3.1. Change in serum IGF-I after rhIGF-I/rhIGFBP-3 administration for 28 days in 56 recreational athletes (top panel: women, bottom panel: men). Serum concentrations of IGF-I were measured during the treatment and washout periods. Data shown are results from individuals in the placebo group (blue lines), low dose treatment group (red lines) and high dose treatment group (black lines).

In women in the low dose group, mean (± SD) IGF-I concentration increased from 284 ± 94 ng/ml on Day 0 to a maximum of 1116 ± 214 ng/ml on Day 28, approximately a four-fold increase. In women in the high dose group, mean IGF-I concentration increased from 279 ± 87 ng/ml on Day 0 to a maximum of 1237 ± 262 ng/ml on Day 21, approximately a 4.4-fold increase. There was no significant difference in mean

maximum IGF-I concentration between low and high dose groups ($P = 0.063$). Mean IGF-I concentration remained significantly increased in both low and high dose groups for two days after the discontinuation of IGF-I (low dose IGF-I group, $P = 0.017$ on Day 30 versus Day 0; high dose IGF-I group, $P = 0.016$ on Day 30 versus Day 0). Thereafter there were no differences in mean IGF-I concentration, compared with Day 0.

In men in the low dose group, mean IGF-I concentration increased from 238 ± 42 ng/ml on Day 0 to a maximum of 807 ± 203 ng/ml on Day 28, approximately a 3.5-fold increase. In men in the high dose group, mean IGF-I concentration increased from 245 ± 53 ng/ml on Day 0 to a maximum of 992 ± 167 ng/ml on Day 7, approximately a four-fold increase. The mean maximum IGF-I concentration was significantly higher in the high dose group compared with the low dose group ($P = 0.018$). Mean IGF-I concentration remained significantly increased in both low and high dose groups for two days after the discontinuation of IGF-I (low dose IGF-I group, $P = 0.004$ on Day 30 versus Day 0; high dose IGF-I group, $P = 0.002$ on Day 30 versus Day 0). Thereafter there were no differences in mean IGF-I concentration, compared with Day 0.

There were no significant changes in IGF-I concentrations in the placebo group in either men or women throughout the study period, compared with Day 0.

Pharmacokinetics of rhIGF-I/rhIGFBP-3

The estimated pharmacokinetic parameters of the rhIGF-I/rhIGFBP-3 complex are summarised in Table 3.2. The pattern of serum IGF-I changes in three individuals (two women and one man) was markedly different from the rest of the participants. These three athletes were excluded from the statistical analyses of pharmacokinetic parameters and their results are discussed below in section 3.4.2. In women, MCR was significantly lower in the low dose group compared with the high dose group ($P = 0.001$). In men, estimated IGF-I steady-state concentration ($P = 0.026$), MCR ($P = 0.001$), and V_d ($P = 0.002$) were significantly lower in the low dose group compared with the high dose group. In both women and men, there were no significant differences in estimated IGF-I $t_{1/2}$ between the two treatment groups.

When women and men were compared (low dose and high dose treatment groups combined), estimated IGF-I steady-state concentration was significantly higher in

women than in men (P<0.001) but there were no significant differences in estimated MCR, $t_{1/2}$ or V_d.

Table 3.2. Estimated pharmacokinetic parameters of rhIGF-I/rhIGFBP-3 complex in recreational athletes. Three participants were excluded from the statistical analyses (see text). Results shown are mean (SD). MCR = metabolic clearance rate, K_{el} = elimination rate constant, $t_{1/2}$ = serum half-life, V_d = apparent volume of distribution.

Sex	Group	Steady-state concentration (mcg/L)	MCR (mL/hr/kg)	K_{el} (hrs^{-1})	$t_{1/2}$ (hrs)	V_d (mL/kg)
Women	Low dose (30 mg/day)	1079 (195)	4.0 (1.3)	0.0179 (0.0066)	44.1 (18.0)	247.0 (95.8)
	High dose (60 mg/day)	1294 (189)	6.7 (1.5)	0.0194 (0.0070)	40.5 (15.6)	394.1 (185.2)
Men	Low dose (30 mg/day)	801 (168)	4.4 (1.0)	0.0173 (0.0058)	44.2 (14.1)	266.0 (66.1)
	High dose (60 mg/day)	970 (122)	6.6 (0.9)	0.0167 (0.0034)	43.4 (10.0)	412.6 (122.8)

Figures 3.2 to 3.4 show the relationships between MCR and V_d (Fig. 3.2), between $t_{1/2}$ and V_d (Fig. 3.3) and between MCR and $t_{1/2}$ (Fig. 3.4). There was a significant positive relationship between MCR and V_d (r = 0.627, P < 0.001) and between $t_{1/2}$ and V_d (r = 0.607, P < 0.001). There was no significant relationship between MCR and $t_{1/2}$ (r = -0.189, P = 0.276).

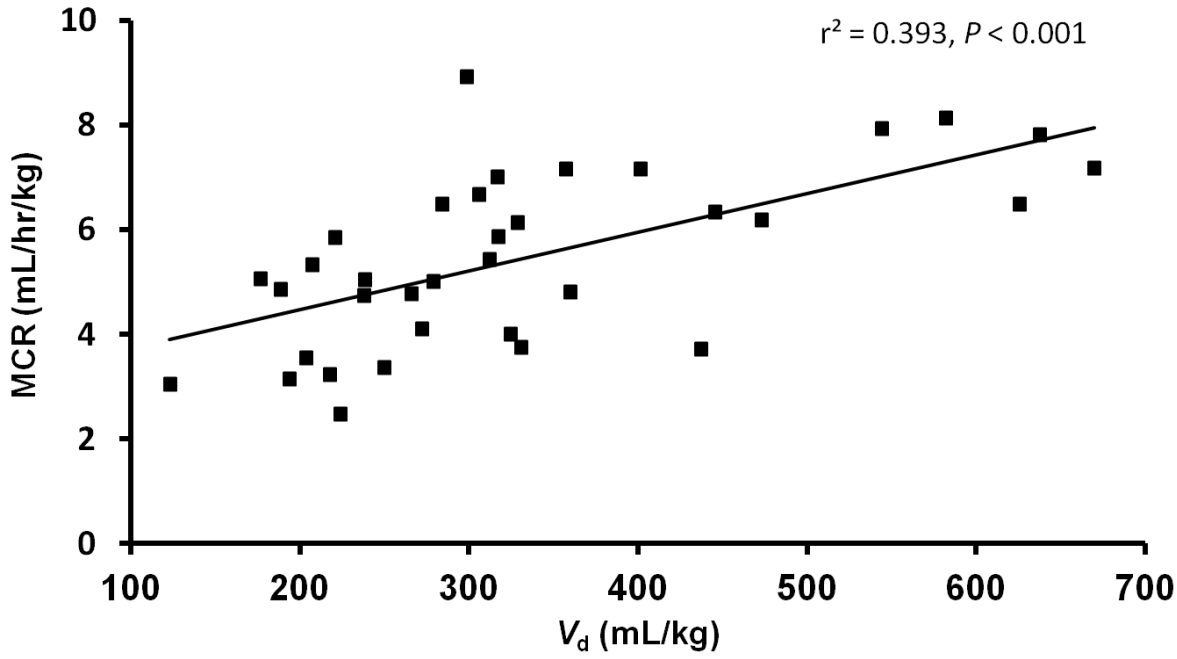

Figure 3.2. The relationship between MCR and V_d for rhIGF-I/rhIGFBP-3. MCR = metabolic clearance rate, V_d = apparent volume of distribution.

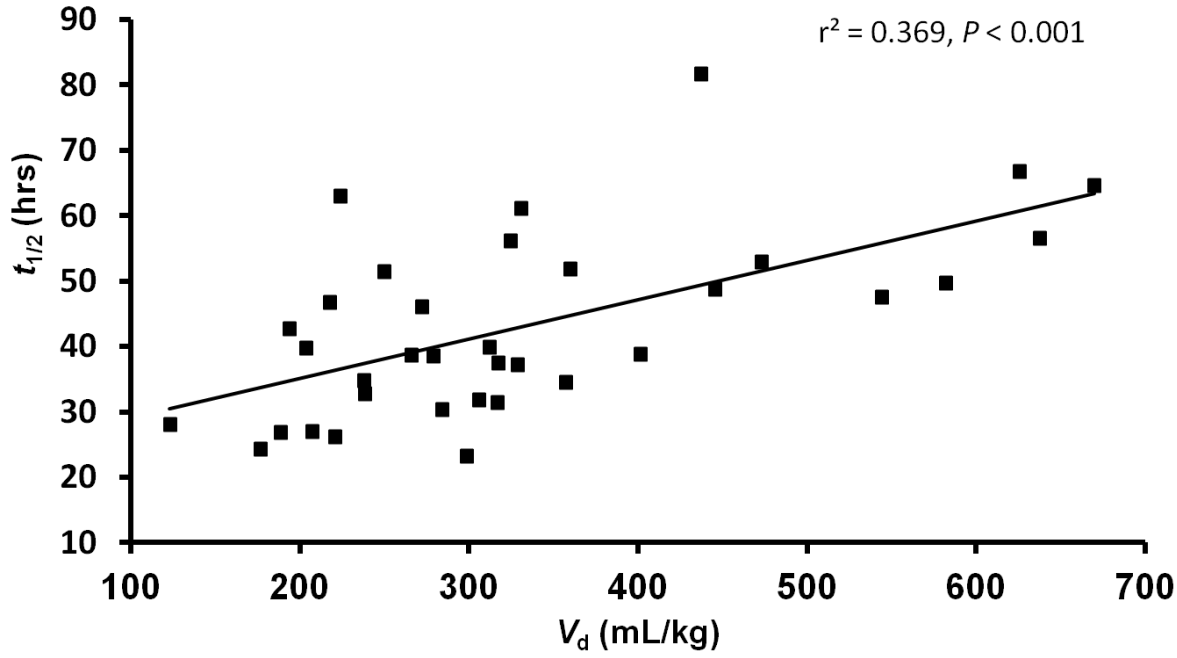

Figure 3.3. The relationship between $t_{1/2}$ and V_d for rhIGF-I/rhIGFBP-3. $t_{1/2}$ = serum half-life, V_d = apparent volume of distribution.

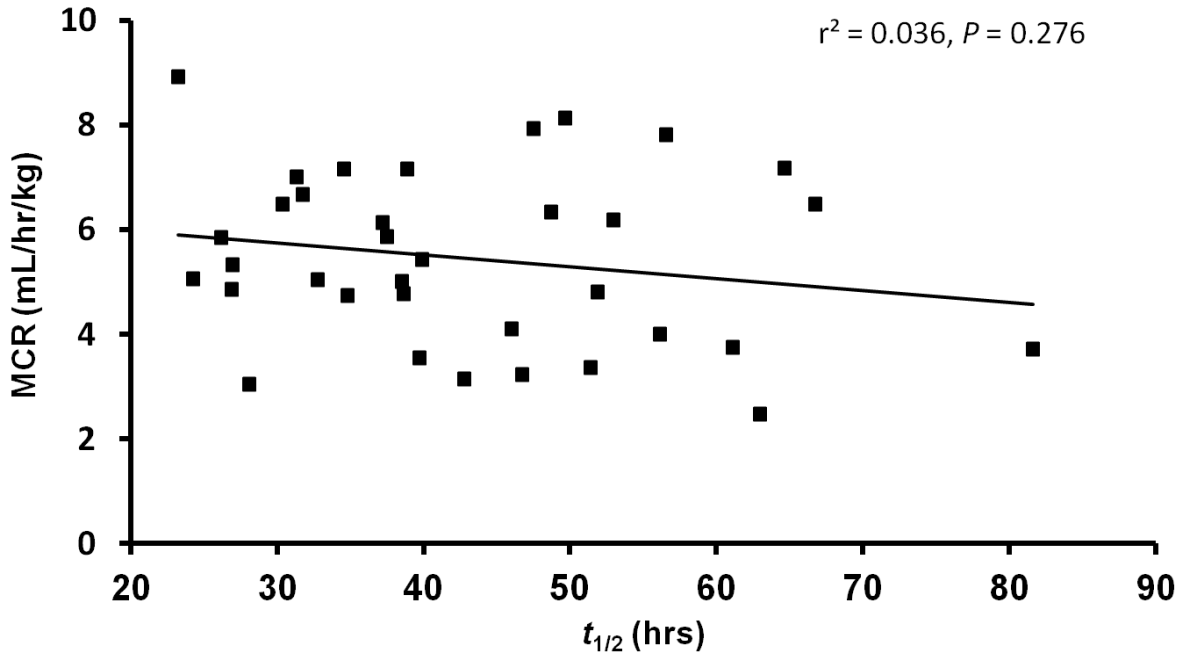

Figure 3.4. The relationship between MCR and $t_{1/2}$ for rhIGF-I/rhIGFBP-3. MCR = metabolic clearance rate, $t_{1/2}$ = serum half-life.

3.3.2 *P-III-NP concentrations*

Figure 3.5 shows the response in serum P-III-NP concentration to the administration of rhIGF-I/rhIGFBP-3 or placebo in recreational athletes. P-III-NP concentration increased in response to both low and high dose rhIGF-I/rhIGFBP-3 administration in both women and men.

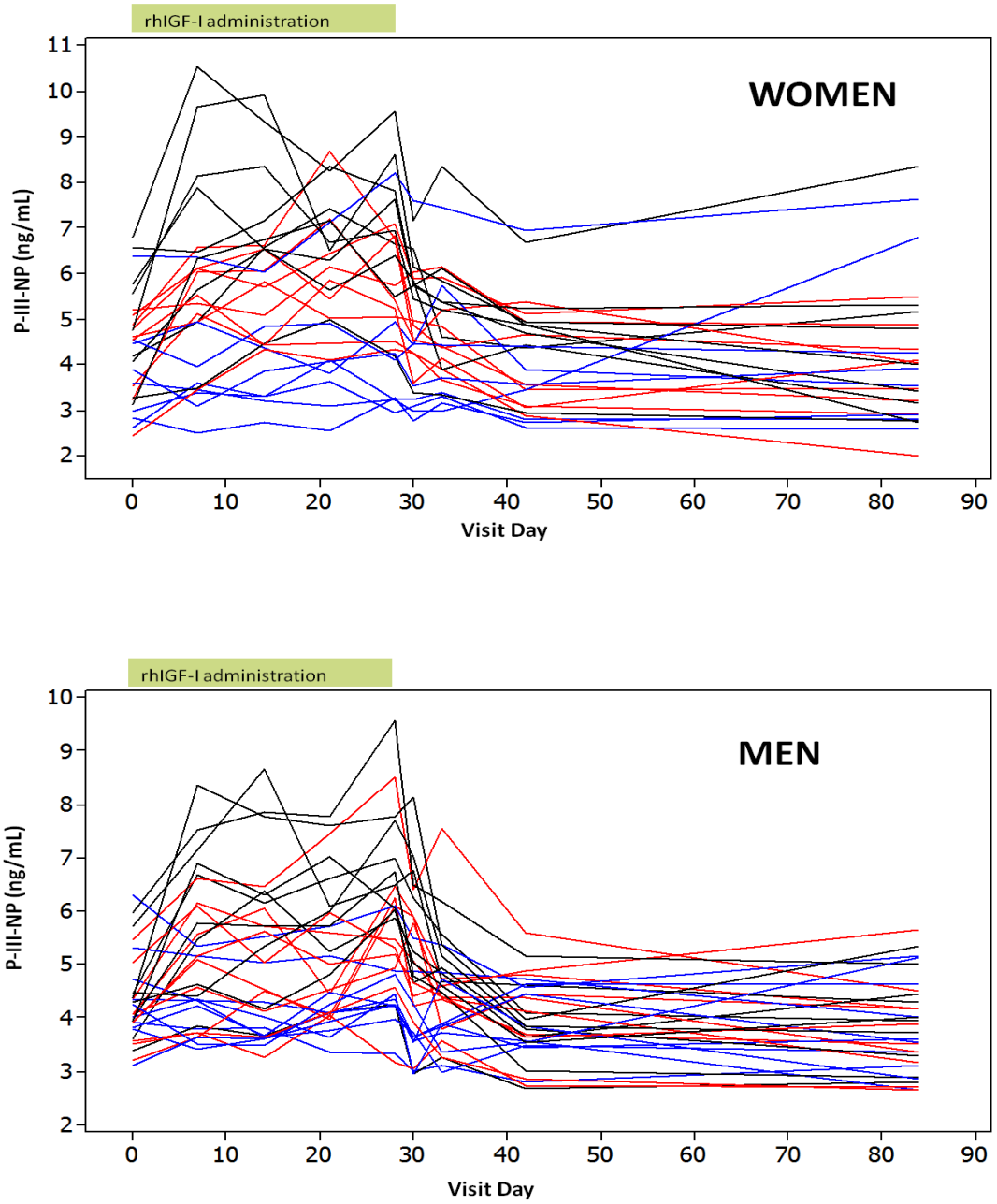

Figure 3.5. Response in serum P-III-NP to rhIGF-I/rhIGFBP-3 administration for 28 days in 56 recreational athletes (top panel: women, bottom panel: men). Serum concentrations of P-III-NP were measured during the treatment and washout periods. Data shown are results from individuals in the placebo group (blue lines), low dose treatment group (red lines) and high dose treatment group (black lines).

In women in the low dose group, mean P-III-NP concentration increased from 4.25 ± 0.96 ng/ml on Day 0 to a maximum of 6.12 ± 1.40 ng/ml on Day 21, an approximately 45% increase. In women in the high dose group, mean P-III-NP concentration increased from 4.91 ± 1.36 ng/ml on Day 0 to a maximum of 7.37 ± 1.77 ng/ml on Day 14, an approximately 50% increase. The mean maximum P-III-NP concentration was

significantly higher in the high dose group compared with the low dose group (P = 0.049). Mean P-III-NP concentration remained significantly increased in both low and high dose groups throughout the treatment period (Day 28 low dose IGF-I group, P = 0.001 versus Day 0; Day 28 high dose IGF-I group, $P < 0.0001$ versus Day 0). Thereafter there were no differences in mean P-III-NP concentration, compared with Day 0.

In men in the low dose group, mean P-III-NP concentration increased from 4.11 ± 0.69 ng/ml on Day 0 to a maximum of 5.60 ± 1.39 ng/ml on Day 28, an approximately 35% increase. In men in the high dose group, mean P-III-NP concentration increased from 4.42 ± 0.83 ng/ml on Day 0 to a maximum of 6.76 ± 1.42 ng/ml on Day 28, an approximately 53% increase. There was no significant difference in mean maximum P-III-NP concentration between low and high dose groups (P = 0.079). Mean P-III-NP concentration remained significantly increased in both low and high dose groups for two days after the discontinuation of IGF-I (Day 30 low dose IGF-I group, P = 0.013 versus Day 0; Day 30 high dose IGF-I group, P = 0.006 versus Day 0). Thereafter there were no differences in mean P-III-NP concentration, compared with Day 0.

There were no significant changes in P-III-NP concentrations in the placebo group in either men or women throughout the study period, compared with Day 0.

P-III-NP half-life

Table 3.3 shows the estimated steady-state concentration, elimination rate constant and serum half-life of P-III-NP in response to rhIGF-I/rhIGFBP-3 administration. In both women (P = 0.044) and men (P = 0.044), estimated steady-state P-III-NP concentration was significantly lower in the low dose group compared with the high dose group. In both women and men, there were no significant differences in estimated P-III-NP $t_{1/2}$ between the two treatment groups.

When women and men were compared (low dose and high dose treatment groups combined), there were no significant differences in estimated steady-state P-III-NP concentration or $t_{1/2}$.

Table 3.3. Estimated steady-state concentration, elimination rate constant and serum half-life of P-III-NP in response to rhIGF-I/rhIGFBP-3 administration in recreational athletes. Results shown are mean (SD). K_{el} = elimination rate constant, $t_{1/2}$ = serum half-life.

Sex	Group	Steady-state concentration (mcg/L)	K_{el} (hrs^{-1})	$t_{1/2}$ (hrs)
Women	Low dose (30 mg/day)	5.64 (0.95)	0.0051 (0.0022)	162.7 (78.2)
	High dose (60 mg/day)	7.03 (1.52)	0.0054 (0.0011)	134.8 (35.8)
Men	Low dose (30 mg/day)	5.14 (1.00)	0.0042 (0.0019)	194.1 (81.4)
	High dose (60 mg/day)	6.28 (1.29)	0.0058 (0.0019)	138.0 (63.7)

The estimated serum half-life of P-III-NP was significantly longer than the half-life of IGF-I. In women, estimated P-III-NP half-life was approximately 3.6 times longer than IGF-I half-life ($P < 0.001$). In men, estimated P-III-NP half-life was approximately 3.9 times longer than IGF-I half-life ($P < 0.001$).

3.3.3 *GH-2000 score*

Figure 3.6 shows the response in GH-2000 score to the administration of rhIGF-I/rhIGFBP-3 or placebo in recreational athletes. GH-2000 score increased in response to both low and high dose rhIGF-I/rhIGFBP-3 in both women and men.

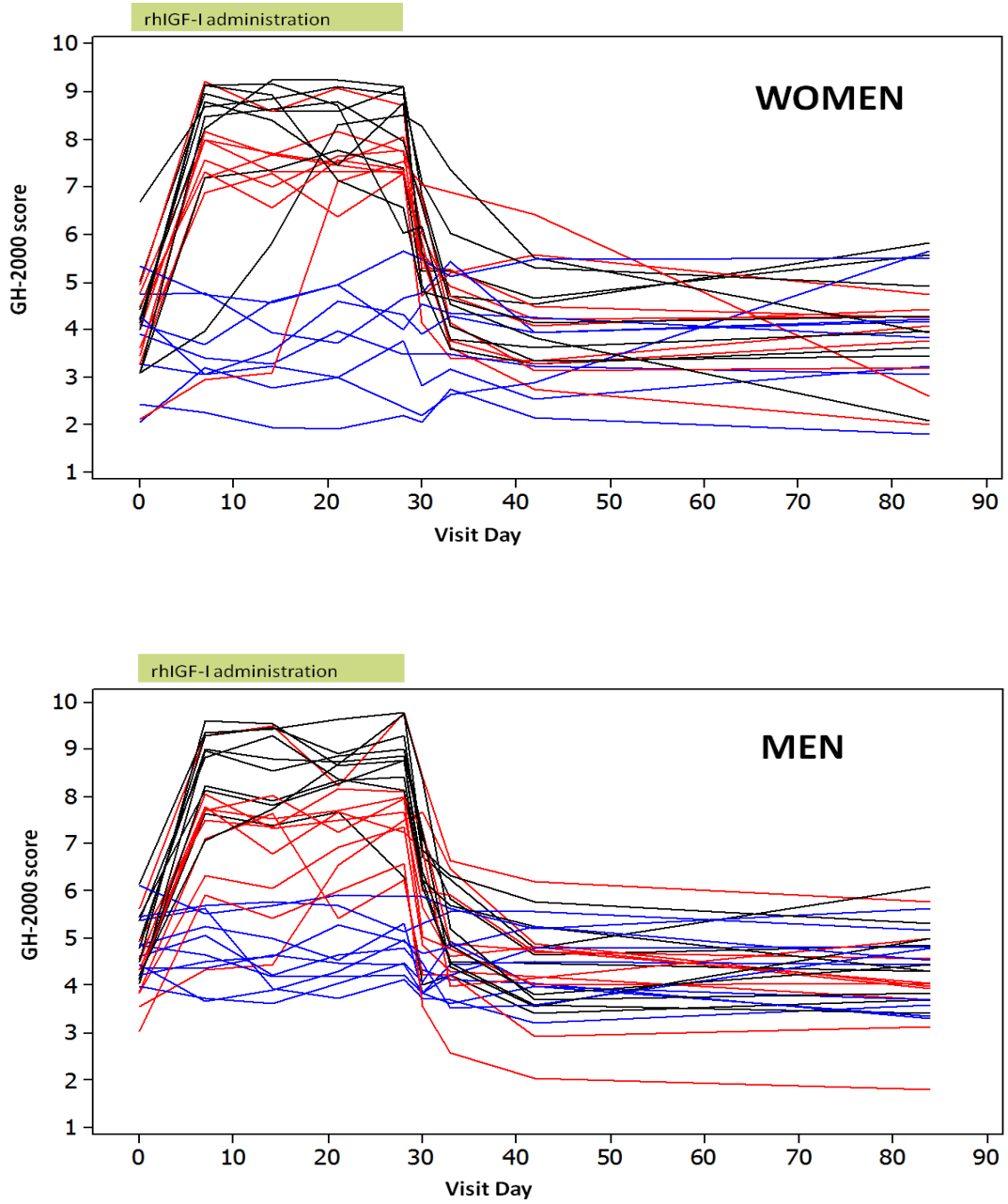

Figure 3.6. Response in GH-2000 score to rhIGF-I/rhIGFBP-3 administration for 28 days in 56 recreational athletes (top panel: women, bottom panel: men). GH-2000 scores were calculated using the published GH-2000 discriminant function formulae (Powrie et al. 2007). Data shown are results from individuals in the placebo group (blue lines), low dose treatment group (red lines) and high dose treatment group (black lines).

In women in the low dose group, mean GH-2000 score increased from 3.87 ± 0.89 on Day 0 to a maximum of 7.67 ± 0.48 on Day 28. In women in the high dose group, mean GH-2000 score increased from 4.19 ± 1.15 on Day 0 to a maximum of 8.34 ± 0.74 on Day 21. The mean maximum GH-2000 score was significantly higher in the high dose group compared with the low dose group ($P = 0.002$). Mean GH-2000 score remained

significantly increased in both low and high dose groups for two days after the discontinuation of IGF-I (low dose IGF-I group, $P = 0.025$ on Day 30 versus Day 0; high dose IGF-I group, $P = 0.014$ on Day 30 versus Day 0). Thereafter there were no differences in the mean GH-2000 score, compared with Day 0.

In men in the low dose group, mean GH-2000 score increased from 4.24 ± 0.73 on Day 0 to a maximum of 7.62 ± 0.96 on Day 28. In men in the high dose group, mean GH-2000 score increased from 4.76 ± 0.71 on Day 0 to a maximum of 8.69 ± 0.99 on Day 28. The mean maximum GH-2000 score was significantly higher in the high dose group compared with the low dose group ($P = 0.002$). Mean GH-2000 score remained significantly increased in both low and high dose groups for two days after the discontinuation of IGF-I (low dose IGF-I group, $P = 0.002$ on Day 30 versus Day 0; high dose IGF-I group, $P = 0.002$ on Day 30 versus Day 0). Thereafter there were no differences in the mean GH-2000 score, compared with Day 0.

There were no significant changes in GH-2000 score in the placebo group throughout the study period in either men or women, compared with Day 0.

Figure 3.7 shows individual values of GH-2000 scores for recreational athletes who received rhIGF-I/rhIGFBP-3, compared with the placebo group. This gives an indication of the sensitivity of the GH-2000 discriminant function formulae for detecting rhIGF-I/rhIGFBP-3 misuse. In women, the mean GH-2000 score for "clean" samples (all placebo group samples and Day 0 samples from the low and high dose IGF-I groups) was 3.78 with standard deviation 1.04. The 99.99% upper threshold level (mean + 3.72 SD) calculated using this population of female recreational athletes is therefore 7.66. In men, the mean GH-2000 score for "clean" samples was 4.56 with SD 0.72. The 99.99% upper threshold level calculated using this population of male recreational athletes is therefore 7.25. Table 3.4 shows the sensitivity of the GH-2000 score for detecting rhIGF-I/rhIGFBP-3 administration during and after the drug administration period.

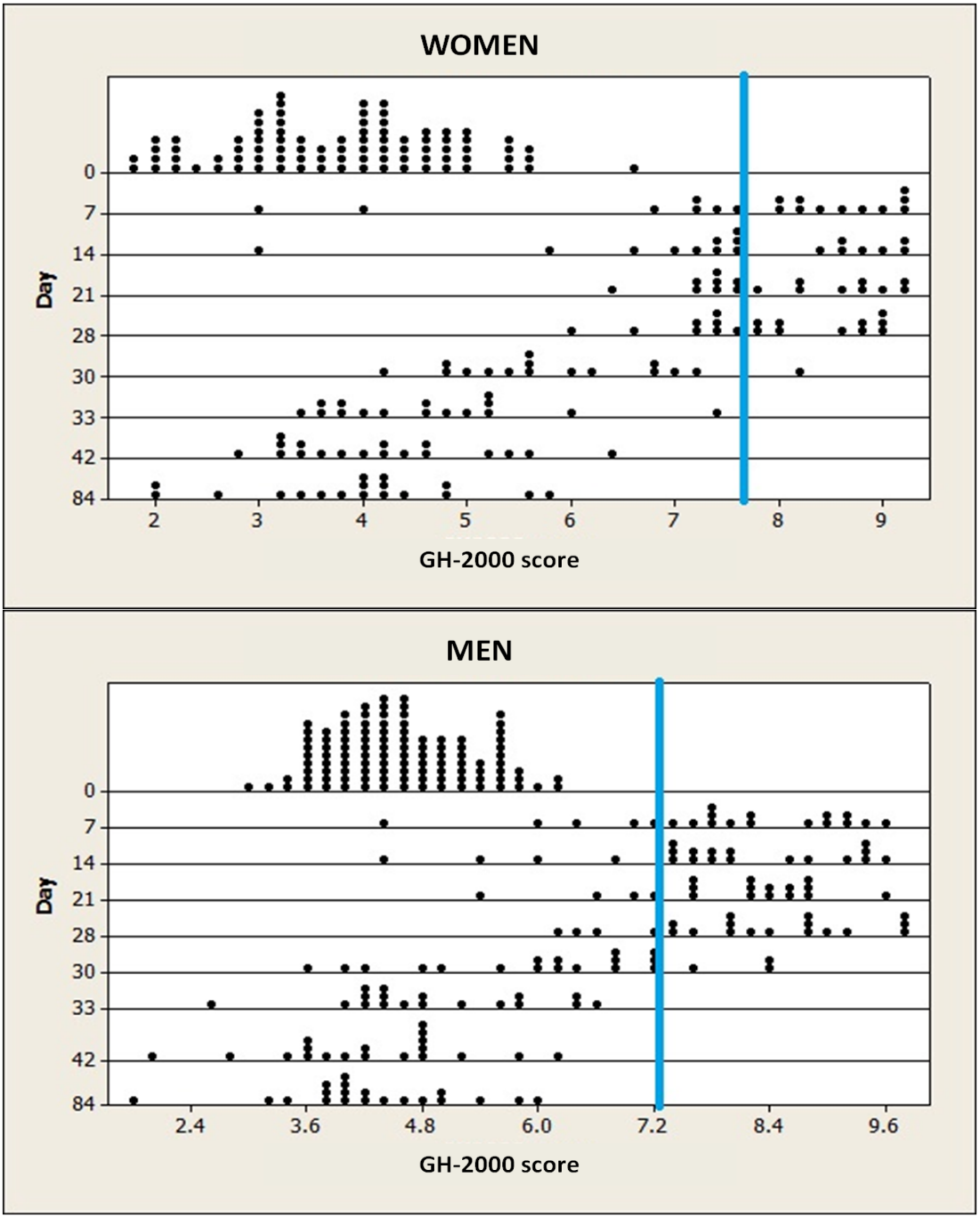

Figure 3.7. GH-2000 scores for women (upper panel) and men (lower panel) after rhIGF-I/rhIGFBP-3 administration, compared with values in the placebo group. "Day 0" includes all samples from the placebo group along with Day 0 samples from the low and high dose IGF-I groups. The vertical lines indicate a GH-2000 score of 7.66 in women and 7.25 in men, above which the chances of a false positive result would be less than 1 in 10,000.

Table 3.4. Sensitivity of GH-2000 score for detecting rhIGF-I/rhIGFBP-3 administration in recreational athletes. Table shows the number of athletes testing positive during and after the drug administration period using the 99.99% upper threshold levels shown in Figure 3.4. rhIGF-I/rhIGFBP-3 was administered between Days 0 and 28. It was not possible to collect blood samples from every participant on every study day because of scheduling difficulties.

Day	Sensitivity in women Positive tests/total in group (%)			Sensitivity in men Positive tests/total in group (%)		
	Low dose	High dose	Overall	Low dose	High dose	Overall
7	4/9 (44)	7/9 (78)	11/18 (61)	6/10 (60)	9/10 (90)	15/20 (75)
14	3/9 (33)	6/8 (75)	9/17 (53)	6/10 (60)	10/10 (100)	16/20 (80)
21	2/8 (25)	7/9 (78)	9/17 (53)	4/8 (50)	10/10 (100)	14/18 (78)
28	4/9 (44)	6/9 (67)	10/18 (56)	7/10 (70)	9/10 (90)	16/20 (80)
30	0/8 (0)	1/8 (13)	1/16 (6)	3/10 (30)	2/10 (20)	5/20 (25)
33	0/8 (0)	0/8 (0)	0/16 (0)	0/9 (0)	0/9 (0)	0/18 (0)
42	0/9 (0)	0/9 (0)	0/18 (0)	0/10 (0)	0/9 (0)	0/19 (0)
84	0/9 (0)	0/9 (0)	0/18 (0)	0/10 (0)	0/10 (0)	0/20 (0)

Figure 3.8 and Table 3.5 show the sensitivity of IGF-I results alone for detecting rhIGF-I/rhIGFBP-3 administration. In women, the calculated 99.99% upper threshold level using log-transformed IGF-I concentrations is 6.40. In men, the calculated 99.99% upper threshold level using log-transformed IGF-I concentrations is 6.45.

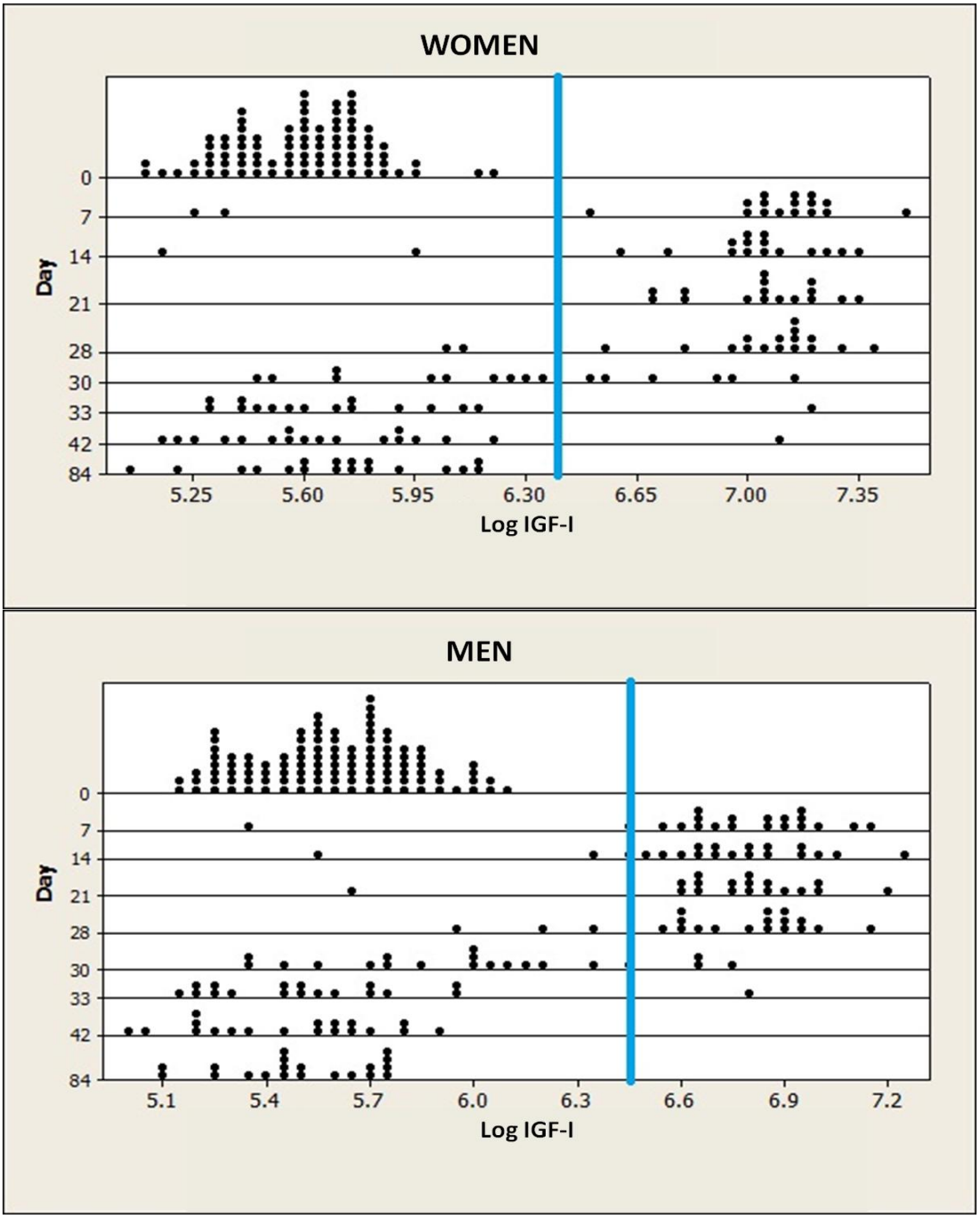

Figure 3.8. Log-transformed IGF-I results for women (upper panel) and men (lower panel) after rhIGF-I/rhIGFBP-3 administration, compared with values in the placebo group. Day 0 includes all samples from the placebo group along with Day 0 samples from the low dose and high dose IGF-I groups. The vertical lines indicate the 99.99% upper thresholds (6.40 in women, 6.45 in men), above which the chance of a false positive result would be less than 1 in 10,000.

Table 3.5. Sensitivity of IGF-I alone for detecting rhIGF-I/rhIGFBP-3 administration in recreational athletes. Table shows the number of athletes testing positive during and after the administration period, using the 99.99% upper threshold levels shown in Figure 3.5. rhIGF-I/rhIGFBP-3 was administered between Days 0 and 28.

Day	Sensitivity in women Positive tests/total in group (%)			Sensitivity in men Positive tests/total in group (%)		
	Low dose	High dose	Overall	Low dose	High dose	Overall
7	8/9 (89)	8/9 (89)	16/18 (89)	8/10 (80)	10/10 (100)	18/20 (90)
14	8/9 (89)	7/8 (88)	15/17 (88)	7/10 (70)	10/10 (100)	17/20 (85)
21	7/8 (88)	9/9 (100)	16/17 (94)	7/8 (88)	10/10 (100)	17/18 (94)
28	9/9 (100)	7/9 (78)	16/18 (89)	8/10 (80)	9/10 (90)	17/20 (85)
30	3/8 (38)	3/8 (38)	6/16 (38)	3/10 (30)	1/10 (10)	4/20 (20)
33	0/8 (0)	1/8 (13)	1/16 (6)	1/9 (11)	0/9 (0)	1/18 (6)
42	1/9 (11)	0/9 (0)	1/18 (6)	0/10 (0)	0/9 (0)	0/19 (0)
84	0/9 (0)	0/9 (0)	0/18 (0)	0/10 (0)	0/10 (0)	0/20 (0)

Stepwise discriminant analysis was performed using data from the high dose IGF-I groups. In both women and men, IGF-I was a useful discriminant marker on Days 7, 14, 21, 28 and 30. In women, P-III-NP was not a useful discriminant marker in addition to IGF-I on any of the days studied. In men, P-III-NP was only useful in addition to IGF-I on Day 30 but was not useful as an additional marker on any other day.

3.3.4 *Comparison between rhIGF-I/rhIGFBP-3 administration and GH-2000 rhGH administration studies*

Figures 3.9 and 3.10 and Tables 3.6 and 3.7 compare the effects of rhIGF-I/rhIGFBP-3 administration in the current study and rhGH administration in the GH-2000 study, on serum IGF-I and P-III-NP concentrations.

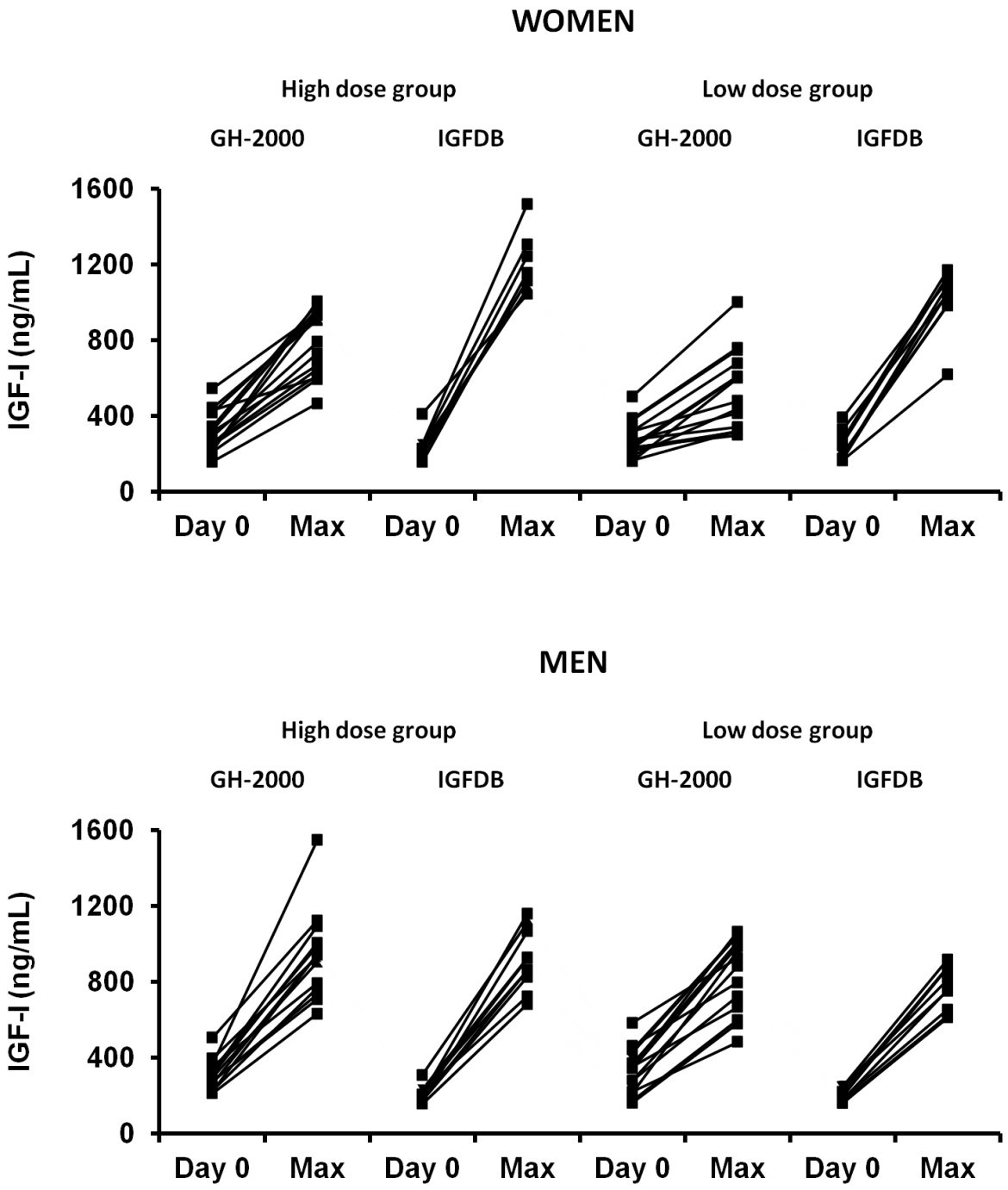

Figure 3.9. Comparison between the effects of rhIGF-I/rhIGFBP-3 administration and rhGH administration on serum IGF-I concentrations in female recreational athletes (top panel) and male recreational athletes (bottom panel). IGF-I results from both studies were aligned with the Nichols IGF-I scale. GH-2000 = GH-2000 rhGH administration study, IGFDB = rhIGF-I/rhIGFBP-3 double-blind administration study, Max = maximum IGF-I concentration.

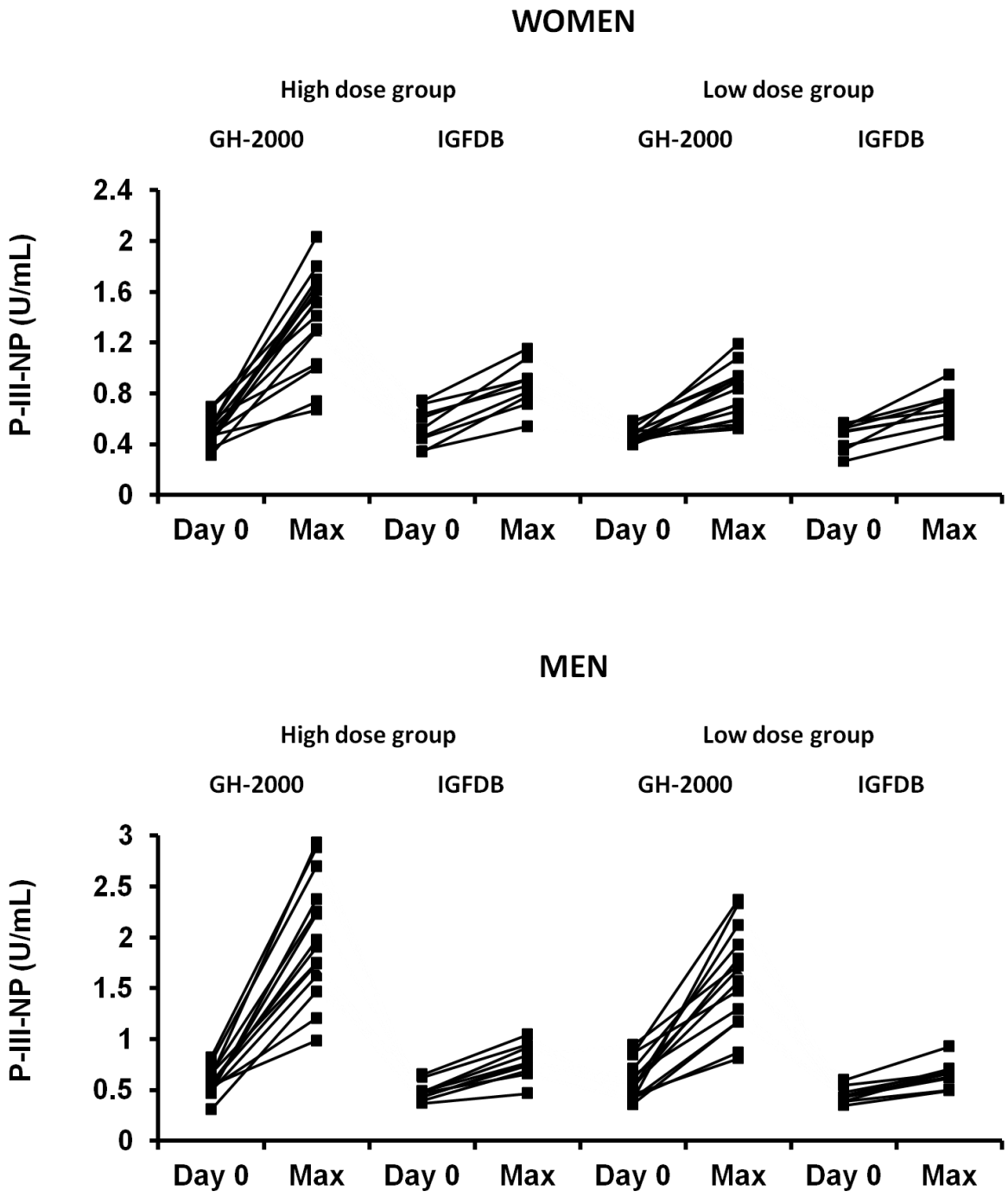

Figure 3.10. Comparison between the effects of rhIGF-I/rhIGFBP-3 administration and rhGH administration on serum P-III-NP concentrations in female recreational athletes (top panel) and male recreational athletes (bottom panel). P-III-NP results from both studies were aligned with the Cisbio P-III-NP scale. GH-2000 = GH-2000 rhGH administration study, IGFDB = rhIGF-I/rhIGFBP-3 double-blind administration study, Max = maximum P-III-NP concentration.

Table 3.6. Comparison between the effects of rhIGF-I/rhIGFBP-3 administration and rhGH administration on serum IGF-I concentrations in female and male recreational athletes. IGF-I results from both studies were aligned with the Nichols IGF-I scale. GH-2000 = GH-2000 rhGH administration study, IGFDB = rhIGF-I/rhIGFBP-3 double-blind administration study.

WOMEN	High dose group		Low dose group	
	GH-2000 (n=15)	IGFDB (n=9)	GH-2000 (n=15)	IGFDB (n=9)
Mean Day 0 IGF-I (SD), ng/mL	311 (108)	235 (73)	277 (93)	239 (79)
Mean maximum IGF-I (SD), ng/mL	787 (179)	1183 (153)	529 (209)	1022 (164)
Mean maximum percentage increase (SD), %	172 (86)	437 (141)	96 (65)	354 (118)
MEN	**High dose group**		**Low dose group**	
	GH-2000 (n=14)	IGFDB (n=10)	GH-2000 (n=15)	IGFDB (n=10)
Mean Day 0 IGF-I (SD), ng/mL	309 (79)	207 (45)	335 (116)	200 (36)
Mean maximum IGF-I (SD), ng/mL	937 (228)	914 (159)	852 (195)	750 (118)
Mean maximum percentage increase (SD), %	211 (68)	354 (98)	172 (76)	278 (42)

Table 3.7. Comparison between the effects of rhIGF-I/rhIGFBP-3 administration and rhGH administration on serum P-III-NP concentrations in female and male recreational athletes. P-III-NP results from both studies were aligned with the Cisbio P-III-NP scale. GH-2000 = GH-2000 rhGH administration study, IGFDB = rhIGF-I/rhIGFBP-3 double-blind administration study.

WOMEN	High dose group		Low dose group	
	GH-2000 (n=15)	IGFDB (n=9)	GH-2000 (n=15)	IGFDB (n=9)
Mean Day 0 P-III-NP (SD), ng/mL	0.50 (0.11)	0.53 (0.15)	0.47 (0.06)	0.46 (0.10)
Mean maximum P-III-NP (SD), ng/mL	1.39 (0.39)	0.86 (0.18)	0.78 (0.21)	0.69 (0.14)
Mean maximum percentage increase (SD), %	185 (89)	66 (34)	66 (42)	54 (34)
MEN	**High dose group**		**Low dose group**	
	GH-2000 (n=14)	IGFDB (n=10)	GH-2000 (n=15)	IGFDB (n=10)
Mean Day 0 P-III-NP (SD), ng/mL	0.57 (0.13)	0.48 (0.09)	0.59 (0.19)	0.45 (0.08)
Mean maximum P-III-NP (SD), ng/mL	2.00 (0.60)	0.78 (0.16)	1.56 (0.49)	0.65 (0.12)
Mean maximum percentage increase (SD), %	255 (95)	62 (19)	186 (110)	46 (15)

Baseline IGF-I concentrations in men in the GH-2000 study were significantly higher than baseline IGF-I concentrations in men in the current rhIGF-I/rhIGFBP-3 double-blind administration ("IGFDB") study in both high dose ($P < 0.001$) and low dose ($P < 0.001$) groups. There were no significant differences in baseline IGF-I concentrations in women in either high dose or low dose groups, when the two studies were compared.

In women, the percentage increase in serum IGF-I was significantly greater in the IGFDB study compared with the GH-2000 study in both high dose ($P < 0.001$) and low dose ($P < 0.001$) groups. Similarly in men, the percentage increase in serum IGF-I was significantly greater in the IGFDB study compared with the GH-2000 study in both high dose ($P < 0.001$) and low dose ($P = 0.001$) groups.

In the GH-2000 study, the percentage increase in serum IGF-I in men was significantly greater than the increase in women in the low dose group ($P = 0.006$) but not in the high dose group ($P = 0.106$). In the IGFDB study, there were no significant differences in percentage increase in serum IGF-I between women and men in either high dose ($P = 0.286$) or low dose ($P = 0.151$) groups and this result was not altered by correcting the participant's dose relative to their weight.

Baseline P-III-NP concentrations in men in the GH-2000 study were significantly higher than baseline P-III-NP concentrations in men in the IGFDB study in the low dose ($P = 0.022$) but not high dose ($P = 0.071$) group. There were no significant differences in baseline P-III-NP concentrations in women in either high dose or low dose groups, when the two studies were compared.

In women, the percentage increase in serum P-III-NP was significantly greater in the GH-2000 study compared with the IGFDB study in the high dose ($P < 0.001$) but not the low dose ($P = 0.668$) group. In men, the percentage increase in serum P-III-NP was significantly greater in the GH-2000 study compared with the IGFDB study in both high dose ($P < 0.001$) and low dose ($P < 0.001$) groups.

In the GH-2000 study, the percentage increase in serum P-III-NP in men was significantly greater than the increase in women in the low dose group ($P < 0.001$) but not in the high dose group ($P = 0.058$). In the IGFDB study, there were no significant differences in percentage increase in serum P-III-NP between women and men in either high dose ($P = 0.972$) or low dose ($P = 0.890$) groups and this result was not altered by correcting the participant's dose relative to their weight.

3.4 Discussion

This randomised, double-blind, placebo-controlled trial has shown that serum IGF-I, serum P-III-NP and GH-2000 score increase in response to rhIGF-I/rhIGFBP-3 administration in recreational athletes. IGF-I showed a much larger incremental change after rhIGF-I/rhIGFBP-3 administration, compared with P-III-NP. Both IGF-I and P-III-NP increased during the first week of the administration period and remained elevated throughout the administration period. The use of the GH-2000 score will detect a significant number of athletes receiving rhIGF-I/rhIGFBP-3 but the use of serum IGF-I alone provides a greater sensitivity to detect rhIGF-I/rhIGFBP-3 misuse.

3.4.1 *IGF-I concentrations*

Previous studies have shown that serum IGF-I concentrations increase in response to rhIGF-I administration in adults with GH deficiency and IGF-I deficiency (Mauras et al. 2000; Mauras et al. 2000), as would be expected. Furthermore, studies have shown that rhIGF-I administration to people with Type 1 diabetes mellitus results in increased IGF-I concentrations (Cheetham et al. 1995; Acerini et al. 1997). Studies involving administration of rhIGF-I/rhIGFBP-3 complex have also shown increases in circulating IGF-I concentrations (Clemmons et al. 2005; Camacho-Hubner et al. 2006).

In the current study, IGF-I concentrations showed a rapid approximately four-fold increase in the majority of participants randomised to rhIGF-I/rhIGFBP-3 administration and decreased sharply after treatment withdrawal. Three participants, however, demonstrated a different response to rhIGF-I/rhIGFBP-3 administration. IGF-I remained raised two weeks after withdrawal of treatment in one woman in the low dose group and remained raised five days after withdrawal of treatment in a further two participants (one woman in the high dose group and one man in the low dose group). In all three of these participants, there was a delayed rise in IGF-I of between 14 and 21 days after the start of the administration period (the maximum increase in other participants occurred in the first sample taken during the administration period on Day 7).

One explanation for the delayed rise would be that the participants were not compliant with the study protocol though all three participants declared that they

started treatment on the correct day and did not miss any drug doses. Furthermore it is not possible that these participants continued to administer rhIGF-I/rhIGFBP-3 after Day 28 because all drug vials were collected at the end of the administration period. A more likely explanation is that these findings reflect the pharmacokinetics of the rhIGF-I/rhIGFBP-3 complex as discussed below in section 3.4.2.

3.4.2 *rhIGF-I/rhIGFBP-3 pharmacokinetics*

The pharmacokinetics of rhIGF-I alone and rhIGF-I/rhIGFBP-3 have been investigated in previous studies. When rhIGF-I was administered to a patient with partial deletion of the IGF-I gene (six daily subcutaneous injections at 40 μg/kg/day and 80 μg/kg/day), the half-life of rhIGF-I was approximately 15 hrs (Camacho-Hubner et al. 1999). When rhIGF-I (40 μg/kg, single subcutaneous dose) was administered to young adults with GHIS and to healthy volunteers, the half-lives were 5.7 ± 2.4 hrs and 17 ± 8.8 hrs respectively. The metabolic clearance rate in participants with GHIS was 36 ml/hr/kg, compared with 12 ml/hr/kg in healthy volunteers and the rapid clearance in adults with GHIS was attributed to the decreased production of IGFBP-3 and ALS (Grahnen et al. 1993). In a more recent study, rhIGF-I/rhIGFBP-3 was administered to four children with GHIS as a single subcutaneous injection at 0.5 and 1.0 mg/kg. The IGF-I half-life was estimated at 21 ± 4 hrs and the authors proposed that rhIGF-I/rhIGFBP-3 can bind to endogenous ALS, extending the half-life of IGF-I in these patients, compared with administering rhIGF-I alone (Camacho-Hubner et al. 2006). This longer half-life could reduce the frequency of injections required during the treatment of GHIS. Treatment with this complex could also reduce the frequency of adverse effects, such as hypoglycaemia, associated with sudden rises in free IGF-I concentrations after rhIGF-I injection.

In the current study of 28 days' rhIGF-I/rhIGFBP-3 administration, the mean estimated IGF-I half-life (approximately 40 to 44 hrs) was longer than in the previous studies described above. This would be expected after multiple subcutaneous doses because IGF binding proteins act as a reservoir from which IGF-I is slowly released and then metabolised and excreted from the body. In the three participants who demonstrated an unusual pattern of serum IGF-I changes, the estimated IGF-I half-lives were between 150 and 530 hours and these values were considerably higher than in the other participants. The calculated apparent volumes of distribution in these three participants were between 791 and 2824 mL/kg and these values were also higher than in the rest of the study population (mean values between 247 and

413 mL/kg). It appears that in these three participants, the rhIGF-I from the rhIGF-I/rhIGFBP-3 complex accumulated in tissues with delayed absorption into the circulation and subsequent excretion. In the remaining 35 participants who were administering rhIGF-I/rhIGFBP-3, the rhIGF-I exhibited a higher degree of plasma protein-binding. This would explain the difference in V_d though it is not clear why this difference between individuals in plasma/tissue distribution should occur.

The impact of apparent volume of distribution on the relationship between the calculated metabolic clearance rate and the estimated half-life is interesting. It might be expected that half-life would decrease as metabolic clearance rate increases but in this study there was no significant correlation between half-life and metabolic clearance rate. There was, however, a direct correlation between IGF-I metabolic clearance rate and apparent volume of distribution and between IGF-I half-life and apparent volume of distribution, in keeping with earlier studies with insulin and proinsulin (Sonksen et al. 1973). Thus the delay in rise of IGF-I and the longer half-life in the three atypical responders reflects the slower filling and clearance from a larger volume of distribution in these participants. It is possible that as the apparent volume of distribution increases (suggesting a higher proportion of extravascular IGF-I distribution), degradation of IGF-I outside of the circulation also increases and this accounts for the increase in metabolic clearance rate, as has been suggested for insulin and growth hormone (Owens et al. 1973; Sonksen et al. 1973). It is also interesting that although the absorption rate in the high dose group was double that in the low dose group, the mean steady state concentration increased by only approximately 20% in both women and men. This finding reflects the increased metabolic clearance rate of IGF-I with increasing IGF-I concentrations. As IGF-I concentrations increase and IGF binding proteins are saturated, free IGF-I diffuses more easily into the extravascular space where it is degraded after binding with IGF receptors within tissues, and this limits the rise in measured serum IGF-I concentrations.

3.4.3 *P-III-NP concentrations*

No previous studies have investigated the effects of rhIGF-I/rhIGFBP-3 administration on serum P-III-NP. Type III collagen is found in connective tissue throughout the body and is also present in bone post-fracture when it is found in the callus (Kurdy et al. 1998). It is known that the GH-IGF axis affects soft tissue collagen turnover and that GH deficiency in childhood reduces collagen production

and is associated with a significant reduction in serum P-III-NP (Sartorio et al. 1993). Furthermore in previous studies by the GH-2000, GH-2004 and other research groups, P-III-NP was the most sensitive collagen marker of rhGH administration (Longobardi et al. 2000; Nelson et al. 2008; Holt et al. 2010) and we hypothesised that rhIGF-I/rhIGFBP-3 administration would have similar effects on serum P-III-NP. In this study, we showed that serum P-III-NP increased in athletes treated with rhIGF-I/rhIGFBP-3 but the relative incremental response (approximately 40-50%) was less than the increase in serum IGF-I concentrations.

The pattern of P-III-NP response to rhIGF-I/rhIGFBP-3 administration was similar in most participants with an increase in P-III-NP by Day 7, followed by a plateau during the administration period and subsequent decline during the washout period. The estimated elimination half-life of P-III-NP was nearly four times longer than the estimated half-life of IGF-I and this explains why P-III-NP remained significantly elevated for up to two weeks after rhGH treatment was withdrawn in the GH-2000 and GH-2004 studies (Powrie et al. 2007; Holt et al. 2010). This long half-life contributes to the "window of detection" of the marker method: the ability to detect athletes that have stopped taking rhGH some time before the test is performed.

Three participants (all women, two in the placebo group and one in the high dose group) had relatively high P-III-NP results on Day 84, compared with the other participants. The cause of these high P-III-NP values is unclear; none of the participants declared an injury during the study period that might explain these high results. The high P-III-NP values did not result in high GH-2000 scores.

3.4.4 *GH-2000 score*

The GH-2000 score rose significantly in response to rhIGF-I/rhIGFBP-3 administration in both women and men, and athletes who have administered rhIGF-I/rhIGFBP-3 may test "positive" using the GH-2000 marker method. The GH-2000 score increased rapidly during the first week of administration, remained elevated throughout the administration period and then declined. The pattern of change in GH-2000 score (Fig. 3.6) was very similar to that of serum IGF-I changes (Fig. 3.1). Discriminant analysis showed that the addition of P-III-NP to IGF-I did not help to identify those athletes taking rhIGF-I/rhIGFBP-3. In fact, the sensitivity of using IGF-I alone for detecting rhIGF-I/rhIGFBP-3 administration was higher than that of the GH-2000 score. The higher sensitivity of IGF-I alone suggests that the addition of P-III-NP actually interfered with the identification of rhIGF-I/rhIGFBP-3

administration. It is possible, however, that P-III-NP will prove to be a useful marker of IGF-I misuse in more complex combinations including other GH-IGF axis and bone/collagen markers; the possibility of using other serum markers to detect IGF-I misuse is discussed further in Chapter 9.

3.4.5 *Comparison between rhIGF-I/rhIGFBP-3 administration and GH-2000 rhGH administration studies*

The marker results from the current study and the previous GH-2000 study were compared by aligning current assay results with the Nichols IGF-I and Cisbio P-III-NP assay scales used in the GH-2000 study. Caution is required when assay results from different assays are aligned because the conversion factors used were derived from one study, as discussed further in Chapter 5. It was not possible to use the same assays in both studies, however, because the Nichols IGF-I assay is no longer available. It is also important to note that the low dose and high dose treatment groups in the current rhIGF-I/rhIGFBP-3 administration study were compared with the equivalent groups in the GH-2000 study. We do not know if these rhIGF-I/rhIGFBP-3 doses (30 mg/day and 60 mg/day) and rhGH doses (0.1 IU/kg/day and 0.2 IU/kg/day) were equivalent and therefore I have only been able to draw conclusions based on the doses used in these studies. A further limitation in this comparison is that although a population of recreational athletes was recruited for each study, there were some differences in the baseline characteristics of these athletes including baseline marker concentrations in men.

At the doses administered in these two studies, the relative increase in P-III-NP in response to rhIGF-I/rhIGFBP-3 administration was considerably less than the P-III-NP response observed in the GH-2000 study in all treatment groups except in women in the low dose group. This is in contrast with the relative increase in IGF-I concentration, which was greater in the current study compared with the GH-2000 study, at each of the selected doses of rhIGF-I/rhIGBP-3 or rhGH. This finding suggests that GH has effects on collagen metabolism both directly and indirectly (through the production of endocrine IGF-I) and that the direct effect of GH is greater than its indirect effect via IGF-I. This provides further evidence that the original somatomedin hypothesis is incorrect and supports the alternative hypothesis that circulating IGF-I is actually a marker of GH action on the liver rather than an essential intermediary in its action (Sonksen 2001).

It is also notable that the relative increase in both IGF-I and P-III-NP observed in the GH-2000 study was greater in men than in women in the low dose group but not in the high dose group. No such gender differences were observed in the current rhIGF-I/rhIGFBP-3 administration study. This illustrates the relative GH resistance of women when compared with men, as has also been shown in the treatment of adults with GH deficiency (Burman et al. 1997). The GH-2000 group suggested that although the marker response might be attenuated in women as a result of this resistance, this would be balanced by the need for higher GH doses in women to obtain significant performance-enhancing effects (Powrie et al. 2007).

3.4.6 *Limitations*

It is difficult to estimate the sensitivity of an anti-doping test in this type of study because doping regimens of rhIGF-I/rhIGFBP-3 administration are unknown and it is possible that athletes are administering doses that are higher than those used in this study. The doses used in this study were proposed by the drug manufacturer (Insmed Incorporated) based on the doses used in clinical practice and by using safety data from their own clinical trials. Another limitation is that athletes may be misusing rhIGF-I alone rather than the rhIGF-I/rhIGFBP-3 complex used in this study, or misusing rhGH in combination with rhIGF-I, and marker responses may differ in those scenarios.

A further limitation is that the athletes recruited were recreational athletes rather than elite athletes; it would not be possible for elite athletes to take part in this type of study involving administration of a prohibited substance. The recreational athletes who participated in the current study had lower GH-2000 scores than the population of elite athletes who were used to determine the current WADA decision limits for the GH-2000 method (Chapter 7 and Erotokritou-Mulligan et al. 2012). Lower thresholds were therefore used to assess the sensitivity of the test in this population of recreational athletes.

In conclusion, the increase in serum IGF-I after rhIGF-I/rhIGFBP-3 administration was greater than the increase in serum P-III-NP in this study of 56 recreational athletes. Serum P-III-NP rises in response to rhIGF-I/rhIGFBP-3 administration but to a smaller extent than in response to rhGH administration. Although

rhIGF-I/rhIGFBP-3 administration can be detected using the GH-2000 score, a test based on IGF-I alone provides better sensitivity.

CHAPTER 4: THE EFFECTS OF IGF-I ON LIPID METABOLISM, CARBOHYDRATE METABOLISM, BODY COMPOSITION AND PHYSICAL FITNESS

4.1 Introduction

For many years there was no clear evidence that growth hormone improves athletic performance (Liu et al. 2008) but recent studies have been able to demonstrate performance-enhancing effects of growth hormone in athletes, particularly when combined with other anabolic agents (Graham et al. 2007; Meinhardt et al. 2010). There is no evidence to suggest that administering IGF-I to healthy athletes will improve physical performance or alter body composition. Furthermore there are limited data on the effects of IGF-I on lipid and carbohydrate metabolism in healthy volunteers, though it has been shown that IGF-I treatment enhances insulin sensitivity and improves glycaemic control in people with diabetes (Simpson et al. 1998). The aim of this study was to investigate the effects of rhIGF-I/rhIGFBP-3 administration over 28 days on lipid and carbohydrate metabolism and on body composition and physical fitness in recreational athletes.

4.2 Methods

This study formed part of the randomised, double-blind, placebo-controlled trial of rhIGF-I/rhIGFBP-3 administration described in Chapter 3. 30 male and 26 female recreational athletes were recruited and randomised to three treatment groups (low dose (30 mg/day) rhIGF-I/rhIGFBP-3 complex, high dose (60 mg/day) rhIGF-I/rhIGFBP-3 complex or placebo) as described in sections 2.1.1.1 and 2.1.1.2.

The following assessments were performed before treatment (baseline) and at the end of treatment (Day 28) and further details on these assessments are provided in sections 2.1.1.5 to 2.1.1.11.

- Anthropometric measurements and body composition (sections 2.1.1.5 and 2.1.1.6)
- Physical fitness assessed by cardiopulmonary exercise testing (section 2.1.1.7)
- Fasting lipids and HbA_{1c} (section 2.1.1.8)

- Oral glucose tolerance test and indirect calorimetry (sections 2.1.1.9 and 2.1.1.10)

Growth hormone assays were performed on all serum samples collected for analysis of IGF-I and P-III-NP, as described in section 2.1.1.11. GH results that were below the Limit of Quantitation (LOQ) of the assay must lie in the range from 0 to 0.05 mcg/L but were formally viewed as censored. For statistical analyses, I followed Bidlingmaier *et al.* (Bidlingmaier et al. 2009) and assigned an arbitrary value of 0.05 mcg/L. I repeated the statistical analyses using an arbitrary GH value of 0.025 mcg/L to ensure that the choice of value did not affect the results.

We based sample size calculations on predicted responses in serum biomarkers to exogenous IGF-I administration (Chapter 3). Power calculations were not performed for substrate utilisation, body composition or physical fitness outcomes because no previous studies have examined these variables to provide data on which to base a power calculation. Area under the curve (AUC) calculations for glucose, insulin and NEFA were performed using the trapezoidal method. Body composition and physical fitness data from participants in low and high dose treatment groups were analysed separately and combined. Within-group changes after treatment were assessed using paired *t*-tests. The relative effects of rhIGF-I/rhIGFBP-3 administration on male and female treatment groups were compared using unpaired *t*-tests and analysis of covariance (ANCOVA). Analyses were performed on log-transformed data and $P < 0.05$ was considered statistically significant.

My role in this part of the study was as follows: I recruited all participants to the study through the methods described in section 2.1.1.1. I supervised the Baseline and Day 28 assessments; all data during these assessments were collected by myself and by University of Southampton medical students under my supervision. I performed all GH assays at the Oxford University Hospitals NHS Trust Clinical Biochemistry Department. I maintained the project results database and performed statistical analyses of the data with the exception of the AUC analyses, which were performed by Dr Erotokritou-Mulligan, GH-2004 project statistician.

4.3 Results

Table 4.1 shows the baseline characteristics of the groups. The 30 male volunteers comprised 29 white Europeans and 1 Asian. The 26 female volunteers comprised 20 white Europeans, 2 Asians, 1 African and 3 Mixed Race. In women, there were

significant differences at baseline between placebo, low dose and high dose groups in mean cholesterol and fasting glucose. In men, there were significant differences at baseline between treatment groups in mean weight, lean body mass and HbA_{1c}. There were no significant differences at baseline between treatment groups in age, height, body mass index, fat mass, triglycerides, fasting insulin or VO_2 max.

4.3.1 *Lipid metabolism*

The effects of rhIGF-I/rhIGFBP-3 administration on lipid measurements and lipid oxidation rates are shown in Tables 4.2 to 4.5 and in Figures 4.1 and 4.2. In women treated with high dose rhIGF-I/rhIGFBP-3, there was a 25% reduction in fasting triglycerides ($P = 0.038$) but no significant changes in fasting NEFA ($P = 0.374$) or glycerol ($P = 0.166$) concentrations. There were also significant increases in the high dose group in total cholesterol (approximately 15% increase, $P = 0.003$), LDL cholesterol (25% increase, $P = 0.002$) and HDL cholesterol (approximately 15% increase, $P = 0.001$), but no significant changes in total cholesterol:HDL ratio ($P = 0.582$). There were no significant changes in any of these lipid concentrations in either the low dose IGF-I or placebo groups. There were also no significant changes in NEFA AUC or lipid oxidation rates (fasting or post-glucose challenge, Table 4.4) in any of the three treatment groups.

In men treated with high dose rhIGF-I/rhIGFBP-3, there was a 40% reduction in fasting triglycerides ($P = 0.027$) but no significant changes in fasting NEFA ($P = 0.351$) or glycerol ($P = 0.145$) concentrations. There was also a 12.5% increase in LDL cholesterol in the high dose group ($P = 0.013$) but no significant changes in total cholesterol ($P = 0.289$), HDL cholesterol ($P = 0.976$) or total cholesterol:HDL ratio ($P = 0.242$). There were no significant changes in any of these lipid concentrations in either the low dose IGF-I or placebo groups. There were also no significant changes in NEFA AUC or lipid oxidation rates (fasting or post-glucose challenge, Table 4.5) in any of the three treatment groups.

In the high dose group, the relative increase in HDL cholesterol in women was significantly greater ($P = 0.013$), compared with the effect in men. This difference remained significant ($P = 0.035$) after correcting the participant's dose relative to their weight. There were no other significant differences in the relative effects of high dose or low dose rhIGF-I/rhIGFBP-3 on lipid metabolism, when women and men were compared.

Table 4.1. Baseline characteristics of 56 recreational athletes. BMI = Body Mass Index, IGF-I = rhIGF-I/rhIGFBP-3 administration, VO_2 max = maximal oxygen consumption (measured by incremental treadmill test). Fat mass and lean body mass were measured using Dual Energy X-ray Absorptiometry (DEXA). *Significant difference ($P < 0.05$) between placebo, low dose and high dose groups.

	Women			Men		
	Placebo (n=8)	Low dose IGF-I (n=9)	High dose IGF-I (n=9)	Placebo (n=10)	Low dose IGF-I (n=10)	High dose IGF-I (n=10)
Mean age (SD), years	21.9 (2.2)	21.7 (3.4)	21.4 (1.7)	22.0 (2.8)	21.9 (2.7)	23.2 (2.7)
Mean height (SD), cm	167.5 (7.7)	165.2 (2.3)	169.0 (6.6)	185.0 (5.8)	179.2 (10.2)	181.3 (6.2)
Mean weight (SD), kg	61.7 (7.0)	60.2 (4.9)	60.5 (7.4)	92.4 (16.2)*	76.9 (12.0)*	80.7 (12.9)*
Mean BMI (SD), kg/m^2	22.0 (1.6)	22.0 (1.8)	21.2 (2.4)	27.0 (4.3)	23.8 (2.5)	24.6 (3.9)
Mean fat mass (SD), kg	17.8 (6.0)	16.5 (5.0)	16.5 (3.8)	17.2 (10.4)	12.3 (4.1)	15.6 (8.0)
Mean lean body mass (SD), kg	42.0 (2.9)	40.4 (3.2)	40.7 (4.2)	69.6 (6.4)*	60.2 (7.9)*	63.7 (7.0)*
Mean triglycerides (SD), mmol/L	0.9 (0.3)	0.9 (0.4)	0.8 (0.2)	1.2 (0.4)	0.9 (0.3)	1.0 (0.5)
Mean cholesterol (SD), mmol/L	4.1 (0.6)*	4.6 (0.5)*	3.8 (0.4)*	4.3 (0.6)	4.6 (1.3)	4.1 (0.7)
Mean fasting glucose (SD), mmol/L	4.4 (0.3)*	4.9 (0.4)*	4.5 (0.2)*	4.8 (0.4)	4.7 (0.4)	4.7 (0.3)
Mean fasting insulin (SD), mU/L	4.1 (1.4)	5.4 (2.7)	5.2 (2.9)	5.5 (2.3)	4.0 (2.0)	4.5 (2.0)
Mean HbA_{1c} (SD), %	5.3 (0.3)	5.4 (0.2)	5.4 (0.1)	5.4 (0.2)*	5.2 (0.2)*	5.1 (0.3)*
Mean VO_2 max (SD), ml/min/kg	47.0 (7.5)	48.1 (8.8)	46.2 (5.6)	48.0 (8.3)	51.9 (12.3)	48.0 (10.6)

Table 4.2. Changes in lipid measurements after 28 days of treatment in 26 female recreational athletes. IGF-I = rhIGF-I/rhIGFBP-3 administration, NEFA = non-esterified fatty acids, AUC = area under the curve. *Significant difference ($P < 0.05$) compared with Day 0.

WOMEN	Treatment group					
	Placebo (n=8)		Low dose IGF-I (n=9)		High dose IGF-I (n=9)	
	Day 0	Day 28	Day 0	Day 28	Day 0	Day 28
Mean fasting triglycerides (SD), mmol/L	0.9 (0.3)	0.8 (0.3)	0.9 (0.4)	0.8 (0.4)	**0.8 (0.2)**	**0.6 (0.2)***
Mean fasting NEFA (SD), micromol/L	516 (134)	441 (111)	490 (202)	415 (173)	422 (110)	380 (75)
Mean fasting glycerol (SD), micromol/L	78.8 (29.7)	69.5 (35.6)	71.4 (38.5)	64.3 (30.8)	72.4 (40.7)	57.0 (35.0)
Mean fasting total cholesterol (SD), mmol/L	4.1 (0.6)	3.9 (0.5)	4.6 (0.5)	4.9 (0.8)	**3.8 (0.4)**	**4.4 (0.5)***
Mean fasting HDL cholesterol (SD), mmol/L	1.6 (0.2)	1.5 (0.4)	1.5 (0.4)	1.5 (0.4)	**1.4 (0.3)**	**1.6 (0.2)***
Mean fasting total cholesterol : HDL ratio (SD)	2.6 (0.4)	2.8 (0.7)	3.4 (1.1)	3.5 (1.3)	2.8 (0.4)	2.8 (0.5)
Mean fasting LDL cholesterol (SD), mmol/L	2.1 (0.5)	2.1 (0.4)	2.8 (0.8)	3.1 (1.1)	**2.0 (0.3)**	**2.5 (0.6)***
Mean fasting NEFA AUC (SD), micromol/L x min	15,086 (5447)	14,624 (3040)	17,229 (8535)	16,622 (8450)	17,051 (8125)	12,748 (3919)

Table 4.3. Changes in lipid measurements after 28 days of treatment in 30 male recreational athletes. IGF-I = rhIGF-I/rhIGFBP-3 administration, NEFA = non-esterified fatty acids, AUC = area under the curve. *Significant difference ($P < 0.05$) compared with Day 0.

MEN	Treatment group					
	Placebo (n=10)		Low dose IGF-I (n=10)		High dose IGF-I (n=10)	
	Day 0	Day 28	Day 0	Day 28	Day 0	Day 28
Mean fasting triglycerides (SD), mmol/L	1.2 (0.4)	1.3 (0.8)	0.9 (0.3)	0.8 (0.3)	**1.0 (0.5)**	**0.6 (0.2)***
Mean fasting NEFA (SD), micromol/L	354 (125)	358 (224)	441 (226)	463 (173)	482 (158)	636 (282)
Mean fasting glycerol (SD), micromol/L	40.8 (15.6)	41.9 (28.0)	44.1 (27.9)	47.3 (23.3)	41.4 (19.0)	59.4 (25.9)
Mean fasting total cholesterol (SD), mmol/L	4.3 (0.6)	4.4 (0.6)	4.6 (1.3)	4.3 (0.9)	4.1 (0.7)	4.3 (0.6)
Mean fasting HDL cholesterol (SD), mmol/L	1.1 (0.3)	1.1 (0.2)	1.4 (0.4)	1.3 (0.3)	1.3 (0.2)	1.3 (0.2)
Mean fasting total cholesterol : HDL ratio (SD)	4.1 (0.9)	4.2 (0.9)	3.4 (0.7)	3.3 (0.6)	3.3 (0.8)	3.4 (0.8)
Mean fasting LDL cholesterol (SD), mmol/L	2.7 (0.4)	2.7 (0.4)	2.8 (1.1)	2.6 (0.8)	**2.4 (0.7)**	**2.7 (0.6)***
Mean NEFA AUC (SD), micromol/L x min	15,677 (5021)	16,480 (5638)	15,695 (4481)	17,572 (5528)	16,159 (4263)	18,565 (6339)

Table 4.4. Changes in lipid oxidation rates after 28 days of treatment in 26 female recreational athletes. IGF-I = rhIGF-I/rhIGFBP-3 administration. Data from low and high dose treatment groups were combined as it was not possible to perform indirect calorimetry on all participants at all time-points.

WOMEN	Placebo (n=6)		Low and High dose IGF-I (n=9)	
	Day 0	Day 28	Day 0	Day 28
Mean basal lipid oxidation rate (SD), mg/min	44.7 (19.3)	40.5 (23.5)	28.1 (14.4)	27.2 (16.9)
Mean post-glucose (60 mins) lipid oxidation rate (SD), mg/min	-1.5 (19.6)	-8.2 (24.0)	-7.3 (14.3)	-27.8 (17.4)
Mean post-glucose (120 mins) lipid oxidation rate (SD), mg/min	-12.5 (13.4)	-9.3 (20.2)	-4.4 (16.1)	-15.6 (9.8)

Table 4.5. Changes in lipid oxidation rates after 28 days of treatment in 30 male recreational athletes. IGF-I = rhIGF-I/rhIGFBP-3 administration. Data from low and high dose treatment groups were combined as it was not possible to perform indirect calorimetry on all participants at all time-points.

MEN	Placebo (n=9)		Low and High dose IGF-I (n=14)	
	Day 0	Day 28	Day 0	Day 28
Mean basal lipid oxidation rate (SD), mg/min	41.4 (14.9)	31.9 (22.6)	51.5 (18.8)	48.9 (36.1)
Mean post-glucose (60 mins) lipid oxidation rate (SD), mg/min	10.7 (21.0)	13.5 (29.8)	14.4 (26.6)	15.9 (28.6)
Mean post-glucose (120 mins) lipid oxidation rate (SD), mg/min	6.8 (42.6)	-7.9 (31.6)	16.1 (43.3)	24.0 (25.9)

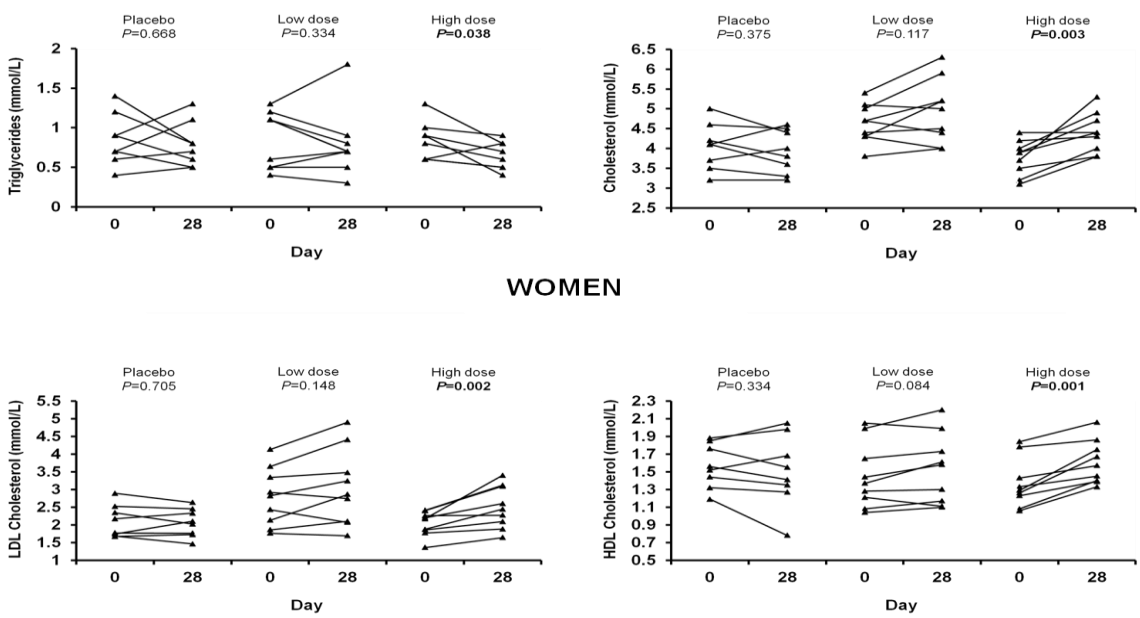

Figure 4.1. The effects of rhIGF-I/rhIGFBP-3 administration on fasting lipid profile in 26 female recreational athletes.

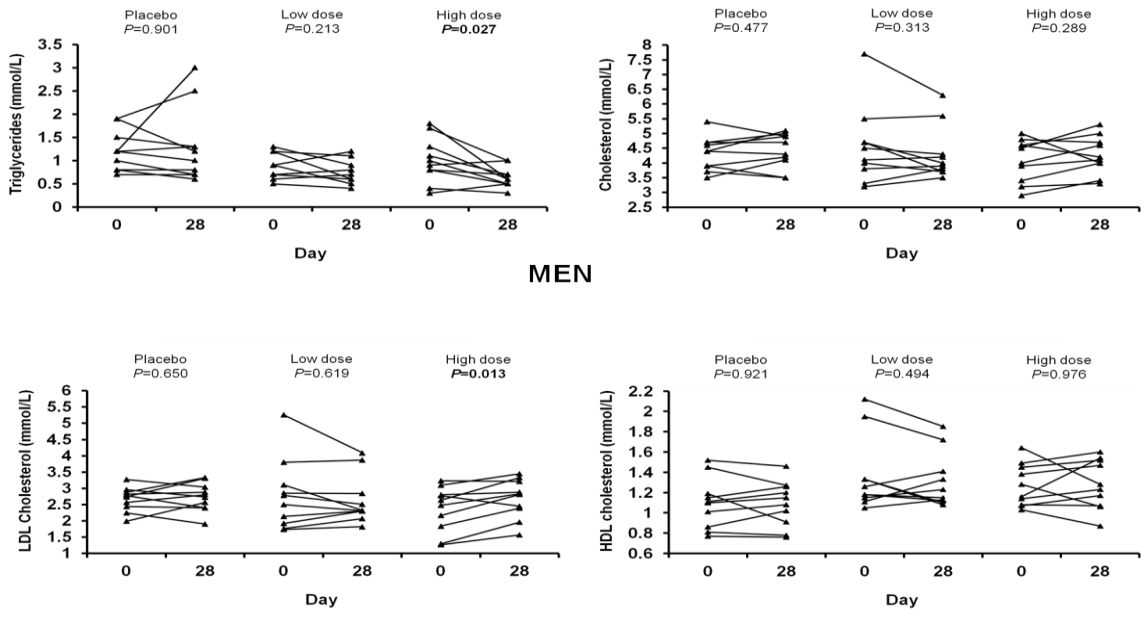

Figure 4.2. The effects of rhIGF-I/rhIGFBP-3 administration on fasting lipid profile in 30 male recreational athletes.

4.3.2 *Carbohydrate metabolism*

The effects of rhIGF-I/rhIGFBP-3 administration on carbohydrate metabolism and carbohydrate oxidation rates are shown in Tables 4.6 to 4.9 and in Figures 4.3 to 4.5. In women treated with low dose and high dose rhIGF-I/rhIGFBP-3, there was a significant decrease in fasting insulin, C-peptide, HOMA-IR and HbA_{1c}. In the low dose group, there was a 54% reduction in fasting insulin ($P = 0.001$), 47% reduction in fasting C-peptide ($P = 0.001$), 58% reduction in HOMA-IR ($P = 0.001$) and 6% reduction in HbA_{1c} ($P = 0.016$). In the high dose group, there was a 54% reduction in fasting insulin ($P = 0.009$), 46% reduction in fasting C-peptide ($P = 0.004$), 50% reduction in HOMA-IR ($P = 0.011$) and 6% reduction in HbA_{1c} ($P = 0.018$). There was also a significant decrease (8% reduction, $P = 0.020$) in fasting glucose in women treated with low dose but not high dose rhIGF-I/rhIGFBP-3. There were no significant changes in glucose AUC, insulin AUC or carbohydrate oxidation rates (fasting or post-glucose challenge) in any of the female treatment groups.

In men treated with low dose and high dose rhIGF-I/rhIGFBP-3, there was a significant decrease in fasting C-peptide concentrations (36% reduction in the low dose group, $P = 0.002$; 50% reduction in the high dose group, $P = 0.001$)). There were also significant decreases in fasting glucose (4% reduction, $P = 0.022$), insulin (49% reduction, $P = 0.001$), HOMA-IR (50% reduction, $P = 0.001$) and insulin AUC (35% reduction, $P = 0.011$) in men treated with high dose but not low dose rhIGF-I/rhIGFBP-3. There were no significant changes in HbA_{1c} or carbohydrate oxidation rates (fasting or post-glucose challenge) in any of the male treatment groups.

When the relative effects of rhIGF-I/rhIGFBP-3 administration on carbohydrate metabolism in women and men were compared, there were no significant differences in either the low dose or high dose groups.

Table 4.6. Changes in carbohydrate metabolism after 28 days of treatment in 26 female recreational athletes. IGF-I = rhIGF-I/rhIGFBP-3 administration, AUC = area under the curve. *Significant difference ($P < 0.05$) compared with Day 0.

WOMEN	Treatment group					
	Placebo (n=8)		Low dose IGF-I (n=9)		High dose IGF-I (n=9)	
	Day 0	Day 28	Day 0	Day 28	Day 0	Day 28
Mean fasting glucose (SD), mmol/L	4.4 (0.3)	4.4 (0.3)	**4.9 (0.4)**	**4.5 (0.3)***	4.5 (0.2)	4.4 (0.2)
Mean fasting insulin (SD), mU/L	4.1 (1.4)	4.4 (0.8)	**5.4 (2.7)**	**2.5 (1.8)***	**5.2 (2.9)**	**2.4 (0.9)***
Mean fasting C-peptide (SD), pmol/L	412 (96)	461 (32)	**418 (162)**	**223 (78)***	**461 (135)**	**249 (123)***
Mean HOMA-IR (SD), $(mU*mmol)/L^2$	0.8 (0.3)	0.9 (0.2)	**1.2 (0.7)**	**0.5 (0.4)***	**1.0 (0.5)**	**0.5 (0.2)***
Mean HbA_{1c} (SD), %	5.3 (0.3)	5.1 (0.2)	**5.4 (0.2)**	**5.1 (0.3)***	**5.4 (0.1)**	**5.1 (0.2)***
Mean glucose AUC (SD), mmol/L x min	494 (287)	551 (166)	585 (217)	567 (261)	484 (245)	464 (147)
Mean insulin AUC (SD), mU/L x min	3035 (2557)	3160 (975)	2211 (1000)	1785 (727)	2027 (991)	1428 (519)

Table 4.7. Changes in carbohydrate metabolism after 28 days of treatment in 30 male recreational athletes. IGF-I = rhIGF-I/rhIGFBP-3 administration, AUC = area under the curve. *Significant difference ($P < 0.05$) compared with Day 0.

MEN	Treatment group					
	Placebo (n=10)		Low dose IGF-I (n=10)		High dose IGF-I (n=10)	
	Day 0	Day 28	Day 0	Day 28	Day 0	Day 28
Mean fasting glucose (SD), mmol/L	4.8 (0.4)	4.8 (0.4)	4.7 (0.4)	4.7 (0.2)	**4.7 (0.3)**	**4.5 (0.3)***
Mean fasting insulin (SD), mU/L	5.5 (2.3)	5.0 (3.8)	4.0 (2.0)	2.7 (1.8)	**4.5 (2.0)**	**2.3 (1.2)***
Mean fasting C-peptide (SD), pmol/L	494 (170)	427 (186)	**349 (81)**	**222 (76)***	**347 (82)**	**172 (65)***
Mean HOMA-IR (SD), $(mU*mmol)/L^2$	1.2 (0.5)	1.1 (0.9)	0.8 (0.4)	0.6 (0.4)	**1.0 (0.4)**	**0.5 (0.3)***
Mean HbA_{1c} (SD), %	5.4 (0.2)	5.3 (0.2)	5.2 (0.2)	5.1 (0.3)	5.1 (0.3)	5.1 (0.4)
Mean glucose AUC (SD), mmol/L x min	709 (102)	672 (93)	684 (102)	680 (90)	771 (162)	722 (190)
Mean insulin AUC (SD), mU/L x min	3558 (2091)	3056 (2393)	2045 (1033)	1955 (696)	**3259 (1488)**	**2106 (1073)***

Table 4.8. Changes in carbohydrate oxidation rates after 28 days of treatment in 26 female recreational athletes. IGF-I = rhIGF-I/rhIGFBP-3 administration. Data from low and high dose treatment groups were combined as it was not possible to perform indirect calorimetry on all participants at all time-points.

WOMEN	Placebo (n=6)		Low and High dose IGF-I (n=9)	
	Day 0	Day 28	Day 0	Day 28
Mean basal carbohydrate oxidation rate (SD), mg/min	117.5 (24.8)	128.0 (37.3)	168.8 (27.9)	150.3 (43.0)
Mean post-glucose (60 mins) carbohydrate oxidation rate (SD), mg/min	265.3 (38.6)	295.6 (55.7)	281.0 (54.6)	317.3 (49.0)
Mean post-glucose (120 mins) carbohydrate oxidation rate (SD), mg/min	257.3 (38.3)	259.3 (43.0)	271.7 (38.0)	286.9 (39.5)

Table 4.9. Changes in carbohydrate oxidation rates after 28 days of treatment in 30 male recreational athletes. IGF-I = rhIGF-I/rhIGFBP-3 administration. Data from low and high dose treatment groups were combined as it was not possible to perform indirect calorimetry on all participants at all time-points.

MEN	Placebo (n=9)		Low and High dose IGF-I (n= 14)	
	Day 0	Day 28	Day 0	Day 28
Mean basal carbohydrate oxidation rate (SD), mg/min	191.0 (55.4)	221.3 (74.5)	146.6 (69.9)	149.4 (97.0)
Mean post-glucose (60 mins) carbohydrate oxidation rate (SD), mg/min	275.6 (51.7)	282.1 (71.1)	287.3 (69.1)	273.4 (89.4)
Mean post-glucose (120 mins) carbohydrate oxidation rate (SD), mg/min	276.9 (95.4)	319.5 (120.4)	267.2 (99.6)	233.5 (63.4)

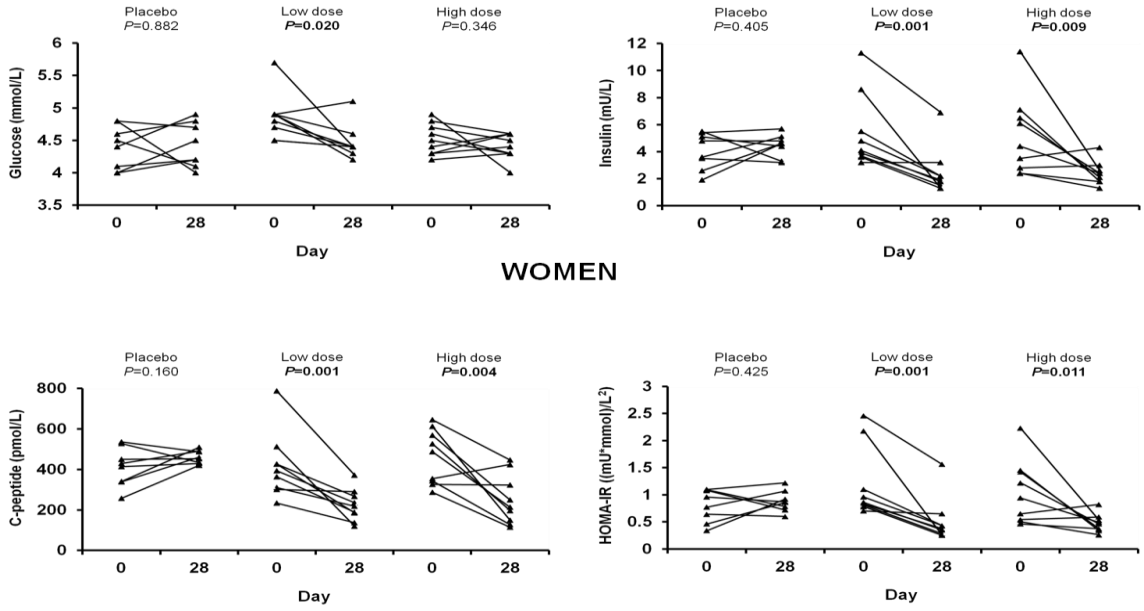

Figure 4.3. The effects of rhIGF-I/rhIGFBP-3 administration on carbohydrate metabolism in 26 female recreational athletes. All samples were collected in the fasting state.

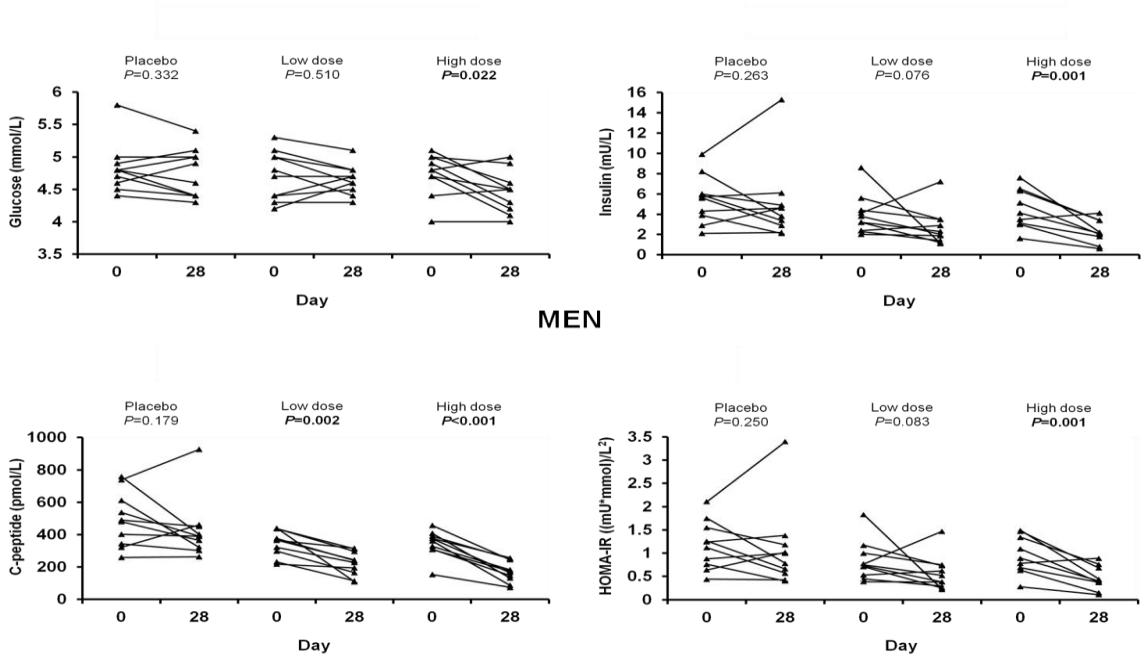

Figure 4.4. The effects of rhIGF-I/rhIGFBP-3 administration on carbohydrate metabolism in 30 male recreational athletes. All samples were collected in the fasting state.

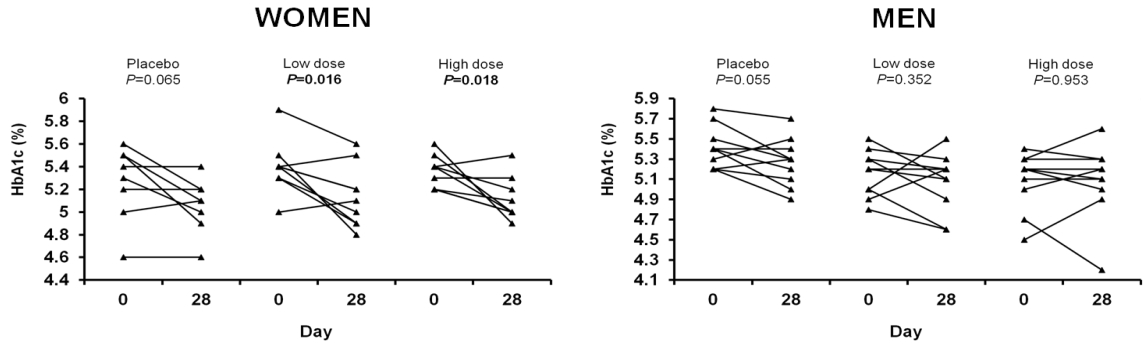

Figure 4.5. The effects of rhIGF-I/rhIGFBP-3 administration on glycated haemoglobin (HbA_{1c}) in 26 female (left panel) and 30 male (right panel) recreational athletes.

4.3.3 *Serum GH concentrations*

The effects of rhIGF-I/rhIGFBP-3 administration on serum GH concentrations are shown in Figures 4.6 (women) and 4.7 (men). Serum GH concentrations were lower during rhIGF-I/rhIGFBP-3 administration than at baseline. In women, serum GH was significantly decreased in both high and low dose groups on Day 7 (high dose $P = 0.006$, low dose $P < 0.001$), Day 14 (high dose $P = 0.002$, low dose $P < 0.001$) and Day 42 (high dose $P = 0.013$, low dose $P = 0.001$), compared with Day 0. GH was significantly decreased in the high dose but not the low dose group on Day 21 ($P = 0.001$), Day 30 ($P = 0.032$) and Day 84 ($P < 0.001$). In the placebo group, GH was lower on Day 42 ($P = 0.047$), compared with Day 0.

In men, serum GH was significantly decreased in both high and low dose groups on Day 14 (high dose: $P = 0.004$, low dose: $P = 0.004$). GH was significantly decreased in the high dose but not the low dose group on Day 21 ($P = 0.035$). GH was significantly decreased in the low dose but not the high dose group on Day 7 ($P = 0.01$). There were no significant changes in serum GH in the placebo group.

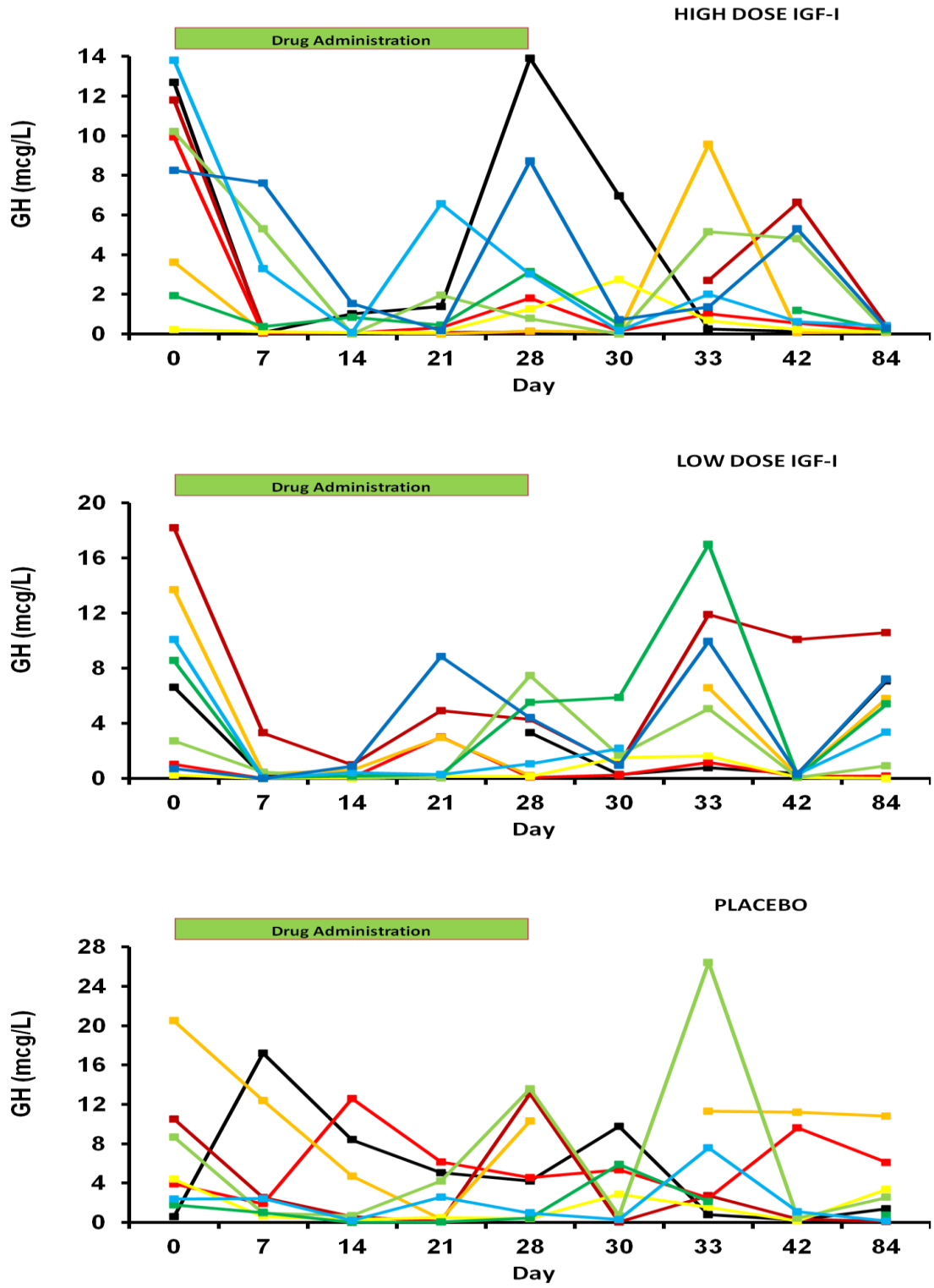

Figure 4.6. Response in serum GH to rhIGF-I/rhIGFBP-3 or placebo administration for 28 days in 26 female recreational athletes.

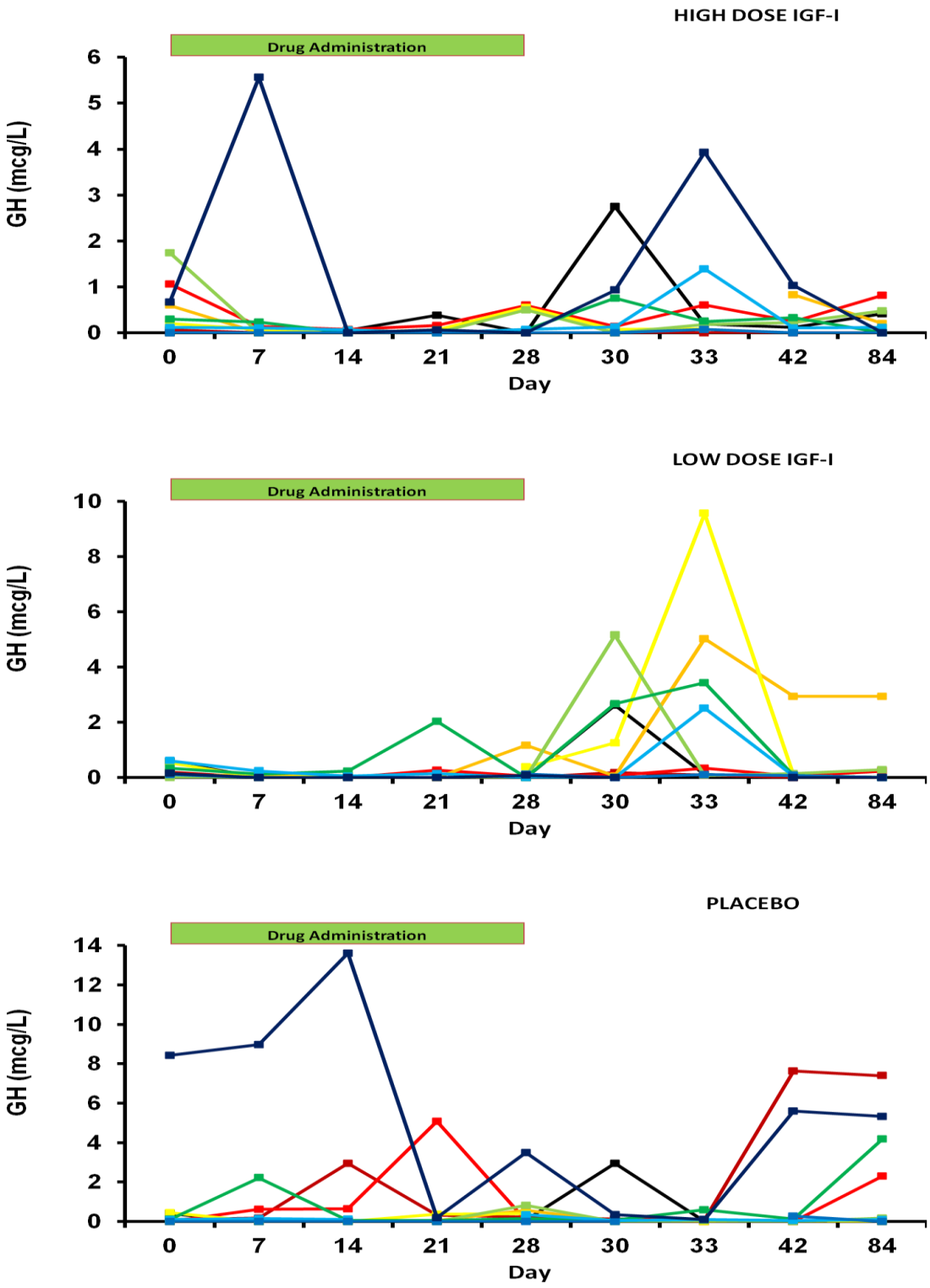

Figure 4.7. Response in serum GH to rhIGF-I/rhIGFBP-3 or placebo administration for 28 days in 30 male recreational athletes.

4.3.4 *Body composition*

There were no significant changes in fat mass or lean body mass in women or men after administration of either rhIGF-I/rhIGFBP-3 complex or placebo (Table 4.10). The body composition data shown are from DEXA assessments only but no significant changes in body composition were observed from skinfold assessments or from bioelectrical impedance analysis.

4.3.5 *Physical fitness*

rhIGF-I/rhIGFBP-3 administration significantly increased VO_2 max (Table 4.10 and Figure 4.8). When the low and high dose treatment groups were combined, there was approximately a 9% increase in mean VO_2 max in women and 6% increase in men. No significant changes in VO_2 max were observed in the placebo group in either women or men.

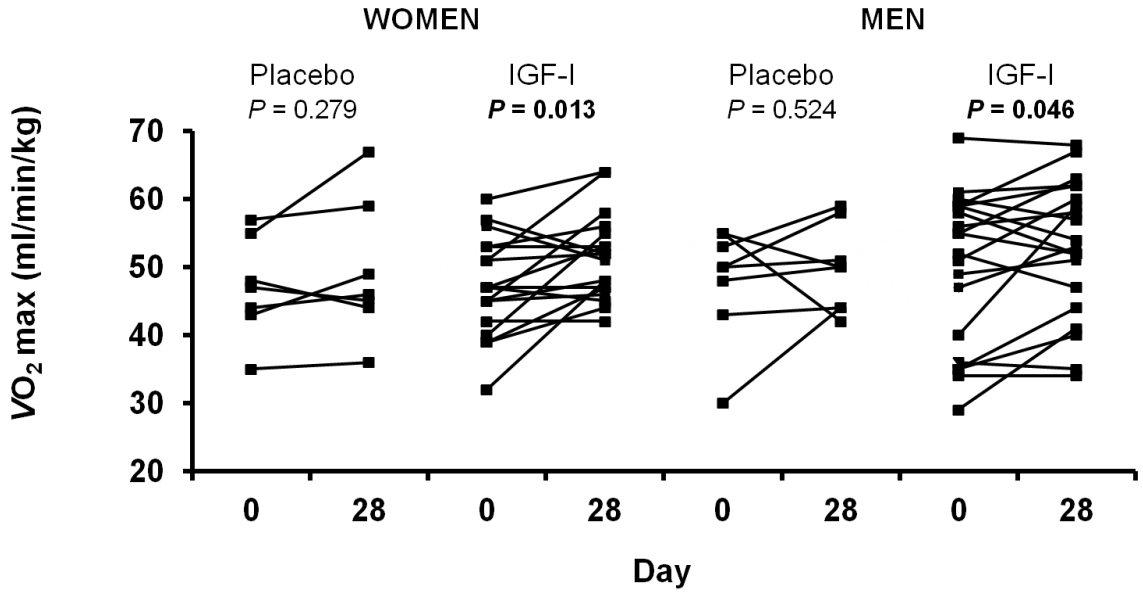

Figure 4.8. The effects of rhIGF-I/rhIGFBP-3 administration on physical fitness in 26 female and 30 male recreational athletes. Maximal oxygen uptake (VO_2 max) was measured before and after 28 days of treatment. Data from high and low dose treatment groups were combined.

Table 4.10. Changes in body composition and physical fitness after 28 days of treatment in 56 recreational athletes. BMI = Body Mass Index, IGF-I = rhIGF-I/rhIGFBP-3 administration, VO_2 max = maximal oxygen consumption. Data from high and low dose treatment groups were combined.
$ Data from 2 women (both placebo group) and 3 men (all IGF-I group) were excluded because of scheduling difficulties.
† Data from 1 woman (placebo group) and 2 men (both placebo group) were excluded because of technical difficulties with the equipment. *Significant difference ($P < 0.05$) compared with Day 0.

	Women		Men	
	Placebo (n=8)	IGF-I (n=18)	Placebo (n=10)	IGF-I (n=20)
Weight, kg				
Mean (SD) baseline	61.7 (7.0)	60.3 (6.1)	92.4 (16.2)	78.8 (12.3)
Mean (SD) Day 28	62.2 (6.3)	60.2 (6.2)	92.3 (16.2)	79.0 (12.8)
BMI, kg/m^2				
Mean (SD) baseline	22.0 (1.6)	21.6 (2.1)	27.0 (4.3)	24.2 (3.2)
Mean (SD) Day 28	22.2 (1.6)	21.5 (2.3)	26.9 (4.4)	24.2 (3.2)
Fat mass, kg $				
Mean (SD) baseline	17.8 (6.0)	16.5 (4.3)	17.2 (10.4)	13.7 (6.0)
Mean (SD) Day 28	17.8 (6.0)	16.1 (4.5)	17.1 (9.9)	13.7 (5.7)
Lean body mass, kg $				
Mean (SD) baseline	42.0 (2.9)	40.6 (3.6)	69.6 (6.4)	61.6 (7.5)
Mean (SD) Day 28	42.8 (4.1)	40.7 (4.2)	69.9 (7.3)	61.8 (8.3)
VO_2 max, ml/min/kg †				
Mean (SD) baseline	47.0 (7.5)	**47.2 (7.2)**	48.0 (8.3)	**50. 0 (11.3)**
Mean (SD) Day 28	49.4 (10.3)	**51.4 (6.3)***	49.8 (6.3)	**53.0 (10.1)***

4.4 Discussion

This part of the study was designed to investigate the effects of rhIGF-I/rhIGFBP-3 administration on lipid and glucose metabolism and on body composition and physical fitness in recreational athletes. Fasting triglycerides decreased and LDL cholesterol increased after rhIGF-I/rhIGFBP-3 administration. Total and HDL cholesterol increased in women after treatment with rhIGF-I/rhIGFBP-3, but not in men. These changes in serum lipid levels were associated with suppression of GH concentrations, reduced insulin secretion and increased insulin sensitivity. rhIGF-I/rhIGFBP-3 administration did not affect substrate utilisation as measured by lipid or carbohydrate oxidation rates. Furthermore, rhIGF-I/rhIGFBP-3 administration improved aerobic performance but there were no significant effects on body composition.

4.4.1 *Lipid metabolism*

There was a significant reduction in fasting triglycerides in response to rhIGF-I/rhIGFBP-3 administration but there were no significant changes in glycerol or NEFA concentrations. Previous IGF-I administration studies have demonstrated mixed effects on lipid profiles, depending on the population studied. A decrease in triglycerides was also observed when rhIGF-I/rhIGFBP-3 complex was administered to people with type 2 diabetes (mean age 56 years) for seven days (Clemmons et al. 2007). The authors also found that total cholesterol decreased after rhIGF-I/rhIGFBP-3 administration whereas in the current study of healthy young adults, total, LDL and HDL cholesterol increased in women, while only LDL cholesterol increased in men. When rhIGF-I/rhIGFBP-3 was administered to people with type 1 diabetes (mean age 27 years) for two weeks, there was a significant reduction in total cholesterol but no change in triglyceride concentrations (Clemmons et al. 2000). In comparison with the current study population, the participants in these previous studies were older and had altered physiology in terms of reduced insulin secretion (type 1 diabetes) or reduced insulin sensitivity (type 2 diabetes), and therefore it is difficult to compare the results between these studies. It is known that insulin lowers triglycerides through the stimulation of lipoprotein lipase (LPL) (Rosato et al. 1997); indeed insulin can be used as an emergency treatment in severe hypertriglyceridaemia (Poonuru et al. 2011). It appears that rhIGF-I/rhIGFBP-3 administration has similar triglyceride-lowering effects; the mechanism of this is unclear as previous studies have demonstrated differing effects between IGF-I and insulin on LPL activity in adipose tissue (Oscarsson et al. 1999).

When interpreting the effects of rhIGF-I administration on lipid profile, it is important to take into account the influence of GH on lipid metabolism. It is known that rhGH therapy in GH deficient adults has beneficial effects on lipid profile in terms of improving cardiovascular risk (decreased total and LDL cholesterol along with decreased Apo B-100, the main apoprotein constituent in LDL cholesterol particles) (Carroll et al. 1998). The effects of rhGH administration on lipid profile in healthy adults are not well characterised but when rhGH was administered to a group of abstinent anabolic steroid users for six days, there was a significant reduction in total cholesterol but no change in triglycerides (Graham et al. 2007). The effects of rhIGF-I administration on lipid profile in GH deficiency have also been investigated; when rhIGF-I was administered to eight adults with GH deficiency for eight weeks, there were no significant changes in triglycerides, total cholesterol, HDL cholesterol or LDL cholesterol (Mauras et al. 2000). When rhGH was administered to the same group for eight weeks, however, there was a significant decrease in LDL cholesterol and increase in triglycerides.

In the current study, GH concentrations were suppressed by rhIGF-I/rhIGFBP-3 administration in both women and men, as would be expected. Furthermore rhIGF-I/rhIGFBP-3 administration resulted in reduced insulin secretion and increased insulin sensitivity (discussed in section 4.4.2 below). It is possible that the increase in cholesterol observed in the current study occurred as a result of suppressed GH and reduced insulin secretion rather than through direct effects of IGF-I on cholesterol metabolism.

It has been shown previously that IGF-I has different effects on lipid oxidation, depending on the duration of treatment, the dose administered and the population studied. When rhIGF-I was administered to eight healthy volunteers via a continuous subcutaneous infusion of 10 μg/kg.h for five days, an increase in lipid oxidation rates was observed (Hussain et al. 1993). Similarly, rhIGF-I administration increased lipid oxidation rates after seven days of treatment in adults with GH deficiency (Hussain et al. 1994) but lipid oxidation rates were unchanged after eight weeks of rhIGF-I administration in a subsequent study in adults with GH deficiency (Mauras et al. 2000). The difference in the results of these studies was attributed to an acute reduction in insulin production in the earlier short-term studies, resulting in increased lipolysis and lipid oxidation. When rhIGF-I was administered to a group of ten adults with GH insensitivity syndrome (GHIS) for eight weeks, increased lipolysis (measured using stable isotope glycerol tracers) and increased lipid oxidation rates (measured by indirect calorimetry) were observed (Mauras et al. 2000). In the current

study, no significant changes in lipid oxidation rates were observed using indirect calorimetry after 28 days of rhIGF-I/rhIGFBP-3 administration. It appears that prolonged IGF-I administration (at the doses given in this study) does not stimulate lipid oxidation in healthy volunteers with normal physiology, in contrast with its effects in participants with longstanding deficiencies in GH or IGF-I. An alternative explanation is that the indirect calorimetry method used during this study was not sensitive enough to demonstrate subtle changes in lipid oxidation rates.

When the relative effects of rhIGF-I/rhIGFBP-3 administration on lipid metabolism in women and men were compared, the only difference was the increase in HDL cholesterol in women treated with high dose rhIGF-I/rhIGFBP-3. The body weight of participants needed to be considered when comparing women and men because at baseline, the mean weight in the female low dose and high dose groups was lower than that in the equivalent male groups. The rhIGF-I/rhIGFBP-3 complex in this study was administered at fixed doses (either 30 mg/day or 60 mg/day) and the relative dose within each treatment group was therefore higher in the female groups than in the male groups. This factor, however, did not explain the differential effect on HDL cholesterol between women and men.

4.4.2 *Carbohydrate metabolism*

rhIGF-I/rhIGFBP-3 administration increased insulin sensitivity in this study and insulin secretion was suppressed. The effects on carbohydrate metabolism included a reduction in fasting glucose, insulin, C-peptide and HOMA-IR in both women and men and a reduction in HbA_{1c} in women but not in men. Furthermore there was a significant reduction in insulin AUC during OGTT in men in the high dose treatment group. The suppression of GH secretion is an important part of the explanation for the increased insulin sensitivity and, as with lipid metabolism (section 4.4.1 above), the effects on carbohydrate metabolism are a result of the complex interplay between the actions of IGF-I, GH and insulin.

Experimental mouse models lacking the liver-specific IGF-I gene showed a 75% reduction in circulating IGF-I levels along with marked insulin resistance but it was not clear whether this resistance was a consequence of low IGF-I levels or the elevated GH levels observed in these mice (Yakar et al. 2001). Simpson *et al.* investigated this in people with type 1 diabetes; after suppressing endogenous GH secretion with the somatostatin-analogue octreotide, IGF-I administration reduced hepatic glucose output and increased peripheral glucose uptake (Simpson et al. 2004).

This suggested that IGF-I has a direct effect on glucose metabolism, independent of its role in suppressing GH secretion. Other studies have demonstrated a direct role of IGF-I in increasing glucose transport into skeletal muscle and increasing muscle glycogen synthesis (Dimitriadis et al. 1992; Frick et al. 2000).

The results of the current study are in keeping with those of previous studies in which rhIGF-I/rhIGFBP-3 was administered to people with diabetes. Fifteen participants with type 1 diabetes were given rhIGF-I/rhIGFBP-3 complex for two days, which resulted in decreased overnight insulin requirements to maintain euglycaemia and improved insulin sensitivity (Saukkonen et al. 2004). Seven days' administration of rhIGF-I/rhIGFBP-3 to 39 people with type 2 diabetes caused reductions in fasting glucose and mean daily glucose calculated from four glucose readings per day (Clemmons et al. 2007). Administration of this complex has also been associated with improved glycaemic control in people with severe insulin resistance (Regan et al. 2010).

No significant changes in carbohydrate oxidation rates were observed in any of the treatment groups in this study. This agrees with the findings from previous studies in which rhIGF-I was administered to eight healthy volunteers for five days (Hussain et al. 1993), eight adults with GH deficiency for seven days (Hussain et al. 1994) and eight adults with GH deficiency for eight weeks (Mauras et al. 2000). When rhIGF-I was administered to ten adults with GHIS for eight weeks, however, there was a decrease in carbohydrate oxidation rates along with an increase in hepatic glucose production (Mauras et al. 2000). These findings were attributed to insulinopaenia in the portal circulation secondary to the suppression of insulin secretion by IGF-I (Mauras et al. 2005). In the current study, the absence of any changes in carbohydrate oxidation rates probably reflects the combined effects of IGF-I acting directly on carbohydrate oxidation along with the suppression of GH and insulin secretion that occurred during rhIGF-I/rhIGFBP-3 administration.

4.4.3 *Body composition*

rhIGF-I/rhIGFBP-3 administration had no significant effects on fat mass or lean body mass in this study. Previous IGF-I administration studies have yielded conflicting results in terms of effects on body composition, again depending on the population studied. One study investigated the effects of rhGH and rhIGF-I administration in a group of 33 obese post-menopausal women who were undertaking a diet and exercise programme over 12 weeks. The administration of rhGH alone and rhGH combined

with rhIGF-I resulted in an increase in fat-free mass in these women, while the administration of rhIGF-I alone had no effect on fat-free mass (Thompson et al. 1998). Furthermore, substantial changes in body composition were observed when rhIGF-I was administered to adults with GH deficiency (Mauras et al. 2000) and to adults with GHIS (Mauras et al. 2000). In both of these studies, rhIGF-I administration was associated with increased lean body mass and decreased adiposity. The findings in the latter study were attributed to the stimulatory effects of rhIGF-I on lipolysis and lipid oxidation in adults with GHIS, described above in section 4.4.1. When rhIGF-I was administered to a group of 16 healthy post-menopausal women for one year, however, there was no increase in lean body mass or decrease in adipose tissue after treatment (Friedlander et al. 2001), similar to the findings in the current study. It is possible that the positive effect of IGF-I administration on body composition in patients with GHIS reflects the severe nature of their IGF-I deficiency (Mauras et al. 2005) whereas the healthy recreational athletes in the current study with normal endogenous IGF-I production are less likely to respond.

The effects of rhIGF-I administration on body composition in young healthy athletes have not been studied previously but a systematic review of the effects of rhGH administration on body composition and athletic performance was published in 2008 (Liu et al. 2008). The authors included 27 studies of rhGH administration in young, lean, physically fit participants. The conclusion of the review was that rhGH administration increases lean body mass though strength and exercise capacity did not seem to improve. The authors noted that GH administration protocols in these studies may not reflect the regimens used by elite athletes (Liu et al. 2008) and this limitation is relevant to the current study, as discussed in section 4.4.5 below.

4.4.4 *Physical fitness*

rhIGF-I/rhIGFBP-3 administration resulted in improvements in maximal oxygen consumption (VO_2 max) in both women and men. The use of VO_2 max as a measure of physical fitness in clinical studies has been debated but it has been suggested that small increments in VO_2 max can have an important influence on the outcome of endurance events (Shephard 2009). VO_2 max can be improved by physical training and it has been shown that major factors determining the level of improvement in aerobic fitness include the volume, intensity and frequency of training as well as the initial level of fitness (Mujika 1998). Highly trained athletes might pursue alternative

ways of improving VO_2 max when no further improvements can be attained through training alone, and the effects of IGF-I may therefore be attractive to this population.

The mechanisms of VO_2 max improvement have not been investigated in this study. Oxygen consumption during exercise is dependent on many factors including efficient inspiration by the respiratory system, transport of oxygen in the circulation to skeletal muscles and effective aerobic metabolism by skeletal muscle fibres. One potential explanation for the improvement is that IGF-I treatment increases respiratory muscle strength as has been shown in a previous rhGH administration study in abstinent anabolic steroid users, in which both maximal oxygen uptake and mean inspiratory pressure were increased after rhGH treatment (Graham et al. 2007). It has also been shown previously that serum IGF-I concentrations are positively correlated with haemoglobin concentrations (Kong et al. 2011). An increase in haemoglobin might explain improved aerobic performance after IGF-I treatment through enhanced oxygen delivery to exercising skeletal muscle. Haemoglobin concentrations were not measured in our participants so it was not possible to determine the contribution of this factor to the improvement observed in this study. It is also possible that effects on the cardiovascular system contributed to improved aerobic performance; it has been shown previously that intravenous IGF-I infusion caused an increase in cardiac output, heart rate and stroke volume in healthy volunteers (Russell-Jones et al. 1995). These cardiovascular variables were not monitored in this study and future studies into the effects of IGF-I on athletic performance should include evaluation of effects on the cardiovascular, respiratory and haematological systems.

4.4.5 *Limitations*

The first limitation of this study is that it included recreational rather than elite athletes because it is not possible to administer prohibited substances to elite athletes. The baseline physical fitness levels and body composition of an elite athlete population would be different to the athletes in the current study and we do not know if rhIGF-I/rhIGFBP-3 administration would have the same effect on an elite athlete as on a recreational athlete. Second, we do not know the doses of IGF-I that are being used by elite athletes nor the typical duration of treatment. It is likely that the drug would be taken for a longer period than the 28 days employed in this study and it is possible that prolonged administration could lead to more marked changes in body composition as well as physical fitness. Third, we did not control the training

intensity of the participants during the drug administration period and this may have contributed to the changes in physical fitness observed in the treatment group, though it is reassuring that no significant changes were observed in the placebo groups.

In conclusion, the administration of rhIGF-I/rhIGFBP-3 to recreational athletes caused a reduction in insulin secretion, improved insulin sensitivity and had significant effects on lipid profile including a decrease in fasting triglycerides. These changes are largely explained by the suppression of GH secretion as well as the direct effects of IGF-I. Furthermore, there were significant improvements in aerobic performance in both women and men, though no changes in body composition were observed. This performance-enhancing effect of IGF-I has not been demonstrated previously and the athletic significance of the improvement in VO_2 max is discussed in Chapter 9. The findings of this study support the inclusion of IGF-I on the WADA list of prohibited substances and highlight the need for methods to detect IGF-I misuse in athletes as discussed further in Chapters 1, 3 and 9.

CHAPTER 5: CROSS-SECTIONAL STUDY OF ELITE ADOLESCENT ATHLETES

5.1 Introduction

The GH-2000 formulae include a correction based on the reciprocal of age to allow for the normal age-related decline in GH secretion in adulthood (Powrie et al. 2007). This correction, however, has never been validated in adolescent athletes and may be inappropriate for this group. GH secretion is low during the pre-pubertal phase of growth but during puberty, alterations in the hypothalamic control of the GH-IGF axis and increased gonadal sex steroids lead to a rise in GH secretion that peaks during mid to late puberty (Martha et al. 1992; Veldhuis et al. 2005). The timing of puberty thus affects the concentration of GH-sensitive biomarkers (Mauras et al. 2007). Adolescent athletes compete at national and international events and it is believed that GH is misused by high school athletes (Rickert et al. 1992). It is therefore important that any test for GH misuse is applicable to adolescent athletes.

The original GH-2000 study included few athletes younger than 18 years and therefore the primary aim of this study was to investigate serum IGF-I and P-III-NP in elite adolescent athletes in order to determine how a test based on the measurement of GH-dependent markers would perform in younger athletes. A further aim of the study was to compare two IGF-I and P-III-NP assays using serum samples from elite adolescent athletes.

5.2 Methods

Sixty-three national and regional sporting organisations were contacted to obtain permission to approach elite athletes (representing county level or above) aged between 12 and 20 years. Permission was subsequently obtained to attend training sessions at the organisations detailed in section 2.1.2.1. Individual participants were also recruited through personal contacts of the research team. If the athlete agreed to participate, written informed consent was obtained together with parental consent if the athlete was younger than 18 years. 157 (85 male, 72 female) elite adolescent athletes were included in the study. Volunteers were excluded if they had a history of endocrine pathology or a recent musculoskeletal injury. Demographic data on gender, age, ethnic origin, sport, training hours, diet, injuries, medications and menstrual history were recorded. Height was measured to the nearest centimetre using a

portable stadiometer (Raven Equipment, Essex) and weight was measured to the nearest 0.1kg using electronic scales.

Venous blood samples were collected from adolescent athletes either before or after exercise as described in section 2.1.1.3. All samples were analysed at the Drug Control Centre, King's College London. Serum IGF-I was measured by the DSL-5600 ACTIVE IGF-I assay (Diagnostics Systems Laboratories, Texas, USA) and the Immunotech A15729 IGF-I IRMA (Immunotech SAS, Marseille, France). P-III-NP was measured by the RIA-gnost P-III-NP assay (Cisbio, Gif-sur-Yvette, France) and UniQ™ P-III-NP RIA (Orion Diagnostica, Espoo, Finland). These assays are described in detail in sections 2.1.1.4 and 2.1.2.3.

Height, weight and BMI measurements were converted to standard deviation scores (SDS) for chronological age (UK standards 1990) (Freeman et al. 1995). Anthropometric data were compared using unpaired *t* tests. All statistical comparisons were two-tailed. $P<0.05$ was considered statistically significant. Analyses of IGF-I, P-III-NP results and discriminant function scores were performed by Dr Erotokritou-Mulligan, GH-2004 Project statistician. Data were analysed using SAS® (SAS Institute Inc., Cary, NC, USA) and SPSS (SPSS Inc., Chicago, IL, USA) software. Results are given as mean ± standard error.

In order to calculate discriminant function scores for the adolescent athletes in this study, it was necessary to convert the results from the assays used into equivalent results on the GH-2000 scales using the methods described in section 2.2.1. Inter-assay agreement between DSL and Immunotech IGF-I assays and between the Cisbio and Orion P-III-NP assays was assessed by analyzing 124 samples from the elite adolescent athletes using all four assays. Inter-assay agreement was evaluated using simple linear regression and modified Bland-Altman plots.

My role in this study was as follows: I was responsible for attending some of the training sessions described in section 2.1.2.1 and collected approximately one-third of the serum samples used in the study, along with athlete demographic data. The remaining serum samples were collected by medical staff from the participating sporting organisations and by medical student members of the GH-2004 research group under my supervision. Data from this study have been published in "Guha N *et al.* Serum insulin-like growth factor-I and pro-collagen type III N-terminal peptide in adolescent elite athletes: implications for the detection of growth hormone abuse in sport, *J Clin Endocrinol Metab*, **95**(6): 2969-76". I am the first author on this paper

and was responsible for drafting and editing the manuscript along with other members of the GH-2004 team.

5.3 Results

Samples were collected from 85 male (aged 16.0 ± 0.2 yrs, range 12.1 – 19.9) and 72 female (aged 16.0 ± 0.2 yrs, range 12.0 – 20.4) white European elite athletes. Participants represented 14 sporting disciplines in 8 sporting categories (Table 5.1).

5.3.1 *Height, weight and BMI*

On average, male participants were significantly taller (1.74 ± 0.01m vs. 1.64 ± 0.01m, $P<0.001$) and heavier (67.3 ± 1.76kg vs. 58.2 ± 1.15kg, $P<0.001$) than females. There was no difference in BMI between male and female participants (22.0 ± 0.4 vs. 21.7 ± 0.3kg/m^2, $P=0.60$). Male and female adolescent athletes were taller and had larger BMI than the age-matched control 1990 UK population (Table 5.1).

Table 5.1. Frequency numbers for adolescent athletes by sporting discipline showing height, weight, BMI and SDS for chronological age (mean ± SEM).

Sporting Category	Sports	Gender		TOTAL (n)	Height (m)	Height SDS	Weight (kg)	Weight SDS	BMI (kg/m^2)	BMI SDS
		Male	Female							
Athletics	**Athletics**	4	3	7	1.69 ± 0.03	0.51 ± 0.35	58.5 ± 4.1	0.29 ± 0.35	20.3 ± 0.8	0.10 ± 0.26
Power	**Bodybuilding**	2	0	2	1.77 ± 0.09	0.52 ± 0.79	69.4 ± 9.0	0.87 ± 0.79	22.1 ± 0.7	0.83 ± 0.07
Endurance Sports	**Cycling, Rowing, Canoeing**	5	2	7	1.78 ± 0.03	1.02 ± 0.29	71.3 ± 3.9	1.01 ± 0.27	22.4 ± 0.7	0.64 ± 0.21
Gymnastics	**Trampolining**	0	1	1	1.64	0.11	56.7	0.11	21.1	0.20
Racket Sports	**Squash**	2	2	4	1.58 ± 0.04	-0.14 ± 0.46	45.0 ± 3.4	-0.46 ± 0.38	18.0 ± 0.8	-0.51 ± 0.31
Sailing	**Sailing**	11	9	20	1.66 ± 0.03	-0.15 ± 0.20	60.4 ± 2.9	0.23 ± 0.18	21.6 ± 0.6	0.39 ± 0.15
Swimming	**Swimming, Diving**	26	29	55	1.69 ± 0.01	0.75 ± 0.11	60.2 ± 1.4	0.64 ± 0.09	20.9 ± 0.3	0.38 ± 0.10
Team Ball Sports	**Hockey, Lacrosse, Netball, Rugby**	35	26	61	1.70 ± 0.01	0.36 ± 0.12	67.5 ± 2.2	0.89 ± 0.13	23.1 ± 0.5	0.83 ± 0.13
TOTAL		85	72	157	1.69 ± 0.01	0.46 ± 0.07	63.1 ± 1.1	0.66 ± 0.07	21.8 ± 0.3	0.54 ± 0.07

5.3.2 *IGF-I concentration*

In male and female adolescent athletes, mean serum IGF-I concentration rose in the early teenage years and then declined. Figure 5.1 shows the IGF-I concentration plotted with the mean and 99% prediction limits obtained from the older athletes in the previous GH-2000 study (Healy et al. 2005). All observations from adolescent athletes lay below the upper 99% prediction limit extrapolated from the adult data but approximately 14% lay beneath the lower 99% prediction interval. Although there are insufficient data for an accurate analysis, peak levels appeared between 15 and 16 years in boys and may have been slightly earlier in girls.

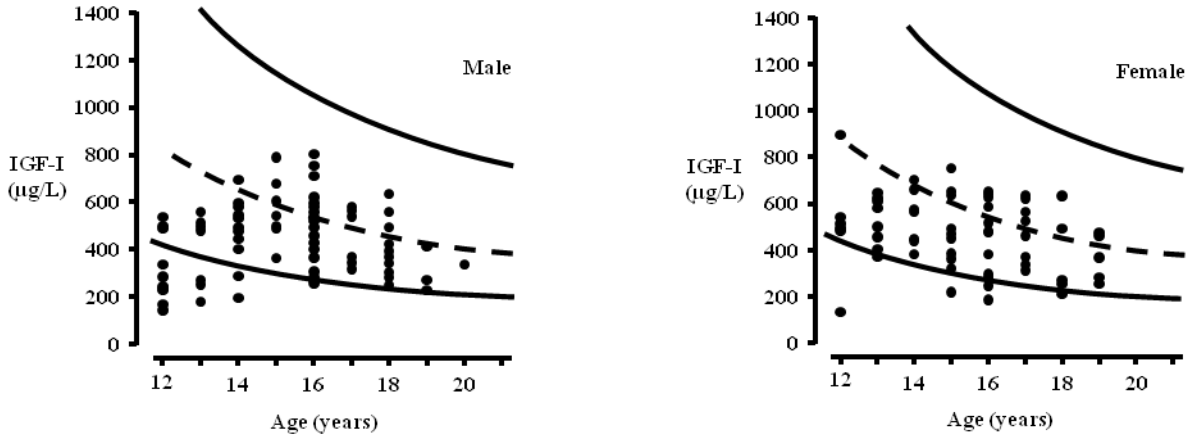

Figure 5.1. Age-dependent change in serum IGF-I concentrations from 85 elite male adolescent athletes (left panel) and 72 elite female adolescent athletes (right panel). The solid and dotted lines indicate the mean and 99% prediction intervals extrapolated from data collected from adult elite athletes in the GH-2000 study.

5.3.3 *P-III-NP concentration*

Figure 5.2 shows the P-III-NP concentration plotted with the mean and 99% prediction limits obtained from the previous GH-2000 study (Healy et al. 2005). In boys, mean serum P-III-NP concentration increased in early teenage years, peaked between 14 and 16 years and then declined (Fig 5.2, left panel). Mean P-III-NP concentration in female athletes rose between 12 and 13 years and declined from the age of 13 years onwards (Fig. 5.2, right panel). In 10 (11.8%) male and 3 (4.2%) female adolescent athletes, P-III-NP concentrations were greater than the upper 99% prediction limit extrapolated from the adult data. Furthermore, 18 (11.5%) observations lay beneath the lower 99% prediction interval.

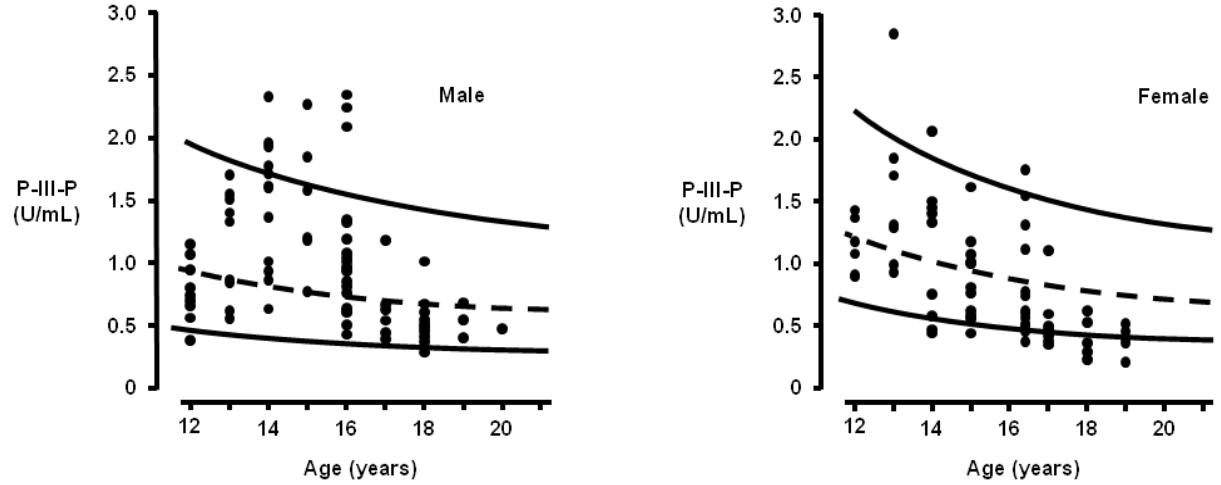

Figure 5.2. Age-dependent change in serum P-III-NP concentrations from 85 elite male adolescent athletes (left panel) and 72 elite female adolescent athletes (right panel). The solid and dotted lines indicate the mean and 99% prediction intervals extrapolated from data collected from adult elite athletes in the GH-2000 study.

5.3.4 *GH discriminant function scores for adolescent athletes*

The mean GH-2000 score for male and female adolescent athletes was -1.2 ± 0.2 (range -6.0 to 3.2) and -0.7 ± 0.2 (range -4.5 to 3.1) respectively (Table 5.2). There was a clear relationship between mean GH-2000 score and age. In boys, the GH-2000 score rose to a peak at age 15 years and then declined. The GH-2000 score showed a similar pattern in girls except the peak was at age 13 years. No adolescent athlete exceeded the previously proposed cut-off score of 3.72 indicating that none of these athletes would be accused of GH doping if the GH-2000 discriminant formulae had been used (Fig. 5.3).

Table 5.2. GH-2000 scores in male and female adolescent athletes. Scores were calculated using results from the DSL IGF-I assay and Cisbio P-III-NP assay.

	Number	GH-2000 score (mean ± SD)	Range
Male Athletes			
12-13 years	19	-3.0 ± 2.0	-6.0 to -0.1
14-15 years	20	0.2 ± 1.7	-4.1 to 2.3
16-17 years	28	-0.3 ± 1.7	-3.8 to 3.2
≥18 years	18	-1.7 ± 1.0	-3.2 to 0.8
Female Athletes			
12-13 years	14	0.0 ± 1.7	-4.1 to 3.1
14-15 years	21	-0.4 ± 1.4	-2.5 to 2.0
16-17 years	23	-0.9 ± 1.5	-3.5 to 2.5
≥18 years	14	-1.8 ± 1.3	-4.5 to 0.0

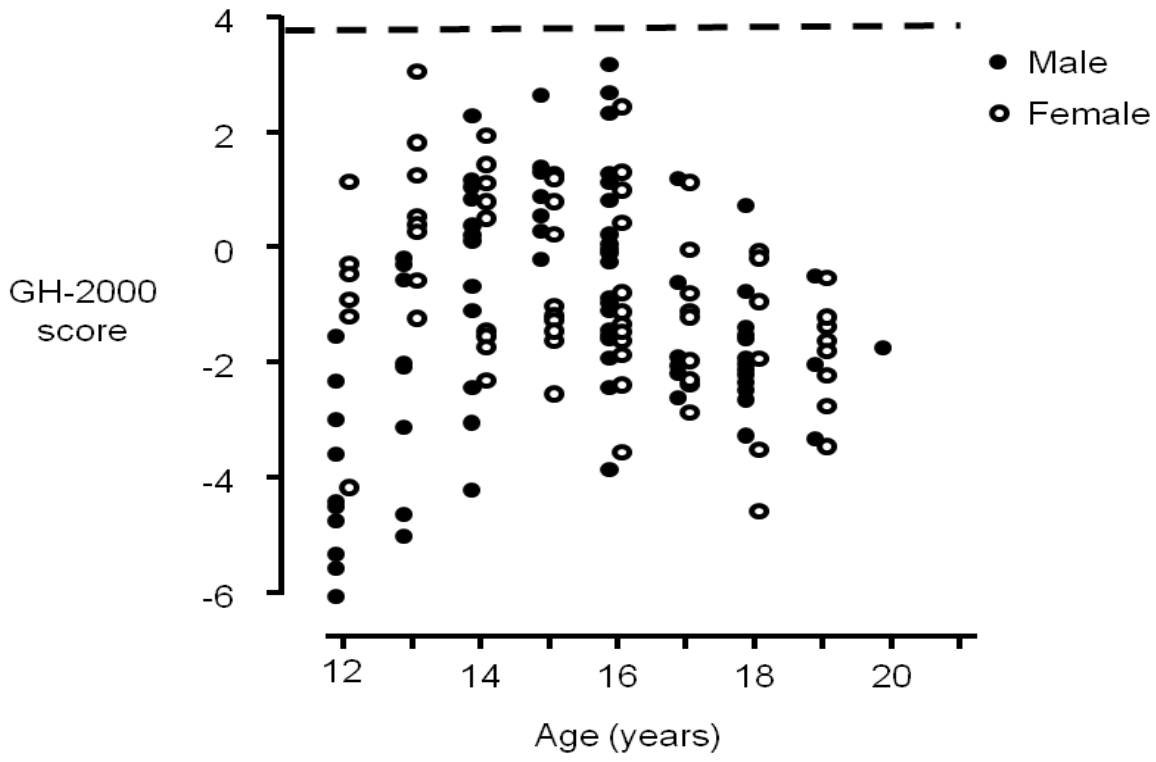

Figure 5.3. Calculated GH-2000 scores for 85 elite male and 72 elite female adolescent athletes. Scores were calculated using the results from the DSL IGF-I assay and Cisbio P-III-NP assay. The horizontal dotted line shows GH-2000 score of 3.72 (the previously proposed cut-off that suggests doping with GH).

5.3.5 *Inter-assay validation*

5.3.5.1 IGF-I assays

There was a strong correlation between Immunotech and DSL IGF-I assays with no constant bias (r = 0.909, Fig. 5.4). The equation used to convert IGF-I results between the DSL and Immunotech assays is: Immunotech IGF-I = 0.783 * DSL-5600 IGF-I.

The published conversion between DSL and the original Nichols assay used in the GH-2000 studies is: Nichols RIA IGF-I = 0.660 * DSL-5600 IGF-I (Erotokritou-Mulligan et al. 2008).

The conversion between Immunotech and Nichols assays is therefore:

Nichols RIA IGF-I = 0.843 * Immunotech IGF-I

A modified Bland-Altman plot showing the relationship between the Immunotech and DSL-5600 IGF-I assays is shown in Figure 5.5. This demonstrates that on average, the IGF-I measurements by the Immunotech assay are 20% lower than the DSL assay but that most readings lie within 2 SDs of the mean and there is no systematic bias at any point in the measured range. This is almost identical to the conversion derived from the correlation data.

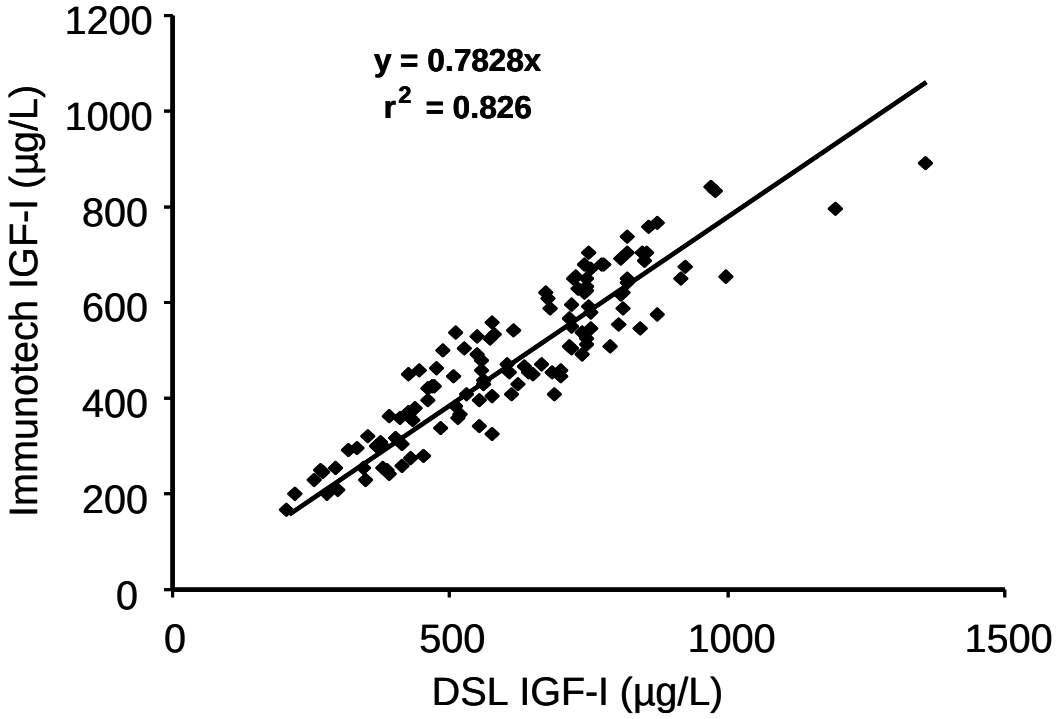

Figure 5.4. Inter-technique comparison of IGF-I assays, Immunotech and DSL-5600 IRMA; linear regression.

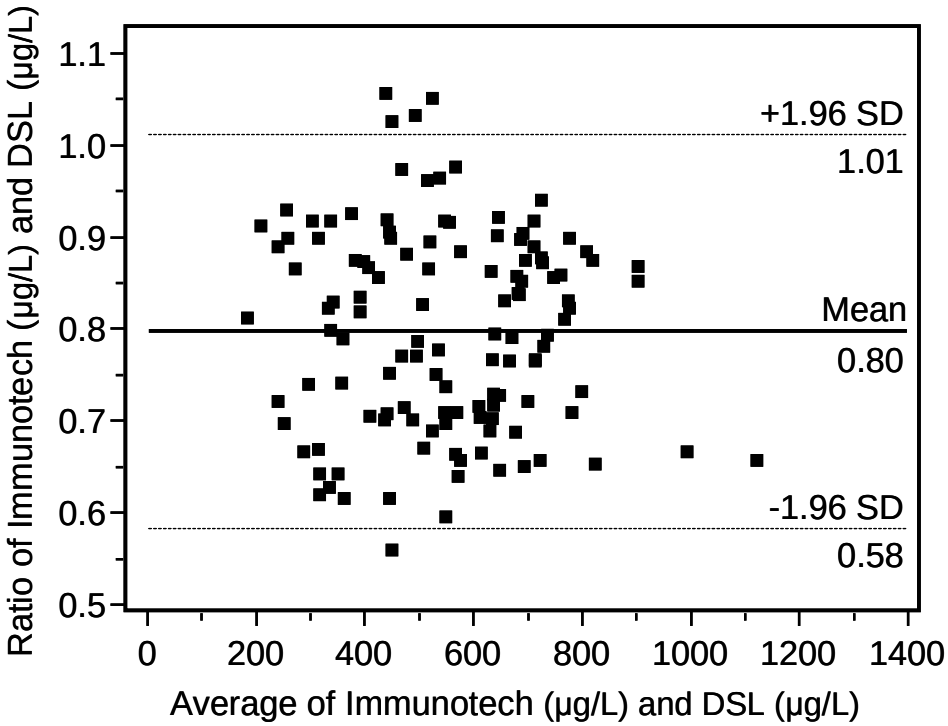

Figure 5.5 Inter-technique comparison of IGF-I assays, Immunotech and DSL-5600 IRMA; modified Bland-Altman plot.

5.3.5.2 P-III-NP assays

The strong correlation between the Cisbio and Orion P-III-NP assays (r = 0.876) is shown in Figure 5.6. As there is no international reference preparation, the two assays use different units of measurement (μg/L vs. U/mL). Using linear regression, the equation for converting P-III-NP results from Orion units to Cisbio units is:

Cisbio P-III-NP = 0.109 * Orion P-III-NP

A modified Bland-Altman plot showing the relationship between the Cisbio and Orion assays is shown in Figure 5.7. This demonstrates that on average, the P-III-NP measurements by the Orion assay are 10.5 fold greater than the Cisbio assay. Most readings lie within 2 SDs of the mean and there is no systematic bias at any point in the measured range. Furthermore this figure is similar to the conversion derived from the correlation data.

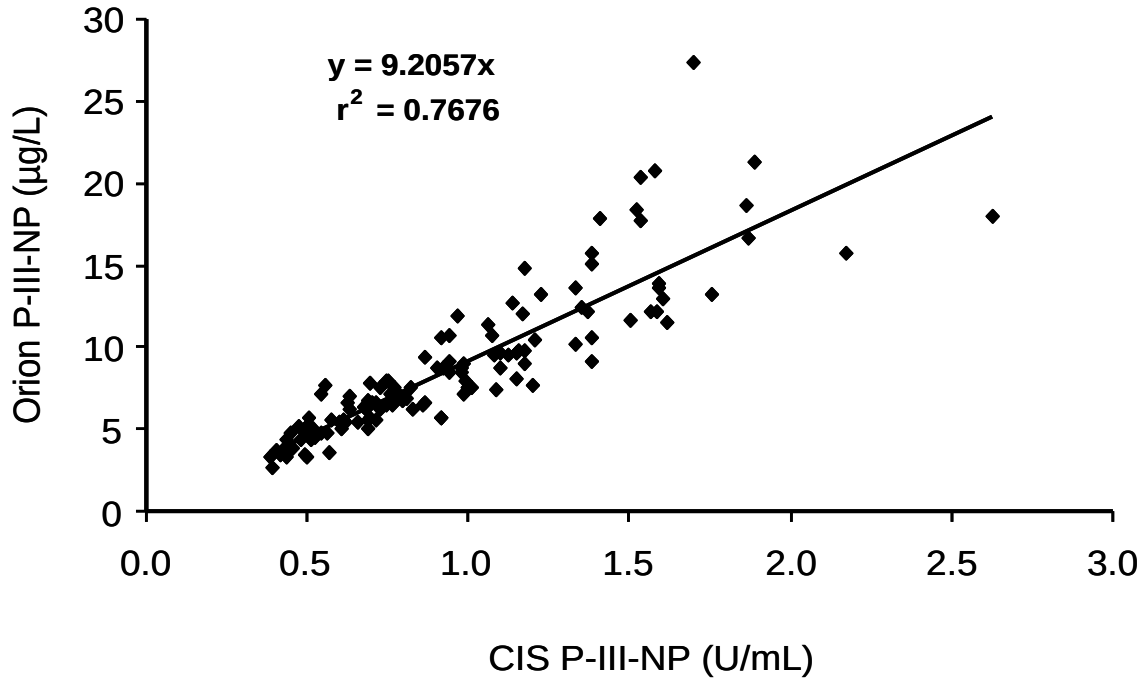

Figure 5.6. Inter-technique comparison of P-III-NP assays, Orion and Cisbio; linear regression.

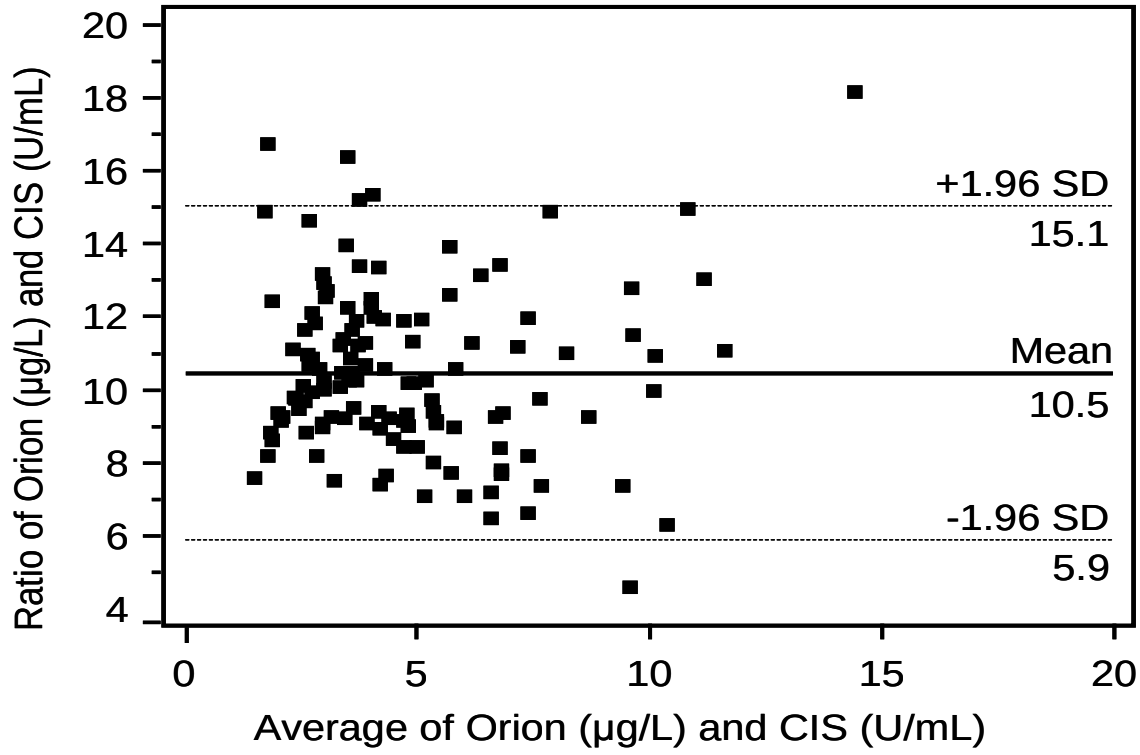

Figure 5.7. Inter-technique comparison of P-III-NP assays, Orion and Cisbio; modified Bland-Altman plot.

5.4 Discussion

This cross-sectional study demonstrates the relationship between age and serum IGF-I and P-III-NP in elite adolescent athletes. Serum IGF-I increased to a maximum in early puberty in both male and female athletes. A pubertal peak in P-III-NP concentration was also found and mean P-III-NP concentration in female athletes declined from the age of 13 years onwards, while in boys the adolescent peak was between 14 and 16 years.

The adolescent athletes were taller than average and had a higher than average BMI when compared with the age-matched cross-sectional growth standards for children from 1990. The extent to which this reflects secular changes in height and weight over the last 20 years is unknown. It is also difficult to compare the average BMI of athletes with the general population as the body composition of athletes differs markedly from normal participants and between different sporting disciplines. Athletes have an increased lean body mass to fat mass ratio and lower body fat percentage compared with a non-athletic group (Healy et al. 2005).

The GH-IGF axis plays an important role in pubertal development and deficiencies delay the onset of puberty and reduce the pace of pubertal maturation (Arsenijevic et al. 1989; Stanhope et al. 1992; Laron 1999). In non-athletic adolescents, serum IGF-I rises during childhood with a peak during puberty in girls aged 14.5 years and boys aged 15.5 years (Juul et al. 1994; Juul et al. 1997; Lofqvist et al. 2001). A similar pattern for IGF-I was observed in this study of adolescent athletes, with the peak IGF-I concentration occurring approximately two years earlier in girls than in boys.

P-III-NP is an indicator of connective tissue growth, particularly in tendons, ligaments and skin. Serum P-III-NP changes significantly with age in both male and female adolescents with a peak between 12-16 years in boys and 11-12 years in girls (Crofton et al. 1997). In the current study, the variation of P-III-NP with age in male adolescent athletes showed a similar pattern to that reported in the untrained population. As serum P-III-NP correlates with maximal height velocity in children (Trivedi et al. 1989), it is likely that the higher P-III-NP concentrations in our adolescent athletes occurred in those participants experiencing their pubertal growth spurt. The pattern of P-III-NP concentration in female athletes, compared with male athletes, demonstrated an earlier peak and subsequent decline with age.

The adolescent athlete results were compared with the 99% prediction intervals for white European adult athletes calculated by extrapolation from the GH-2000

cross-sectional study. IGF-I concentrations in adolescent athletes aged 14 and younger are significantly lower than would be predicted from the reference ranges constructed for elite adult athletes. P-III-NP concentrations, however, are higher in some adolescent athletes than would have been predicted by extrapolation from the adult reference data. As expected, the relationship between age and the two biomarkers in adolescent athletes is different from that observed in adult athletes. This was inevitable since both IGF-I and P-III-P are low in early childhood, rising to a peak during puberty and declining with age thereafter.

The GH-2000 scoring system incorporates both markers and the athlete's age into gender-specific formulae. In this study, the GH-2000 score increased in early adolescence, reached a broad peak in male athletes aged around 15 years and female athletes aged around 13 years, and then decreased. This reflects pubertal effects on serum IGF-I and P-III-NP concentrations and the difference in the timing of puberty between genders. It also demonstrates that the age-correction factors derived from the GH-2000 data involving athletes aged 18 and over do not adequately deal with the rapidly changing IGF-I and P-III-NP levels that occur around puberty. Previous studies have demonstrated that pubertal stage, rather than chronological age, is a better predictor of changes in the GH-IGF axis and P-III-NP concentrations in children (Sorva et al. 1997; Lofqvist et al. 2001) and one limitation of our study was the lack of formal pubertal staging. This was not possible because many of the training venues where the athletes were recruited were unsuitable for this examination and it would have been unlikely that such a study would receive ethics approval. Furthermore, it would not be feasible to assess the pubertal stage of athletes if this test were to be used in an anti-doping context.

From an empirical "anti-doping" perspective, no athlete exceeded the previously proposed GH-2000 cut-off score of 3.72 (suggestive of doping with GH). A number of adolescent athletes, however, had scores above 3.0 and caution would be required if the test were used around the time of peak growth velocity. In addition, the standard deviation of the GH-2000 score in the adolescent athletes was greater than 1.0, suggesting that the variability of the score is higher in this population than in adult athletes. It is therefore possible that a higher cut-off value would be required in adolescent athletes to decrease the risk of false-positive results. Another approach could be to investigate the pattern of change in these biomarkers within individual athletes throughout puberty (the "Athlete's Passport" approach (Erotokritou-Mulligan et al. 2009)). Suspicions of doping could then be raised if dramatic increases in marker concentration, in excess of normal pubertal peaks for athletes, were observed.

The use of immunoassays in analyzing markers of GH misuse gives rise to a number of problems (reviewed in Chapter 1, section 1.14). One method of dealing with changes to commercial IGF-I and P-III-NP immunoassays is to compare the results of current assays with those of the Nichols IGF-I RIA and Cisbio P-III-NP assay used in the original GH-2000 studies. In the current study, 124 samples from elite adolescent athletes were analysed using two IGF-I and two P-III-NP immunoassays that were available in 2008, when the assays were performed. There was a close correlation between the IGF-I assays despite fundamental differences in methodology. Both immunoassays are two-site immunoradiometric assays and use monoclonal antibodies but the antigen specificity of these assays is not provided by the manufacturers. The DSL method uses an acid-ethanol extraction process to separate IGF-I from IGFBPs, whereas the Immunotech assay incorporates acidification with addition of excess IGF-II to prevent further interference from IGFBPs. The small numerical differences between the two IGF-I assays is best attributed to misalignment with the current WHO standard and are likely to become less significant if the manufacturers use a pure recombinant IGF-I standard to calibrate their assays (Clemmons 2011).

The two immunoassay kits available for measuring serum P-III-NP during this study also demonstrated a very close correlation between results. The factor we derived to convert results between the Cisbio and Orion assays is very similar to that described in a previous study (Abellan et al. 2005) using 54 samples from recreational and elite athletes: (Orion P-III-NP = 9.2057 x Cisbio P-III-NP) *vs*. (Orion P-III-NP = 10.02 x Cisbio P-III-NP - 2.43). In addition, the conversion factor is consistent with the results of our subsequent study using samples from 496 elite adult athletes, described in Chapter 7. The main concern when comparing results from these P-III-NP assays is that no international reference standard is available and the exact nature of the standards used by manufacturers for calibration of their assays is not described.

In conclusion, this study demonstrated that both IGF-I and P-III-NP rise to a peak during adolescence in elite athletes in a similar pattern to that in the general population. The GH-2000 score derived from these markers rises in early adolescence, reaches a peak in athletes aged 13-16 years and then falls. We have found no evidence that using the equations derived to calculate the GH-2000 score (developed in adults aged 18 and over) would lead to an unacceptable rate of false positive results in adolescent athletes, although caution will be prudent when using this test around the time of peak growth velocity.

CHAPTER 6: THE EFFECTS OF A FREEZE-THAW CYCLE AND PRE-ANALYTICAL STORAGE TEMPERATURE ON THE STABILITY OF IGF-I AND P-III-NP CONCENTRATIONS

6.1 Introduction

The stability of GH-dependent markers over time and under different storage conditions must be established before they can be used as part of a doping test. The variability of IGF-I and P-III-NP concentrations in serum stored at -80°C and during transport at 4°C had previously been established (Holt et al. 2009). Anti-doping laboratories may not, however, have access to -80°C storage facilities. In addition, during major sporting events, blood samples are taken at sites where access to chilled storage or centrifugation facilities may not be available; blood samples may also be analysed immediately without prior freezing. It is important that these sources of pre-analytical variability are investigated to ensure the reliability of assay results.

The aims of this study were to investigate the stability of IGF-I and P-III-NP concentrations in serum stored at -20°C and to establish the effects of a freeze-thaw cycle on immunoassay results.

6.2 Methods

20 healthy volunteers (12 men, 8 women; mean age 27.8 ± 0.8 years, range 22-34 years) were recruited to the study through personal contacts at UK Anti-Doping. Exclusion criteria included previous history of endocrinopathy and previous use of performance-enhancing drugs. Demographic data on gender, age, ethnic origin, physical activity, diet, injuries, medications, menstrual history, self-reported height and weight were recorded. 20mL of whole blood was collected and processed as described in section 2.1.1.3. After centrifugation, the serum was divided into 1 mL aliquots. 1 aliquot from 10 volunteers was analysed immediately. There was insufficient time to analyse samples from all 20 participants on the day of sample collection. The remaining aliquots and those from the other 10 participant were stored overnight at 4°C and then frozen at -20°C. Aliquots from all 20 participants were analysed after storage for 1 day at 4°C, 1 day at -20°C, 1 week at -20°C, 5 weeks at -20°C and 3 months at -20°C.

All serum samples were analysed at the Drug Control Centre, King's College London. The 10 "fresh" samples assayed immediately after centrifugation were analysed using the DSL 10-5600 IGF-I ELISA and the Orion UniQ™ P-III-NP RIA as described in section 2.1.3.3. Samples from all remaining time-points were analysed using the DSL 10-5600 IGF-I ELISA, the Immunotech A15729 IGF-I IRMA, the Orion UniQ™ P-III-NP RIA and the RIA-gnost P-III-NP assay from Cisbio as described in section 2.1.4.3. All samples in this study were analysed in duplicate.

Assay results were converted to GH-2000 assay scales as described in section 2.2.1 and then incorporated into the GH-2000 discriminant function formulae. Analysis of variance (ANOVA) was performed to estimate intra-sample variability in IGF-I and P-III-NP concentrations and in GH-2000 score. Intra-sample variability describes the variability in results from aliquots exposed to different storage conditions. Inter-assay variability was determined by analyzing one quality control (QC) sample on the same day as the participant samples. Analysis of variance was performed by Dr Erotokritou-Mulligan using SAS software (SAS Institute, Cary, NC, USA).

My role in this study was as follows: I contributed to the study design and protocol when the study was proposed to WADA. I was responsible for collecting all of the serum samples used in the study, along with participant demographic data. Data from this study have been published in "Guha N *et al.* The effects of a freeze-thaw cycle and pre-analytical storage temperature on the stability of insulin-like growth factor-I and pro-collagen type III N-terminal propeptide concentrations: Implications for the detection of growth hormone misuse in athletes. Drug Test Anal. **2012** Jun; 4(6):455-9." I am the first author on this publication and I was responsible for drafting and editing the manuscript along with other members of the GH-2004 team.

6.3 Results

6.3.1 *IGF-I concentration*

Figure 6.1 shows the individual changes in IGF-I concentration with time and storage conditions as measured by the DSL ELISA and Immunotech A15729 IRMA.

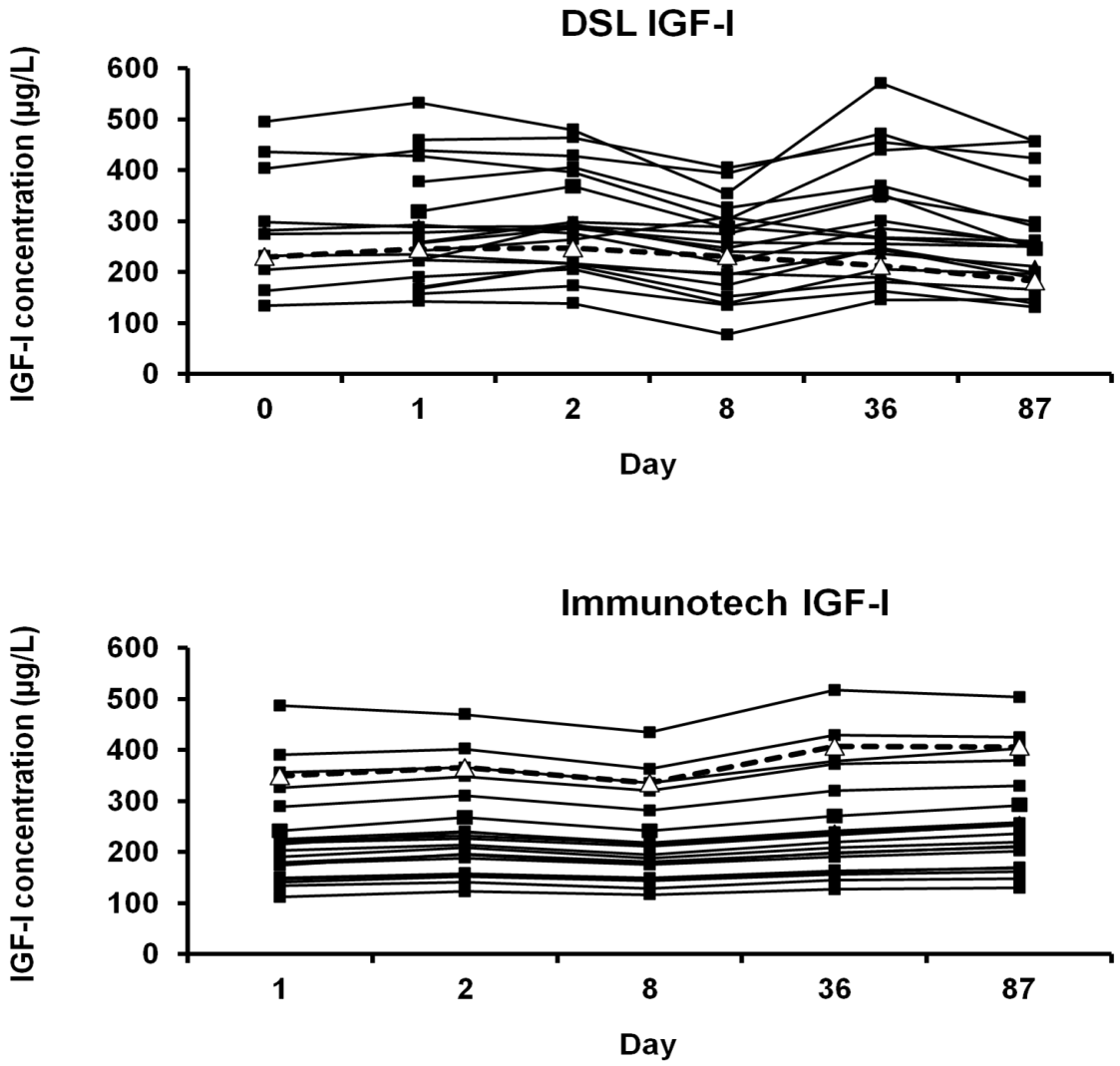

Figure 6.1. The effects of one freeze-thaw cycle and storage temperature on individual values of IGF-I. Samples were analysed using the DSL 10-5600 ELISA (upper panel) immediately after centrifugation (Day 0), after 1 day at 4°C (Day 1), after 1 day at -20°C (Day 2), after 7 days at -20°C (Day 8), after 35 days at -20°C (Day 36) and after 86 days at -20°C (Day 87). Samples were also analysed using the Immunotech A15729 IRMA (lower panel) at all time-points except immediately after centrifugation. One quality control sample (Δ and dashed line) was analysed at all time-points.

A single freeze-thaw cycle, storage of serum at 4°C for one day and at -20°C for up to three months had no significant effect on IGF-I results. The estimated intra-sample variability for IGF-I concentration along with the corresponding inter-assay variability (determined by analysing one QC sample at each time-point) is shown in Table 6.1.

6.3.2 *P-III-NP concentration*

A single freeze-thaw cycle, storage of serum at 4°C for one day and at -20°C for up to three months had no significant effect on P-III-NP results (Fig. 6.2). The estimated intra-sample variability for P-III-NP concentration along with the corresponding inter-assay variability (determined by analysing one QC sample at each time-point) is shown in Table 6.1.

Table 6.1. The intra-sample variability (Coefficient of Variation, CV) for IGF-I and P-III-NP results and corresponding inter-assay CV. Inter-assay CV was estimated from quality control (QC) results.

	Intra-sample CV	Inter-assay CV
IGF-I Assay		
DSL 10-5600 ELISA	12.9 %	10.7%
Immunotech A15729 IRMA	6.8 %	8.8%
P-III-NP Assay		
Orion UniQ™ P-III-NP	13.7%	5.0%
Cisbio RIA-gnost P-III-NP	10.9%	11.7%

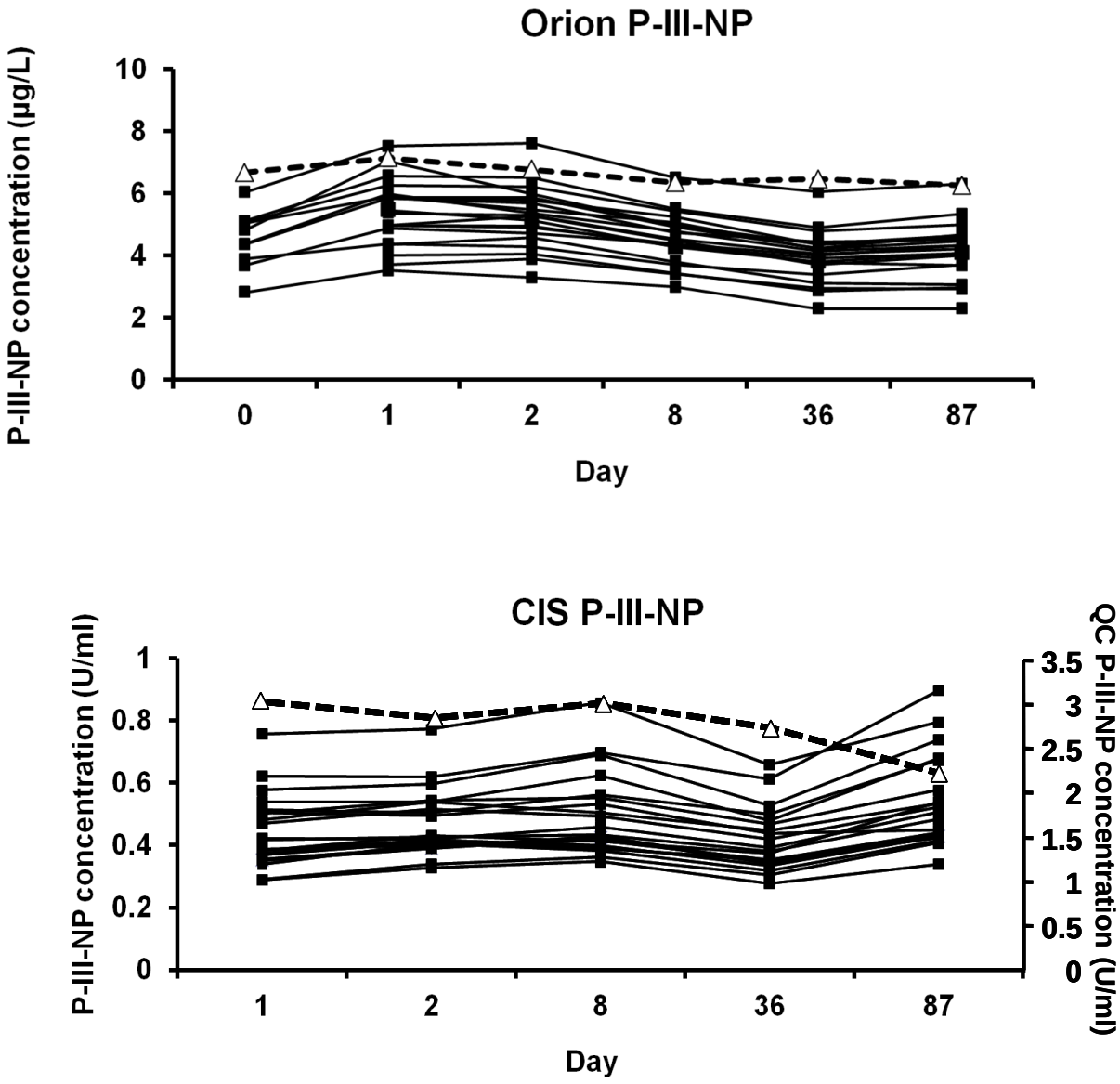

Figure 6.2. The effects of one freeze-thaw cycle and storage temperature on individual values of P-III-NP. Samples were analysed using the Orion UniQ™ RIA (upper panel) immediately after centrifugation (Day 0), after 1 day at 4°C (Day 1), after 1 day at -20°C (Day 2), after 7 days at -20°C (Day 8), after 35 days at -20°C (Day 36) and after 86 days at -20°C (Day 87). Samples were also analysed using the Cisbio RIA-gnost assay (lower panel) at all time-points except immediately after centrifugation. One quality control sample (Δ and dashed line) was analysed at all time-points.

6.3.3 *GH-2000 discriminant function scores*

GH-2000 discriminant function scores were calculated using combinations of IGF-I and P-III-NP assay results (Fig. 6.3). The estimated intra-sample variability (expressed as standard deviations) for the GH-2000 score was 0.50 (Orion P-III-NP and DSL IGF-I combination), 0.35 (Orion P-III-NP and Immunotech IGF-I combination), 0.31 (Cisbio P-III-NP and DSL IGF-I combination) and 0.35 (Cisbio P-III-NP and Immunotech IGF-I combination). None of the GH-2000 scores were more than 3.72 SDs above the mean (a cut-off point which would give a false-positive rate of approximately 1 in 10,000 tests).

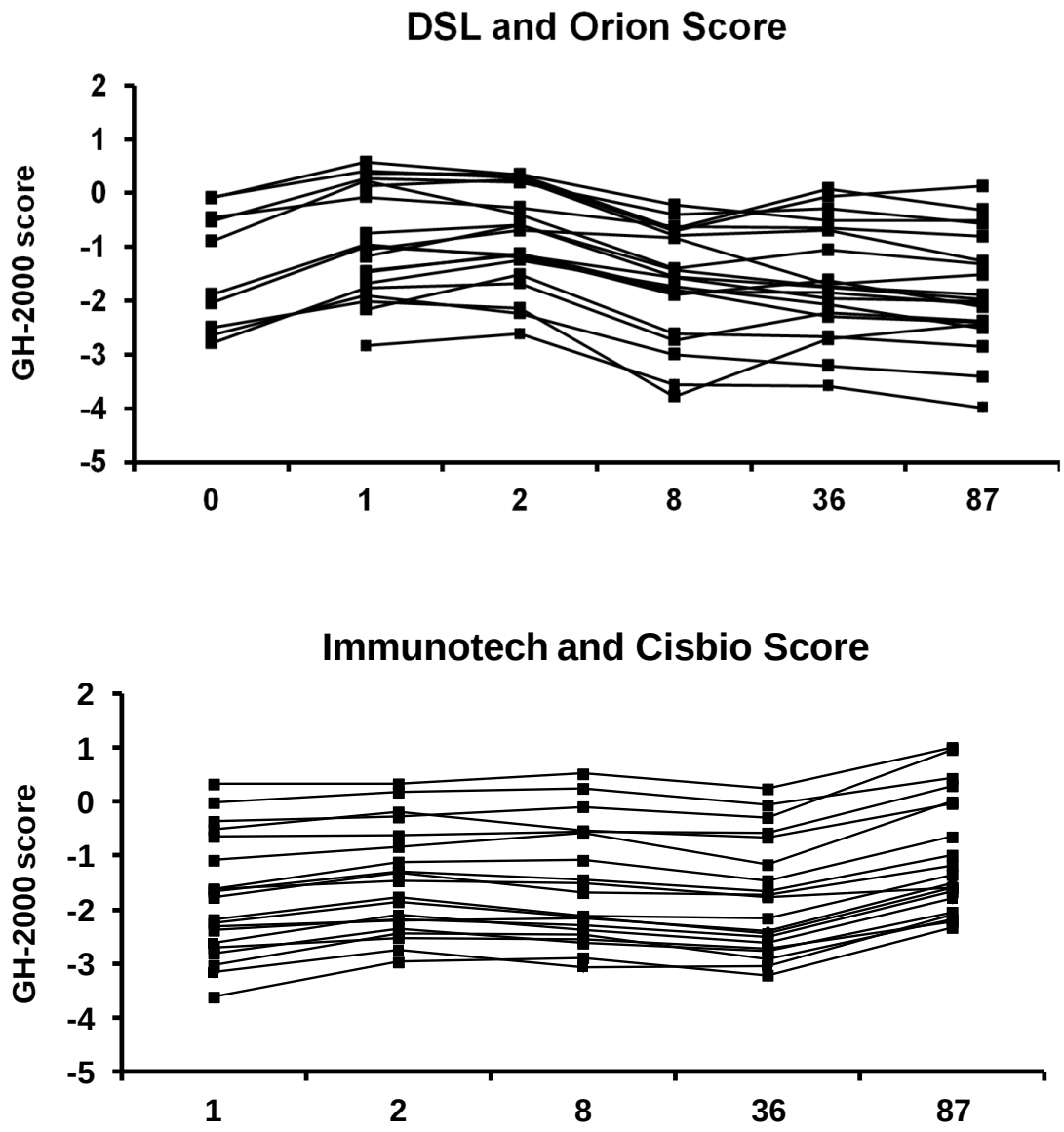

Figure 6.3. The effects of one freeze-thaw cycle and storage temperature on individual values of GH-2000 score. Scores were calculated by combining results from the DSL IGF-I and Orion P-III-NP assays (upper panel) and from the Immunotech IGF-I and Cisbio P-III-NP assays (lower panel) using the GH-2000 discriminant function formulae (Powrie et al. 2007).

6.4 Discussion

The results of this study show that the variability in IGF-I and P-III-NP results of stored samples is largely determined by the precision of the assays. IGF-I and P-III-NP concentrations and GH-2000 discriminant function scores were unaffected by a single freeze-thaw cycle, by storage at 4ºC for one day or by storage at -20ºC for up to three months.

These results compliment the findings of the previous GH-2004 study, which showed that storage of serum or clotted blood samples at 4ºC for up to five days did not result in any significant changes in IGF-I and P-III-NP concentrations (Holt et al. 2009). Several other studies have investigated the effects of pre-analytical storage conditions on the stability of IGF-I results in blood samples. Delays in centrifugation of whole blood samples stored at room temperature can lead to increased IGF-I concentrations as IGF-I is released from lysed or dying blood cells and also dissociates from IGF binding proteins (Hartog et al. 2008). Harris *et al.* showed that serum IGF-I increased significantly if whole blood was stored at room temperature for 24 hrs before centrifugation, but if the samples were centrifuged soon after blood collection and stored as serum aliquots, there was no significant change in results after 24 hrs (Harris et al. 2006). By contrast, Kristal *et al.* showed a decrease in IGF-I concentrations in blood samples collected into tubes containing the anticoagulant ethylenediaminetetraacetic acid (EDTA) after delays in centrifugation of between 32 and 144 hrs (Kristal et al. 2005). The previous GH-2004 study showed that EDTA is not a suitable collection medium for GH-sensitive markers because it exerts a significant matrix effect on P-III-NP analysis (Holt et al. 2009). Ideally delays in centrifugation should be avoided but if this test is used in an out-of-competition anti-doping setting with sample collection at the athlete's training venue or home, immediate access to a centrifuge will be difficult. Since this may result in delayed centrifugation, it is essential that the samples are kept chilled during transportation. This is reflected in the WADA guidelines for blood sample collection, which state that samples should be transported to the laboratory in a refrigerated state. No sample should be allowed to freeze, and should ideally be kept at a temperature of approximately 4°C (WADA 2011).

IGF-I concentrations are highly stable in serum stored at -20ºC for up to three months and therefore it appears these storage conditions are acceptable for serum samples collected for detection of GH misuse. In comparison with -80ºC storage facilities, -20ºC freezers are widely available both in laboratories and also in other locations

potentially closer to the site of blood collection, which could be an advantage for sample transport and storage.

None of the pre-analytical storage conditions investigated in the present study resulted in a significant change in P-III-NP concentration. This is in contrast to the previous GH-2004 study in which serum P-III-NP concentrations rose significantly if blood samples were stored at room temperature either as serum or clotted blood (Holt et al. 2009). This emphasised the need for anti-doping authorities to ensure that blood samples are kept chilled during transportation to the laboratory. The rise in P-III-NP at room temperature may result from cleavage of the P-III-NP molecule by collagenases to produce P-III-NP fragments and exposure of new antigenic sites (Holt et al. 2009). Storage of samples at -20ºC appears to inhibit these processes and therefore the rise in P-III-NP.

Freezing and thawing serum can alter the measured concentrations of serum proteins (Petrakis 1985) because protein denaturation may occur during the freeze-thaw cycle (Pinsky et al. 2003). In the previous GH-2000 and GH-2004 studies, all samples underwent at least one freeze-thaw cycle but in anti-doping testing at major sporting events, samples are often analysed soon after collection without being frozen. This study has shown that one freeze-thaw cycle has no significant effect on IGF-I or P-III-NP results and that it is acceptable to measure fresh, unfrozen samples to detect GH doping.

The storage of serum at 4ºC overnight or frozen at -20ºC for up to three months had no significant effect on the GH-2000 score. The standard deviation of the GH-2000 score is dependent on the CVs of IGF-I and P-III-NP values. Score variability cannot be expressed as a percentage because it is impossible to calculate a percentage change from zero. The intra-sample standard deviation of GH-2000 scores varied between 0.31 and 0.50, depending on the assay combination used. This indicates that the GH-2000 score of a sample may vary by up to 1.0 (two standard deviations) from the mean score for that sample, when exposed to these pre-analytical conditions. This score variability is largely determined by the inter-assay variability of the IGF-I and P-III-NP assays.

It is of considerable interest that the apparent variability in GH-2000 score in this study is very similar to that found in elite athletes when multiple blood samples were taken and analysed over the course of a year (Erotokritou-Mulligan et al. 2009). It is likely that this apparent variability is entirely accounted for by assay variability and

in fact, marker concentrations may not vary at all. When more precise mass spectrometry assays become available for these protein analytes, this apparent variability will fall.

It is important to note that this study was performed using healthy volunteers rather than elite athletes. The IGF-I and P-III-NP concentrations and therefore GH-2000 scores in elite athletes are likely to be higher than in these healthy volunteers and this has been taken into account when setting the decision limits described in Chapter 7. We have found no evidence, however, that the variability in results is dependent on analyte concentration and therefore we have proposed that these findings are applicable to samples taken from elite athletes.

In conclusion, this study shows that both IGF-I and P-III-NP concentrations are stable in serum stored at 4°C overnight and at -20ºC for up to three months. Furthermore, a single freeze-thaw cycle has no significant effect on assay results. It is therefore acceptable for anti-doping laboratories to analyse samples immediately after centrifugation or for samples to be transported at 4ºC and serum stored at -20ºC for up to three months, prior to analysis.

CHAPTER 7: CROSS-SECTIONAL STUDY OF ELITE ATHLETES AND DEVELOPMENT OF DECISION LIMITS FOR THE IMPLEMENTATION OF THE GH-2000 DETECTION METHOD

7.1 Introduction

The GH-2000 discriminant formulae incorporating IGF-I and P-III-NP results were based on measurements from the Nichols Institute Diagnostics IGF-I radioimmunoassay (RIA), which is no longer available, and Cisbio P-III-NP immunoradiometric assay. In 2008, WADA recommended that the calculation of GH-2000 scores using the assay conversion methods described in Chapters 5 and 6 should not be used for forensic purposes. Their preference was that GH-2000 scores should be calculated using results from current immunoassays and that decision limits for determining GH misuse should be defined for each combination of IGF-I and P-III-NP assay. Furthermore, WADA rules state that any analyte measured by immunoassay should be measured by two separate assays that recognise different epitopes (WADA 2008). Thus it was necessary to validate two IGF-I assays that are currently available to anti-doping laboratories and a further P-III-NP assay before the test could be introduced.

The aims of this study were to measure IGF-I and P-III-NP in elite athletes using two commercial assays for each analyte and to develop appropriate decision limits to determine whether an athlete has been misusing GH.

7.2 Methods

In collaboration with UK Anti-Doping (UKAD), twenty-four sporting organisations were contacted to obtain permission to approach elite athletes (representing national level or above). Permission was obtained to attend training sessions at the organisations detailed in section 2.1.4.1. Participants were required to confirm that they had not taken performance-enhancing drugs prior to taking part. 260 athletes agreed to participate in the study. Demographic data on gender, age, ethnic origin, sport, training hours, diet, injuries, medications, menstrual history, self-reported height and weight were recorded. Venous blood samples were collected either before or after exercise according to WADA guidelines as described in section 2.1.1.3. All samples were anonymised before analysis. A further 238 samples were collected from

elite athletes as part of the UK Anti-Doping testing programme. These athletes had provided consent for their samples to be used for research purposes and these samples were also collected according to WADA guidelines for blood sample collection.

Serum aliquots were transported frozen on dry ice to the Drug Control Centre, King's College in London for analysis of serum IGF-I and P-III-NP concentrations. Serum IGF-I was measured using the Siemens Immulite IGF-I assay (Siemens Medical Solutions Diagnostics Limited, Llanberis, UK) and the Immunotech A15729 IGF-I IRMA (Immunotech SAS, Marseille, France). Serum P-III-NP was measured using the RIA-gnost P-III-NP assay from Cisbio (Gif-sur-Yvette, France) and the UniQ™ P-III-NP RIA (Orion Diagnostica, Espoo, Finland). These four assays are described in detail in section 2.1.1.4. Analyses were performed in duplicate for all assays except the Siemens Immulite IGF-I assay for which analysis was performed in singlicate, according to the manufacturer's recommendations.

The statistical analysis of these results was carried out by Dr Erotokritou-Mulligan, GH-2004 Project statistician. Inter-assay agreement between the Siemens Immulite and Immunotech IGF-I assays and between the Cisbio and Orion P-III-NP assays was evaluated using simple linear regression. Discriminant function scores were calculated using the methods described in section 2.2.1. Decision limits for a combined test using two assay pairings were calculated as described in section 2.2.6.2. The proposed decision limits were based on data from our sample of elite athletes and therefore it was necessary to estimate a degree of uncertainty around these limits as described in section 2.2.6.2.

My role in this study was as follows: I contributed to the study design and protocol when the study was proposed to WADA. I was responsible for attending the majority of the training sessions described in section 2.1.4.1 and recruiting the athletes. I collected the majority of the 260 serum samples we provided for the study, along with athlete demographic data. The remaining samples were collected by Professor Richard Holt (Principal Investigator), medical staff from the participating sporting organisations and UK Anti-Doping Doping Control Officers. Data from this study have been published in "Erotokritou-Mulligan, I *et al*. The development of decision limits for the implementation of the GH-2000 detection methodology using current commercial insulin-like growth factor-I and amino-terminal pro-peptide of type III collagen assays, *Growth Horm IGF Res*, **22**(2): 53-8". I am the second author on this paper and contributed to the writing and editing of the manuscript throughout the submission process.

7.3 Results

Blood samples were collected from 404 men and 94 women. 168 men and 92 women were recruited by our research team and 236 men and 2 women were recruited through the UK Anti-Doping testing programme. The men had an average age of 23.9 yrs (range 12-37 yrs) and women had an average age of 24.5 yrs (range 18-34 yrs). Of the 260 athletes recruited by our research team, 219 were white European, 18 Mixed Race, 13 Caribbean, 7 African, 2 Polynesian and 1 Maori. No ethnicity information was available for the athletes recruited through the UK Anti-Doping programme. One male participant was excluded from the analysis because there was insufficient serum to complete all four assays. Furthermore, during the preliminary analysis of these data, one male participant was identified with results that were incompatible with normal physiology. These results were reported to UK Anti-Doping who informed us that this participant's sample had tested positive with the WADA isoform GH test. The results for this participant are included in the figures below to demonstrate how this individual's sample would have been declared as an adverse analytical finding; however, his results were excluded from the calculations used to estimate the GH-2000 score decision limits.

Consequently results from 496 athletes (402 men, 94 women) were used to determine the decision limits.

7.3.1 *IGF-I inter-assay comparison*

Paired analysis of the 496 elite athlete samples showed that there is a good correlation (R=0.85) between the Immulite and Immunotech IGF-I assays (Fig. 7.1), in keeping with expected differences resulting from assay variation. Results from the Immunotech assay were approximately 5.0 ± 0.7% higher than the Immulite assay.

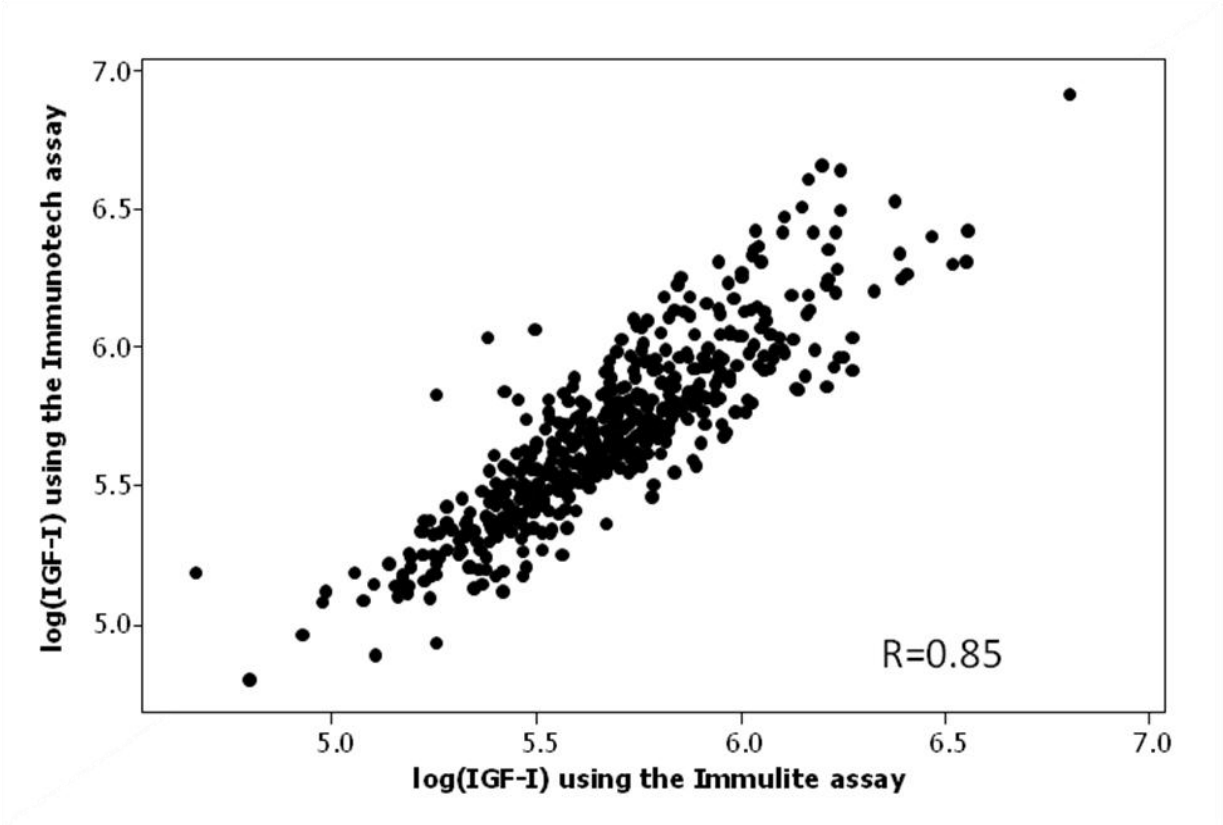

Figure 7.1. Correlation of IGF-I using log-transformed measurements from the Immunotech and Immulite IGF-I immunoassays.

7.3.2 *P-III-NP inter-assay comparison*

Paired analysis of the elite athlete samples showed that there is a good correlation (R=0.76) between the Orion and Cisbio P-III-NP assays (Fig.7.2), again in keeping with known assay imprecision. The numerical differences in results relate to the differences in measurement units (micrograms/litre *vs.* Units/millilitre) but systematic changes in Cisbio assay results were accompanied by systematic changes in Orion assay results.

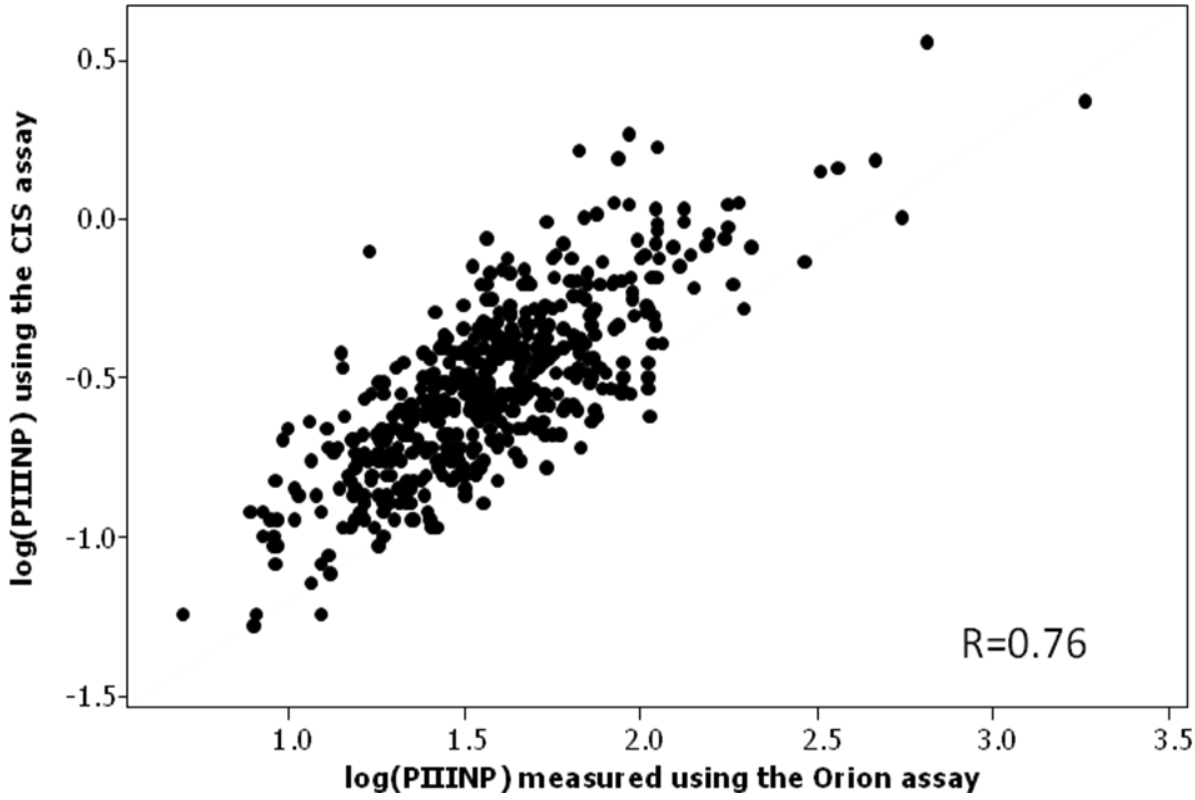

Figure 7.2. Correlation of P-III-NP using log-transformed measurements from the Cisbio and Orion P-III-NP immunoassays.

7.3.3 *GH-2000 scores and proposed decision limits*

GH-2000 scores were calculated for each elite athlete using the four possible combinations of IGF-I and P-III-NP assays. Figures 7.3 and 7.4 and Tables 7.1 and 7.2 show the distributions and corresponding decision limits derived for each assay pairing, to detect GH misuse with a false positive rate of 1 in 10,000 (99.99% specificity) in men and women respectively.

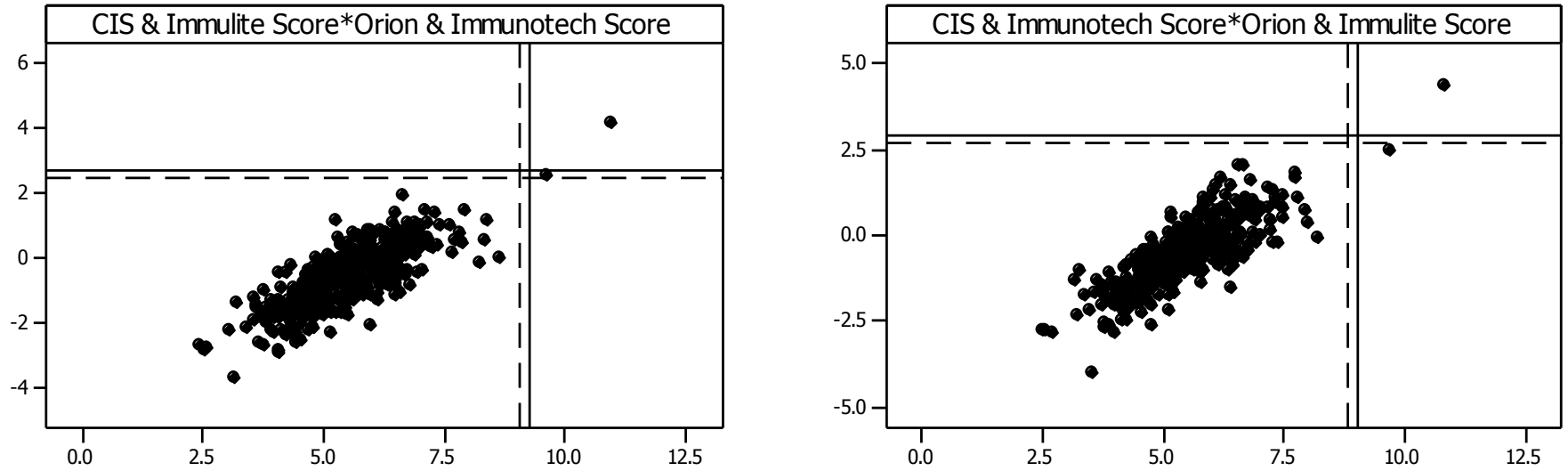

Figure 7.3. GH-2000 scores for each of the possible assay combinations for 403 male elite athletes. Left panel shows the combination of Orion and Immunotech results on the *x* axis and Cisbio and Immulite results on the *y* axis. Right panel shows the combination of Orion and Immulite results on the *x* axis and Cisbio and Immunotech results on the *y* axis. The upper 99.99% thresholds are drawn using dashed lines. The sample size uncertainty limits are drawn using solid lines (these are the proposed thresholds for use in an anti-doping test). These diagrams include the male athlete who was excluded from estimation of the cut-offs because of a suspected protocol violation.

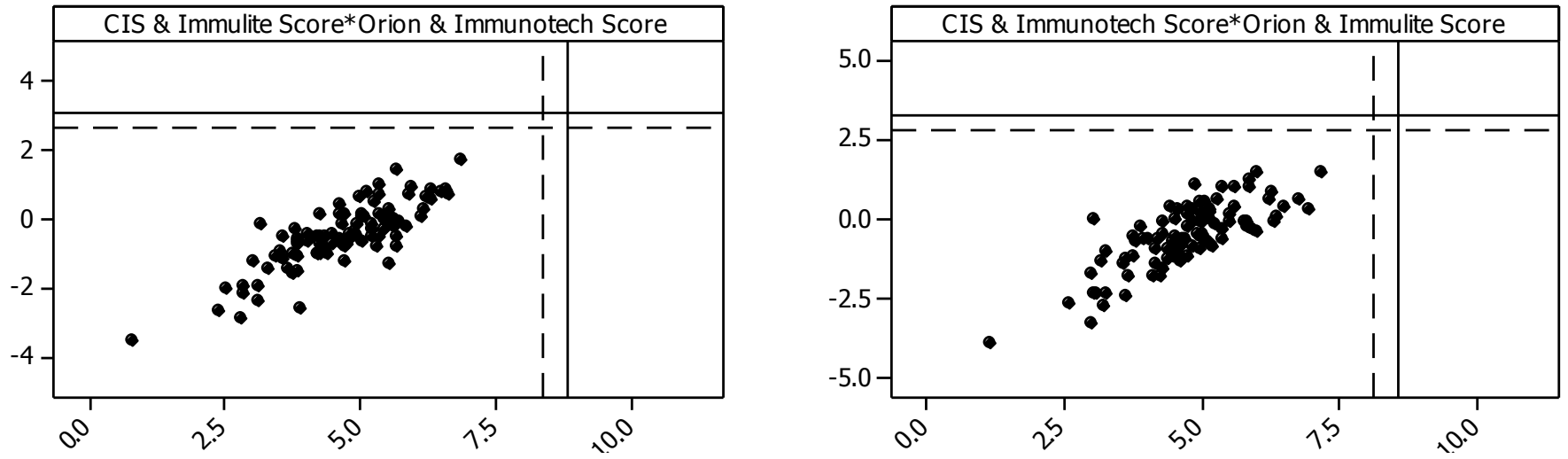

Figure 7.4. GH-2000 scores for each of the possible assay combinations for 94 female elite athletes. Left panel shows the combination of Orion and Immunotech results on the *x* axis and Cisbio and Immulite results on the *y* axis. Right panel shows the combination of Orion and Immulite results on the *x* axis and Cisbio and Immunotech results on the *y* axis. The upper 99.99% thresholds are drawn using dashed lines. The sample size uncertainty limits are drawn using solid lines (these are the proposed thresholds for use in an anti-doping test).

Table 7.1. The mean GH-2000 scores, SD, 99.99% upper threshold and sample size uncertainty limits in 402 male elite athletes using an individual and combined assay specificity of 1 in 10,000 for each assay pairing. The values in the final column are those proposed for use in the anti-doping test.

MEN				Individual test specificity of 1 in 10,000			Combined test specificity of 1 in 10,000		
Assays	n	GH-2000 score mean	GH-2000 score SD	99.99% upper threshold level	Sample size uncertainty	Decision Limit	99.99% upper threshold level	Sample size uncertainty	Decision Limit
Cisbio & Immulite	402	-0.55	0.92	2.89	0.21	3.10	2.50	0.19	**2.69**
Cisbio & Immunotech	402	-0.51	0.96	3.06	0.22	3.28	2.70	0.20	**2.91**
Orion & Immulite	402	5.49	0.99	9.19	0.23	9.42	8.82	0.21	**9.03**
Orion & Immunotech	402	5.52	1.07	9.49	0.25	9.73	9.04	0.22	**9.26**

Table 7.2. The mean GH-2000 scores, SD, 99.99% upper threshold and sample size uncertainty limits in 94 female elite athletes using an individual and combined assay specificity of 1 in 10,000 for each assay pairing. The values in the final column are those proposed for use in the anti-doping test.

WOMEN				Individual test specificity of 1 in 10,000			Combined test specificity of 1 in 10,000		
Assays	n	GH-2000 score mean	GH-2000 score SD	99.99% upper threshold level	Sample size uncertainty	Decision Limit	99.99% upper threshold level	Sample size uncertainty	Decision Limit
Cisbio & Immulite	94	-0.52	0.94	2.99	0.45	3.44	2.66	0.41	**3.07**
Cisbio & Immunotech	94	-0.57	1.03	3.25	0.49	3.74	2.83	0.44	**3.27**
Orion & Immulite	94	4.72	1.03	8.56	0.49	9.05	8.14	0.45	**8.59**
Orion & Immunotech	94	4.68	1.09	8.73	0.52	9.25	8.35	0.48	**8.82**

Tables 7.1 and 7.2 include the "sample size uncertainty limits" which reflect the adjustment required to take account of the finite sample size in this study. As this test is used as part of an anti-doping programme and more data become available, the extent of this uncertainty will decrease and we anticipate that the "sample size uncertainty limits" will fall towards the estimated 99.99% upper threshold level.

We calculated the decision limits to produce a combined test with an estimated specificity of 99.99%. Using the combined approach, a sample can only be declared as positive if it exceeds the thresholds using both pairs of assays. These thresholds are shown in Tables 7.1 and 7.2.

7.4 Discussion

In this study we determined decision limits for the GH-2000 biomarker detection method using two commercial assays. These decision limits were calculated using samples from an elite athlete population, the population in which the test is designed to be used. A sample size uncertainty correction was included in the proposed decision limits to take account of the finite sample size included in the study.

There were two abnormal samples within this dataset. One sample which exceeded the decision limits for both assays pairings was from a man whose sample was obtained through the UK Anti-Doping testing programme. This result was communicated to UK Anti-Doping so that the athlete could be informed of these findings for medical reasons, for example he may have developed acromegaly. We were informed that the same sample had tested positive with the current GH isoform test and appropriate action had been taken. This sample was therefore excluded from the estimation of decision limits.

A second sample was positive using one assay pairing and was close to the decision limit of the other assay pairing (Fig. 7.3). This sample was also obtained from a male athlete through the UK Anti-Doping testing programme. His P-III-NP result was markedly elevated but his IGF-I result was within the normal range. No explanation was available for the elevated P-III-NP and his results are compatible with those expected from an athlete who had been misusing GH previously but discontinued treatment several days prior to the test. Since this result was obtained during a research project, it was not communicated formally to UK Anti-Doping. In a real life anti-doping context, this result could be used as intelligence for further testing of the same individual. As there was no definitive evidence that this athlete had been doping, his results were included in the estimation of decision limits (further analysis

showed that excluding this result had only a trivial effect on these limits). The two highest P-III-NP results were from adolescent athletes and may reflect pubertal changes in serum P-III-NP concentrations, as described in more detail in Chapter 5. Their corresponding IGF-I results and GH-2000 scores were within the normal range.

The sample size of 496 elite athletes provides a good estimation of appropriate decision limits but a further safeguard against false positive results was added by considering "sample size uncertainty". Collecting more normative data from elite athletes will allow a more precise assessment of the decision limits by reducing this uncertainty. In Table 7.3 below, the combination of Orion and Immunotech assays is used to illustrate how increasing the number of samples reduces the sample size uncertainty adjustment. We have proposed to WADA that the sensitivity of the test will be improved by employing a dynamic approach where the decision limits are continually refined as more normative data are obtained through the UK Anti-Doping and other national testing programmes.

Table 7.3. The relationship between the sample size required and sample size uncertainty (using the Orion and Immunotech assay combination in women as an example). Increasing the number of samples reduces the sample size uncertainty adjustment that is required.

	Sample size uncertainty			
	0.10	**0.15**	**0.20**	**0.30**
Sample size required for combined test specificity of 1 in 10,000	2139	951	535	238
Sample size required for individual test specificity of 1 in 10,000	2542	1130	636	283

One limitation of this study is that the majority of volunteers were white European and therefore it was not possible to determine if threshold levels were affected by ethnicity. Results from previous research, however, show that people from non-white European ethnic backgrounds do not have statistically different GH-dependent marker results or GH-2000 scores, compared with white Europeans (Nelson et al. 2006; Erotokritou-Mulligan et al. 2008). Therefore it is unlikely that alternative decision limits will be required for athletes from different ethnic groups. A further

consideration is that we cannot be certain that all samples in this study came from "clean" athletes (i.e. athletes who were not misusing GH). In the event of participants in this study using GH, the current decision limits would have reduced sensitivity. The dynamic approach, where decision limits are continuously refined, will counter this possibility by decreasing the impact of any doping athletes on the calculated limits.

In conclusion, we have calculated GH-2000 score decision limits using currently available commercial assays to measure IGF-I and P-III-NP in elite athletes. This has allowed the introduction of a test for detecting GH misuse based on GH-dependent markers. The test was implemented by WADA for the London Olympic Games 2012 through the anti-doping laboratory at King's College London and will now be introduced in other laboratories around the world.

CHAPTER 8: THE USE OF BLOOD mRNA TECHNOLOGY TO DETECT GH AND IGF-I MISUSE IN ATHLETES

8.1 Introduction

The use of peptide markers and immunoassays in detecting GH and IGF-I misuse has created a number of problems, as described in the preceding chapters. One major issue is that this requires the development and validation of two assays for each marker in order to fulfil WADA requirements, as described in Chapter 7. Furthermore, samples for marker analysis cannot be kept at room temperature for prolonged periods and must be transported to the laboratory at approximately 4°C, as described in Chapter 6.

The investigation of circulating nucleic acids in plasma and serum (CNAPS), including various messenger RNA (mRNA) species, is an expanding scientific field. The detection of endogenous GH production using circulating nucleic acids was investigated in a preliminary study at St. Thomas' Hospital, London (Thakkar et al. 2008). This preliminary study demonstrated mRNA for GH and GHRH in the circulation and raised the possibility of using mRNA technology in the detection of GH and IGF-I misuse. The intra-individual variability of circulating mRNA for peptides in the GH-IGF axis has not been investigated previously and it is unknown whether concentrations of these mRNA species are affected by exogenous hormone administration.

We hypothesised that injection of exogenous rhGH or rhIGF-I/rhIGFBP-3 complex would act through negative feedback mechanisms to modify serum levels of endogenous peptides and that synthesis of mRNA encoding these peptides, and therefore circulating mRNA concentrations, would also be altered.

The specific aims of this pilot study were:

1. To assess the intra-individual variability of circulating mRNA for GH, GHRH, IGF-I and IGFBP-3.

2. To assess the acute changes in circulating mRNA concentrations for GH, GHRH, IGF-I and IGFBP-3 in response to four injections of rhGH (0.1 units/kg).

3. To assess the changes in circulating mRNA concentrations for GH, GHRH, IGF-I and IGFBP-3 in response to the administration of rhIGF-I/rhIGFBP-3 complex for 28 days.

8.2 Methods

This study was divided into three sub-studies as outlined below.

8.2.1 *The intra-individual variability of mRNA for GH, GHRH, IGF-I and IGFBP-3*

10 healthy recreational athletes (8 men, 2 women) aged between 19-29 years were recruited as described in section 2.1.5.1.1. At the beginning of the study (Day 0), demographic data were collected (section 2.1.5.1.2) and body composition and physical fitness were assessed as described in sections 2.1.1.6 and 2.1.1.7. Venous blood samples were collected for mRNA analysis at baseline (Day 0) and then at two-weekly intervals over 6 weeks, as described in section 2.1.5.1.4. Samples were anonymised before analysis and all analyses were performed by Dr Asif Butt at the Department of Chemical Pathology, St Thomas' Hospital, London. RNA was extracted from whole blood as described in section 2.1.5.1.5 and total RNA was quantified as described in section 2.1.5.1.6. Reverse transcription and quantitative real-time polymerase chain reaction (qPCR) were performed as described in sections 2.1.5.1.7 to 2.1.5.1.9. Venous blood samples for GH-dependent markers were collected at baseline (Day 0) as described in section 2.1.5.1.3 and were analysed at the Drug Control Centre, King's College London using the IGF-I and P-III-NP assays described in section 2.1.1.4. This was a pilot study and so formal power calculations were not possible. A sample size of 10 participants was agreed in collaboration with WADA. mRNA concentrations for target genes were corrected for total RNA concentrations, as well as for the housekeeping gene beta-actin.

8.2.2 *Acute changes in blood mRNA concentrations for GH, GHRH, IGF-I and IGFBP-3 in response to four injections of rhGH*

10 healthy male recreational athletes aged 20-23 years were recruited as described in section 2.1.5.2.1. This study was a non-randomised intervention study. At the beginning of the study (Day 1), demographic data were collected (section 2.1.5.2.2) and body composition and physical fitness were assessed as described in sections

2.1.1.6 and 2.1.1.7. I administered a daily injection of rhGH (0.1 units/kg/day) to all participants between 8am and 10am on Days 1, 2, 3 and 4 as described in section 2.1.5.2.2. Venous blood samples were collected for mRNA analysis at baseline (prior to the first injection of rhGH on Day 1) and 30 minutes, 1 hour, 2 hours, 4 hours and 8 hours after the first injection. Subsequent blood samples were taken after 1 day (prior to the second injection), 2 days (prior to the third injection), 4 days and 1 week as described in section 2.1.5.2.3. mRNA quantitation for GH, GHRH, IGF-I and IGFBP-3 was performed as described in sections 2.1.5.1.5 to 2.1.5.1.9. Venous blood samples for GH-dependent markers were collected at all time-points and analysed as described in section 2.1.5.2.4. This was a pilot study and so formal power calculations were not possible. A sample size of 10 participants was agreed in collaboration with WADA. mRNA concentrations for target genes were corrected for total RNA concentrations as well as for the housekeeping genes beta-actin and beta-globin. The relationship between mRNA concentrations and IGF-I peptide concentrations was assessed using simple linear regression. GH-2000 scores were calculated using the methods described in section 2.2.2.

8.2.3 *The effects of rhIGF-I/rhIGFBP-3 administration on circulating mRNA for GH, GHRH, IGF-I and IGFBP-3*

This study was undertaken as part of the rhIGF-I/rhIGFBP-3 administration study described in Chapters 3 and 4. 30 healthy male recreational athletes aged between 19-29 years were recruited, as described in section 2.1.1.1. Participants were randomly assigned to receive low dose (30 mg/day) rhIGF-I/rhIGFBP-3 complex, high dose (60 mg/day) rhIGF-I/rhIGFBP-3 complex or placebo for 28 days, as described in section 2.1.1.2. Venous blood samples were collected for mRNA analysis at baseline (Day 0), during the treatment period (Day 7) and during the washout period on Days 28, 30, 33, 42 and 84, as described in section 2.1.5.3.1. mRNA quantitation for GH, GHRH, IGF-I and IGFBP-3 was performed as described in sections 2.1.5.1.5 to 2.1.5.1.9. Venous blood samples for GH-dependent markers were collected at all time-points as described in Chapter 3. mRNA concentrations for target genes were corrected for total RNA concentrations, as well as for the housekeeping genes beta-actin and beta-globin. Changes in mRNA concentrations and peptide concentrations between time-points were assessed using paired *t*-tests. The relationship between mRNA concentrations and IGF-I peptide concentrations was assessed using simple linear regression. GH-2000 scores were calculated using the methods described in section 2.2.2.

My role in these studies was as follows: I recruited all participants to the study using the methods described in section 2.1.5. I supervised all baseline assessments; all data during these assessments were collected by me and by University of Southampton medical students under my supervision. I administered daily rhGH injections to participants as described in section 8.2.2 above. I maintained the project results database and prepared all figures presented in section 8.3 below. Dr Erotokritou-Mulligan (GH-2004 Project statistician) estimated intra-individual variability of mRNA concentrations using analysis of variance (ANOVA) and assessed changes in mRNA concentrations and peptide concentrations between time-points using paired *t*-tests.

8.3 Results

8.3.1 *The intra-individual variability of mRNA for GH, GHRH, IGF-I and IGFBP-3*

The baseline characteristics of the 10 participants in the intra-individual variability study are shown in Table 8.1.

8.3.1.1 Changes in mRNA concentrations for GH, IGF-I and IGFBP-3

mRNA for GH, GHRH, IGF-I and IGFBP-3 were detectable in whole blood samples. Figures 8.1 to 8.3 demonstrate time traces for concentrations of mRNA for GH, IGF-I and IGFBP-3 in the 10 participants. mRNA concentrations for each gene are expressed relative to the total RNA concentration measured in each sample.

Table 8.1. Baseline characteristics of participants in the intra-individual variability study. BMI = Body Mass Index, VO_2 max = maximal oxygen consumption (measured by incremental treadmill test using the Bruce Protocol). Fat percentage was measured using bioelectrical impedance analysis.

Age (yrs)	Gender	Ethnicity	Sport	BMI (kg/m^2)	Body Fat (%)	VO_2 max (ml/min/kg)	IGF-I (ng/ml)	P-III-NP (ng/ml)
23	Female	Caucasian	Running	22.9	21.1	48	486	5.95
24	Female	Caucasian	Running	21.4	20.5	45	251	2.72
27	Male	Caucasian	Surfing	23.6	9.6	51	160	5.04
24	Male	African	Football	21.5	15.8	47	130	3.48
23	Male	African	Football	21.0	11.8	38	347	4.06
23	Male	Asian	Football	24.6	15.2	42	429	5.48
23	Male	Asian	Hockey	26.1	15.2	51	212	4.94
19	Male	Caucasian	Football	21.5	9.3	55	334	4.72
21	Male	Caucasian	Football	20.2	8.7	61	300	4.02
29	Male	Caucasian	Rugby	27.3	17.7	51	252	3.61

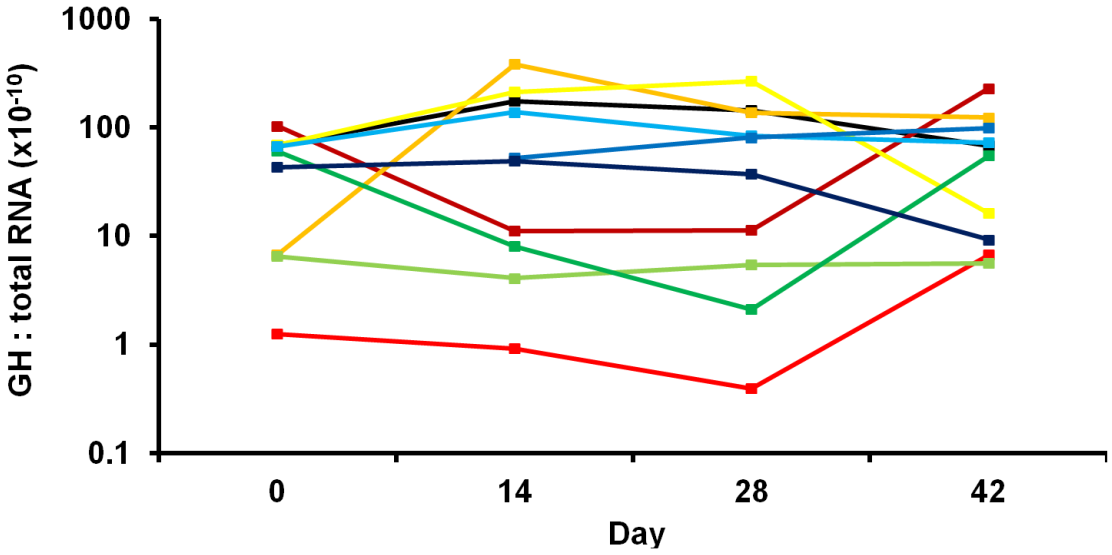

Figure 8.1. Ratio of GH mRNA concentration to total RNA. Each line connects the samples collected from each of the 10 participants; each symbol represents the GH mRNA:total RNA ratio.

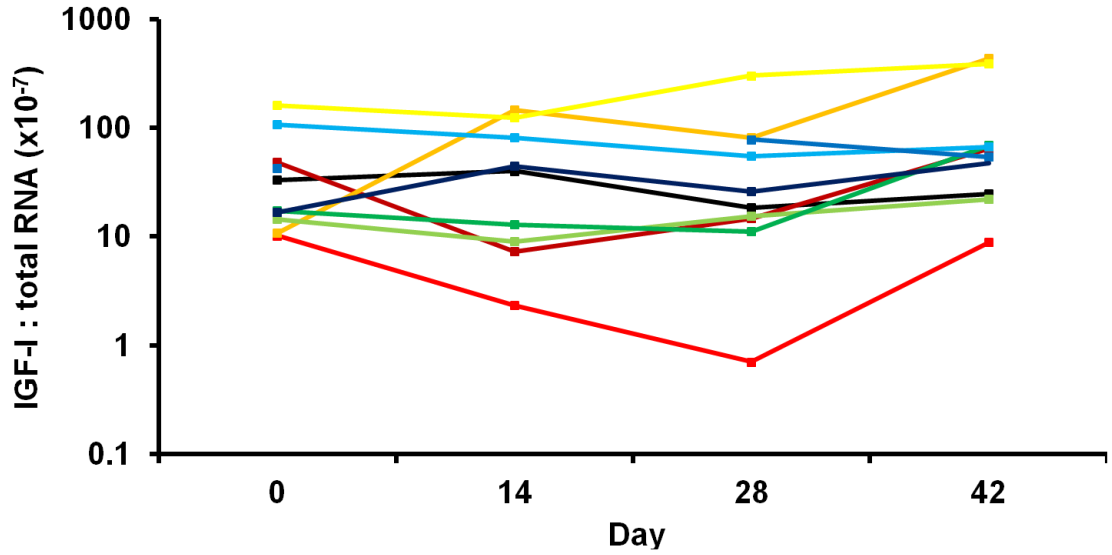

Figure 8.2. Ratio of IGF-I mRNA concentration to total RNA. Each line connects the samples collected from each of the 10 participants; each symbol represents the IGF-I mRNA:total RNA ratio.

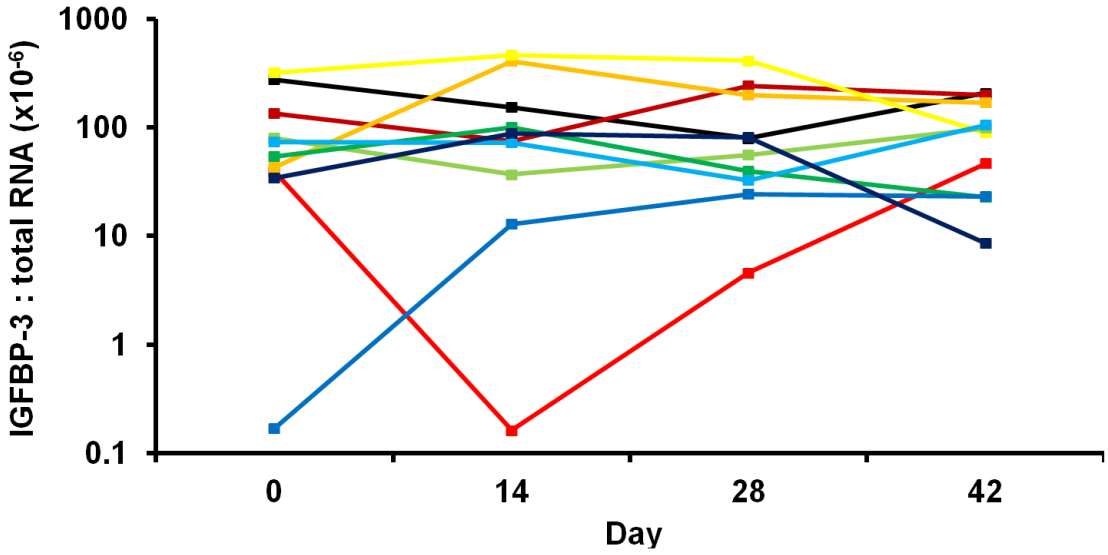

Figure 8.3. Ratio of IGFBP-3 mRNA concentration to total RNA. Each line connects the samples collected from each of the 10 participants; each symbol represents the IGFBP-3 mRNA:total RNA ratio.

Table 8.2 shows the estimated intra-individual variability of GH, IGF-I and IGFBP-3 mRNA concentrations corrected for total RNA and for beta-actin mRNA.

Table 8.2. The intra-individual variability of mRNA concentrations for GH, IGF-I and IGFBP-3. Results are expressed using target gene mRNA concentrations and also corrected for total RNA and for beta-actin mRNA concentrations.

mRNA	Intra-individual variability (%)	Ratio	Intra-individual variability (%)	Ratio	Intra-individual variability (%)
GH	110	GH:total RNA	112	GH:beta-actin	194
IGF-I	83	IGF-I:total RNA	83	IGF-I:beta-actin	169
IGFBP-3	127	IGFBP-3:total RNA	129	IGFBP-3:beta-actin	192

Intra-individual variability estimates for total RNA and for beta-actin mRNA concentrations were 26% and 97% respectively. The intra-individual variability

estimates for all target gene mRNA species were greater than 80%; this variability was not improved by correcting for total RNA or beta-actin mRNA.

8.3.1.2 Changes in GHRH mRNA

Figure 8.4 shows the time-trace for GHRH mRNA threshold cycle (C_T) numbers. Human hypothalamic cDNA was not available for the development of standard curves for GHRH mRNA and therefore it was not possible to convert these C_T numbers to mRNA concentrations.

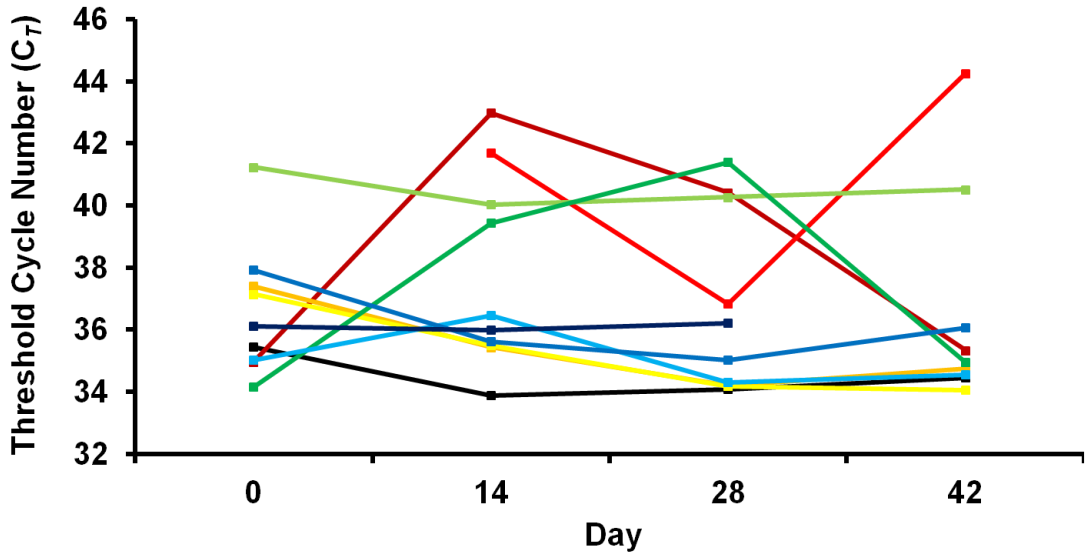

Figure 8.4. Threshold cycle numbers for GHRH mRNA. Each line connects the samples collected from each of the 10 participants; each symbol represents the threshold cycle (C_T) number. C_T numbers could not be determined in two blood samples because of failed mRNA amplification.

8.3.2 *Acute changes in blood mRNA concentrations for GH, GHRH, IGF-I and IGFBP-3 in response to four injections of rhGH*

The baseline characteristics of the 10 participants in the rhGH administration study are shown in Table 8.3.

8.3.2.1 Acute changes in mRNA concentrations for GH, IGF-I and IGFBP-3

Figures 8.5 to 8.7 show the changes in mRNA concentrations for GH, IGF-I and IGFBP-3 over the course of Day 1 (before and after the first dose of rhGH). mRNA

concentrations for each gene are expressed relative to the total RNA concentration measured in each sample. There were no significant changes in mRNA concentrations (corrected for total RNA, beta-actin or beta-globin) at any time-point on Day 1, compared with baseline samples.

Table 8.3. Baseline characteristics of participants in the GH administration study. BMI = Body Mass Index, VO_2 max = maximal oxygen consumption (measured by incremental treadmill test using the Bruce Protocol). Fat percentage was measured using bioelectrical impedance analysis.

Age (yrs)	Gender	Ethnicity	Sport	BMI (kg/m^2)	Body Fat (%)	VO_2 max (ml/min/kg)	IGF-I (ng/ml)	P-III-NP (ng/ml)
20	Male	Caucasian	Football	20.1	8.9	58	314	5.55
23	Male	Caucasian	Martial arts	23.7	17.9	49	197	4.03
20	Male	Asian	Football	22.5	7.8	53	348	5.33
21	Male	Caucasian	Triathlon	21.6	7.4	51	256	4.38
20	Male	Caucasian	Triathlon	22.5	6.0	61	483	8.97
21	Male	Caucasian	Rugby	27.7	18.4	50	343	5.20
23	Male	Caucasian	Athletics	20.1	11.9	83	238	4.09
21	Male	Asian	Rugby	23.9	9.8	66	278	4.54
23	Male	Caucasian	Football	26.2	17	57	223	2.97
22	Male	Caucasian	Football	22.6	12.6	62	252	4.10

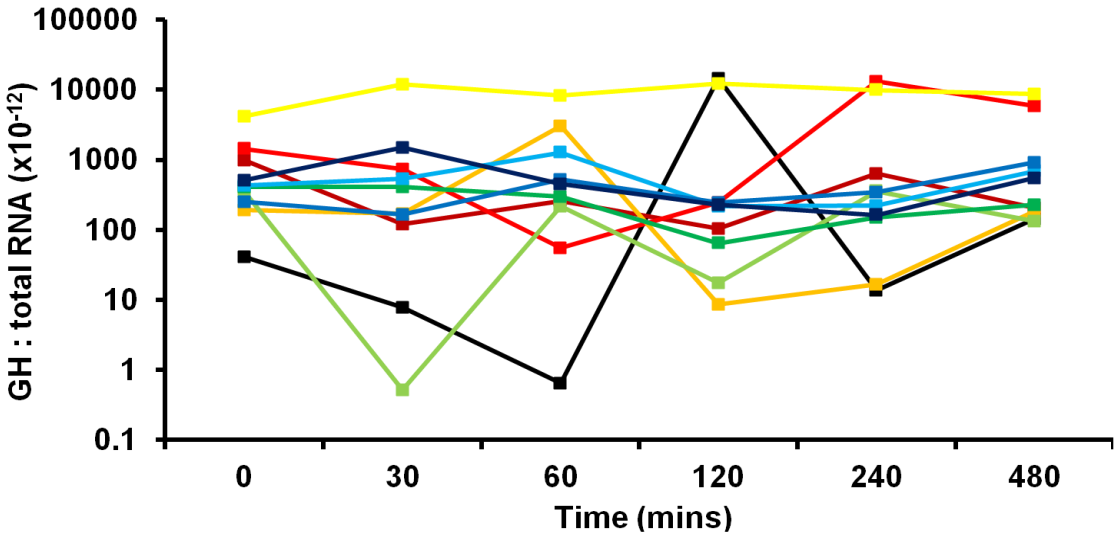

Figure 8.5. Changes in GH mRNA concentrations during Day 1 in 10 participants. rhGH was administered after the baseline blood sample was taken at time 0. Each symbol represents the GH mRNA:total RNA ratio.

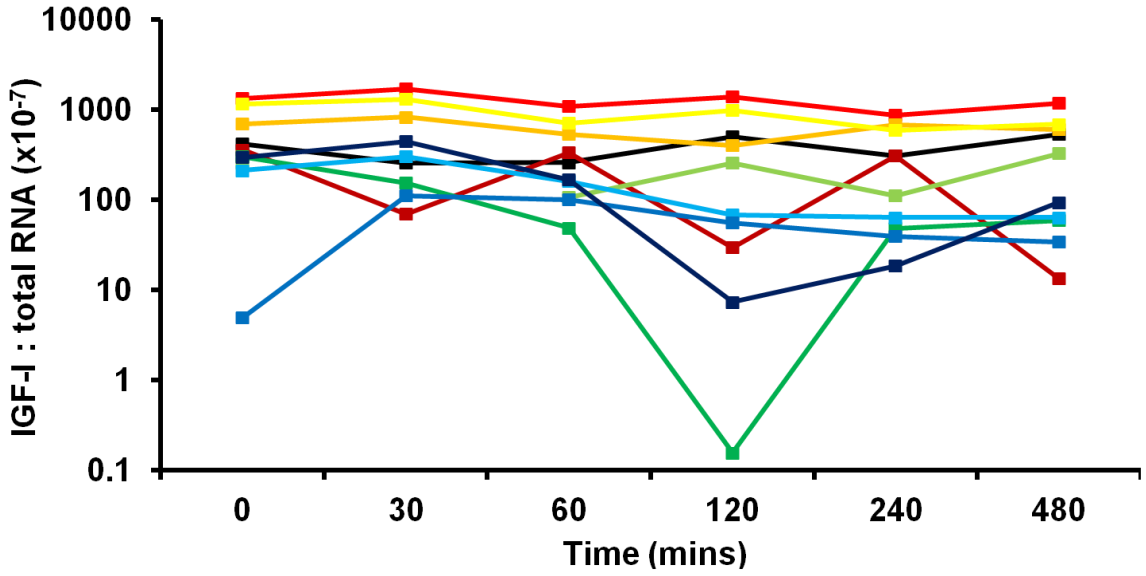

Figure 8.6. Changes in IGF-I mRNA concentrations during Day 1 in 10 participants. rhGH was administered after the baseline blood sample was taken at time 0. Each symbol represents the IGF-I mRNA:total RNA ratio.

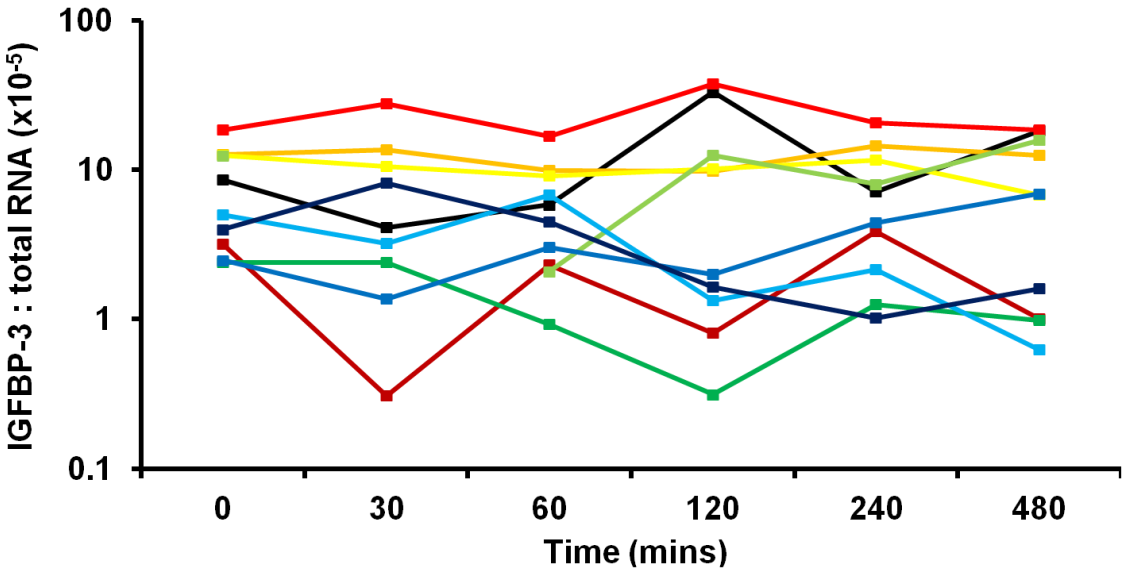

Figure 8.7. Changes in IGFBP-3 mRNA concentrations during Day 1 in 10 participants. rhGH was administered after the baseline blood sample was taken at time 0. Each symbol represents the IGFBP-3 mRNA:total RNA ratio.

8.3.2.2 Changes in mRNA concentrations for GH, IGF-I and IGFBP-3 over one week

Figures 8.8 to 8.10 show the changes in mRNA concentrations for GH, IGF-I and IGFBP-3 over the course of one week in response to four daily injections of rhGH. mRNA concentrations for each gene are expressed relative to the total RNA concentration measured in each sample. There were no significant changes in mRNA concentrations (corrected for total RNA, beta-actin or beta-globin) on any day, compared with baseline samples on Day 1.

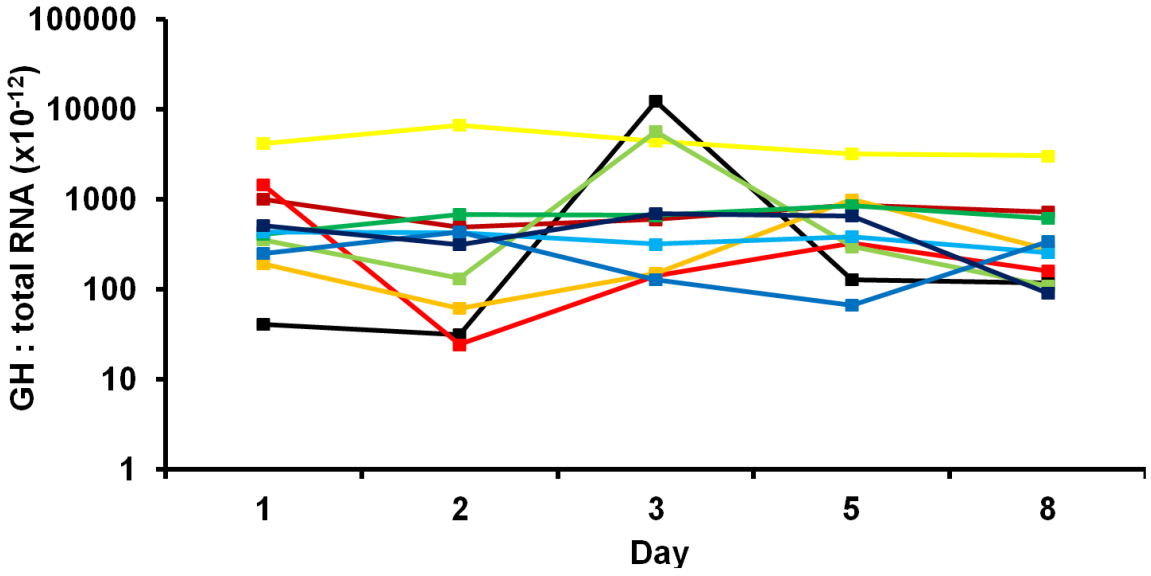

Figure 8.8. Changes in GH mRNA concentrations over one week in 10 participants. rhGH was administered on Days 1, 2, 3 and 4. Each symbol represents the GH mRNA:total RNA ratio. Results from Day 1 are from samples taken at baseline, prior to the first injection of rhGH.

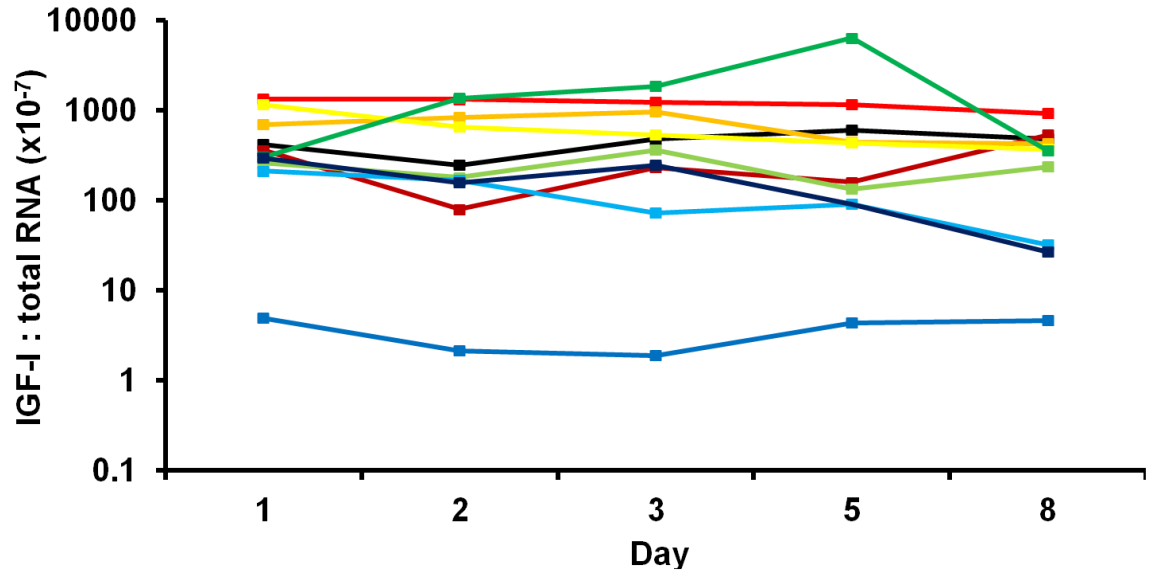

Figure 8.9. Changes in IGF-I mRNA concentrations over one week in 10 participants. rhGH was administered on Days 1, 2, 3 and 4. Each symbol represents the IGF-I mRNA:total RNA ratio. Results from Day 1 are from samples taken at baseline, prior to the first injection of rhGH.

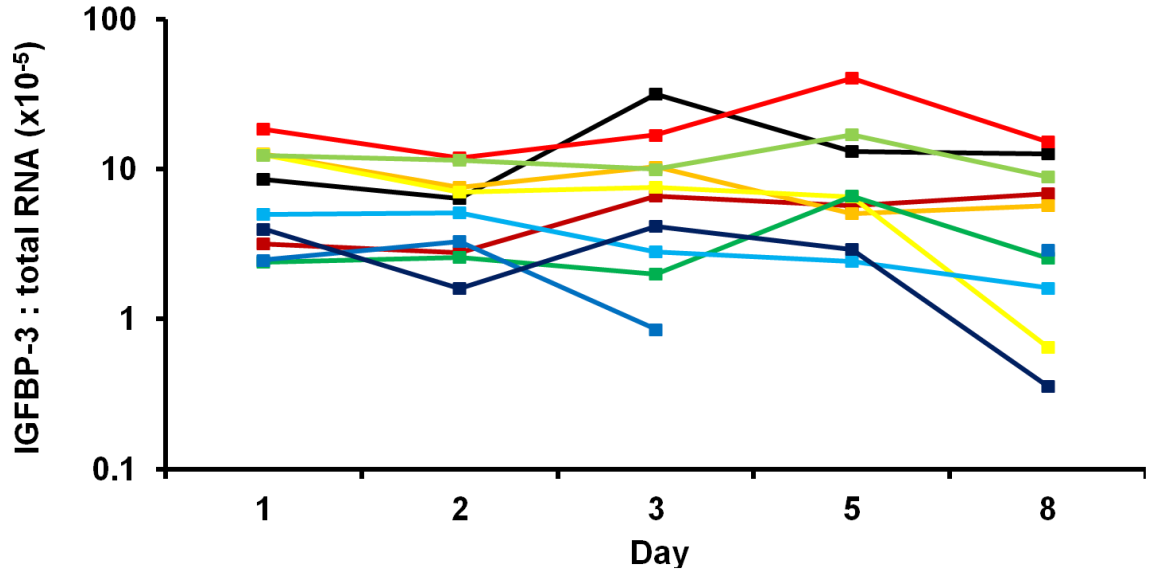

Figure 8.10. Changes in IGFBP-3 mRNA concentrations over one week in 10 participants. rhGH was administered on Days 1, 2, 3 and 4. Each symbol represents the IGFBP-3 mRNA:total RNA ratio. Results from Day 1 are from samples taken at baseline, prior to the first injection of rhGH.

8.3.2.3 Changes in GHRH mRNA

Figures 8.11 and 8.12 show the time-traces for GHRH mRNA threshold cycle (C_T) numbers in response to one dose of rhGH on Day 1 (Fig. 8.11) and over the course of one week in response to four daily injections of rhGH (Fig. 8.12). Human hypothalamic cDNA was not available for the development of standard curves for GHRH mRNA and therefore it was not possible to convert these C_T numbers to mRNA concentrations.

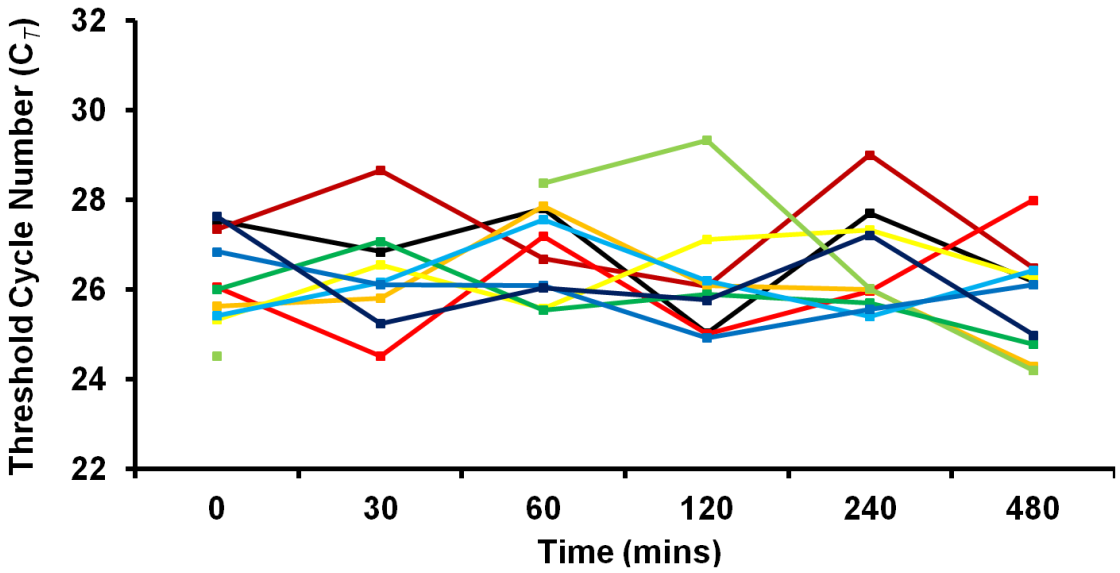

Figure 8.11. Threshold cycle numbers for GHRH mRNA on Day 1. Each line connects the samples collected from each of the 10 participants; each symbol represents the threshold cycle (C_T) number. C_T number could not be determined in one blood sample because of failure of mRNA amplification.

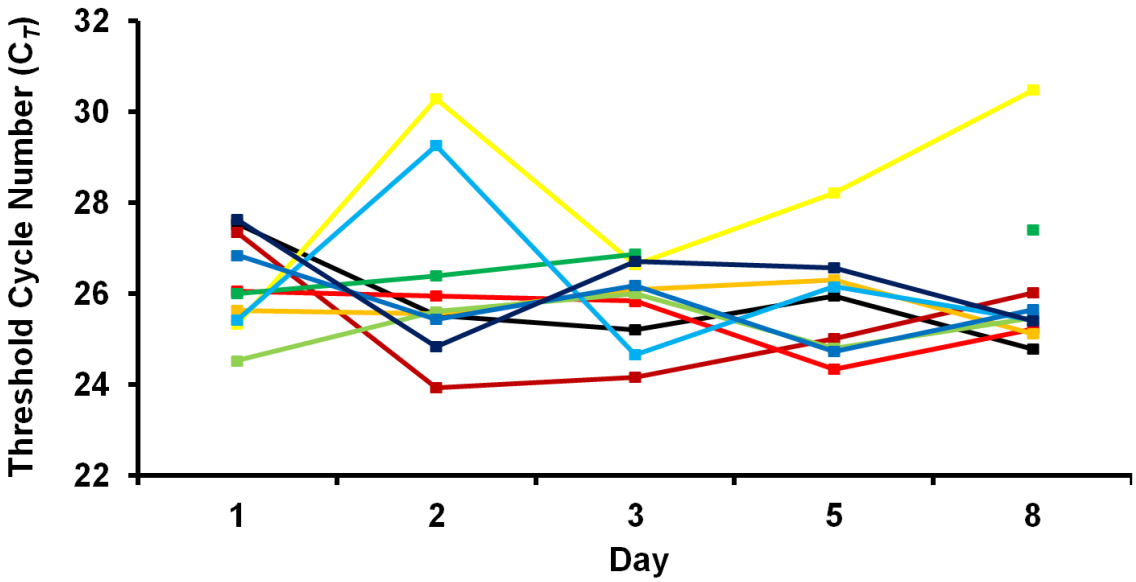

Figure 8.12. Threshold cycle numbers for GHRH mRNA over one week. rhGH was administered on Days 1, 2, 3 and 4. Results from Day 1 are from samples taken at baseline, prior to the first injection of rhGH. Each line connects the samples collected from each of the 10 participants; each symbol represents the threshold cycle (C_T) number. C_T number could not be determined in one blood sample because of failure of mRNA amplification.

8.3.2.4 *Comparison between mRNA concentrations and serum peptide concentrations*

Figures 8.13 to 8.15 show the changes in IGF-I peptide concentrations, P-III-NP peptide concentrations and GH-2000 scores over the course of one week in response to four daily injections of rhGH.

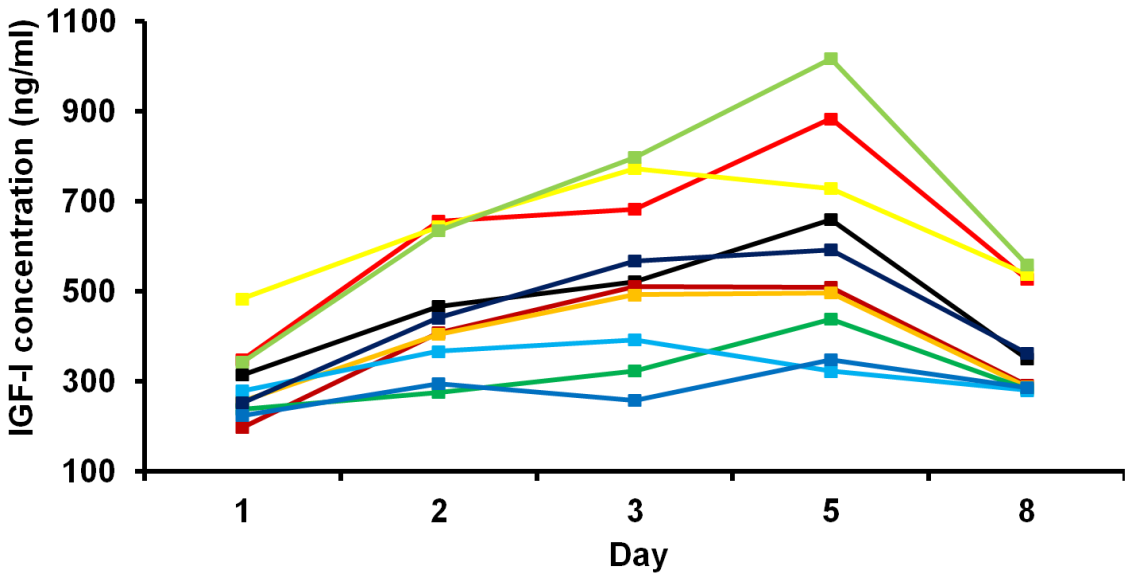

Figure 8.13. Changes in IGF-I peptide concentrations (measured by the Immunotech IGF-I assay) over one week in 10 participants. rhGH was administered on Days 1, 2, 3 and 4. Results from Day 1 are from samples taken at baseline, prior to the first injection of rhGH.

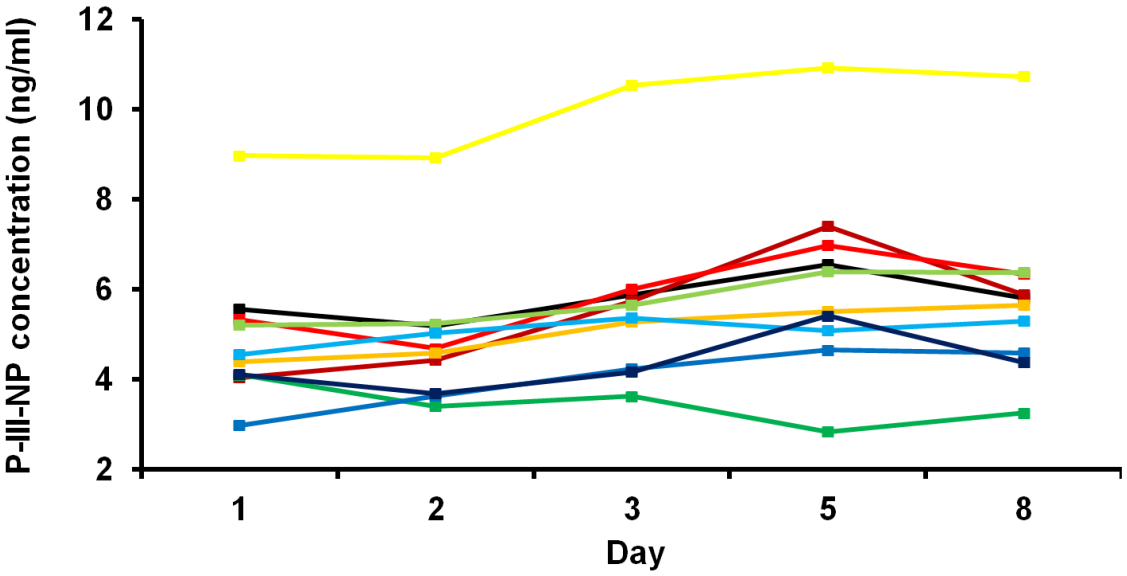

Figure 8.14. Changes in P-III-NP peptide concentrations (measured by the Orion P-III-NP assay) over one week in 10 participants. rhGH was administered on Days 1, 2, 3 and 4. Results from Day 1 are from samples taken at baseline, prior to the first injection of rhGH.

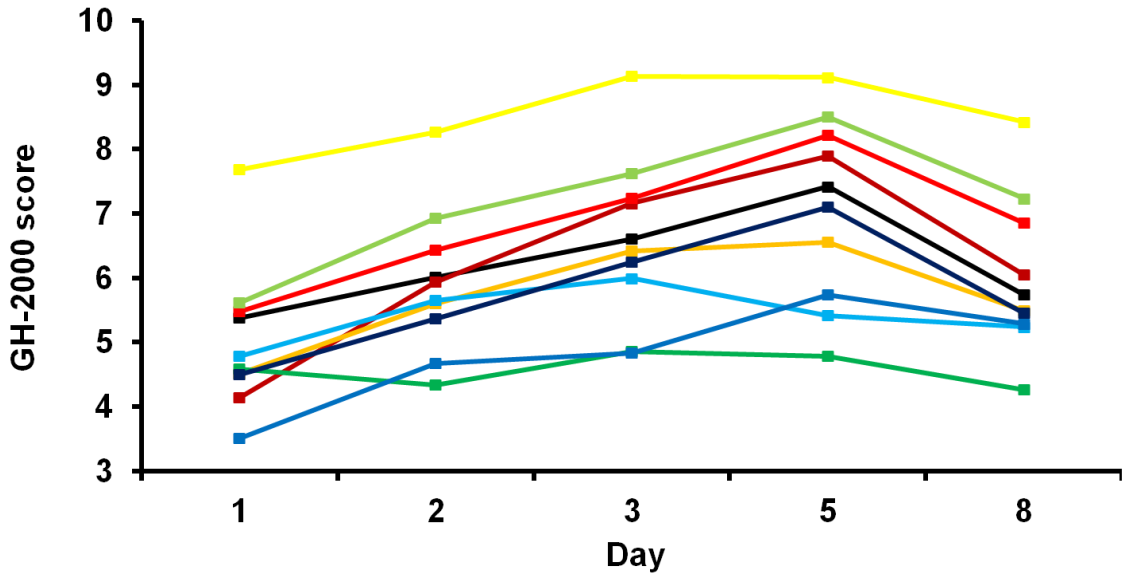

Figure 8.15. Changes in GH-2000 score (calculated using results from the Immunotech IGF-I and Orion P-III-NP assays) over one week in 10 participants. rhGH was administered on Days 1, 2, 3 and 4. Results from Day 1 are from samples taken at baseline, prior to the first injection of rhGH.

IGF-I, P-III-NP and GH-2000 scores increased in response to rhGH administration. There was a significant increase in mean IGF-I concentration on Day 2 ($P < 0.0001$), Day 3 ($P < 0.0001$), Day 5 ($P < 0.0001$) and Day 8 ($P = 0.012$), compared with Day 1. There was also a significant increase in mean P-III-NP concentration on Day 3 ($P = 0.0157$), Day 5 ($P = 0.0250$) and Day 8 ($P = 0.0202$), compared with Day 1. Finally, there was a significant increase in GH-2000 score on Day 2 ($P = 0.0005$), Day 3 ($P < 0.0001$), Day 5 ($P = 0.0002$) and Day 8 ($P = 0.0017$), compared with Day 1.

Figures 8.16 to 8.18 show the relationship between log-transformed GH, IGF-I and IGFBP-3 mRNA concentrations (corrected for total RNA) and log-transformed IGF-I peptide concentrations. There was a weak but significant correlation between GH (P=0.01), IGF-I (P<0.001) and IGFBP-3 (P<0.001) mRNA concentrations and IGF-I peptide concentrations.

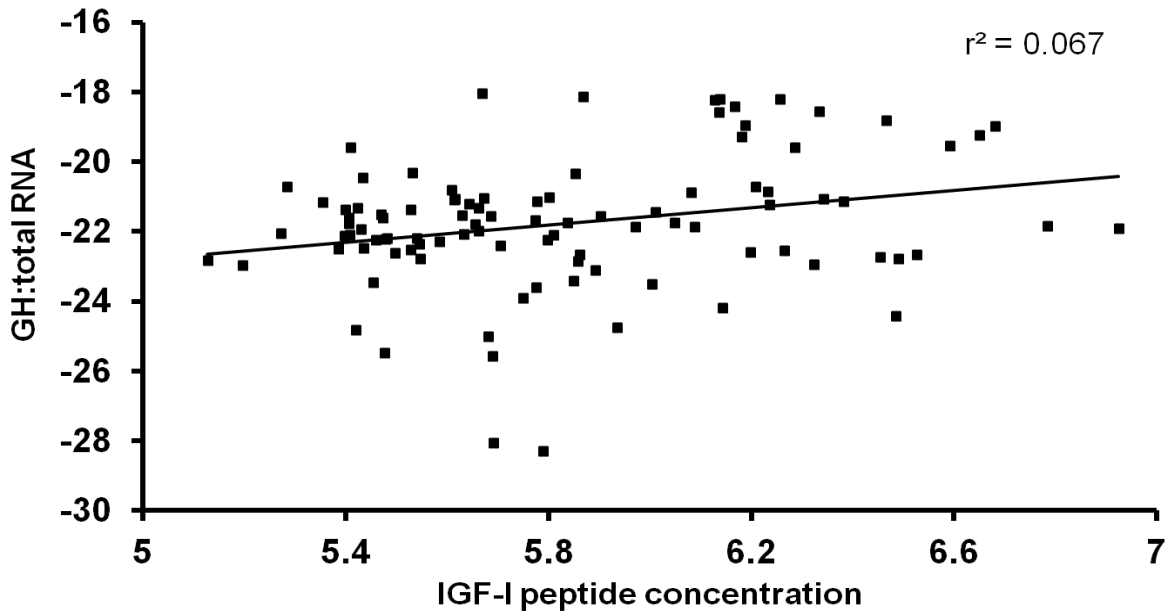

Figure 8.16. The relationship between log-transformed GH mRNA:total RNA ratio and log-transformed IGF-I peptide concentrations. Results are from 10 participants (10 blood samples per participant). The linear regression line is shown.

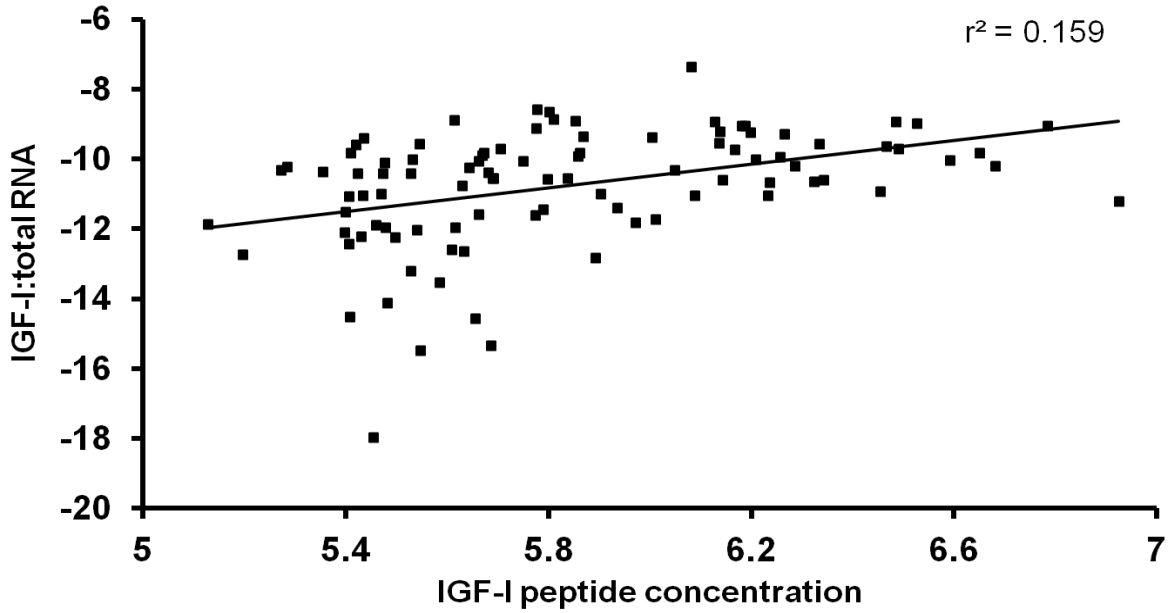

Figure 8.17. The relationship between log-transformed IGF-I mRNA:total RNA ratio and log-transformed IGF-I peptide concentrations. Results are from 10 participants (10 blood samples per participant). The linear regression line is shown.

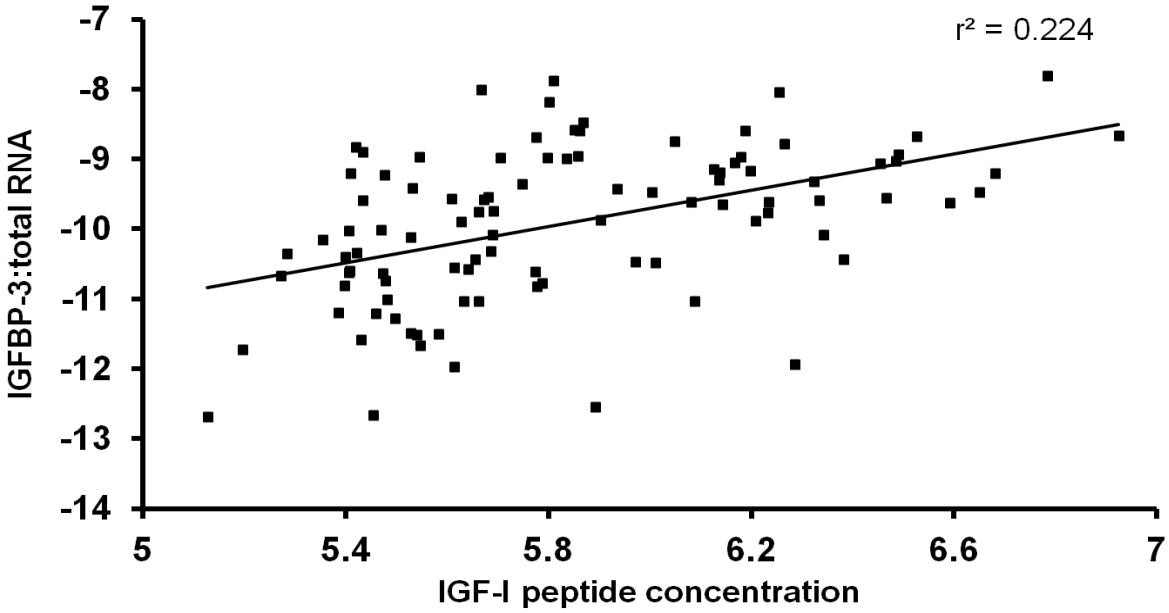

Figure 8.18. The relationship between log-transformed IGFBP-3 mRNA:total RNA ratio and log-transformed IGF-I peptide concentrations. Results are from 10 participants (10 blood samples per participant). The linear regression line is shown.

8.3.3 *The effects of rhIGF-I/rhIGFBP-3 administration on circulating mRNA for GH, GHRH, IGF-I and IGFBP-3*

This study was undertaken as part of the rhIGF-I/rhIGFBP-3 administration study described in Chapters 3 and 4 and the baseline characteristics of the participants in this study are shown in Table 3.1, Chapter 3 and Table 4.1, Chapter 4.

8.3.3.1 Changes in GH, IGF-I and IGFBP-3 mRNA concentrations

Table 8.4 shows the differences in mRNA concentrations for GH, IGF-I and IGFBP-3 between Day 0 (pre-treatment) and Day 28 (post-treatment). There were no statistically significant changes in circulating GH mRNA concentrations over time in either the low dose or high dose treatment groups, regardless of whether results were normalised using total RNA, beta-actin mRNA or beta-globin mRNA (Figure 8.19 and Table 8.4). Similarly there were no changes in IGF-I mRNA concentrations (Figure 8.20 and Table 8.4) or IGFBP-3 mRNA concentrations (Figure 8.21 and Table 8.4) in either the low dose or high dose treatment groups.

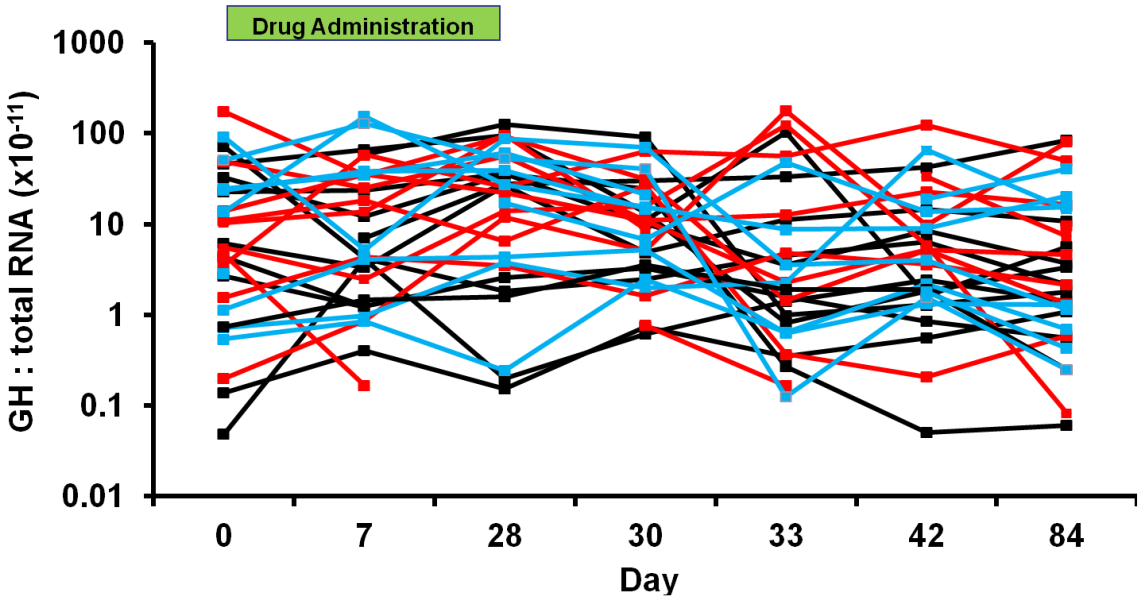

Figure 8.19. Circulating GH mRNA concentrations in participants receiving placebo (blue), low dose rhIGF-I/rhIGFBP-3 (red) and high dose rhIGF-I/rhIGFBP-3 (black). Each symbol represents the GH mRNA:total RNA ratio.

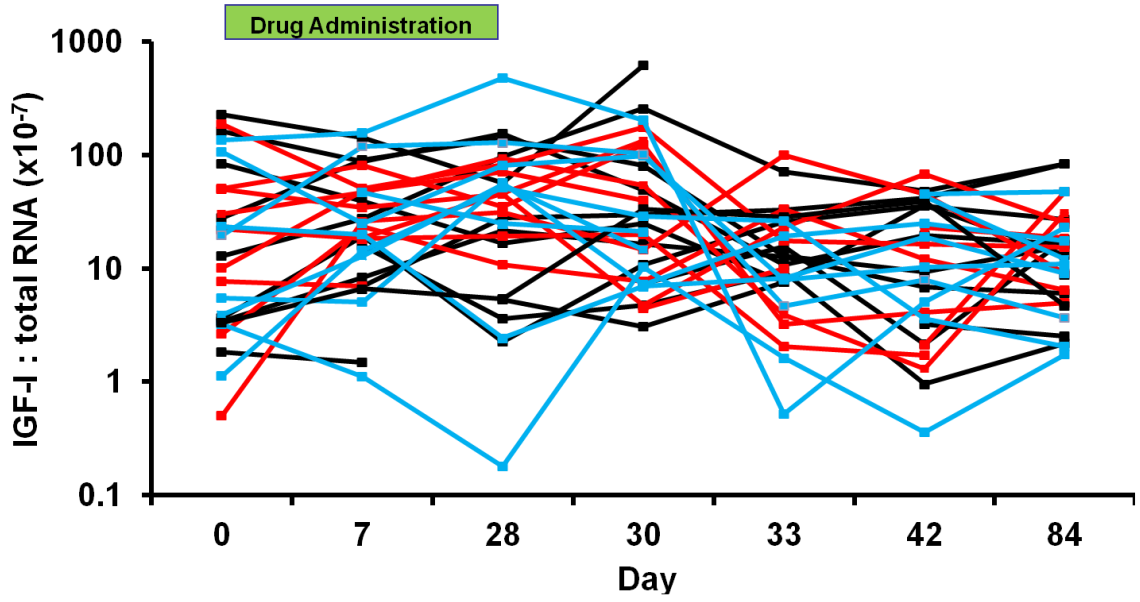

Figure 8.20. Circulating IGF-I mRNA concentrations in participants receiving placebo (blue), low dose rhIGF-I/rhIGFBP-3 (red) and high dose rhIGF-I/rhIGFBP-3 (black). Each symbol represents the IGF-I mRNA:total RNA ratio.

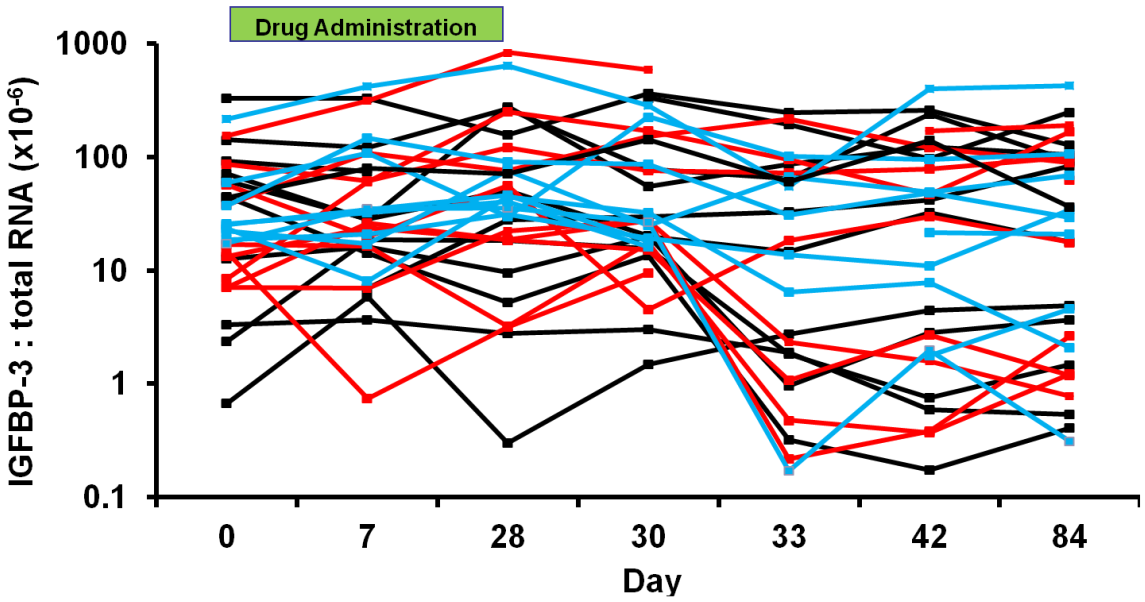

Figure 8.21. Circulating IGFBP-3 mRNA concentrations in participants receiving placebo (blue), low dose rhIGF-I/rhIGFBP-3 (red) and high dose rhIGF-I/rhIGFBP-3 (black). Each symbol represents the IGFBP-3 mRNA:total RNA ratio.

Table 8.4. Within-group differences from baseline in log-transformed mRNA concentrations for GH, IGF-I and IGFBP-3 corrected for beta-actin, beta-globin and total RNA.

Ratio	Treatment group	Mean difference between Days 0 and 28	95% CI for mean difference	*P* value
GH:beta-actin	**Placebo**	-0.1	-1.4 to 1.2	0.858
	Low dose IGF-I	-1.0	-2.6 to 0.7	0.204
	High dose IGF-I	0.7	-1.4 to 2.9	0.431
GH:beta-globin	**Placebo**	-0.2	-1.3 to 0.9	0.680
	Low dose IGF-I	-0.6	-2.1 to 1.0	0.421
	High dose IGF-I	0.8	-1.1 to 2.6	0.365
GH:total RNA	**Placebo**	-0.9	-1.4 to -0.3	**0.006**
	Low dose IGF-I	-0.8	-2.3 to 0.8	0.267
	High dose IGF-I	0.4	-1.0 to 1.8	0.557
IGF-I:beta-actin	**Placebo**	-0.3	-1.0 to 0.6	0.485
	Low dose IGF-I	-0.8	-1.9 to 0.5	0.186
	High dose IGF-I	0.4	-1.5 to 2.4	0.626
IGF-I:beta-globin	**Placebo**	-0.6	-2.0 to 0.8	0.332
	Low dose IGF-I	-0.7	-1.9 to 0.5	0.197
	High dose IGF-I	0.3	-1.7 to 2.3	0.712
IGF-I:total RNA	**Placebo**	-1.0	-2.2 to 0.2	0.089
	Low dose IGF-I	-0.9	-2.1 to 0.3	0.122
	High dose IGF-I	-0.1	-1.4 to 1.3	0.905
IGFBP-3:beta-actin	**Placebo**	-0.1	-1.0 to 0.8	0.772
	Low dose IGF-I	-0.8	-1.8 to 0.3	0.121
	High dose IGF-I	0.2	-1.0 to 1.5	0.662
IGFBP-3:beta-globin	**Placebo**	-0.5	-1.4 to 0.5	0.286
	Low dose IGF-I	-0.4	-1.4 to 0.6	0.362
	High dose IGF-I	0.2	-1.3 to 1.7	0.798
IGFBP-3:total RNA	**Placebo**	-0.7	-0.9 to -0.5	**<0.001**
	Low dose IGF-I	-0.6	-1.8 to 0.6	0.274
	High dose IGF-I	-0.1	-1.1 to 1.0	0.866

8.3.3.2 Changes in GHRH mRNA

Figure 8.22 shows the time-trace for GHRH mRNA threshold cycle (C_T) numbers. Human hypothalamic cDNA was not available for the development of standard curves for GHRH mRNA and therefore it was not possible to convert these C_T numbers to mRNA concentrations.

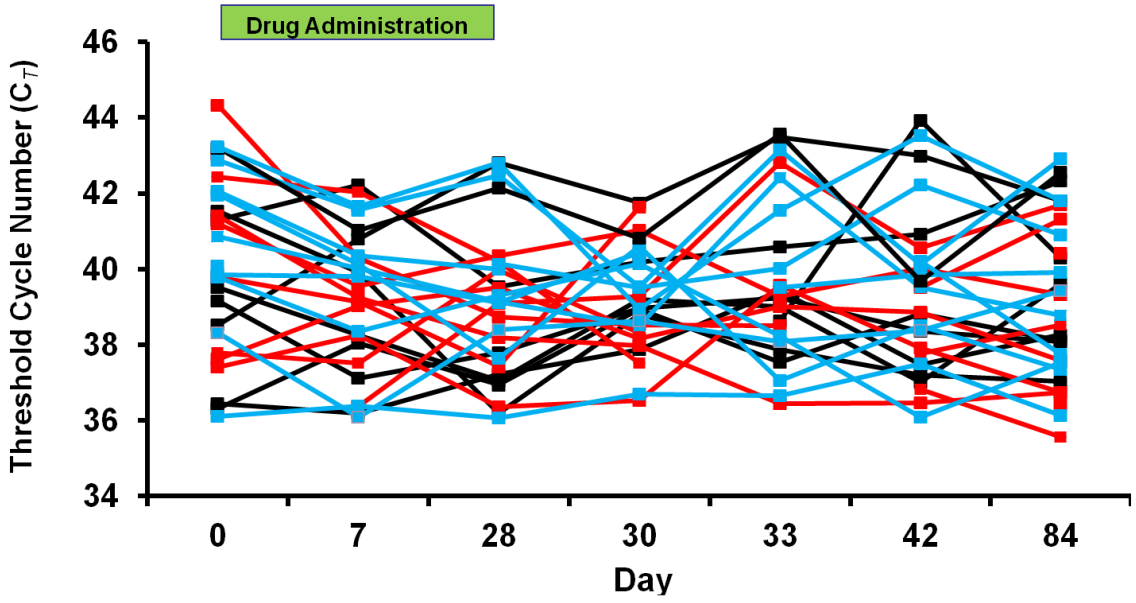

Figure 8.22. Threshold cycle (C_T) numbers for GHRH mRNA in participants receiving placebo (blue), low dose rhIGF-I/rhIGFBP-3 (red) and high dose rhIGF-I/rhIGFBP-3 (black).

8.3.3.3 Comparison between mRNA concentrations and serum peptide concentrations

As discussed in Chapter 3, IGF-I, P-III-NP and GH-2000 scores increased in response to both low and high dose rhIGF-I/rhIGFBP-3 administration (Chapter 3, Figures 3.1 to 3.3). Figures 8.23 to 8.25 show the relationship between log-transformed GH, IGF-I and IGFBP-3 mRNA concentrations (corrected for total RNA) and log-transformed IGF-I peptide concentrations. There was a weak correlation between IGF-I mRNA (P=0.008) and IGFBP-3 mRNA (P=0.004) and IGF-I peptide concentrations but no significant correlation between GH mRNA and IGF-I peptide concentrations (P=0.055).

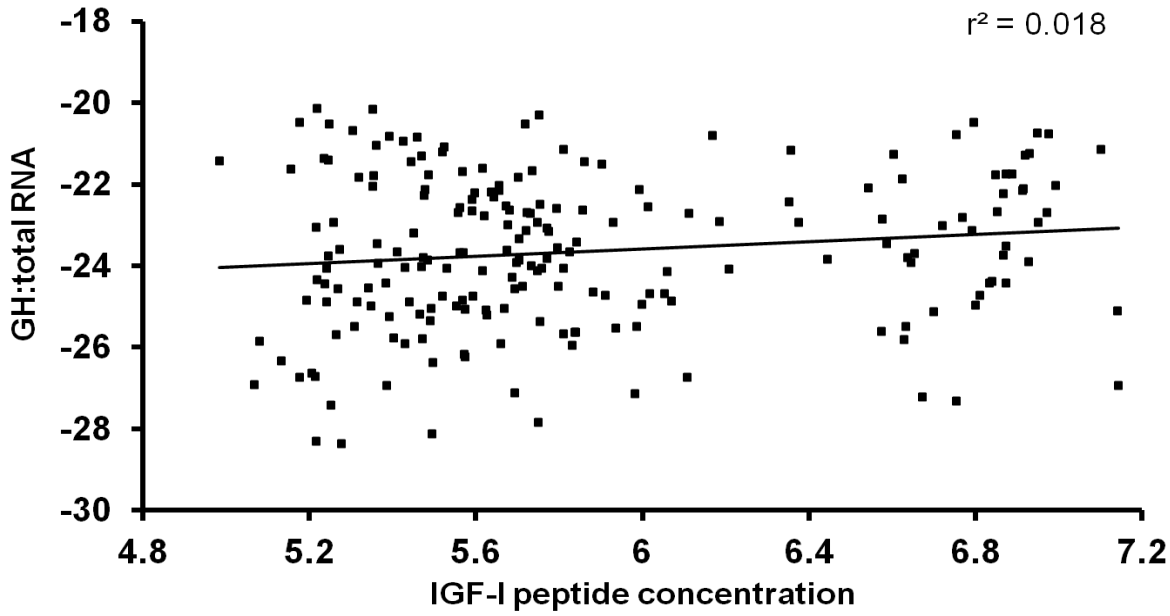

Figure 8.23. The relationship between GH mRNA:total RNA ratio and IGF-I peptide concentrations. All data are log-transformed. Results are from 30 participants (7 blood samples per participant). The linear regression line is shown.

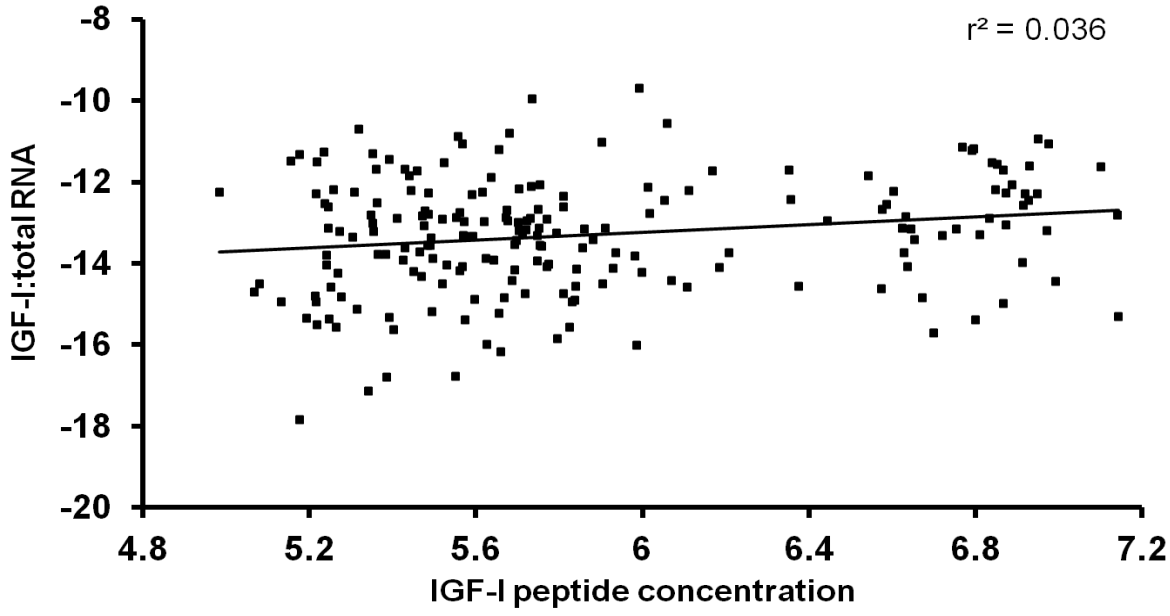

Figure 8.24. The relationship between IGF-I mRNA:total RNA ratio and IGF-I peptide concentrations. All data are log-transformed. Results are from 30 participants (7 blood samples per participant). The linear regression line is shown.

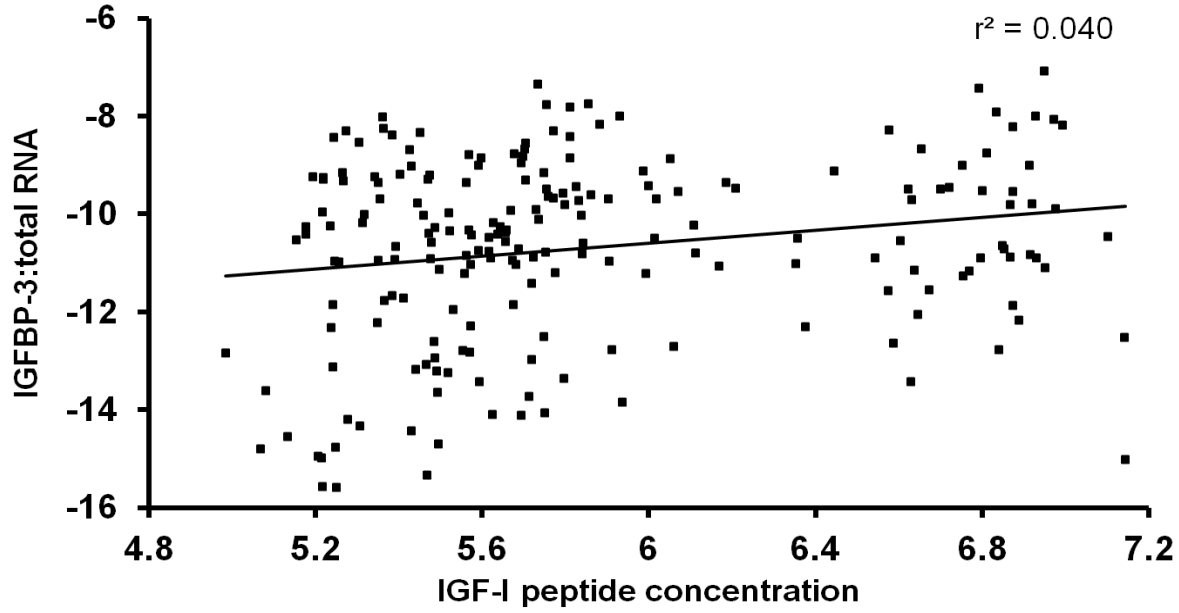

Figure 8.25. The relationship between IGFBP-3 mRNA:total RNA ratio and IGF-I peptide concentrations. All data are log-transformed. Results are from 30 participants (7 blood samples per participant). The linear regression line is shown.

8.4 Discussion

This study confirmed the presence of GH and GHRH mRNA in the circulation and is the first study to demonstrate the presence of small but quantifiable concentrations of circulating IGF-I and IGFBP-3 mRNA. The intra-individual variability of mRNA concentrations for GH, IGF-I and IGFBP-3 was assessed and found to be high. Furthermore, there were no significant changes in circulating mRNA for GH, IGF-I or IGFBP-3 in response to rhGH administration for 4 days in 10 male recreational athletes or in response to rhIGF-I/rhIGFBP-3 administration for 28 days in 30 male recreational athletes.

8.4.1 *Intra-individual variability of circulating mRNA concentrations*

It is important that all sources of variability are investigated in any anti-doping detection method. Ideally, the intra-individual variability of a detection marker will be low so that administration of exogenous substances can be detected by comparatively large changes in that marker. The intra-individual variability of mRNA concentrations for markers in the GH-IGF axis had not been investigated and these were found to be 112%, 83% and 129% for GH, IGF-I and IGFBP-3 respectively. These figures suggest that circulating mRNA concentrations for these peptides vary widely in an individual over time. It contrasts with the low intra-individual

variability in serum peptide markers that the GH-2004 group have shown in previous studies, approximately 14% for both IGF-I and P-III-NP peptide concentrations in both amateur and elite athletes (Erotokritou-Mulligan et al. 2009).

There are a number of potential explanations for the large intra-individual variability in mRNA concentrations. Normalisation of results is an essential process during qPCR because it controls for variations in extraction yield, reverse transcription yield and also efficiency of amplification, allowing comparisons of mRNA concentrations across different samples. Normalisation involves reporting the ratios of mRNA concentrations of target genes to those of reference genes or total RNA. Reference genes should be stably expressed in the tissue of interest and multiple reference genes should be used to find the optimal ratio for the given experimental conditions (Bustin et al. 2009). In these studies, the concentrations of GH, IGF-I and IGFBP-3 mRNA were normalised using three commonly employed RNA species: total RNA, beta-actin mRNA and beta-globin mRNA (Vandesompele et al. 2002; Bustin et al. 2009). The variability of these reference RNA species was higher than expected in both the intra-individual variability study (26% for total RNA and 97% for beta-actin) and in the rhGH administration study (44% for total RNA, 101% for beta-actin and 111% for beta-globin). This finding increased the intra-individual variability estimates for the target gene mRNA concentration ratios, though the variability in results was less when total RNA was used to normalise concentrations compared with the two housekeeping genes.

The high intra-individual variability in both target gene and housekeeping gene mRNA concentrations consists of both biological and analytical variability. Analytical variability in the laboratory procedures had been investigated during the preliminary studies at St Thomas' Hospital, London (Thakkar et al. 2008). The variability was found to be acceptable but it appears in the current studies that a high degree of analytical variability for both target genes and reference genes along with highly variable levels of reference gene expression in the circulation, have contributed to the high intra-individual variability estimates.

The origin of the circulating mRNA species detected in this study is unclear and may have contributed to the high degree of intra-individual variability. The sample type used in this study was whole blood though it is also possible to measure free circulating mRNA species in plasma. The advantages of measuring mRNA transcripts in whole blood compared with plasma are that transcript levels for some genes are up to 22-fold higher in whole blood compared with plasma (Okazaki et al. 2006). In

addition, the PAXgene blood RNA system has been designed to stabilise cellular RNA as well as enable its extraction from whole blood, allowing the collection of blood in tubes that can remain at room temperature for up to 72 hours before freezing. This has obvious advantages in an anti-doping context where transport of athlete samples from remote locations to the anti-doping laboratory may be delayed. It is possible, however, that mRNA for GH, GHRH, IGF-I and IGFBP-3, as well as for the housekeeping genes, could be expressed by lymphocytes, granulocytes or other cells within the circulation. Variations in the number of circulating cells (within and between individuals) would therefore influence the measured concentration of mRNA both of the target genes and reference genes, contributing to the high variability. It would not be feasible, however, to use plasma mRNA concentrations in an anti-doping context at present because these blood samples must be processed in the laboratory immediately after sample collection.

8.4.2 *The effects of rhGH and rhIGF-I/rhIGFBP-3 administration on circulating mRNA concentrations*

No previous studies have investigated the response in circulating mRNA concentrations to exogenous hormone administration. In our present studies, there were no significant changes in mRNA concentrations for any of the target genes in response to the administration of rhGH for four days or rhIGF-I/rhIGFBP-3 for 28 days, regardless of the method used to normalise mRNA concentrations. The statistically significant decreases in GH and IGFBP-3 mRNA concentrations (corrected for total RNA) in the placebo group of the rhIGF-I/rhIGFBP-3 administration study could have occurred by chance because multiple comparisons were performed on the data. Another explanation for these changes in the placebo group is the high intra-individual variability in levels of these mRNA species, discussed above in section 8.4.1. Indeed it is not possible to differentiate any effect of the administered treatment in these studies from the underlying physiological variability.

It is important to note that in both the rhGH and rhIGF-I/rhIGFBP-3 administration studies there was only a weak correlation between GH, IGF-I and IGFBP-3 mRNA concentrations and IGF-I peptide concentrations. This is in contrast to previous studies in which plasma and whole blood mRNA concentrations for the placental protein human chorionic gonadotrophin (hCG) strongly correlated with plasma hCG peptide concentrations (Okazaki et al. 2006). We hypothesised that rhGH and rhIGF-I/rhIGFBP-3 administration would act through the negative feedback

mechanisms that regulate endogenous hormone production and that changes in hormone production would be reflected by changes in mRNA concentrations. It appears, however, that circulating mRNA concentrations for GH-IGF axis peptides are a poor marker of changes in IGF-I peptide concentrations. Indeed the significant changes in serum IGF-I peptide concentrations, P-III-NP peptide concentrations and GH-2000 scores observed in response to rhGH administration and rhIGF-I/rhIGFBP-3 administration are not reflected by changes in the corresponding mRNA concentrations.

8.4.3 *Limitations*

It is possible that mRNA concentrations would demonstrate greater responses if higher doses of rhGH or rhIGF-I/rhIGFBP-3 were administered or if these hormones were administered for longer periods than 4 days and 28 days respectively. Drug dose and treatment duration were based on previous investigations of markers of GH misuse and also on recommendations from the rhIGF-I/rhIGFBP-3 manufacturer, Insmed Incorporated (based on previous clinical trials with this complex). Compliance with treatment was 100% in the rhGH administration study because I administered the rhGH injections. In the rhIGF-I/rhIGFBP-3 administration study, participants self-administered their treatment but the results of the GH-dependent markers described in Chapter 3 suggest that participants were compliant with the study protocol. It seems unlikely therefore that poor compliance was the explanation for the negative results in this part of the study.

The rhGH and rhIGF-I/rhIGFBP-3 administration studies only involved male participants and it is possible that the response in mRNA concentrations would be different in women. It is unlikely, however, that this would significantly affect the results because previous studies have shown that IGF axis and collagen peptide markers demonstrate a greater response to rhGH administration in men than in women (Dall et al. 2000; Longobardi et al. 2000; Nelson et al. 2008). It was for this reason that men were chosen as the ideal study population for these pilot studies. In addition, the majority of participants in these studies were white Europeans but we have found no evidence that the response in mRNA concentrations is affected by ethnicity. A final consideration is that all participants were recreational athletes rather than elite athletes; it would not be possible for elite athletes to take part in studies involving the administration of a prohibited substance. It is unlikely, however,

that the response in mRNA concentrations would be different in elite athletes compared with our current study population.

In conclusion, the results of this study demonstrate the presence of mRNA for GH, GHRH, IGF-I and IGFBP-3 in whole blood samples. The intra-individual variability of these mRNA species is high and this limits their utility as a marker of GH and IGF-I misuse. The administration of rhGH and rhIGF-I/rhIGFBP-3 to recreational athletes does not result in significant changes in these mRNA concentrations and therefore it is unlikely that the mRNA quantitation techniques described in this chapter will prove a useful method for detecting GH or IGF-I misuse.

CHAPTER 9: DISCUSSION, CONCLUSIONS AND FUTURE WORK

Athletes have been using performance-enhancing drugs to gain an advantage over their opponents throughout the history of competitive sports. It is clear that GH has been a popular substance of misuse for many years because of its anabolic and lipolytic properties. The anabolic effects of IGF-I on muscle protein synthesis, collagen metabolism and glycogen storage also make this an attractive doping agent, though there is no published evidence to suggest that IGF-I has a performance-enhancing effect in athletes. The increasing availability of rhIGF-I preparations for clinical use will increase the chances of athletes and their support teams gaining access to this peptide for illicit purposes. The use of GH and IGF-I is not only unfair to the athlete's competitors but in the long term could result in detrimental effects to the athlete's health. For these reasons, both GH and IGF-I are included in the WADA list of prohibited substances (WADA 2012).

The GH-2000 and GH-2004 research teams developed a method for detecting GH misuse based on the serum concentrations of GH-dependent markers. They devised gender-specific discriminant function equations, which incorporated the markers IGF-I and P-III-NP along with a correction factor for age (Powrie et al. 2007). These equations can be used to distinguish a group of athletes who are administering rhGH from those administering placebo. This 'marker method' was shown to function effectively in athletes regardless of their ethnicity (Erotokritou-Mulligan et al. 2008; Holt et al. 2010) and the results were not significantly affected by injury (Erotokritou-Mulligan et al. 2008). The intra-individual variability of marker levels was established (Erotokritou-Mulligan et al. 2009) and preliminary investigations into the stability of these markers in serum were performed (Holt et al. 2009).

9.1 Biomarkers of IGF-I misuse in recreational athletes

While significant progress has been made in the detection of GH misuse, no previous studies have investigated the detection of IGF-I misuse in athletes. The primary aim of my research was therefore to investigate methods for detecting IGF-I misuse, based on the principles of the GH-2000 marker method. Our hypothesis was that IGF-I misuse would be expected to cause changes in GH-sensitive serum markers, as had been observed after GH administration.

We first investigated whether the administration of rhIGF-I/rhIGFBP-3 complex induces changes in serum IGF-I and P-III-NP (the markers that proved most useful in detecting GH misuse) and in GH-2000 score. As described in Chapter 3, I supervised a randomised, double-blind, placebo-controlled rhIGF-I/rhIGFBP-3 administration study in 56 recreational athletes (26 women and 30 men). Participants were randomly assigned to receive placebo, low dose rhIGF-I/rhIGFBP-3 (30 mg/day) or high dose rhIGF-I/rhIGFBP-3 (60 mg/day). Venous blood samples for IGF-I and P-III-NP were collected prior to treatment, during the treatment period and up to 8 weeks after treatment had been completed.

In this study, IGF-I, P-III-NP and GH-2000 score rose in response to both low and high dose rhIGF-I/rhIGFBP-3 in both women and men. IGF-I was a more responsive marker than P-III-NP but using the GH-2000 score method, we were able to detect a significant number of athletes receiving rhIGF-I/rhIGFBP-3 complex. The major limitation of this study was that the doses of rhIGF-I/rhIGFBP-3 currently misused by elite athletes are unknown and may be much higher than the doses chosen for this study. Furthermore athletes may be using rhIGF-I alone rather than rhIGF-I/rhIGFBP-3 complex and the two compounds may have different effects on GH-dependent markers (though it is likely that both compounds will cause increased serum IGF-I concentrations).

The P-III-NP response to rhIGF-I/rhIGFBP-3 administration was substantially less than that observed previously in response to rhGH administration, while the relative increase in IGF-I concentration was greater in the current study than in the GH-2000 study. This supports the idea that GH has both direct effects on collagen metabolism and indirect effects through IGF-I, and that the direct effect of GH is greater than its indirect effect.

In the original GH-2000 studies, although IGF-I and P-III-NP were selected as the best markers of GH administration, other markers of GH action also responded to GH administration. These markers were IGFBP-2, IGFBP-3, acid-labile subunit (ALS) (Dall et al. 2000), osteocalcin, procollagen type I carboxy-terminal propeptide (PICP) and type I collagen cross-linked carboxy-terminal telopeptide (ICTP) (Longobardi et al. 2000). It is possible that these markers could be used instead of, or in addition to, IGF-I and P-III-NP to improve the performance of a test for detecting IGF-I misuse.

The next stage in developing a test for detecting IGF-I misuse therefore relies on investigating the response of further serum markers to IGF-I administration. We

have in fact started this further work supported by a grant from the Partnership for Clean Competition (PCC), an anti-doping research collaboration that combines the expertise of the United States Anti-Doping Agency (USADA) and major professional sporting organisations in the USA. We aim to measure the serum markers described above, in the samples collected during the rhIGF-I/rhIGFBP-3 administration study described in Chapter 3. Initial results suggest that serum IGFBP-2 increases and IGF-II decreases in response to rhIGF-I/rhIGFBP-3 administration in both male and female recreational athletes, and that ALS decreases in women but not in men. The initial analyses have been performed using samples from before and during the drug administration period (Days 0, 21 and 28) and in the next phase of the study, we plan to investigate the response of these three additional markers throughout the 84-day study period.

It is hoped that one or more of these markers could be combined with serum IGF-I to provide a more sensitive and specific test for detecting IGF-I misuse. It is also possible that the inclusion of P-III-NP could further improve the specificity of a test for detecting IGF-I misuse, in combination with these other markers. The overall aim of these studies is to combine the best markers of IGF-I misuse in an "IGF-I discriminant function", analogous to the GH-2000 score for detecting GH misuse. We hope that this formula could be adopted by WADA for detecting IGF-I misuse, alongside the current marker and isoform methods for detecting GH misuse.

9.2 The effects of IGF-I on lipid metabolism, carbohydrate metabolism, body composition and physical fitness

There are limited data on the effects of IGF-I administration on substrate metabolism in healthy volunteers and there is no published evidence to suggest that IGF-I alters body composition or enhances performance in athletes. As part of the randomised, double-blind, placebo-controlled study described in Chapter 3, we investigated the effects of rhIGF-I/rhIGFBP-3 administration on lipid and glucose homeostasis and on body composition and physical fitness in 56 recreational athletes. The hypothesis tested in Chapter 4 was that IGF-I administration would result in improved insulin sensitivity, enhanced triglyceride breakdown, increased lean body mass and improved aerobic performance.

This results of this part of the study showed that rhIGF-I/rhIGFBP-3 administration caused improvements in insulin sensitivity along with decreased fasting triglycerides and increased LDL cholesterol. The most likely mechanism of these effects is through

suppression of GH secretion. As described in my Introduction (Chapter 1), rhIGF-I/rhIGFBP-3 has been investigated as a treatment for people with diabetes and its administration is associated with decreased insulin requirements and improved insulin sensitivity (Saukkonen et al. 2004; Clemmons et al. 2007). The further development of both rhIGF-I alone and rhIGF-I/rhIGFBP-3 complex as a treatment for diabetes has been hampered by concerns about potential side-effects, in particular the transient worsening of retinopathy. The results of the current study in healthy recreational athletes show that rhIGF-I/rhIGFBP-3 administration does indeed have glucose-lowering effects. I believe that longer term studies of the effects of rhIGF-I on people with diabetes are merited and that it may yet prove a useful adjunct to current treatments.

The effects of rhIGF-I/rhIGFBP-3 administration on body composition and physical fitness were also interesting. There were no significant changes in body composition assessed by DEXA scan, skinfold assessments or bioelectrical impedance analysis. This is in contrast with the decrease in fat mass and increase in lean body mass observed after rhGH administration to recreational athletes (Meinhardt et al. 2010). This finding supports the theory that GH acts directly on body composition rather than acting only indirectly, through the production of hepatic IGF-I. There were, however, significant improvements in aerobic performance measured by maximal oxygen consumption (VO_2 max) in both female and male recreational athletes after rhIGF-I/rhIGFBP-3 administration. The relative increase in VO_2 max in women was approximately 9% while that in men was approximately 6%. The mechanisms underlying this improvement require further research. Previous studies of the effects of rhGH administration on athletic performance have demonstrated improvements in respiratory muscle strength and lung capacity, along with peak oxygen uptake (Graham et al. 2007). Future studies investigating the potential benefits of IGF-I on athletic performance therefore should include assessments of respiratory muscle and cardiovascular function.

The significance of this improvement in VO_2 max to elite athletic performance is unclear; we do not know if an elite athlete would benefit in the same way as the recreational athletes in this study. To put this in the context of athletic performance, a 9% improvement in aerobic performance translates into approximately 12 minutes gained over the course of a 2 hours 15 minutes marathon race (the current world record time for women). The effects of IGF-I on skeletal muscle strength in athletes are also unknown; future studies should investigate the effects of IGF-I administration on variables such as maximal strength, explosive power and sprint

capacity, as have previously been investigated in rhGH administration studies (Meinhardt et al. 2010). If significant improvements in these aspects of physical performance are demonstrated, it would suggest potential benefits of IGF-I administration to athletes in power sports such as sprinting and weight-lifting. It would also, however, support the further investigation of IGF-I compounds for the treatment of muscle-wasting diseases such as muscular dystrophy and frailty associated with loss of muscle mass in elderly people.

9.3 Cross-sectional study of elite adolescent athletes

It is important that tests for detecting GH misuse are applicable to adolescent athletes because increasing numbers of athletes in this age group compete at national and international events. The original GH-2000 studies included few athletes younger than 18 years and the age adjustment included in the GH-2000 formulae was valid over age range 18-50 years (Powrie et al. 2007). The hypothesis tested in Chapter 5 was that the relationship between age and serum GH-dependent markers would be different in adolescent athletes compared with older athletes because of known changes in GH secretion that occur around puberty. We measured serum IGF-I and P-III-NP concentrations in 157 elite adolescent athletes aged between 12-20 years and calculated their GH-2000 scores. Both IGF-I and P-III-NP rose to a peak during adolescence in elite athletes and then declined and this confirmed our hypothesis. The variability of the GH-2000 score was higher in adolescents than in adult athletes. We found no evidence, however, that the use of the GH-2000 equations would lead to an unacceptable rate of false positive results in adolescent athletes. We have suggested that caution will be required when using this test around the time of peak growth velocity and we have currently recommended to WADA that the test is not used to sanction athletes under 18 years old. It will be important to collect further samples from adolescent athletes (either during anti-doping sample collections or through future research studies) to add to the data generated in this study and further characterise the relationship between GH-2000 score and age in younger elite athletes. It is possible that the "Athlete's Passport" approach, where biomarker concentrations are monitored longitudinally, may prove useful in this age group.

9.4 The effects of a freeze-thaw cycle and pre-analytical storage temperature on the stability of IGF-I and P-III-NP concentrations

It was necessary to establish the pre-analytical storage and treatment conditions required to ensure accurate IGF-I and P-III-NP measurements, before the marker method was implemented by anti-doping organisations. Since anti-doping laboratories do not always have access to -80°C storage facilities, we investigated the stability of these analytes in serum stored at -20°C and also investigated the effects of a single freeze-thaw cycle on analyte results. The hypothesis tested in Chapter 6 was that these pre-analytical factors would not significantly affect IGF-I and P-III-NP results. Blood samples were collected from 20 healthy volunteers; IGF-I and P-III-NP were each measured by two assays and GH-2000 scores were calculated. We found that the analysis of samples immediately after centrifugation and the storage of samples at -20°C for up to three months had no significant effects on analyte results. Indeed the variability in results was largely determined by the inter-assay precision of the assays. The previous GH-2004 study of pre-analytical factors showed that storage of serum or clotted blood samples at 4ºC for up to five days did not result in any significant changes in IGF-I and P-III-NP concentrations (Holt et al. 2009). We have used the results of these two studies to recommend to WADA that samples for GH testing should be kept chilled after collection; these samples should be delivered to the anti-doping laboratory and analysed within five days. Separated serum fractions can be stored at -20°C for up to three months and then should be stored at -80°C.

9.5 Cross-sectional study of elite athletes and development of decision limits for the implementation of the GH-2000 detection methodology

The GH-2000 discriminant function formulae for detecting GH misuse were initially validated using a commercial IGF-I assay that is no longer available and, in addition, WADA rules state that any analyte measured by immunoassay should be measured by two separate assays that recognise different epitopes. It was therefore necessary to validate two IGF-I and two P-III-NP assays that are currently available, using samples from elite athletes. The hypothesis tested in Chapter 7 was that the GH-2000 formulae would function effectively, regardless of the assays used to measure the analytes. We collected venous blood samples from 498 elite athletes and measured IGF-I and P-III-NP in these samples using two commercial assays for each marker. We calculated GH-2000 scores using all possible combinations of assay results and

then used the mean and standard deviation of these scores to estimate decision limits, above which the false positive rate would be approximately 1 in 10,000.

During the analysis of these results, one participant was excluded because there was insufficient serum for all four assays and another was excluded because his GH-2000 score was incompatible with normal physiology. UK Anti-Doping confirmed that this athlete had tested positive for GH misuse using the WADA isoform GH test and I included the results from this athlete in the figures in Chapter 7 to demonstrate how the marker test can be used to declare a "positive" or adverse analytical finding. It provided further evidence that the test can be used to detect athletes who are misusing GH. We used the results from 496 elite athletes to propose decision limits to WADA based on the combination of Orion P-III-NP and Immunotech IGF-I assays and the Cisbio P-III-NP and Immulite IGF-I assays.

The results of this study and the proposed decision limits were accepted by WADA in June 2012. The final step in the implementation of the marker method was for the King's College London anti-doping laboratory to obtain accreditation for the test from the UK Accreditation Service (UKAS), which assesses the competence of laboratory testing against international standards. This process required the London laboratory to take part in a successful inter-laboratory study in which test results from London were compared with those from two other European laboratories. Accreditation was obtained in July 2012 and this enabled the test to be implemented at the London 2012 Olympic and Paralympic Games. A number of blood samples were analysed using the marker method before and during the Games and two athletes were found to have test results above the decision limits for both assay pairings or "Adverse Analytical Findings". Both athletes were Paralympian powerlifters who subsequently admitted to misuse of rhGH and have since been banned from competing for two years.

Despite the resounding success of the test, the method remains vulnerable to changes in the four commercial immunoassays used to measure IGF-I and P-III-NP. As described in my Introduction, the GH-2000 team anticipated this risk and advised the IOC and WADA to develop their own assays but this work was not completed. Problems have recently arisen with the supply of the Siemens Immulite IGF-I reagents and the GH-2004 team is therefore continuing to develop mass spectrometric assays for IGF-I. These methods will not only provide a highly accurate and precise estimation of IGF-I concentrations but will also improve the long-term viability of the test, regardless of manufacturer reagent alterations.

9.6 The use of blood mRNA technology to detect GH and IGF-I misuse in athletes

It had been shown previously that it is possible to detect mRNA for GH and GHRH in the peripheral circulation and this raised the possibility of using mRNA technology to detect misuse with GH and IGF-I in athletes. We investigated the intra-individual variability of circulating mRNA concentrations for GH, GHRH, IGF-I and IGFBP-3 and also the response of these mRNA concentrations to exogenous administration of rhGH and rhIGF-I/rhIGFBP-3 complex. The hypotheses tested in Chapter 8 were that the administration of rhGH would suppress mRNA concentrations for GH and GHRH while increasing mRNA concentrations for IGF-I and IGFBP-3, and that the administration of rhIGF-I/rhIGFBP-3 would suppress mRNA concentrations for all four target genes. We collected blood from 10 recreational athletes on four occasions over six weeks to assess intra-individual variability of mRNA concentrations and we assessed the acute changes in mRNA concentrations in response to four daily doses of rhGH, in a separate group of 10 recreational athletes. Finally, we assessed the changes in mRNA concentrations in the 30 male recreational athletes who were taking part in the rhIGF-I/rhIGFBP-3 administration study described in Chapter 3.

The results of this study confirmed the presence of GH and GHRH mRNA in the circulation and we were also able to detect circulating mRNA for IGF-I and IGFBP-3. The intra-individual variability of these circulating mRNA concentrations was extremely high and it was not possible to demonstrate a significant change in circulating mRNA concentrations in response to either rhGH or rhIGF-I/rhIGFBP-3 administration. These disappointing results were due in part to the nature of the sample type used: whole blood was chosen because of the apparent stability of nucleic acids in this sample type. Plasma mRNA concentrations may well show lower intra-individual variability but this would require immediate processing in a laboratory, which would not be feasible in an anti-doping setting.

Since this study was completed, we have been in communication with a research group from the University of Virginia, USA who have also been investigating the expression patterns of GH mRNA in circulating white blood cells (Kelly et al. 2012). The intra-individual variability of mRNA concentrations in their study of healthy volunteers appears to be much lower than in our mRNA study. One explanation for the difference in results is that the Virginia research group employed a different method of reverse-transcription quantitative PCR including nested primers used in

successive PCR runs. This technique reduces the amplification of unwanted PCR products and this might explain the lower variability observed. The next stage in their investigations will be to determine if changes in GH mRNA concentrations, in response to rhGH administration or rhIGF-I administration, can be quantified using the nested primer PCR technique.

9.7 Conclusions

During this research, I aimed to provide further evidence for the validity of the GH-2000 method for detecting GH misuse in athletes and to begin work on developing similar methods for detecting IGF-I misuse. The work I performed with the GH-2004 research group on adolescent athletes, on the stability of marker results and on elite adult athletes contributed to the implementation of the GH-2000 marker method at the London 2012 Olympic and Paralympic Games and our first successes in detecting GH misuse in two athletes. The results of the rhIGF-I/rhIGFBP-3 administration study suggest that a similar marker method should prove successful in detecting IGF-I misuse in athletes but further work is necessary to find the optimal combination of markers for detecting this type of doping. Furthermore, this administration study in healthy young volunteers provided insights into the effects of IGF-I on glucose and lipid metabolism that could have implications in a clinical setting and also demonstrated flaws in the original somatomedin hypothesis. Finally, the studies on circulating mRNA species demonstrated the presence of GH, GHRH, IGF-I and IGFBP-3 mRNA in whole blood but did not seem to provide an alternative method for detecting GH and IGF-I misuse, though it is possible that this technique could be improved with further development of the quantitative PCR protocol.

The battle against doping will continue as athletes and their support teams find ways to circumvent new tests as they are implemented. We are already aware, for example, of the threat of athletes using growth hormone secretagogues to mimic the effects of rhGH administration; the marker method should also prove successful in detecting such compounds because we use downstream markers in the GH-IGF axis but this will require investigation. The major challenge to the success of our method remains the ability to control the consistency of reagents provided by commercial manufacturers and our research team continues to work with WADA to provide solutions to these types of problems as they arise.

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