

Research Article

# The Proteomic Signature of Recombinant Growth Hormone in Recreational Athletes

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**Abbreviations:** ECM, extracellular matrix; GDF, growth differentiation factor; GH, growth hormone; GHR, growth hormone receptor; hGH, human growth hormone; IGF1, insulin-like growth factor-1; IGF1R, insulin-like growth factor 1 receptor; KEGG, Kyoto Encyclopedia of Genes and Genomes; MAP2K4, mitogen-activated protein kinase 4; P-III-NP, amino-terminal propeptide of type III collagen; PI3K-AKT, phosphatidylinositol 3'-kinase–protein kinase B; RFU, relative fluorescent units; SOMAmer, slow-off rate-modified DNA aptamers; TNF, tumor necrosis factor; WADA, World Anti-Doping Agency.

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## Abstract

**Objective:** Administration of human growth hormone (hGH) is prohibited in competitive sport and its detection in an athlete's sample triggers an adverse analytical finding. However, the biological processes that are modulated by recombinant hGH are not well characterized and associated blood serum proteins may constitute new biomarkers for hGH misuse.

**Methods:** Thirty-five recreational athletes were enrolled in a study to investigate the time- and dose-dependent response of serum protein levels to recombinant hGH administration. Participants were randomly assigned to 4 groups, receiving 1 of 3 different doses of recombinant hGH or a placebo. Bio samples were collected at 22 time points over a period of 13 weeks, starting 4 weeks before treatment, during 3 weeks of treatment, and at 6 weeks' follow-up. A total of 749 serum samples were analyzed for 1305 protein markers using the SOMAscan proteomics platform.

**Results:** We identified 66 proteins that significantly associated with recombinant hGH administration and dosage, including well known hGH targets, such as IGF1, but also previously unknown hGH-related proteins (eg, protease inhibitors, WFIKKN1, and

chemokines, CCL2). Network analysis revealed changes in specific biological pathways, mainly related to the immune system and glucose metabolism.

**Conclusion:** Our analysis suggests that hGH administration affects biological processes more strongly than previously acknowledged. Some of the proteins were dysregulated even after hGH treatment and could potentially be developed into biomarkers for hGH misuse. Moreover, our findings suggest new roles for hGH-associated proteins in the etiology of hGH-related diseases and may indicate new risks that may be associated with hGH misuse.

**Key Words:** proteomics, antidoping, human growth hormone, glucose metabolism

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Growth hormone (GH) is secreted by cells of the pituitary gland [1]. The target of GH signaling is the GH receptor (GHR), which is expressed by many cell types [2], whereby the response and sensitivity to GH differs substantially between tissues [3]. Its actions are either mediated indirectly through insulin-like growth factor 1 (IGF1), which is released from GHR-expressing cells [1], or directly by phosphorylation of a tyrosine kinase after GHR binding [2]. Initially implicated as a regulator of postnatal growth and development, GH also was found to play a regulatory role in energy homeostasis [4, 5] and immune response [6, 7]. Since the late 1980s, the therapeutic use of recombinant human GH (hGH) has also been possible; for example, GH-deficient patients treated with hGH benefit from a reduction in fat mass, an increase in muscle mass and strength, and increased bone mineral density [8]. However, these patients also present reduced insulin sensitivity and higher plasma glucose levels [9], although these changes did not necessarily increase their risk for type 2 diabetes [10-12]. Whether long-term use of hGH may be associated with other adverse events, especially a higher risk of developing primary or secondary cancers, remains controversial [13-15]. Nevertheless, the therapeutic use of hGH is considered safe and satisfactory, as stated by the GH safety workshop in 2016 [16].

Owing to its putative effects on the human body, hGH also became an attractive doping agent, and while doses of hGH used in the treatment of GH-deficient adults range from 0.47 to 1.56 IU/day to reach physiological levels of hGH [12], doses of hGH abused in sports are estimated to be 4 to 14 IU/day, although reliable sources for these values are scarce [17]. The chronic use of hGH by healthy individuals potentially increases the risk of cardiovascular and metabolic diseases, according to a statement from the Endocrine Society [18].

Because of the supposed performance-enhancing properties and potential health risks of hGH, the World Anti-Doping Agency (WADA) in 1999 included it in their list of prohibited substances, and substantial efforts were made to develop a method to detect hGH misuse [19].

WADA-accredited laboratories are currently using 2 different methods: i) an isoform-differential immunoassay, which distinguishes between the isoforms of GH that are naturally released by the pituitary gland [20] and exogenously administered recombinant hGH [21], and ii) an “indirect” biomarker-based approach, which depends on the serum concentrations of 2 GH-responsive proteins, namely the IGF1 and the amino-terminal propeptide of type III collagen (P-III-NP). However, the interindividual variation of these biomarkers attributed to factors such as age and sex reduces the sensitivity of these biomarker tests, especially because the decision limits to determine hGH misuse are currently based on reference ranges derived for the general population [22]. On the other hand, the isoform-differential immunoassay method, while more successful so far than the biomarker method in detecting hGH doping, has a narrow time window of detection due to the short half-life (~ 4 hours after subcutaneous injection) [23] of hGH in circulation, which makes it challenging to reach adequate sensitivity [24, 25].

In light of the performance of currently available detection methods, a key priority for antidoping research is the application of novel technologies, such as proteomics, to discover biomarkers of hGH doping with sufficient sensitivity and specificity [21, 26, 27]. The approximately 5000 serum proteins that could potentially be found in circulation spans more than 8 orders of magnitude in the concentration range, which makes it challenging to reach acceptable reproducibility by conventional mass spectrometry-based proteomics technologies. Affinity-based proteomics, such as the SOMAscan assay (Somalogic), provide an alternative with excellent sensitivity together with a low coefficient of variation. The assay uses chemically modified nucleotides that mimic amino acid side chains to bind with high affinity to protein epitopes for an extended time, referred to as slow-off rate-modified DNA aptamers (SOMAmer). An iterative selection and amplification process of aptamers to the native folded proteins, called SELEX (Systematic Evolution of Ligands by EXponential enrichment), provides the desirable protein-nucleic acid interactions in the development

of SOMAmer reagents. Selective binding transforms the concentration of a protein in a serum sample into a defined SOMAmer concentration, which allows the simultaneous quantification of proteins by conventional DNA hybridization methods, such as quantitative polymerase chain reaction and DNA microarray [28-30].

Using proteomics to unravel which hGH-related proteins are involved in metabolic processes and associated networks [31] will help not only in antidoping research [27, 32, 33], but also patients might benefit when features of hGH actions become more evident [34]; for example, previous studies have used hGH as a treatment option for patients with immunodeficient condition, such as HIV [35], severe burns [36], or ataxia telangiectasia [37]. Also, beneficial effects of GHR antagonism have been reported for several cancer types; however, mainly in vitro models were used [38].

In this single-blind, open-label, randomized study, we used the SOMAscan platform to analyze serum samples from 35 recreational athletes who were administered 3 doses of recombinant hGH or a placebo. Participants were followed for 4 weeks' preadministration to establish baseline values of biomarkers in resting state and after exercise. Individuals were randomly assigned to either a placebo group or to one of the administration groups, which consisted of 3 increasing doses of hGH in a 3-week administration period. At the end of this period, participants were followed for a further 6 weeks to evaluate the hGH-induced changes in their serum protein levels. Using this high-sensitive proteomic screening approach, we identified novel potential biomarkers of hGH misuse and explored hGH-related biologic pathways and their role in health and disease.

## Materials and Methods

### Study Design

This open-label, single-site study (protocol No. IMIMFTCL/GH/4) was performed at the Clinical Trials Unit of the IMIM (Hospital del Mar Medical Research Institute), Barcelona, Spain. The study was approved by the local ethics committee (CEIm-PSMAR) and written informed consent was obtained from all participants. The study was registered in the European Union Drug Regulating Authorities Clinical Trials Database (EudraCT No.: 2014-000563-41). Recreational athletes with at least 5 hours/week of moderate to intense physical activity or with an energy expenditure of at least 5000 kcal/week were recruited during 3 enrollment rounds. Regular use of prescription drugs, if any, was prohibited 1 month before the start of the study. Occasional use of medication was allowed under the

supervision of the principal investigator. Volunteers who took steroids, erythropoietin, IGF1, diuretics, or plasma expanders were not included in this study. The athletes were screened during the study for the presence of drugs in their urine and alcohol on their breath. All participants were allocated randomly to either the placebo group or to 1 of 3 groups with recombinant hGH doses ("doping" groups), which covered a very low dose of 0.016 mg/kg, a low dose of 0.033 mg/kg, or a high dose of 0.066 mg/kg. For an athlete weighing 75 kg, this corresponds to hGH doses of 3.75, 7.5, and 15 IU/day for the very low, low, and high doses of hGH, respectively. Daily treatment by subcutaneous injection of recombinant hGH (NutropinAq, Ipsen Pharma GmbH) or a placebo was administered over a period of 3 weeks. Study treatment was administered on day 1 in the clinical research unit and participants were trained to administer themselves daily during the treatment period. Sufficient NutropinAq vials and administration supplies (NutropinAq pen and needles) needed throughout the treatment period according to weight and dosage were supplied for each individual. The collection of serum samples was carried out for a total of 4 weeks' preadministration (day: -28, -25, -21, -18, -14, -11, -7, -4, -1), the 3-week treatment period (day: 1, 7 before training, 7 after training, 14, 21 before training, 21 after training), and a further 6-week follow-up period (day: 22, 24, 28, 35, 42, 49, 63). Samples were taken under nonfasting conditions with the exception of visits on day -1, 7 before training, 28, and 63, where fasting was needed for further biochemical analysis to monitor the health state. To obtain serum, blood was collected in SST-II tubes from BD Vacutainer, kept for 10 minutes at room temperature, centrifuged over 10 minutes at 1600g in a refrigerated centrifuge, and frozen at -80 °C until analysis. The sex distribution, treatment allocation of the 35 participants, and number of collected samples are shown in Table 1.

All individuals were monitored for regular physical activity during the study period and all completed the study. During the study, some minor adverse effects (eg, paresthesia, mild peripheral edema) were registered in the treatment groups but were not an eliminating factor for study participation, and in total only a few sample collections were missed (see Table 1). The sequence of the main steps of the study are highlighted in Fig. 1.

### Proteomics Analysis

Serum samples were analyzed on the SOMAscan biomarker discovery platform at the proteomics core facility of Weill Cornell Medicine-Qatar as previously described [39, 40]. This method is based on quantifying protein-specific aptamer binding using a DNA microarray. The readout of

the microarray is given in relative fluorescent units (RFU), which are directly proportional to the amount of target protein in the initial sample and reaches a dynamic range of 8 orders of magnitude by using 3 serial dilutions of the sample. Version 3 of the SOMAscan assay covers 1305 unique aptamer probes. The experiments were conducted following Somalogic Inc protocols on dedicated instrumentation also certified by Somalogic. Primary data were sent to Somalogic for processing. This includes a cross-batch calibration and several steps of quality control.

Briefly, sample data were first normalized to remove hybridization variation within a run followed by median normalization across all samples to remove other assay biases within the run and finally calibrated to remove assay differences between runs.

## Data Analysis

To identify protein markers that show a time- and dose-dependent change in response to hGH treatment, linear mixed models were computed using the *lmer* function from the R package *lme4* [41] using the following model equation:

$$\text{Protein} \sim 1 + \text{Period} * \text{Dose} + \text{Time} \\ + \text{Sex} + (1|\text{Subject-ID}) + (1|\text{Plate-ID})$$

The fitted model includes an interaction term between the 3 study periods (period: baseline, treatment, follow-up) and hGH dose (dose: coded as integer number, proportional to the actual dose: 0, 1, 2, 4). Subject-ID and SomaScan Plate-ID were used as categorical random effects, and time of day (time: am, pm) and sex (male, female) as fixed effects. Protein levels were reported as log-scaled RFU ( $\log_{10}(\text{RFU})$ ). The *lmer* function provides a *t* statistic for each fixed effect in the model.

Of particular interest here are the interaction terms treatment-to-dose and follow-up-to-dose as they compare the dose dependence of the protein levels during the treatment period and the follow-up period compared to baseline, respectively. Rather than using an arbitrary *P* value cutoff, we chose an ad hoc *t* value ( $t > 3$ ) to identify a manageable number of specific time-dependent associations for further investigation, which we then further confirmed by visual inspection of

plots of protein levels vs period and dose. Roughly, a *t* value of *t* greater than 3 corresponds to an  $\alpha$  level of significance of *P* equal to approximately .0028 in our study (degree of freedom = ~650). For each protein the receiver operating characteristic curve was calculated using different definitions of “doped” (treatment vs baseline, follow-up vs baseline, treatment and follow-up vs baseline; and using different dosages as the cutoff while setting the remainder to missing). In addition, a time-series plot of the participants’ serum levels were created using the R package *ggplot2* [42].

Next, significant proteins and corresponding *t* values were used as input for the NetworkAnalyst 3.0 software [43, 44] to explore biological pathways associated with hGH treatment. This web-based tool allows a functional enrichment analysis and tests whether there is a significant overlap between a list of proteins and a preselected pathway library. The enrichment analysis was based on hypergeometric distribution followed by false discovery rate correction (threshold = 0.05) of the uploaded proteins and *t* values. Identified pathways are displayed in network form, where pathway nodes with overlapping proteins are connected by edges, which represent protein-protein associations (associations are meant to be specific and meaningful, for example, proteins jointly contribute to a shared function; this does not necessarily mean they are physically binding each other). The size of the pathway node corresponds to the number of proteins of this pathway that are also present in the analyzed input. Functionally similar pathways are grouped together, which helps to navigate through complex data sets. The network shows only proteins that are already linked to distinct biological pathways and protein nodes are colored according to their *t* value from the input table. The pathway library from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used because it combines functional genomic, chemical, and systemic level information provided by high-throughput technologies, such as genomics and proteomics [45, 46].

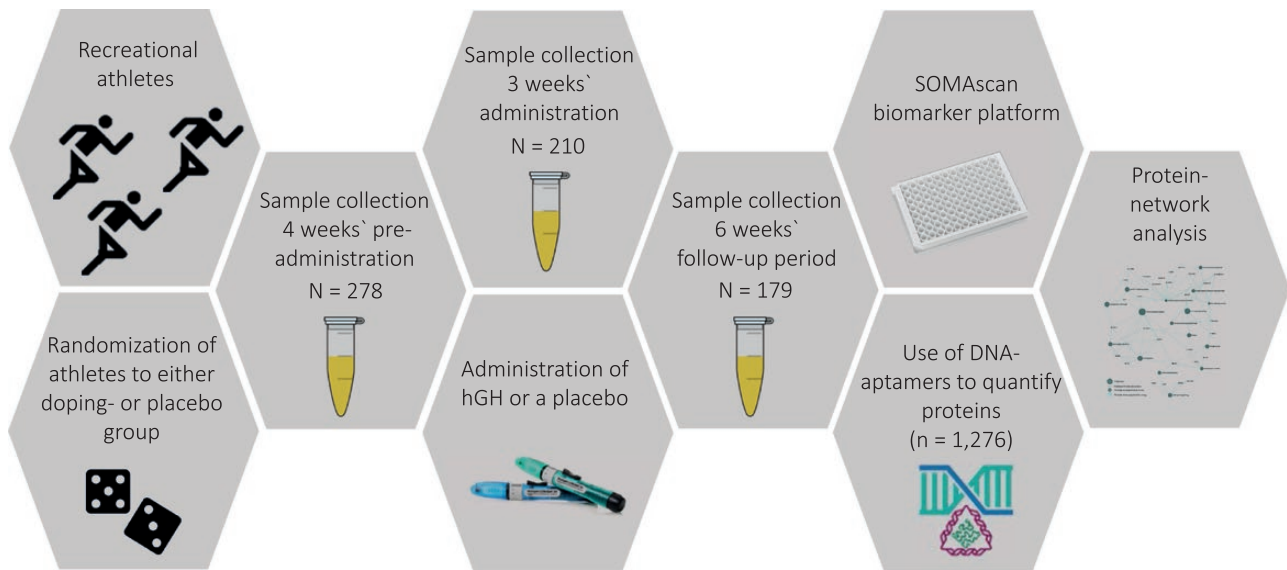
## Results

### Study Cohort

A total of 10 female and 25 male athletes, of mean age  $31.5 \pm 8.5$  years, weight  $70.6 \pm 8.9$  kg, height

**Table 1.** Distribution of participants in study groups and number of samples collected (missing) per group

Study group	Male participants	Female participants	No. of serum samples in group
Control group	6	2	174 (2 missing)
Very low dose, 0.016 mg/kg	7	3	217 (3 missing)
Low dose, 0.033 mg/kg	7	3	219 (1 missing)
High dose, 0.066 mg/kg	5	2	154
Total	25	10	764



**Figure 1.** Schematic view of the study design. Thirty-five recreational athletes were allocated randomly to either the placebo group ( $n = 8$ ) or 1 of the 3 treatment groups with very low human growth hormone (hGH) dose ( $n = 10$ ), low hGH dose ( $n = 10$ ) and high hGH dose ( $n = 7$ ). Collection of serum samples took place over a period of 13 weeks, corresponding to 22 time points. A total of 764 samples were included in the SOMAscan analysis. The SOMAscan technology allows the quantification of proteins through high-affinity binding of proteins by DNA aptamers. Computer interpretation of the output is used to identify significantly changed proteins after hGH administration. In addition, network analysis allows interpretation of associated metabolic and physiologic pathways.

$173.3 \pm 7.7$  cm, and body mass index  $23.5 \pm 2.2$  were enrolled in the study, with a mean exercise time of  $8.5 \pm 3.4$  hours per week. Most participants reported following the Mediterranean diet and 3 of them were vegetarian. Further anthropometric data of the participants and a description of their physical activities are shown in Supplementary Table 1 [47].

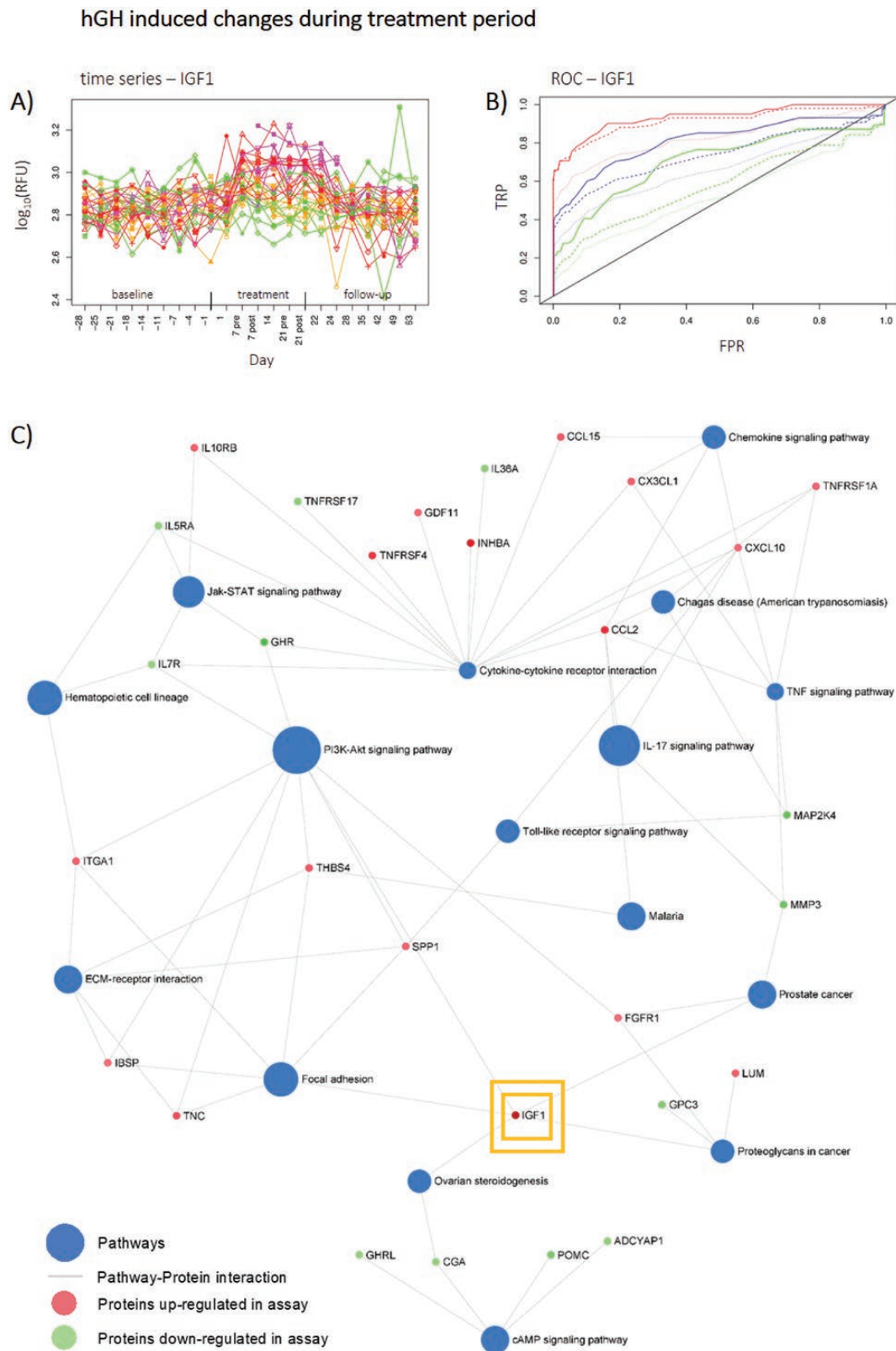
### Results of Proteomic Analysis

Using the SOMAscan platform, we quantified the levels of 1305 proteins in 764 serum samples collected from 35 recreational athletes who received either a 3-week treatment with 1 of 3 different doses of recombinant hGH or a placebo. Fifteen samples were flagged as turbid and/or containing debris and were eliminated from further analysis. These samples were not biased toward any of the parameters used in this study (participant, dose, sex, time of day, study period), but appeared to be randomly distributed across groups. Twenty-nine of the 1305 protein measurements did not pass the SOMAscan quality control and after removal of flagged samples and proteins, 1276 proteins and 749 samples remained for further data analysis. This resulted in the identification of proteins with significantly changed abundance after hGH treatment (requiring  $t > 3$  for the treatment-to-dose interaction), including previously reported hGH doping-related proteins, such as IGF1 (Fig. 2) and insulin-like growth factor-binding proteins (IGFBP2, IGFBP3, IGFBP4, IGFBP5). In addition to

the 5 proteins, which have already been published by the WADA-driven GH-2000 and GH-2004 projects [48-50] and others [27, 51, 52], we identified 61 additional proteins at a significance level of  $t$  greater than 3 proteins that we further validated by visual inspection of the respective box plots (see Table 2 and Supplementary Fig. 1 [47]). Proteins with high  $t$  values for both the treatment and the follow-up phases are potential candidates for detecting hGH doping. Several proteins showed significant sex differences (see Supplementary Table 2) [47].

### Network Analysis of the Treatment Period

Having identified serum proteins regulated by recombinant hGH, we then concentrated on the interpretation of the biological effects of hGH administration. Thus, visual representation of regulated proteins and pathways with the NetworkAnalyst 3.0 software provided a comprehensive overview of the GH biology. Fig. 2C shows related KEGG pathways during the treatment period with hGH. Among the 66 proteins identified as significant for the treatment period, 29 proteins were associated with 16 pathways in the KEGG database. These included cytokine-cytokine receptor interaction (14/294; from 294 proteins known to be relevant for this pathway, the levels of 14 proteins were significantly changed by the hGH treatment), the tumor necrosis factor (TNF) signaling pathway (6/110), the extracellular matrix (ECM)-receptor interaction (5/82), the phosphatidylinositol 3'-kinase (PI3K)-protein



**Figure 2.** Overview of human growth hormone (hGH)-induced changes in the serum levels of proteins of recreational athletes during the treatment period, shown exemplary with insulin-like growth factor 1 (IGF1). A, Time-series plot of IGF1 serum levels for all study participants, colored by dose (placebo group: green, very low hGH dose: orange, low hGH dose: red, high hGH dose: magenta); protein levels measured in the SOMAscan assay are in  $\log_{10}$ (relative fluorescent units [RFU]). Similar plots for all 66 significant proteins are provided in Supplementary Fig. 2 [47]. B, Receiver operating characteristic (ROC) curve of IGF1. ROC curves used different definitions of “doped”: treatment period vs baseline (red), follow-up period vs

kinase B (AKT) signaling pathway (9/354), focal adhesion (6/199), and several others. Some of the key proteins in the presented networks are the immune-related chemokines CCL2 (C-C motif chemokine 2), CXCL10 (C-X-C motif chemokine 10), CX3CL1 (fractalkine), CCL15 (C-C motif chemokine 15) but also the proteins osteopontin (SPP1), thrombospondin-4 (THBS4), and IGF1. For IGF1, the protein with the highest *t* value of 8.79, the abundance was significantly higher after low dose and high dose in the treatment phase compared to the control, baseline, and follow-up period (Fig. 2A). Owing to its significant alteration during hGH treatment, IGF1 is an excellent predictor for treatment effects, at least compared to the baseline (Fig. 2B).

### Network Analysis of the Follow-up Period

Twenty-seven of the 66 proteins that were associated with hGH administration during the treatment period also showed a significant *t* value in the follow-up period ( $t > 3$ ). These 27 proteins and the related *t* values for the follow-up period were used as input for the NetworkAnalyst 3.0 software. According to the KEGG database, 12 proteins from the input were associated with 10 pathways. The most prominent pathways of the follow-up period were again the cytokine-cytokine receptor interaction (5/294), TNF signaling pathway (3/110), and ECM-receptor interaction (2/82) (Fig. 3C).

Some of the key proteins in the presented network are, for example, the TNFs TNFRSF4 (TNF receptor superfamily member 4) and TNFRSF1A (TNF receptor superfamily member 1A) but also the dual specificity mitogen-activated protein kinase 4 (MAP2K4), which is the protein with one of the lowest *t* values, at  $-5.21$ . The abundance of MAP2K4 was lower in the follow-up period (Fig. 3A), but the prediction power was weak compared to baseline (Fig. 3B).

### Discussion

To our knowledge, few studies have used affinity proteomics to gain a broad view of how hGH affects protein biology, and in particular protein levels that are measurable in blood. Here, we identified 66 proteins, of which 20

proteins showed lower abundance and 46 proteins showed higher and dose-dependent abundances during 3 weeks of treatment with recombinant hGH. Twenty-seven of these proteins remained further dysregulated during a 6-week follow-up period.

Tan et al [27] report on a quantitative approach (2 differential gel electrophoresis and iTRAQ liquid chromatography–tandem mass spectrometry) to search for novel protein biomarkers associated with hGH administration in nonelite athletes. In their study, participants received either a placebo or recombinant hGH for 8 weeks, and were followed over a 6-week follow-up period. Eight hGH-dependent serum proteins were identified, of which we replicate 3: IGFBP3 (rank 3 in Table 2), afamin (AFM, rank 15), and lumican (LUM, rank 33). Three proteins were measured here but were not associated in our analyses with significant changes after hGH treatment: apolipoprotein-L1 (APOL1), alpha-HS-glycoprotein (FETUA), and ECM protein 1 (ECM1). Two proteins were not on the SOMAscan assay: vitamin D-binding protein (VTDB) IGFBP complex acid labile subunit (IGFBP-ALS). Proteins identified with significant changes after hGH treatment by both studies (IGFBP3, AFM, LUM) showed identical directionality after the treatment with recombinant hGH and can therefore be considered as replicated biomarkers of hGH doping.

Among the 16 pathways found in the network analysis of the treatment period, only the focal adhesion pathway (see Fig. 2C) was clearly related to skeletal muscle growth. Interestingly, evidence of hGH as a performance enhancer in healthy athletes has been given only for anaerobic sprint capacity, but hGH was not associated with increased muscle strength and power, nor with improved aerobic capacity as shown by Meinhardt et al in a double-blind and placebo-controlled study [53]. A meta-analysis supported these results and underpinned further the missing effects of hGH treatment on muscle strength and aerobic capacity in healthy adults. Nevertheless, the study exhibited the potential anabolic and lipolytic properties of hGH on body composition, but again, these changes were not associated with improved performance in a competition setting [54].

The 6-week follow-up period in our study allowed us to monitor protein abundances as they returned to individual baseline levels, and therefore the identification of proteins that respond to hGH treatment with long-lasting changes

#### Figure 2: continued

baseline (green), and treatment and follow-up vs baseline (blue). Different dosages were used as cutoff, considering all samples collected at baseline and all controls as untreated, and all samples taken during the treatment and follow-up period from treated individuals (dotted), from individuals treated with low and high doses (dashed), from individuals treated with a high dose alone (solid) as doped; remaining samples were excluded from analysis. Similar ROC plots for all 66 significant proteins are provided in Supplementary Fig. 2 [47]. C, Functional network illustrating regulatory Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways involved by treatment with recombinant hGH during the treatment period. Proteins based on an ad hoc criterion of *t* greater than 3 for the treatment-to-dose interaction ( $P < .0028$ ) were used as input for the NetworkAnalyst 3.0 software [44]. Red circles denote upregulated proteins, green circles downregulated proteins. Blue nodes represent sets of KEGG pathways, where the size of the nodes corresponds to the number of proteins associated with a distinct pathway that were included in the analyzed protein list.

**Table 2.** The 66 proteins that are most strongly associated with the treatment-to-dose interaction, using an ad hoc significance level of  $t$  greater than 3;  $t$  values for the interactions treatment-to-dose and follow-up-to-dose, ordered by  $t$  for treatment-to-dose interaction

Rank	Entrez gene	Protein Name	$t$ value (treatment:dose)	$t$ value (follow-up:dose)
1	IGF1	Insulin-like growth factor 1	8.79	2.47
2	IGFBP5	Insulin-like growth factor-binding protein 5	8.51	3.15
3	IGFBP3	Insulin-like growth factor-binding protein 3	8.04	1.59
4	INHBA	Inhibin beta A chain	6.35	0.72
5	GHR	Growth hormone receptor	-6.23	-6.03
6	FTH1	Ferritin heavy chain	-6.20	-7.39
7	HAMP	Hepcidin	-5.42	-4.20
8	CCL2	C-C motif chemokine 2	5.28	1.99
9	PTN	Pleiotrophin	5.27	7.95
10	MAP2K4	Dual specificity mitogen-activated protein kinase 4	-5.21	-4.12
11	ADAM12	Disintegrin and metalloproteinase domain-containing protein 12	5.15	4.23
12	CDON	Cell adhesion molecule-related/down-regulated by oncogenes	5.13	4.45
13	TNFRSF4	Tumor necrosis factor receptor superfamily member 4	5.06	6.03
14	MMP3	Stromelysin-1	-5.05	-5.39
15	AFM	Afamin	4.79	1.90
16	MBL2	Mannose-binding protein C	4.76	2.87
17	IGHM	Immunoglobulin M	-4.67	-2.41
18	RET	Proto-oncogene tyrosine-protein kinase receptor Ret	4.34	4.40
20	HPX	Hemopexin	4.26	2.48
21	POMC	Beta-endorphin	-4.23	-2.61
22	TIMP2	Metalloproteinase inhibitor 2	4.14	3.73
23	TNC	Tenascin	4.13	2.64
24	GPC3	Glypican-3	-4.00	-2.97
25	CCDC80	Coiled-coil domain-containing protein 80	3.97	2.92
26	MRC2	C-type mannose receptor 2	3.95	4.16
27	SELL	L-Selectin	-3.93	-2.68
28	IGFBP2	Insulin-like growth factor-binding protein 2	-3.89	-1.78
	ETHE1	Persulfide dioxygenase ETHE1, mitochondrial	-3.84	-2.14
29	THBS4	Thrombospondin-4	3.74	5.79
30	ACY1	Aminoacylase-1	3.74	3.05
31	CCL15	C-C motif chemokine 15	3.73	-0.09
32	IL10RB	Interleukin-10 receptor subunit beta	3.68	3.23
33	LUM	Lumican	3.68	4.90
34	ITGA1 ITGB1	Integrin alpha-I: beta-1 complex	3.62	5.75
35	CD93	Complement component C1q receptor	3.60	4.73
36	TNFRSF17	Tumor necrosis factor receptor superfamily member 17	-3.58	-4.72
37	WFIKKN1	WAP, kazal, immunoglobulin, kunitz and NTR domain-containing protein 1	3.56	2.51
38	CX3CL1	Fractalkine	3.54	1.93
39	EPHB2	Ephrin type-B receptor 2	3.53	3.22
40	TNFRSF1A	Tumor necrosis factor receptor superfamily member 1A	3.50	3.65
41	IL36A	Interleukin-36 alpha	-3.49	-0.98
42	METAP2	Methionine aminopeptidase 2	3.48	4.00
43	DCTPP1	dCTP pyrophosphatase 1	3.47	1.69
44	STC1	Stanniocalcin-1	3.44	0.93
45	POR	NADPH--cytochrome P450 reductase	3.42	4.35
46	ROR1	Tyrosine-protein kinase transmembrane receptor ROR1	3.39	2.45
47	CST3	Cystatin-C	3.36	3.18
48	SPP1	Osteopontin	3.35	1.91



Table 2. Continued

Rank	Entrez gene	Protein Name	<i>t</i> value (treatment:dose)	<i>t</i> value (follow-up:dose)
49	ADCYAP1	Pituitary adenylate cyclase-activating polypeptide 27	-3.32	-1.63
50	IGFBP4	Insulin-like growth factor-binding protein 4	3.32	1.02
51	CXCL10	C-X-C motif chemokine 10	3.31	1.61
52	GDF11 MSTN	Growth/differentiation factor 11/8	3.31	1.29
53	UNC5C	Netrin receptor UNC5C	3.27	1.43
54	IL5RA	Interleukin-5 receptor subunit alpha	-3.24	-2.00
55	KYNU	Kynureninase	3.20	2.94
56	NRXN1	Neurexin-1-beta	-3.15	-3.96
57	FGFR1	Fibroblast growth factor receptor 1	3.14	2.70
58	CD177	CD177 antigen	-3.13	-0.70
59	IL7R	Interleukin-7 receptor subunit alpha	-3.11	-0.23
60	CTSZ	Cathepsin Z	3.11	0.11
61	GHRL	Appetite-regulating hormone	-3.10	-2.18
62	KIRREL3	Kin of IRRE-like protein 3	3.10	2.98
63	IL17RD	Interleukin-17 receptor D	3.09	2.24
64	CGA LHB	Luteinizing hormone	-3.08	-3.39
65	FSTL1	Follistatin-related protein 1	3.05	2.15
66	IBSP	Bone sialoprotein 2	3.03	0.36

Positive *t* values indicate that the protein levels were increased after human growth hormone administration; *t* values for all models and all proteins are provided in Supplementary Table 2 [47].

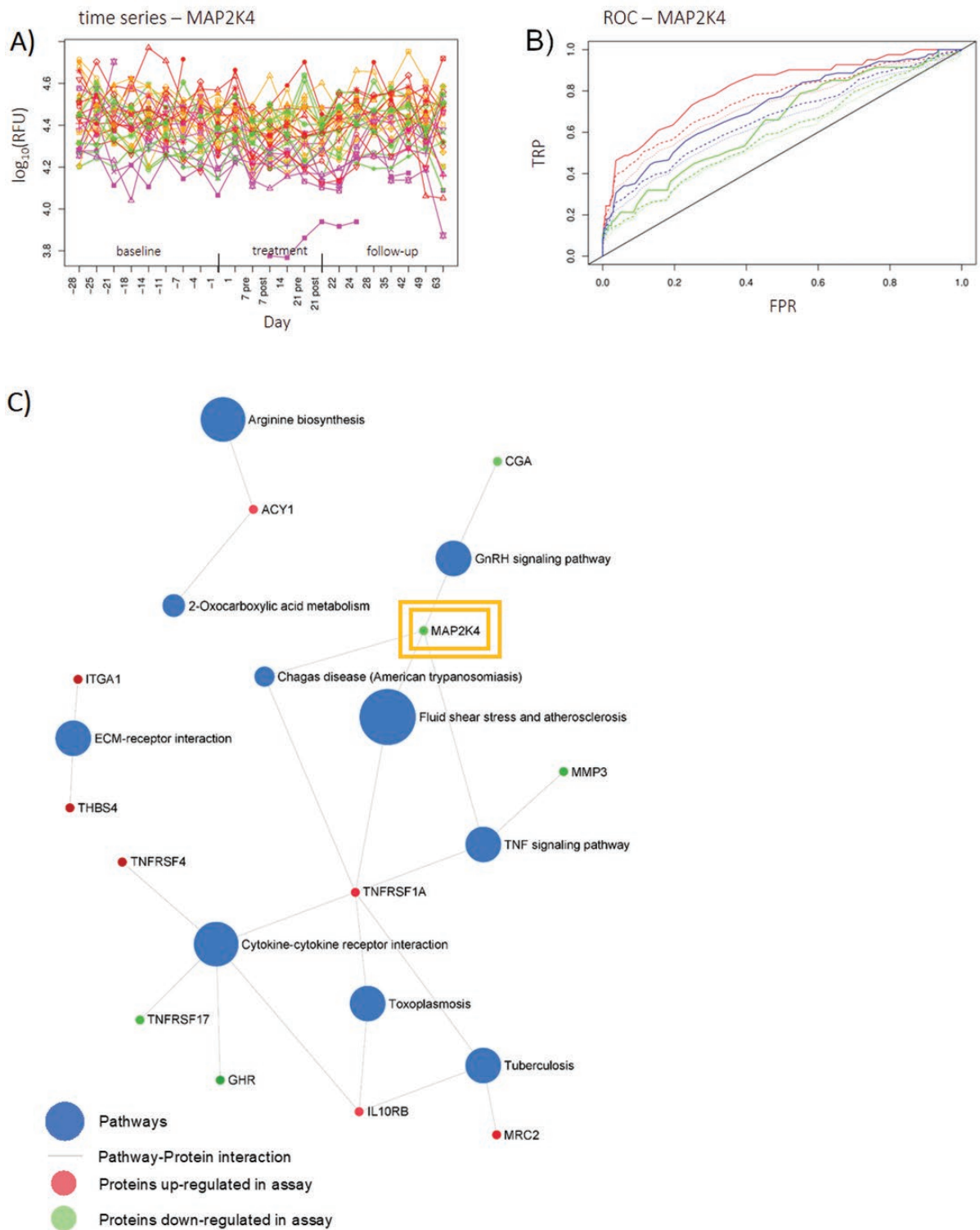
was possible. Levels of 19 proteins were significantly increased ( $t > 3$  for the follow-up-to-dose interaction) after cessation of hGH treatment, and 8 proteins showed prolonged decreased abundances ( $t < -3$  for the follow-up-to-dose interaction) (see Table 2). These 27 proteins are particularly interesting because they may persist as biomarkers after the athletes have stopped the administration of hGH. Especially proteins with increasing serum levels in the follow-up period (see *t*-value), for example, ferritin heavy chain (FTH1, rank 6), pleiotrophin (PTN, rank 9), TNF receptor superfamily member 4 (TNFRSF4, rank 13), and MAP2K4 (rank 10) Fig. 3A and 3B) may have the potential to be used as biomarkers of hGH abuse.

Potential adverse effects of the chronic administration with hGH have been the subject of discussions since the legal use of the drug for therapeutic purposes was approved [8, 18]. Retrospective cohort studies raised concerns about a greater incidence of cancer, particularly colorectal cancer, Hodgkin disease [14], and leukemia [55], after treatment with pituitary-derived hGH, but recent studies could not generally confirm a higher risk for primary cancer after treatment with recombinant hGH [13, 15]. However, most cohort studies had only a short follow-up period that may not include the long-term period of mitogenic effect of hGH treatment, only small group sizes, and patients received hGH in doses to target physiologic GH levels [56]. The role of the GH/IGF1 axis in cancer biology also has been studied elsewhere; for example, patients with endogenously elevated GH and IGF1 levels, usually caused

by pituitary adenoma (acromegaly), had a higher risk of several cancers in a meta-analysis by Dal et al [57]. Also, in an in vivo model, GH-deficient (dw/dw) rats were not vulnerable for mammary tumors when treated with the carcinogen nitrosomethylurea, but when animals received hormone replacement with GH the tumor incidence increased toward normal levels. Surprisingly, when GH treatment was stopped nearly all tumors regressed [58]. Interestingly, in our study, not only was the PI3K-Akt signaling pathway (see Fig. 2C), which mediates mitogenic and antiapoptosis effects of the GH/IGF1 axis, enriched after hGH treatment, but 2 cancer-related pathways were as well, prostate cancer (3/97) and proteoglycans in cancer (4/199), but whether the chronic (mis)use of hGH by healthy athletes may increase the risk for malignant disease cannot be answered by our study.

Another complication of the treatment with hGH is the reduction of insulin sensitivity and increased fasting blood glucose levels, as shown by Maison et al [9]. These effects were independent of the duration and dose of hGH treatment. A recent meta-analysis of 94 randomized, controlled trials could not find clear evidence of an increased risk of diabetes after hGH treatment, despite the lowered insulin sensitivity, but the interpretation of the study results was limited by the small number of participants and missing control groups [11]. While doses of hGH in the treatment of adult GH-deficient patients range from 0.1 to 0.5 mg/day (0.47-1.56 IU/day) according to the current guideline of the American Association of Clinical Endocrinologists

## hGH induced changes during follow-up period



**Figure 3.** Overview of human growth hormone (hGH)-induced changes in the serum levels of proteins of recreational athletes during the follow-up period, shown to be exemplary with dual specificity mitogen-activated protein kinase 4 (MAP2K4). All plots are generated as described in Fig. 2, but requiring additionally  $t$  greater than 3 for the follow-up-to-dose interaction. A, Time-series plot of MAP2K4; B, receiver operating characteristic (ROC) curve of MAP2K4; and C, functional network illustrating regulatory Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways involved by treatment with recombinant hGH during the follow-up period.

and American College of Endocrinology [12], doses abused by athletes are likely higher. Saugy and colleagues [17] estimated that doping athletes use hGH 3 to 4 times per week at doses of 10 to 25 IU/day. Other studies, including ours, that addressed the pharmacological or physiological effects of hGH in healthy individuals administered hGH at an average dose of 7.5 to 19 IU/day [27, 33, 48, 50, 53, 59-61]. Because systematic data on the adverse effects of hGH abuse in healthy individuals are missing, potential health risks are often inferred from studies with patients with acromegaly, in whom supraphysiological hGH levels over many years increases the risk for cardiovascular (hypertension, heart failure, cardiomyopathy) and metabolic disease (type 2 diabetes) [18]. The extent to which chronic (mis) use of hGH increases the risk of developing diabetes remains ambiguous, but some of the proteins identified in our study have recently been described in the context of glucose metabolism; the growth differentiation factor 11 (GDF11) [62] and GDF8 [63] seem to be involved in the pathogenesis of type 2 diabetes. As homologous members of the transforming growth factor- $\beta$  superfamily, both proteins (GDF8/11, rank 52; see Table 2), exhibit high structural similarity [64] and furthermore have a similar high affinity to the protease-inhibitors WFIKKN1 (rank 37) (and WFIKKN2) [65]. Increased serum levels of GDF8 and GDF11 and WFIKKN2 were also seen in patients with increased risk of developing type 2 diabetes [66].

Although the influence of hGH on immunological processes has been postulated since the 1990s when the first patients were treated with hGH [6], none of the studies that addressed hGH treatment in humans reported changes in the serum level of immunoproteins. The heterogeneous group of proteins related to the cytokine-cytokine receptor interaction pathway (see Fig. 2C and 3C) covered several chemokines with increased serum levels, for example, CCL2, CCL15, and CXL10, which have not yet been reported as potential protein biomarkers for hGH misuse, but should be considered in future studies.

## Conclusion and Study limitations

In our study we were able identify (long-term) GH-regulated serum proteins in recreational athletes over a period of 13 weeks after 3 weeks' treatment with recombinant hGH. Using the SOMAscan assay allowed us to target a large panel of serum proteins at high sensitivity and broad coverage. However, the SOMAscan technology is not without limitations. While other methods in antidoping research measure biomarker abundance more or less directly, the amount of SOMAmer reagent captured in the assay expresses their concentration only indirectly as the amount of aptamer-bound protein is first converted into

DNA, which is then quantified using microarray technology. Additionally, aptamers capture proteins based on their inherent 3-dimensional structure. Genetic variance in the aptamer binding sites of the aptamers may lead to unaccounted variations, and aptamers may also potentially cross-react with other proteins with similar binding sites. Furthermore, the targeted preselection of proteins in the panel of the SOMAscan assay has to be considered because only about one-third of all potentially detectable serum proteins are covered by the panel used here; for example, P-III-NP, one of the currently used biomarkers for the detection of hGH misuse, is not covered by our SOMAscan assay. The panel used for this study included 2 other collagen proteins: COL8A1 (collagen alpha-1 chain) and COL23A1 (collagen alpha-1(XXIII) chain). However, these proteins did not show significant differences between the different study groups before hGH administration, during treatment, or during the follow-up period. A more recent version of the SOMAscan assay (4783 SOMAmers binding specifically to 4137 human proteins) includes up to 21 collagen proteins and may be useful for following biomarker discovery studies.

While the main focus of the present study was to address the biological pathways of hGH action, the results can also provide a starting point to establish a group of biomarkers affected by hGH administration and, if possible, from as many different biological pathways as possible (eg, GH/IGF1 axis, glucose metabolism, cell adhesion) that, when combined (in a discriminant function or model), give the highest possible specificity (as close as possible to 100%) and sensitivity of detection of hGH. In particular, it is of interest to extend the detection of doping as long as possible even after the last administration of hGH, ideally at the lowest possible doses.

Overall, treatment with hGH affects a larger number of serum proteins and associated biological pathways, during both the treatment (see Fig. 2C) and follow-up periods (Fig. 3C) as previously acknowledged and supports the hypotheses of GH as hormone with a broader range of functions. Further research is necessary to examine whether related proteins are potential targets for the treatment of disease or useful biomarkers in clinical conditions. Finally, whether the newly identified proteins are applicable as biomarkers for the detection of hGH misuse remains to be investigated, but the results of our study emphasize that proteomics should be considered as a valuable key technology in antidoping research.

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**Data Availability:** The data generated during the present study are not publicly available but are available from the corresponding author on reasonable request.

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