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Procollagen type III amino-terminal propeptide and insulin-like growth factor I as biomarkers of growth hormone administration

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Abstract

The acceptance in 2012 by the World Anti-Doping Agency (WADA) of the biomarker test for human growth hormone (hGH) based on procollagen type III amino-terminal propeptide (P-III-NP) and insulin-like growth factor I (IGF-I) was perhaps the first time that such a method has been used for forensic purposes. Developing a biomarker test to anti-doping standards, where the strict liability principle applies, is discussed. An alternative WADA-accepted approach is based on the measurement of different hGH isoforms, a method that suffers from the very short half-life of hGH limiting the detection period. Modification or withdrawal of the immunoassays, on which the biomarker measurements largely depend, has necessitated revalidation of the assays, remeasurement of samples and adjustment of the decision limits above which an athlete will be assumed to have administered hGH. When a liquid chromatography coupled mass spectrometry (LC–MS) method became a reality for the measurement of IGF-I, more consistency of results was assured. Measurement of P-III-NP is still dependent on immunoassays although work is underway to develop an LC–MS method. The promised long-term detection time for the biomarker assay does not appear to have been realised in practice, and this is perhaps partly the result of decision limits being set too high. Nevertheless, more robust assays are needed before a further adjustment of the decision limit is warranted. In the meantime, WADA is considering using P-III-NP and IGF-I as components of a biomarker passport system recording data from an individual athlete, rather than the population. Using this approach, smaller perturbations in the growth hormone (GH) score would mandate an investigation and possible action for hGH administration.

KEYWORDS
biomarker, growth hormone, IGF-I, P-III-NP, protein quantification

1 | INTRODUCTION & BACKGROUND

The administration of growth hormone (GH) is prohibited in sport, and the detection of the administration of human growth hormone (hGH) is particularly challenging because distinction from normal endogenous production is necessary as discussed in this review. hGH administration was first prohibited by the International Olympic Committee in 1992. This prohibition was continued by the World Anti-Doping Agency

(WADA) when they produced their first prohibited list in 2004, although no analytical test had been approved at that time. Because endogenous hGH is produced episodically, with large fluctuations in circulating concentrations, neither the mere presence nor the concentration is sufficient to provide evidence of administration. Furthermore, the very short half-life of hGH (14–18 min[1]) makes its measurement in blood generally of little evidential value. One method based on the measurement of the relative concentrations of the 22- and 20-kDa isoforms in blood serum has been approved by the WADA[2] as a method to evidence hGH administration, but because of the very short half-life of hGH, this method has a very short detection time. An alternative method is to use suitable biomarkers[3*] affected by hGH administration, and a biomarker method was approved by WADA in 2012. This method is based on a discriminant scoring system, which uses the concentrations of insulin-like growth factor I (IGF-I) and procollagen type III amino-terminal propeptide (P-III-NP). Using these biomarkers gives the possibility of a much longer period of detection because the disappearance half-lives of the two biomarkers are of the order of hours rather than minutes. The disappearance half-life of P-III-NP is reported to be 693 h[4] and that of IGF-I said to be 14–18 h[5,6] although one publication suggests 90 h,[7] but we have been unable to find the primary studies where these values were determined.

Like the isoform method, blood samples are required for the measurement of IGF-I and P-III-NP as they are not normally present in urine. The accepted method is to use only blood serum collected in serum separator tubes because the concentration in plasma, at least of IGF-I, has been shown[8] to be different from that in serum by about 10% less although highly correlated.

This review focuses on the role and measurement of IGF-I and P-III-NP, as the currently used biomarkers for determining hGH administration in sport, and will include how the biomarker assay was developed up to the present state of play.

2 | ESTABLISHMENT OF BIOMARKERS TO DETECT HGH ADMINISTRATION

Kicman et al[9] undertook one of the first studies seeking to find suitable biomarkers of hGH administration. They found that both IGF-I and IGFBP-3 increased whereas IGFBP-2 decreased following hGH administration and proposed a ratio of IGF-I to IGFBP-2 and/or IGFBP-3 to IGFBP-2 as possible biomarkers of hGH administration. However, Wallace et al[10] in their study on possible biomarkers of hGH administration observed only a minor decrease in IGFBP-2, which may be due to the different immunoassays employed for the two studies. A number of collagen bone markers were considered as suitable biomarkers in addition to IGF-I and its binding proteins, and in the normal individual, not using GH, age and sex were shown to be the most important factors affecting their concentrations, whereas sport and ethnicity were minor factors.[11] Considering these different possible biomarkers, Powrie et al[12] published an approach based on a scoring system with P-III-NP, IGF-I and age as the parameters. The score was based on the statistical evaluation of the results of a double-blind, placebo-controlled study with 102 healthy non-competing but trained subjects. Nine markers were measured during administration of hGH for 1 month and during a 56-day washout period. In addition, the study included samples from 813 elite athletes. The scoring system was different for males and females as shown in Figure 1a.

In the original study, a Nichols Institute Diagnostics assay (Nichols Institute Diagnostics, San Juan Capistrano, CA, USA) was used for IGF-I and a CisBio assay (International CIS, Gif sur Yvette, France) for P-III-NP, neither of which is available today. The assays that are used today for IGF-I in the method approved by WADA are the IDS-iSYS IGF-I (Immunodiagnostics Systems Limited, Boldon, UK), the Immunotech A15729 IGF-I IRMA (Immunotech SAS, Marseille, France) or liquid chromatography coupled mass spectrometry (LC–MS). Two studies show the benefits of using LC–MS, one based on digestion of IGF-I with trypsin and quantification of two of the resultant peptides[15] and the other study where IGF-I is measured intact.[16] For P-III-NP, the assays are the Orion UniQ™ P-III-NP RIA (Orion Diagnostica, Espoo, Finland) and the Siemens ADVIA Centaur P-III-NP (Siemens Healthcare Laboratory Diagnostics, Camberley, UK). Recently, an adjustment was made to the score used for males to correct for a minor bias with age, but this did not affect the decision limit for evidence of hGH administration (see Figure 1b).

(a)

GH-2000 score for males:

$$-6.586 + 2.905 \cdot \ln(\textit{P-III-NP}) + 2.100 \cdot \ln(\textit{IGF-I}) - 101.737/ \text{ age}$$

GH-2000 score for females:

$$-8.459 + 2.454 \cdot \ln(\textit{P-III-NP}) + 2.195 \cdot \ln(\textit{IGF-I}) - 73.666/ \text{ age}$$

(b)

GH-2000 score for males adjusted:

$$-6.586 + 2.905 \ln(\text{P-III-NP}) + 2.100 \ln(\text{IGF-I}) - 101.737/\text{age} - 0.02 (\text{age} - 25.09)$$

FIGURE 1 The GH-2000 scoring system, where (a) shows the original score adopted by WADA and (b) shows the adjusted scoring system for males recently implemented by WADA.[13,14] Natural logarithms of the concentrations are used, and age is rounded down to the nearest year

3 | PRODUCTION AND ENDOCRINOLOGY OF THE GH-2000 BIOMARKERS

The production and endocrinology of the two GH-2000 biomarkers will be briefly outlined to help understand some of the issues involved with their analysis.

3.1 | Procollagen type III N-terminal propeptide

P-III-NP is involved in type III collagen synthesis and turnover. Collagens are a vital series of proteins predominantly produced by fibroblast cells, which are found in the interstitial space between cells and are the primary source of extracellular matrix proteins.

Type III collagen is formed by translation from the *COL3A1* gene, which maps to the q24.3–q31 regions of chromosome 2.[17] A signal peptide from 1 to 23 is formed followed by the 130 amino sequences from 24 to 153, which is the N-terminal propeptide, known as P-III-NP. The collagen peptide consists of the amino acid sequence from 154 to 1221 followed by a C-terminal propeptide, P-III-CP, from the remaining amino acid sequence from 1222 to 1466. Each of these monomers (α1 chains) covalently link via disulphide bridges to form homo-trimers directed by the interchain disulphide bridges in P-III-CP.[18] The three α-chains of collagen and P-III-NP have an abundance of a repeating motif of glycine-X-Y where X and Y are any amino acid. However, when Y is proline, the proline undergoes a post-translational modification to hydroxyproline.[19] The individual α1 chains are linked covalently by disulphide bridges at the C-terminus of the protein to form the circulating triple helical procollagen III structure. P-III-CP is cleaved during or after secretion and the resulting molecules, known as pN-collagen, further cross-link to produce collagen fibrils. P-III-NP is cleaved sequentially to help establish stable intramolecular bonds and allow for further collagen precursors, that is with P-III-NP still attached, to build the fibril. Some P-III-NP molecules will remain on the collagen fibril and hence
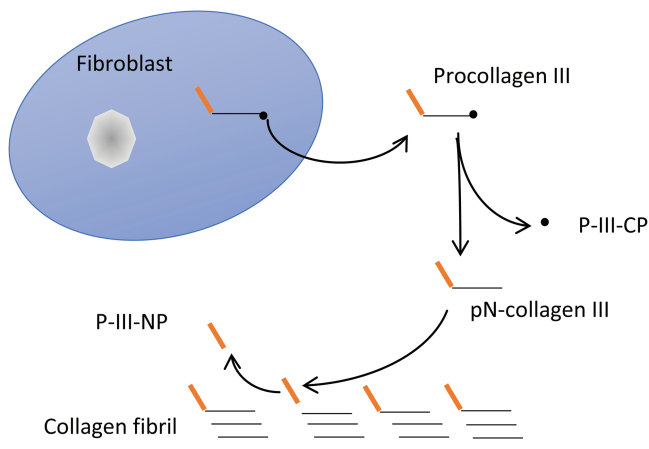
FIGURE 2 Synthesis of type III collagen. Secretion of procollagen from the cell leads to the removal of P-III-CP, leaving type III pN-collagen (P-III-NP and collagen). As pN-collagen is attached to the collagen fibril, P-III-NP from the surface is released into the blood stream

P-III-NP appears in the circulation both during collagen formation and degradation (see Figure 2 below).

It is thought[20,21] that P-III-NP and P-III-CP have an inhibitory role regulating the synthesis of type III collagen, but no other biological activity for P-III-NP is known.

Some variants of type III collagen relating to Ehlers–Danlos syndrome caused by mutations in the *COL3A1* gene have been described.[22] However, although theoretical, there appears to be just one publication[23] showing that it affects P-III-NP. This relates to a change of proline to alanine at position 49 of type III collagen, position 25 of P-III-NP. This has been found in polymicrogyria with or without vascular-type Ehlers–Danlos syndrome (PMGEDSV). The frequency of occurrence of this single nucleotide polymorphism dbSNP: rs1234344050 is given as 1/250968.

This apparent paucity of variants may be due to the lack of limited known connections in relation to the biological activity of P-III-NP and disease such that it has not yet been sufficiently well investigated. As the methods of analysis of P-III-NP improve, it seems likely that more variants will be discovered.

3.2 | Insulin-like growth factor I

IGF-I, previously known as somatomedin-C, is produced primarily in the liver, although most of the body's tissues are capable of this synthesis, mediated by GH release.[24] IGF-I circulates as a complex with specific binding proteins (IGFBPs), mainly IGFBP-3, as well as acid-labile subunit (ALS). Some of these proteins are regulated by GH, and circulating GH and IGF-I both have a negative feedback effect on GH production.[25,26]

IGF-I is formed from the translation of the IGF1 gene, located on chromosome 12q23.2.[27] According to the UniProtKB database, the IGF-I prohormone (accession no. P05019) includes a signalling peptide (1–21), two propeptides (22–48 and 119–195) and the IGF-I chain (49–118). The mRNA sequence when correctly translated yields the 195 amino acid prohormone, but variations of the prohormone are present in humans where four isoforms can be found. This is a result of alternative splicing of the exons in the mature mRNA formed before protein translation.[28–30] The amino acid sequence of expressed IGF-I is identical for all of these isoforms, as the sequence variation occurs in the extension peptide regions of the signalling (isoform 3) and propeptides (isoforms 2–4). However, mutations in the exons of the IGF1 gene have been related to several SNPs that have been associated with disease states including diabetes[31,32] and cancer.[32–36]

The expressed IGF-I protein is comprised of four domains; that is, B (49–77), the amino-terminal domain; C (78–89), A (90–110) and D (111–118), the carboxyl-terminal domain. The domains play different roles in the binding affinity of IGF-I to receptors and binding proteins.[37] The A and B domains are connected by disulphide bonds between Cys-54 and Cys-96 and between Cys-66 and Cys-109. An additional disulphide bond is located in the A-domain between Cys-95 and Cys-100.[38] Oxidation of the Met-129 has been reported; however, this is non-biological and results from sample storage and

handling.[39] Recently, with the move to the use of more modern mass spectrometers (MS) to measure IGF-I, several variants, otherwise indistinguishable from the wild-type protein by immunoassays, have been reported. These result from single nucleotide polymorphisms of the IGF-I gene[40,41] and the level of expression may be complete or partial. The clinical relevance of most of these variants is unknown; however, the R98W variant (dbSNP: rs587779350) has been associated to a patient with primordial dwarfism.[42]

4 | CHEMISTRY

4.1 | Procollagen type III amino-terminal propeptide (P-III-NP)

P-III-NP is comprised of three identical peptide chains and has an approximate molar mass of 42 kDa. Bovine P-III-NP has been best studied having been isolated from foetal calf skin by Brandt et al[43] and sequenced with a commercial version of the 'protein sequenator'[44] and shown to be comprised of 130 amino acids. Bovine P-III-NP has been characterised by ultracentrifugal and circular dichroism studies after treatment with bacterial collagenase into three
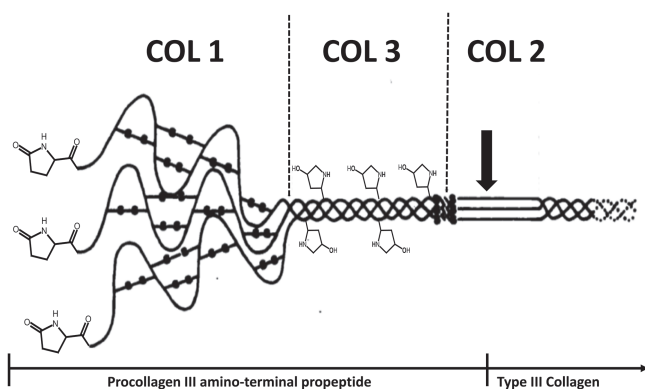
domains.[45,46] Col 1 is at the N-terminal (i.e. amino-terminal) end and has five intrachain disulphide bridges of this cysteine-rich region in each chain, giving three Col 1 domains in the P-III-NP molecule. Col 3 is a middle region of the molecule, with a triple helical structure providing a collagenous non-covalently linked segment. Col 2 is at the C-terminus region with three interchain covalent disulphide bridges. Figure 3 illustrates the structure of P-III-NP.

There does not appear to be an equivalent study for human P-III-NP (hP-III-NP) perhaps because of the difficulty of obtaining sufficient material. However, the predicted protein arising from the human COL3A1 gene is a single strand protein with the UniProt accession number P02461 comprising a signal peptide, P-III-NP, a collagen strand and P-III-CP. The equivalent bovine gene (Bos taurus, accession number Q08E14) produces a P-III-NP (bP-III-NP) with 97% sequence homology but which is commonly used as reference material in the absence of suitable reference hP-III-NP, see Figure 4 for the amino acid sequence of human and bovine P-III-NP, respectively. Interestingly, Bos indicus cattle, also known as humped, Zebus or Brahman cattle originating from south Asia, have a different sequence homology.

4.2 | Insulin-like growth factor I

IGF-I is a 70 amino acid protein with a molecular weight 7.6 kDa. Normally, at least 99% of IGF-I in the circulation is bound to high affinity binding proteins (IGFBPs),[48] with most in the form of a ternary complex. There are six IGFBPs (IGFBP-1 to IGFBP-6) comprising between 200 and 300 highly conserved amino acids that results in a molecular weight range 26–31 kDa. IGFBP-3 has the strongest affinity for IGF-I, and the two form a ternary structure with a glycoprotein called ALS[49] to make a 150-kDa complex that accounts for approximately 80% of circulating IGF-I. IGF-II forms a similar ternary complex.[50] Free IGF-I has a half-life of 10 min; when bound to an IGFBP, this increases to 25 min.[51] In the ternary IGF-I/IGFBP-3/ALS structure, the half-life increases to 16 h.[6] IGFBP-5 can also bind to ALS and IGF-I (or IGF-II) to form an alternate ternary structure, but this is less abundant.[52] As

FIGURE 3 Schematic illustration of P-III-NP showing the Col 1, 2 and 3 domains (from Niemela[47])

P02461 COL3A1 - *Homo sapiens* (Human)

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 24  QQEAVEGGCSHLGQSYADRDVWKPEPCQICVCDSGSVLCDDIICDDQELDCPNPEIPFGE    83
 84  CCAVCPQPPTAPTRPPNGQGPQGPKGDPGPPGIPGRNGDPGIPGQPGSPGSPGPPGICES   143
144  CPTGPQNYSP                                                    153
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Q08E14 COL3A1 – *Bos taurus (Bovine)*

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 24  QQEAVDGGCSHLGQSYADRDVWKPEPCQICVCDSGSVLCDDIICDDQELDCPNPEIPFGE    83
 84  CCAVCPQPPTAPTRPPNGQGPQGPKGDPGPPGIPGRNGDPGPPGSPGSPGSPGPPGICES   143
144  CPTGGQNYSP                                                    153
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FIGURE 4 Amino acid sequence of (a) human and (b) bovine (*Bos taurus*) P-III-NP. *P* (italicized, bold and underlined) indicates proline that is changed to alanine in the known variant of hP-III-NP showing that this is not seen by measurement of T1 or T5 shown in red. The first sequence in red shows position of T1 in relation to the whole peptide and the second sequence in red shows the position of T5 both formed by trypsin digestion, see LC–MS section of this review. The amino acids of bP-III-NP, shown in red, indicate the four different amino acids from human

well as acting as a carrier for IGF-I, the binding proteins also regulate their endocrine actions by controlling receptor bioavailability. Hence, the prolonged half-life and binding capacity of the ternary IGF-I complexes ensures that there is a long-lasting stable supply of protein in circulation to support the many functions of IGF-I in the body. This contrasts with the large number of fluctuations seen with circulating hGH, which is released in a pulsatile manner from the pituitary.

The variables that regulate the IGFBPs control the level of free circulating IGF-I. Administration of IGF-I or GH increases the concentration of IGFBP-5 in circulation, whereas IGFBP-2 concentration is typically very stable but decreases with GH secretion.[9,53] IGFBP-1 and IGFBP-2 form binary complexes with IGF-I, and these complexes have only a short-term effect on the regulation of circulating IGF-I. IGFBP-I is regulated by insulin, which inhibits its secretion.[54] Several studies have shown that individuals with type-1 diabetes have elevated IGFBP-1 levels and as a result have decreased levels of free IGF-I in the circulation[54] and, depending on the measurement approach, these patients can have apparently lower IGF-I concentrations. Many papers have been published on the physiological effects of reduced IGF-I but are outside the scope of this review.

4.3 | Measurement of P-III-NP and IGF-I

4.3.1 | Traceability and commutability of standards

Although the following sections only reference IGF-I, given the lack of a traceable standard for P-III-NP that will be discussed later, the principles discussed in this section are equally relevant. For WADA and modern clinical chemistry, stable traceable standards are essential. Traceability for IGF-I measurements has relied on standards provided via the National Institute for Biological Standards and Controls (NIBSC). The first preparation (as used by Nichols) was an International Reference Reagent (IRR) (87/518) that was replaced by the WHO International Standard IGF-I for bioassay (91/554) followed in 2008 by the WHO 1st International Standard for IGF-I, recombinant, human, for immunoassay (02/254).

The latest standard is the WHO 2nd International Standard (19/166) WHO International Standard IGF-I, recombinant, human, 2nd International Standard released in 2020. This has been checked by modern methods including amino acid analysis.

The National Institute of Standards and Technology (NIST) now supply a frozen solution of IGF-I (Standard Reference Material® 2926) calibrated in molar units per gram, rather than mass per vial as provided by NIBSC, and is based on recombinant IGF-I expressed in *Escherichia coli* supplied by PreproTech. This material has been checked for total concentration by amino analysis using isotope dilution LC–MS/MS.

Interestingly, homogeneously heavy-labelled (^{15}N) IGF-I is available from NIST prepared by expression of the human gene in a microorganism[16] and is a single conformer valuable for use as an internal standard in LC–MS assays for IGF-I as discussed later. Other commercial sources of heavy-labelled IGF are available but generally of unknown purity although still likely to be useful as internal standards.

4.3.2 | P-III-NP immunoassays

As mentioned earlier, WADA has approved two immunoassays[55] for the measurement of P-III-NP, the Orion UniQ™ P-III-NP RIA and the Siemens ADVIA Centaur P-III-NP assay. A comparison of the performance of these two assays has been published by Knudsen et al.[56] Another assay for P-III-NP that has previously been used for the biomarker approach for hGH administration is made by CisBio now part of the PerkinElmer group. Like the Orion assay, the CisBio assay was originally a radio-isotopic assay and discussed by Cowan and Bartlett for hGH biomarker use.[57] The CisBio assay has now been reformatted as an ELISA.

The lack of an international standard or other traceable reference material is a current problem for the measurement of P-III-NP although a number of publications confirm the possibility of producing *h*P-III-NP by the use of genetically engineered microorganisms as reviewed by Baez et al.[58] Similarly, there is no recognised calibration standard for P-III-NP assays. For example, the Centaur P-III-NP assay relies on bovine P-III-NP, which has 97% sequence homology with four different amino acids from the human form.[56] The UniQ PIIINP assay appears, from the company data sheet, to be traceable to an in-house master calibrator from ascites fluid from patients suffering from cancer as described by Risteli et al.[59] Knudsen compared the results obtained by these two assays and found them reasonably well correlated, but the Centaur results were around 1.6-fold greater than those obtained using the UniQ kit. This difference indicates the need for method-specific reference intervals.

A recent paper[60] on the use of the RIA CisBio assay for P-III-NP as a biomarker of cardiovascular events in 244 haemodialysis patients showed a median concentration 1.40 ± 0.08 U/ml against the reference interval for P-III-NP in this assay kit is 0.30–0.80 U/ml for the Japanese population. Unfortunately, these units are method specific and not readily traceable illustrating the problem with the sharing of data obtained using immunoassay.

Overall, the differences between the immunoassays; the different measuring systems used, that is, U/ml or ng/ml; and changes in manufacture over time make their use in a biomarker application difficult. Hence, there is a serious need for, at least, an international standard and a better traceable source of *h*P-III-NP.

4.3.3 | IGF-I immunoassays

Unlike P-III-NP, there have been a multitude of commercial immunoassays for IGF-I. This is probably largely because it is more widely used in clinical chemistry than is P-III-NP. Unfortunately, as described below, these assays lack consistency over long periods of time with manufacturers amending or withdrawing their products, which results in the need to re-establish the decision limits for the biomarker score. The assays previously and currently approved for use by WADA are mentioned elsewhere in this review.

All IGF-I assays measure IGF-I released from the binding protein complex. Importantly, as shown by Daughaday et al,[61] most

immunoassays use acid-ethanol for this displacement often with the addition of a relatively large concentration of IGF-II to stop rebinding occurring.[62] As much as 2 μg/ml (250 pM) of IGF-II has been used for displacement in an LC–MS approach discussed later.[15]

As mentioned earlier, the Nichols assay was calibrated against the IRR, and initially, this was probably the most widely used commercial radioimmunoassay for IGF-I and was used for the establishment of the initial decision limits to evidence hGH misuse. The reference ranges for IGF-I by age were largely based on the Nichols assay results. Unfortunately for Nichols, problems with their immunoradiometric assay for intact parathyroid hormone[63] caused misdiagnosis in a number of patients and ultimately was a factor in the closure of the company and hence non-availability of this and their other assays including the highly popular assay for IGF-I.

Following the withdrawal of the Nichols assay, the GH-2000 team changed to the Immulite 1000 IGF-I assay for the biomarker score. This required the re-establishment of the decision limits based on the re-analysis of 404 samples from males and 94 from females.[64] The change to an automated system was considered useful for its application to IGF-I measurements during the London 2012 Olympic and Paralympic Games. Siemens discovered a problem with their Immulite range of IGF-I assays and issued a recall notice on 19 November 2012 that stated 'As a result of this upcoming major supply disruption, Siemens recommends that customers use the limited inventory available on the IMMULITE/IMMULITE 1000 and IMMULITE 2000/IMMULITE 2000 XPi to perform crossover studies to an alternative IGF-I assay'. Unfortunately, this created a serious problem for the use of the existing decision limits for the biomarker hGH assay. When Siemens reintroduced their IGF-I assay, they still calibrated the IGF-I measurements against the IRR 87/518 rather than either the 91/554 International Standard or the then current 02/254. This recognises the large amount of work required by the assay suppliers whenever an international standard is changed and the need to get a standard that is truly commutable and can stand the test of time. This is still not possible with most biological materials. In the case of the use of the measurements such as for the biomarker score, any change in the assay may require an adjustment of the score and/or decision limits. This is more than simply an adjustment based on correlation but requires remeasuring IGF-I in a large cohort of volunteers to re-establish baseline scores. Of course, the clinical chemist faces similar difficulties in re-establishing the reference intervals to be used for patient investigations. Unfortunately, the Immunotech immunoradiometric assay is still calibrated against the IRR 87/518. This standard has been criticised by Quarmby et al as being impure.[65] Only the Siemens IDS assay for IGF-I is calibrated against the WHO 1st International Standard 02/254.

Challenges to the WADA approach for measuring hGH administration using the isoform method (based then on 352 samples) caused WADA to support the GH-2000 team to undertake work on samples supplied by the IAAF (International Association of Athletics Federations) to increase to 1000 each the number of samples from both males and females to justify better the decision limits for a laboratory to report an adverse analytical finding for hGH administration.[66]

4.3.4 | P-III-NP LC–MS measurement

Given the difficulties in having immunoassay values that are stable over time, a mass spectrometric solution might seem reasonable. IGF-I has been successfully quantified by LC–MS as discussed below and heavy-labelled IGF-I is available from NIST as an internal standard. However, P-III-NP is a much larger molecule being nearly six times larger in mass than IGF-I increasing the difficulty of analysis by LC–MS. This is made even more difficult by the circulating concentration of P-III-NP thought to be around 1/20 to 1/300 of that of IGF-I (1–5 ng/ml compared with 100–300 ng/ml[67] equivalent to 25–125 pM compared with ~13–40 nM for IGF-I). Furthermore, currently P-III-NP is not fully characterised, and although the amino acid sequence is known from the translation of the *COL3A1* gene, post-translational modifications such as hydroxyproline formation and glycosylation are possible confounding factors.

Because of the size of the molecule (42 kDa) and the concentration normally found in blood (1–5 ng/ml), digestion of P-III-NP into suitable peptide units seems to be the most realistic way at present to be able to quantify this analyte. Also it is more practical to make heavy-labelled analogues of the selected digest peptides than to label P-III-NP, at least by current chemical methods.

The peptides currently chosen for the quantification of P-III-NP are T1 and T5 formed by splitting, blocking and digesting the three strands of P-III-NP.[19] This is achieved by heating P-III-NP in the presence of a reducing agent (dithiothreitol) to split the disulphide bridges, thereby separating the three strands, and then blocking the free thiol groups to form S-carbamidomethyl derivatives (with the addition of 57.021 Da for every free thiol derivatised) by treatment with iodoacetamide to avoid the strands recombining, followed by digestion with trypsin. T1 and T5 are comprised of amino acids 1–19 and 86–96 of P-III-NP (or 24–42 and 109–119 of the translated molecules from *COL3A1*), respectively (see Figure 4). T1 (19 amino acids) and T5 (11 amino acids) have the sequences QQEAVEGGCSHLGQSYADR and GDPGPPGIPGR with molar masses of 2034.893 Da and 1019.527 Da, respectively (see Figure 4). The N-terminal glutamine (Q) readily cyclises by deamination to pyroglutamate, which has a lactam structure, decreasing the molar mass to 2018.041 Da. Also, to avoid the cysteine in T1 oxidising or dimerising with another cysteine, the analytical method includes reaction with iodoacetamide to form a stable carbamidomethylated derivative that has a molar mass of 2073.8811 Da. T5 has the generic motif GXY, specifically GDP, GPP and GIP. As mentioned earlier, the proline in the Y-position is hydroxylated to 4-hydroxyproline, Figure 5, by proline 4-hydroxylase.[68] This increases the molar mass of T5 to 1066.5044 Da. Note that the
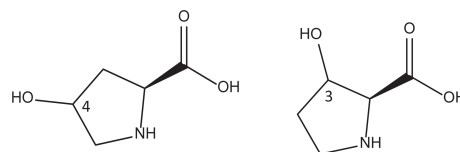

FIGURE 5 4-Hydroxy- and 3-hydroxy-proline

known variant of P-III-NP described earlier, where alanine is substituted for a proline, is not part of either T1 or T5.

Both T1 and T5 form multiply charged ions under nano- and micro-ESI conditions as shown in Table 1.[19] Interestingly, there is a GPP motif present in T5, and theoretically at least, it is possible for the proline next to the glycine to be hydroxylated in the 3-position (II).[69] There has been no report of dihydroxylation in this T5 GPP motif, but this needs to be considered because the knowledge about its formation appears still to be limited.

Moncrieffe has shown that the albumin, present in blood, serum and plasma, is at concentrations typically between 35 and 50 mg/ml $(5.3-7.75 \times 10^{-1}$ mM), interferes in ESI-LC–MS analysis, suppressing the ion signal so that P-III-NP peptides are not detectable. Albumin depletion of more than 99.6% is needed in order to be able to have an LC–MS assay for P-III-NP present in physiological concentrations.[70] Albumin is too similar in size (69 kDa) to P-III-NP either for a protein precipitation method, such as with acetonitrile, or ultra-filtration to be sufficiently effective in separating these two proteins. However, the homo-trimer may be split with dithiothreitol and iodoacetamide into the 3 equal α1-strands of approximately 14 kDa each, whereas albumin is merely elongated by this process. Using this approach to increase the MW difference between P-III-NP and albumin enables separation using molecular weight cut-off (MWCO) ultrafiltration with

a 30-kDa filter. To prevent adsorption losses on MWCO filters, they may be passivated with a variety of different agents such as albumin, salts or surfactants (e.g., Tween 20); however, albumin is clearly unsuitable for this application. The inferred P-III-NP concentration, assessed by LC–MS measurement of T1 and T5, obtained from a single MWCO ultrafiltration where the filter had been passivated with a 5% solution of Tween 20 was of the order of 50% when large concentrations of P-III-NP (approximately 100 μg/ml) were used.[70] At smaller concentrations, residual albumin appeared to suppress LC–MS measurements. A combination of acetonitrile precipitation and double filtration has been shown to produce a good degree of albumin depletion as shown in Table 2.

Although it appears that albumin is removed by the combined ppt and MWCO filtered sample, using targeted LC–MS methods (rather than those for the proteomics experiment shown in Table 2), albumin was still shown to be present. Also, it would appear that, as the human albumin is depleted, even some of the larger plasma proteins are still present. The apparent increase of the larger plasma proteins with reduction in albumin may be linked with reduced ion suppression. Although these methods of depletion did enable T1 and T5 to be detected in the LC–MS instrument, the results were erratic, and this was thought to be due to the variability of the ultrafiltration process. The recovery of P-III-NP by these methods were only of the order of

TABLE 1 Collision energies for selected reaction monitoring (SRM) transitions acquired for P-III-NP peptides by micro- and nano-ESI-MS

P-III-NP precursor peptide	Precursor ion (m/z)	Product ion (m/z)	Collision energy (arbitrary units)
hT1	692	768	10
	1038	240	50
	1038	440	40
T5	534	363	20
	534	448	13
	534	628	27

Abbreviation: P-III-NP, procollagen type III amino-terminal propeptide.

TABLE 2 Efficiency of HSA removal achieved by acetonitrile protein precipitation (ACN ppt) and molecular weight cut-off (MWCO) filtration using a 30-kDa sieve

	Depleted serum albumin (μg/ml)		Proteins identified in depleted serum matrix (peptide threshold > 80%)
	50-mg/ml HSA	Pooled human serum	
ACN ppt	40	1150	ALBU_H, APOA1, APOA2, HBA1, HBB, TRFE, CXCL7, APOA4
MWCO filter	30	80	ALBU_H, APOA1, APOA4
ACN ppt + MWCO filter	—	22	APOA1, APOA2, HBB, TRFE, CXCL7
Double MWCO filter	—	4	APOA2, APOA4, APOC3, HPT_HUMAN

Note: Some of the more abundant proteins remaining in depleted serum matrix (with a threshold > 80%), identified using Mascot algorithm version 2.2.06 searching against the UniProt Swiss-Prot protein database are listed (modified from Moncrieffe[70]). ave = mass obtained from PeptideMass. Some of the above identified proteins exist as dimers or oligomers in solution and hence may not pass through the 30-kDa filter.
Abbreviations: ALBU_H, human albumin (sp|P02768|25-609) ave 66,472.21; APOA1, apolipoprotein A-I (sp|P02647|25-267) ave 28,078.6207; APOA2, apolipoprotein A-II (sp|P02652|24-100) ave 8707.9059; APOA4, apolipoprotein A-IV (sp|P06727|21-396) ave 43,375.5084; APOC3, apolipoprotein C-III (sp|P02656|21-99) ave 8764.67; CXCL7, chemokine (C-X-C motif) ligand (sp|P02775|35-128) ave 10,265.8270; HBA1, haemoglobin subunit alpha (sp|P69905|2-142) ave 15,126.36; HBB, haemoglobin subunit beta (sp|P68871|2-147) ave 15,867.22; HPT, human haptoglobin (sp|P00738|19-406) ave 43,349.01; TRFE, serotransferrin (sp|P02787|20-698) ave 75,195.46.

12% although occasionally recoveries of >50% were reported. This erratic behaviour may in part be because MWCO filters are designed to retain analytes, whereas one wants the albumin to be retained and the P-III-NP (single strand) to be recovered in the ultrafiltrate. The addition of a variety of possible carriers (substance P, insulin and tetracosactide), to reduce the effect of any losses on the filters, apparently did not improve the recovery.

Finally, sufficient recovery has been reported using an immunocapture approach by modification of the ELISA for P-III-NP from CisBio with recoveries estimated to be >70% when assessed using all ion transitions for T1 and T5 with P-III-NP apparent concentrations of 5 and 500 ng/ml.[70] This method was considered to have sufficient sensitivity to be able to detect P-III-NP in a typical serum sample.

4.3.5 | IGF-I LC–MS measurement

Unlike, P-III-NP, several LC–MS methods have been published mainly for the quantification of intact IGF-I or measuring peptides formed by trypsin-digestion of IGF-I. In the case of intact IGF-I different multiply-charged species have been used and rely on the heavy-labelled internal standard behaving in the same manner (personal communication). The earliest papers were by Popot and colleagues measuring IGF-I in the equine.[71–73] The method evolved for human samples initially using des(1–3)-IGF-I[74] and then using fully ^{15}N-labelled IGF-I as internal standard.[75] Further modifications of the method have been published by a number of other workers.[16,76–80] A first interlaboratory study on the measurement of intact IGF-I was published by Moncrieffe et al[16] showing that good interlaboratory precision of <15% was achievable using the published method.

In the case of the analysis of intact IGF-I, factors including possible methionine oxidation[81] and variants, especially those with population frequencies >1%, need to be considered[40,41] because the mass of the analyte will change and not be measured if wild-type IGF-I only is being considered. Care must be taken to appreciate that the paper by Wu and colleagues deals with the frequency of observations from a preselected set of samples from patients as part of their clinical investigations, which does not represent a typical normal population.

There are a number of possible cleavage sites in IGF-I resulting from trypsin digestion (Figure 6). Of the two peptides most commonly used as shown in the figure, T1 (GPETLCGAELVDALQFVCGDR, 1–21) has a 100% cleavage probability whereas that of T2 (GFYFNKPTGYGSSSR, 22–36) is theoretically only 85.5% and also has a missed cleavage point (27-KP-28, i.e., between amino acids

27 and 28). This does not appear to have been discussed by any of the authors using the digest approach although it is reasonable to assume that heavy-labelled IGF-I will compensate appropriately. Also, although T2 is the common abbreviation used for the peptide comprising amino acids 22–36, more correctly, it should be referred to as T2 + T3 to indicate the missed cleavage. Interestingly, Kirsch et al[83] considered the use of T1, T2 and T4 (APQTGIVDECCFR, 38–50) but then quantified using just T2 explaining that it was the only peptide not containing a cysteine. On the other hand, Kay et al used T1 for quantification,[76] whereas Cox et al[15] have chosen to quantify using both T1 and T2 and, assuming reliable quantitative formation, provides a useful check on the performance of the assay. All authors reduce disulphide bonds by heating with dithiothreitol followed by blocking of the thiols by alkylation with iodoacetamide to yield a carbamidomethylated unfolded protein. Like P-III-NP, each carbamidomethylated cysteine requires an adjustment for the resulting mass shift, and because T1 contains two cysteines, an increased mass of +114 Da occurs. Because T2 does not contain any cysteine, no derivatisation or change in mass should occur.

When using the trypsin digestion approach, WADA guidelines[13] stipulate that a minimum complementarity of 20% between T1 and T2 peptide measurements is required for a reliable measurement. Obviously, any incomplete reduction and alkylation can inhibit the generation of the carbamidomethylated T1 peptide, or variable cleavage of the missed-cleavage site at 27-KP-28 affecting T2 mentioned above, could affect the ratio of the peptides unless fully compensated by the calibrants and heavy-labelled internal standard. Furthermore, IGF-I would be expected to be in the ternary complex in the serum sample as described earlier in this review, whereas the internal standard is free and this different environment of the two analytes might affect this compensation.

The use of intact IGF-I for the screening of sport samples is recommended by Moncrieffe et al[16] with the addition of the digest approach, applying the WADA 20% complementarity index between the two digest peptides (T1 and T2), to confirm the final quantitative measurement. This approach is also recommended for use with anomalous clinical samples.

4.4 | Matrix used for measurements

At present, WADA accepts only blood serum as the matrix for the hGH biomarker approach in specified containers (BD Vacutainer® SST™-II, EU ref 367955 or BD Vacutainer® SST™-II Plus *Advance* tubes, EU ref 367954). These tubes contain a proprietary inert
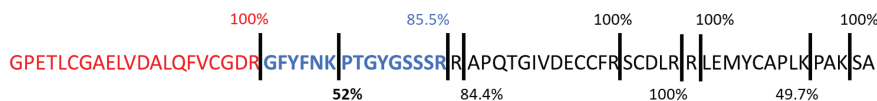
FIGURE 6 Map of cleavage sites after human IGF-I tryptic digestion. The cleavage specificity (% cleavage) is a theoretical value based on a statistical treatment of the accumulated information for trypsin proteases.[82] A possible 10 tryptic peptides (T1–T10) are obtained from IGF-I with no missed cleavage. T1 is indicated in red, and T2 + T3 (commonly called T2) in blue

Heavy *h*T1: Q-NH3-*[^{13}C/^{15}N-Q]*-EAVEGGCSHLG-*[^{13}C/^{15}N-Q]*-SYADR

Heavy T5: GD(*P+OH*)GP(*P+OH*)G-*[^{13}C/^{15}N-I]*-(*P+OH*)GR

FIGURE 7 Heavy-isotope labelling of heavy T1 and heavy T5

polymeric serum separator gel and clotting activation factor and typically will allow the collection of up to 5 ml of blood. The WADA guidelines[13] recommend refrigerated storage from the point of collection to delivery to the laboratory and state that both P-III-NP and IGF-I are stable under these conditions for 5 days.[84] The gel enables the separation of the serum by centrifugation at 2000*g*. The serum may then be removed and stored frozen at −70°C prior to analysis. WADA permits the transport of frozen serum. It specifically states that plasma shall not be accepted for the purposes of hGH analysis with the current assays.

Because whole blood is also collected as part of doping control for the athlete biological passport haematological module,[85] it would seem to be desirable also to be able to use this matrix and/or plasma. A simpler way may be to use dried blood either on a card or using a volumetric absorptive microsampling (VAMS®) device. Some work on IGF-I measurement as dried blood spots on cards[74] and on VAMS®[80] has already been published.

4.5 | Synthesis of reference peptides

Both the digest method for IGF-I and that for P-III-NP rely on the quantification of smaller peptides that need to be made for calibration of the assays. Reference materials of known purity including heavy-labelled equivalents are, of course, valuable for traceability and better assay optimisation purposes. In the case of IGF-I, calibration using the peptides produced by trypsin digestion is possible with intact reference unlabelled and heavy-labelled material, as was used in an inter-laboratory study.[15]

In the absence of a reference standard for *h*P-III-NP and the fact that proteins of this size have not been routinely synthesised, T1 and T5 peptides have been synthesised as the most appropriate peptides for quantification,[19] and solid-phase synthesis has been used[70] with fluoren-9-ylmethyloxycarbonyl-labelled (Fmoc) amino acids. The heavy-labelled equivalents would also be valuable such as is shown in Figure 7 where ^{13}C/^{15}N-glutamine and ^{13}C/^{15}N-isoleucine are used for T1 and T5, respectively.

Crude synthetic peptides require purification, and commercially prepared peptides typically are provided with an estimate of purity. However, this is often merely chromatographic and low molecular weight impurities not seen in the analysis may be present in measurable amounts. Furthermore, mass spectrometric purity is desirable. Indeed, for more accurate quantification, the gravimetric amount of peptide is needed. This is best achieved by amino acid analysis where a sample of the peptide is cleaved with strong acid under pressure[86]

and then several of the liberated amino acids quantified by GC or LC–MS.

5 | CONCLUDING REMARKS

The two biomarkers (IGF-I and P-III-NP) employed for evidencing hGH administration were first proposed to the International Olympic Committee in 1999. Much work was needed to develop the scientific evidence that proved the value of these biomarkers to obtain the level of certainty required for use in anti-doping analysis. In parallel with obtaining the necessary number of samples for establishing suitable decision limits, modifications made by commercial assay manufacturers have meant that revalidation of the modified assay and re-establishment of the limits have been required. Now that two LC–MS based assays (intact and digest) have been developed for IGF-I with evidence of good inter-laboratory (international) reproducibility being achievable, the problem has been largely solved for this analyte. Unfortunately, more work is needed before the same will be the case for P-III-NP and immunoassays for this analyte continue to be modified, possibly requiring further re-establishment of the decision limits.

The use of dried blood has benefits for sample collection (small volume and a phlebotomist generally is not required) and ease of transport and storage. However, a better understanding of the possible effects of the matrix on the measurement of P-III-NP and IGF-I is clearly needed to allow greater flexibility in the use of whole blood, serum or plasma for the measurement of these hGH biomarkers.

The finding of two Russian powerlifters administering hGH at the London Paralympic Games in 2012 confirmed the effectiveness of the biomarker approach, especially because the athletes subsequently admitted the use. However, comparisons with the hGH isoform test have called into question the sensitivity of the biomarker test. Unfortunately, although at least one biomarker positive result has been shown to be isoform negative, no converse comparison appears to have been made. The sensitivity of the biomarker test (and the isoform test) is probably adversely affected by setting populated based decision limits, which are likely set higher than would be needed for an individual (passport) type of approach such as that used by WADA for the blood passport and the steroid module of the same system. It may be that the use of dried blood samples will be a realistic proposition in the near future as instrument (LC–MS) sensitivity continues to improve. It is, of course, essential that good reliability of the quantitative assays and standards employed is assured, preferably over long time scales, to enable biomarker measurements to be used consistently in anti-doping. At the time of writing, WADA is

considering the practicalities of a GH module to their passport system based on measurements of IGF-I and P-III-NP; a MS-based approach for P-III-NP is eagerly awaited.

ENDNOTE

[*] The term 'biomarker' in this review means the use of measurements of an endogenous (not foreign) substance in order to be able to evidence a perturbation of the body system.[3]

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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