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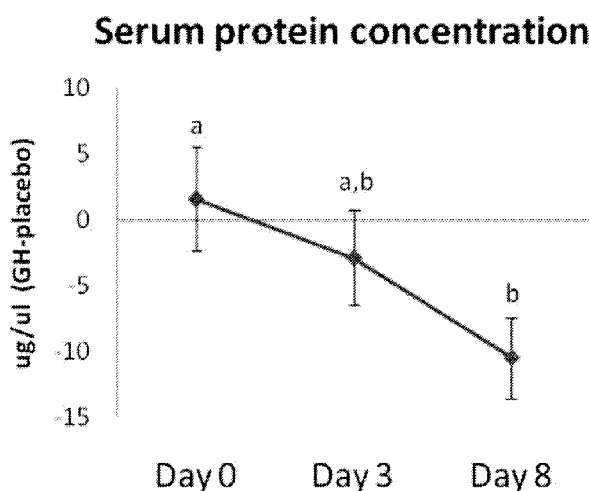


FIG - 1

(57) Abstract: The detection of recombinant human growth hormone (rhGH) is difficult due to its short half-life. The conventional method of detecting rhGH, the isoform approach, has a narrow window of detection. As such, novel biomarkers of GH are needed to facilitate rhGH doping detection. The instant method employs proteins that showed significant changes ($p < 0.05$) due to rhGH treatment and have been identified and confirmed by Western blotting. These included one isoform of alpha-1 antitrypsin and one isoform of transthyretin that significantly increased; one isoform of apolipoprotein A-1, one isoform of hemoglobin beta chain, and one isoform of inter-alpha-trypsin inhibitor heavy chain H4 that significantly decreased. These protein isoforms represent novel biomarkers of short-term exogenous rhGH exposure.



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BIOMARKERS FOR GROWTH HORMONE ACTIONCROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This non-provisional application claims the benefit of U.S. Provisional Application No. 61/384,568 filed on 20 September 2010, the content of which is hereby incorporated by reference as if recited fully herein.

TECHNICAL FIELD

[0002] Embodiments relate to methods for detecting growth hormone (GH) action in a subject, particularly, methods for detecting GH doping.

BACKGROUND

[0003] Recombinant human GH (rhGH) is abused by athletes and is on the prohibited substance list by World Anti-Doping Agency. Positive performance outcomes of GH administration have been controversial, and only recently was the first scientific evidence of GH improving performance in sprint published. Still rhGH abuse is rampant among athletes. It is estimated that the dose of rhGH abuse is 10-25 IU/day, which is much higher than therapeutic doses for adult GH deficiency, which is 0.2-0.3mg/day (~0.6-0.9 IU/day).

[0004] It is difficult to detect GH doping because rhGH is identical to endogenous hGH and has a short plasma half life of about 15 – 20 min. To date, there are two approaches using blood to detect rhGH doping. One is based on the quantification of different hGH isoforms. Endogenous GH, produced in the somatotrophs of the pituitary gland, is composed of several forms including the most abundant 22 kDa isoform, as well as a 20 kDa isoform and several others of varying sizes. Since rhGH is composed of only the 22 kDa isoform, a change in the ratio of 22 kDa isoform over the total GH concentration makes it possible to detect whether exogenous rhGH has been used. The limitation of this approach is that the window of opportunity for detection is narrow. It may be because of this reason that no positive rhGH doping results were found in the 2004 Athens and 2006 Turin Olympic Games where the GH isoform method was adopted. It was only recently (2010) using this method that the first athlete was detected as abusing rhGH.

[0005] A second approach to detect GH doping is to determine GH-dependent biomarkers with longer half lives than GH. Two studies entitled ‘the GH-2000 project’ and ‘the GH-2004 project’ were undertaken to identify and confirm insulin-like growth factor-1 (IGF-1) and procollagen type III (P-III-P) to be two markers of GH in human serum. However, IGF-1 and P-III-P display relatively large variations among individuals or even within the same person. For example, serum samples obtained over a two- to three-week period from 1103 elite athletes have shown that the within-subject coefficient of variation is nearly 21% for

IGF-1 and 15% for the collagen markers. Thus, the discovery of robust and reliable biomarkers for GH action would be valuable.

SUMMARY

[0006] The detection of recombinant human growth hormone (rhGH) is difficult due to its short half-life. Current methods are inadequate because the detection window is exceptionally narrow. Thus, novel biomarkers of GH are needed to facilitate rhGH doping detection. Whether exposure to exogenous GH results from abuse or a therapeutic treatment, this disclosure relates to robust and reliable biomarkers and assays for detecting or monitoring GH action in a subject.

[0007] Aspects include a method for evidencing GH doping in a human subject, comprising: (a) detecting one or more markers in a biological sample of the subject, the markers comprise a protein isoform selected from the group consisting of apolipoproteinA-1 (Swiss-Prot Acc. No. P02647; SEQ ID NO. 1), inter-alpha-trypsin inhibitor heavy chain H4 (Swiss-Prot Acc. No. Q14624; SEQ ID NO. 2), alpha-1 antitrypsin (Swiss-Prot Acc. No. P01009; SEQ ID NO. 3), transthyretin (Swiss-Prot Acc. No. P02766; SEQ ID NO. 4), and hemoglobin beta chain (Swiss-Prot Acc. No. P68871; SEQ ID NO. 5); and (b) determining altered levels of said marker(s), said altered levels indicating a likelihood of GH doping. In various methods, one or more markers are included in a diagnostic panel including at least two of apolipoproteinA-1, inter-alpha-trypsin inhibitor heavy chain H4, alpha-1 antitrypsin, transthyretin, and hemoglobin beta chain. In some embodiments, altered levels are compared to a control sample. The control sample may be taken from the same or a different subject(s). In various embodiments, the method further comprises the step of identifying a subject at risk for GH doping. In some embodiments, the sample is a blood sample.

[0008] In various embodiments, the detecting step is carried out by immunoassay, chromatography, spectrometry, electrophoresis, sedimentation, isoelectric focusing, or any combination thereof. In some embodiments, the detecting step comprises the steps of subjecting the biological sample to two-dimensional gel electrophoresis to yield a stained gel and an increased or decreased concentration of the protein isoform is detected by an increased or decreased intensity of a protein-containing spot on the stained gel, compared with a corresponding control gel. In specific embodiments, the intensity of spot 2502, 4001, 2204, 2304, and 8004 in FIG. 2 is detected. In some embodiments, the increased intensity of spot 2502 or 4001 or the decreased intensity of spot 2204, 2304, and 8004 in FIG. 2 is detected.

[0009] A biomarker of the various embodiments may be defined in terms of its molecular weight (Mw) and isoelectric point (pI). In exemplary embodiments, the protein isoform of

apolipoproteinA-1 has a MW of about 28 and a pI of about 5.7; the protein isoform of inter-alpha-trypsin inhibitor heavy chain H4 has a MW of about 35 and a pI of about 5.8; the protein isoform of alpha-1 antitrypsin has a MW of about 45 and a pI of about 5.8; the protein isoform of transthyretin has a MW of about 14 and a pI of about 6.0; and the protein isoform of hemoglobin beta chain has a MW of about 12 and a pI of about 7.2.

[0010] Other embodiments include a method of screening for GH doping in a diagnostic sample of a valid body tissue taken from a human subject, which comprises detecting an increased concentration of a protein isoform in the diagnostic sample, compared with a control sample, the protein isoform being selected from the group consisting of: alpha-1 antitrypsin; and transthyretin; or a decreased concentration of a protein isoform in the diagnostic sample, compared with a control, normal human sample, the protein isoform being selected from the group consisting of: apolipoproteinA-1; inter-alpha-trypsin inhibitor heavy chain H4; and hemoglobin beta chain; wherein the protein isoform of apolipoproteinA-1 has a MW of about 28 and a pI of about 5.7; the protein isoform of inter-alpha-trypsin inhibitor heavy chain H4 has a MW of about 35 and a pI of about 5.8; the protein isoform of alpha-1 antitrypsin has a MW of about 45 and a pI of about 5.8; the protein isoform of transthyretin has a MW of about 14 and a pI of about 6.0; and the protein isoform of hemoglobin beta chain has a MW of about 12 and a pI of about 7.2. In some embodiments, the valid body tissue may be blood or a blood product such as serum or plasma. In certain embodiments, the body tissue is a body fluid and the body fluid is subjected to an immunoassay. In some embodiments, the control sample may be taken from healthy tissue of a different human subject than the one providing the diagnostic sample.

[0011] Another aspect of the various embodiments includes a method of monitoring the efficacy of GH administration in a subject with growth hormone deficiency, comprising: administering GH to the subject with a growth hormone deficiency; acquiring a diagnostic sample from the subject; detecting an increased concentration of a protein in the diagnostic sample, compared with a control, normal human sample, the protein being selected from the group consisting of: alpha-1 antitrypsin; and transthyretin; or a decreased concentration of a protein in the diagnostic sample, compared with a control sample, the protein being selected from the group consisting of: apolipoproteinA-1; inter-alpha-trypsin inhibitor heavy chain H4; and hemoglobin beta chain.

[0012] Exemplary embodiments also include kits for detecting GH doping in a subject, comprising ligands specific for two or more of apolipoproteinA-1, inter-alpha-trypsin inhibitor heavy chain H4, alpha-1 antitrypsin, transthyretin, and hemoglobin beta chain. In

some embodiments, the ligands are antibodies. In specific embodiments, the antibodies are isoform specific monoclonal antibodies.

[0013] Additional advantages of the disclosed method and compositions are in the description which follows, and in part are understood from the description, or may be learned by practice of the disclosed method and compositions. The advantages of the disclosed method and compositions are realized and attained by means of the elements and combinations particularly pointed out in the appended claims. It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] Included with the filing of this application is a sequence listing file with the file name: OHI2481-003_ST25.txt , the content of which is hereby incorporated by reference as if fully recited herein.

[0015] A better understanding of the embodiments will be obtained from a reading of the following detailed description and the accompanying drawings in which:

[0016] Figure 1. Total serum protein concentration difference between GH and placebo (GH-placebo). One-way repeated measures ANOVA followed by Tukey Test revealed a significant effect of time ($p = 0.047$). Different letters (a and b) denote a significant difference ($p < 0.05$). Error bars represent SEM.

[0017] Figure 2. 2-DE gel image. Spots in circles were significantly altered by GH treatment ($p < 0.05$). Each spot was assigned a unique number by PDQuest, for example, 2502. AAT: alpha-1 antitrypsin; ITIH4: inter-alpha-trypsin inhibitor heavy chain H4; APOA1: apolipoprotein A-1; TTR: transthyretin; HBB: hemoglobin beta chain; Mw: molecular weight; pI: isoelectric point.

[0018] Figure 3. Proteins that were significantly increased by GH treatment. A, AAT. B, TTR. C, 3-D view of intensity of the AAT isoform generated by PDQuest. D, 3-D view of intensity of the TTR isoform generated by PDQuest. The Y axis in Panel A and B represents the intensity difference between GH and placebo phase (intensity of GH phase – intensity of placebo). One-way repeated measures ANOVA followed by Tukey Test revealed significant differences at different treatment days. Different letters (a and b) denote a significant difference ($p < 0.05$). Error bars represent SEM.

[0019] Figure 4. Proteins that were significantly decreased by GH treatment. A, APOA1. B, ITIH4. C, HBB. D, 3-D view of intensity of the ITIH4 isoform generated by PDQuest. The Y axis in Panels A, B, and C represent the intensity difference between GH and placebo

phase (intensity of GH phase – intensity of placebo). One-way repeated measures ANOVA followed by Tukey Test revealed significant differences at different treatment days. Different letters (a and b) denote a significant difference ($p < 0.05$). Error bars represent SEM.

[0020] Figure 5. 1-D Western blotting of AAT, TTR and ITIH4. A, AAT. B, TTR. C, ITIH4. Serum samples from four subjects at day 0 and day 8 of rhGH treatment phase were loaded and immunoblotted with antibodies against AAT, TTR and ITIH4.

[0021] Figure 6. 2-D Western blotting of AAT, TTR and ITIH4. A, AAT. B, TTR. C, ITIH4. Serum samples from the same subject at day 0 and day 8 of rhGH treatment phase were shown for each protein. The isoform corresponding to the one identified by 2-DE was circled and/or pointed to by an arrow.

DETAILED DESCRIPTION OF THE EXEMPLARY EMBODIMENT(S)

[0022] Two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) were used to identify specific protein isoforms for monitoring GH action in a human subject. Various methods and assays described herein are useful for evidencing GH doping (or more generally, exposure to GH) in a human subject. Specifically, altered expression of biomarkers comprising at least one isoform selected from the group consisting of apolipoproteinA-1 (Swiss-Prot Acc. No. P02647; SEQ ID NO. 1), inter-alpha-trypsin inhibitor heavy chain H4 (Swiss-Prot Acc. No. Q14624; SEQ ID NO. 2), alpha-1 antitrypsin (Swiss-Prot Acc. No. P01009; SEQ ID NO. 3), transthyretin (Swiss-Prot Acc. No. P02766; SEQ ID NO. 4), and hemoglobin beta chain (Swiss-Prot Acc. No. P68871; SEQ ID NO. 5) may be used to indicate or evidence exogenous GH exposure. Accordingly, the protein set identified and described herein comprises biomarkers useful to screen or evidence GH doping.

[0023] The Swiss-Prot accession number is provided for the various biomarkers. Sequence information relating to each biomarker is disclosed on The National Center for Biotechnology Information (NCBI) Protein database at <http://www.ncbi.nih.gov/protein>. Additionally, there are a variety of sequences related to biomarkers as well as any other protein disclosed herein that are disclosed on Uniprot (UniProtKB/Swiss-Prot), EMBL, DDBJ, and or Genbank databases, and these sequences and others are herein incorporated by reference in their entireties as well as for individual subsequences contained therein. Those of skill in the art understand how to resolve sequence discrepancies and differences and to adjust the compositions and methods relating to a particular sequence to other related sequences.

[0024] Disclosed herein are biomarkers and methods for identifying and using the biomarkers. The term "marker protein," "marker," or "biomarker" as used herein refers to any assayable characteristic or composition that is used to identify, detect, screen, or monitor

a condition or a therapy for said condition in a subject or sample. Biomarkers include all biologically relevant forms of the proteins identified, including post-translational modifications resulting in isoforms of a given protein. For example, the marker protein can be present in the body tissue in a glycosylated, phosphorylated, multimeric or precursor form. A biomarker is, for example, a protein and or a particular protein isoform, or combination of proteins and or protein isoforms, whose presence, absence, or relative amount is used to identify a condition or status of a condition in a subject or sample. For example, in at least one example, a biomarker is a protein isoform, or a combination of protein isoforms, whose relative concentration in a subject or sample is characteristic of exposure to or treatment with exogenous GH. A biomarker may be any protein or specific protein isoform which can be detected, directly or indirectly (e.g., via an analog, metabolite, fragment or breakdown product) in a biological sample from a subject, an increase or decrease of the amount of which, compared to amounts found in a control subject(s), is indicative that the subject may be exposed to and or treated with GH.

[0025] Marker proteins described herein include any protein listed in Table 1. Relevant biomarkers include one isoform of alpha-1 antitrypsin and one isoform of transthyretin; one isoform of apolipoprotein A-1, one isoform of hemoglobin beta chain, and one isoform of inter-alpha-trypsin inhibitor heavy chain H4. Accordingly, each of these protein isoforms represent a novel biomarker of short-term exogenous GH exposure.

[0026] Various embodiments include methods and materials for monitoring the effect of exogenous GH exposure in a subject. In various embodiments, methods include analyzing the serum proteome of a subject prior to, during, or after sporting competition. Five protein isoforms were differentially expressed following short-term exogenous GH exposure. These proteins included a protein isoform of apolipoproteinA-1 having a MW of about 28 and a pI of about 5.7; a protein isoform of inter-alpha-trypsin inhibitor heavy chain H4 having a MW of about 35 and a pI of about 5.8; a protein isoform of alpha-1 antitrypsin having a MW of about 45 and a pI of about 5.8; a protein isoform of transthyretin having a MW of about 14 and a pI of about 6.0; and a protein isoform of hemoglobin beta chain having a MW of about 12 and a pI of about 7.2. The isoforms represent reliable biomarkers of exogenous GH exposure in a subject, including short-term exposure to exogenous GH, particularly supraphysiological GH.

[0027] Disclosed herein is the use of an isoform of transthyretin as a biomarker for evidencing exogenous GH exposure. Transthyretin is mainly secreted by the liver and transports thyroxine T4 and retinol-binding protein-4 in serum. Also, plasma transthyretin

was found to increase in type II diabetic subjects. The increased isoform of transthyretin discovered may reflect the diabetogenic activity of GH. However, a previous study on healthy subjects treated with GH releasing hormone analog reveals a decrease of another transthyretin isoform. In various embodiments, a protein isoform of transthyretin having a MW of about 14 and a pI of about 6.0 increased at day 8 by exogenous GH treatment. Notably, none of the other isoforms or the total transthyretin levels was significantly different after GH or placebo treatment. Accordingly, this isoform is a biomarker of GH action, but not total transthyretin or any other transthyretin isoform.

[0028] Disclosed herein is the use of an isoform of alpha-1 antitrypsin as a biomarker for evidencing exogenous GH exposure. Alpha-1 antitrypsin is a serine protease inhibitor in plasma. It is also an acute phase protein that is secreted in large amounts by the liver in response to acute infection or injury. Alpha-1 antitrypsin exists as multiple isoforms resulting from the combination of different N-linked glycan structures and different N-termini. Alpha-1 antitrypsin expression in the liver responds to GH in both rats and humans, and GH-deficiency is often associated with deficiency in ATT. In various embodiments, a protein isoform of alpha-1 antitrypsin having a MW of about 45 and a pI of about 5.8 was significantly increased at day 8 by exogenous GH treatment.

[0029] Disclosed herein is the use of an isoform of apolipoproteinA-1 as a biomarker for evidencing exogenous GH exposure. ApolipoproteinA-1 is associated with high-density lipoprotein (HDL) cholesterol. Interestingly, acromegalic patients had lower total apolipoproteinA-1 levels which increased after treatment with somatostatin analogues. In various embodiments, a protein isoform of apolipoproteinA-1 having a MW of about 28 and a pI of about 5.7 was significantly downregulated at day 8 by exogenous GH treatment.

[0030] Disclosed herein is the use of an isoform of hemoglobin beta chain as a biomarker for evidencing exogenous GH exposure. Hemoglobin beta chain is a subunit of hemoglobin, the major protein in red blood cells. Hemoglobin responds to GH positively in that GH deficient children who have lower levels of hemoglobin show elevated hemoglobin levels by GH treatment. Accordingly, it is not known why this particular isoform of hemoglobin beta chain decreases with GH treatment. Interestingly, hemoglobin α -chain has been shown to increase in response to GH using SELDI-TOF MS technique, although the robustness of this marker was brought into question because subsequent analysis found it to be at very low or even undetectable levels, possibly due to a different material used for sample collection. In various embodiments, a protein isoform of hemoglobin beta chain having a MW of about 12 and a pI of about 7.2 was significantly reduced at day 8 by exogenous GH treatment.

[0031] Disclosed herein is the use of an isoform of inter-alpha-trypsin inhibitor heavy chain H4 as a biomarker for evidencing exogenous GH exposure. Inter-alpha-trypsin inhibitor heavy chain H4, also called inter-alpha-trypsin inhibitor family heavy chain-related protein (IHRP) or plasma kallikrein sensitive glycoprotein 120 (PK-120 or Gp120), is an acute phase protein that has serine protease inhibitor activity. ITIH4 is composed of 70 kDa and 35 kDa subunits, and the 35 kDa subunit was the one detected in the 2-D gels (Table 1). Although no relationship to GH has been reported, elevated serum ITIH4 concentrations are observed in patients with different malignant tumors, and reduced levels are reported in patients with liver fibrosis caused by hepatitis C. In various embodiments, a protein isoform of inter-alpha-trypsin inhibitor heavy chain H4 having a MW of about 35 and a pI of about 5.8 was significantly reduced at days 3 and 8 by exogenous GH treatment.

[0032] The term "protein" (also referred to as "polypeptide") is not restricted to the sequences corresponding to the accession numbers provided above, and includes variants and other isoforms thereof. A variant is defined as a naturally occurring variation in the sequence of a polypeptide which has a high degree of homology with the given sequence. A high degree of homology is defined as at least 90%, preferably at least 95% and most preferably at least 99% homology. Protein variants may occur within a single species or between different species. The above proteins are of human origin, but various embodiments encompass use of the corresponding polypeptides from other mammalian species.

[0033] As used herein, the term "isoform" means a molecular form of a given protein, and includes proteins differing at the level of (1) primary structure (such as due to alternate RNA splicing, or polymorphisms); (2) secondary structure (such as due to different co- or post translational modifications); and/or (3) tertiary or quaternary structure (such as due to different sub-unit interactions, homo- or hetero-oligomeric multimerization). For example, differences in mass and/or charge of specific isoforms may be due to posttranslational modifications, including, but not limited to, alkylation, ubiquitination, phosphorylation, and glycosylation.

[0034] "Diagnosing," "detecting," "evidencing," or "screening" as used herein means providing an indication that a subject may have been exposed to or treated with exogenous GH. It will be appreciated that no such technique is perfect and that such detection, screening, or the like may be confirmed by other procedures such as physical examination, imaging, histological examination of tissue samples, etc.

[0035] "Panel test" as described herein refers to a group of individual laboratory tests that are related in some way, including, but not limited to, the condition they are designed to detect, the specimen type, and the methodology employed by the test.

[0036] The term "GH doping," as used herein, broadly refers to any non-therapeutic exposure or treatment with (e.g., injection of, dosing of, etc.) growth hormone, particularly recombinant human growth hormone. In most cases, the term relates to the misuse/abuse of GH as a performance-enhancing drug, particularly with respect to athletic competition. However, the term is also used herein to refer broadly to any non-therapeutic administration of GH to a subject.

[0037] Embodiments may be useful for monitoring the efficacy of a GH therapy in a patient with a growth hormone deficiency or any other condition in which the administration of exogenous GH is indicated. Such conditions include but are not limited to the use of GH in long-term treatment of pediatric patients who have growth failure due to an inadequate secretion of normal endogenous growth hormone; the treatment of short stature associated with Turner Syndrome in patients whose epiphyses are not closed; for the treatment of Small for Gestational Age (SGA); the treatment of short stature homeobox gene defects (SHOX deficiency); patients suffering from AIDS wasting; for replacement of endogenous growth hormone in adults with growth hormone deficiency; and for any other indication of GH.

[0038] The term "growth hormone" refers to (1) growth hormone itself of whatever species, for example, human, bovine, or porcine, although embodiments are particularly applicable to human growth hormone (hGH); (2) precursors to growth hormone, such as reduced (—SH) growth hormone and S-protected growth hormone, for example, growth hormone S-sulfonate; (3) variants of growth hormone or its precursors, for example, structures which have been modified to lengthen and/or shorten the growth hormone amino acid sequence, for example, the 20K variant of growth hormone, methionyl growth hormone, and the like; (4) analogs of growth hormone or its precursors, for example, a molecule having one or more amino acid substitutions, deletions, inversions, or additions compared with growth hormone; and (5) derivatives of growth hormone or its precursors, for example, a molecule having the amino acid sequence of growth hormone or growth hormone analog, but additionally having chemical modification of one or more of its amino acid side groups, alpha-carbon atoms, terminal amino groups, or terminal carboxylic acid groups.

[0039] The term "differentially expressed" means that the stained protein-bearing spots are present at a higher or lower optical density in the gel from the sample taken for diagnosis (the "diagnostic sample") relative to that from the gel from a control or other comparative sample.

In various embodiments, these changes are associated with GH doping. It follows that the proteins are present in the diagnostic sample at a higher or lower concentration than in the control or other comparative sample.

[0040] "Altered level" or "altered levels" as used with respect to marker proteins herein refers to an increased level (e.g., a one or two fold increase, or more) or a decreased level (e.g., a one or two-fold decrease, or more) in the quantity of one or more marker proteins detectable in or via a biological sample from a subject, as compared to a level or levels of one or more marker proteins in a corresponding normal (or placebo treated) subject.

[0041] Some protein "spots" will represent post-translational modifications of the same protein while others may represent heterogeneity due to genetic polymorphisms. For example, 2D gels often reveal a "charge" train representing a difference in isoelectric points of the said protein that may be caused by differential phosphorylation states of the same protein.

[0042] The term "binding partner" includes a substance that recognizes or has affinity for the marker protein. It may or may not itself be labeled.

[0043] The term "antibody" includes polyclonal antiserum, mouse monoclonal antibodies, mouse/human chimeric monoclonal antibodies, humanized monoclonal antibodies, human monoclonal antibodies, and fragments of any of the types of antibodies such as single chain and Fab fragments, and genetically engineered antibodies. The antibodies may be chimeric or of a single species.

[0044] The term "valid body tissue" means any tissue in which it may reasonably be expected that a marker protein would accumulate in relation to GH exposure or GH treatment. For example, it may be a body fluid such as blood or a blood derivative such as plasma or serum, saliva, or urine.

[0045] Suitable methods for determining an amino acid sequence of the proteins and peptides include, but are not limited to, Edman degradation, (tandem) mass spectrometry and the like (see e.g. Edman, P. Mol. Biol. Biochem. Biophys., (1970), 8: 211-255; U.S. Pat. No. 6,799,121). The amino acid sequence of the proteins and peptides may be compared to amino acid sequences of known proteins. The term "mass spectrometry" as used herein includes various methods such as tandem mass spectrometry, matrix assisted laser desorption ionization (MALDI) time-of-flight (TOF) mass spectrometry, MALDI-TOF-TOF mass spectrometry, MALDI Quadrupole-time-of-flight (Q-TOF) mass spectrometry, electrospray ionization (ESI)-TOF mass spectrometry, ESI-Q-TOF, ESI-TOF-TOF, ESI-ion trap mass spectrometry, ESI Triple quadrupole mass spectrometry, ESI Fourier Transform mass

spectrometry (FTMS), MALDI-FTMS, MALDI-Ion Trap-TOF, and ESI-Ion Trap TOF. These mass spectrometry methods are well known in the art (see e.g. Gary Siuzdak, "Mass Spectrometry for Biotechnology", Academic Press, NY, (1996)). At its most basic level, mass spectrometry involves ionizing a molecule and then measuring the mass of the resulting ion. Since molecules ionize in a way that is well known, the molecular weight of the molecule can generally be accurately determined from the mass of the ion. Tandem mass spectrometry, for instance, may be used to identify proteins because it can provide information in addition to parent ion molecular weight. Tandem mass spectrometry involves first obtaining a mass spectrum of the ion of interest, then fragmenting that ion and obtaining a mass spectrum of the fragments. Tandem mass spectrometry thus provides both molecular weight information and a fragmentation pattern that can be used in combination along with the molecular weight information to identify the exact sequence of a peptide or protein (see e.g. Hunt et al. (1986) PNAS USA 83:6233-6237; Shevchenko et al. (1996) PNAS USA 93:14440-14445; Figeys et al. (1996) Anal. Chem. 68:1822-1828 and Wilm et al. (1996) Nature 379:466-469).

[0046] "Subjects" as described herein are generally human subjects and include athletes, competitors, patients, etc. The subjects may be male or female and may be of any race or ethnicity, including but not limited to Caucasian, African-American, African, Asian, Hispanic, Indian, etc. The subjects may be of any age, including newborn, neonate, infant, child, adolescent, adult, and geriatric. Subjects may also include animal subjects, particularly mammalian subjects such as dog, cat, horse, mouse, rat, etc., screened for veterinary medicine or pharmaceutical drug development purposes.

[0047] "Biological sample" as used herein refers to any material taken from the body of a subject that may carry the target compound or compounds of the tests described herein, including both tissue samples and biological fluids such as blood samples, saliva samples, urine samples, etc. The sample can be taken from any valid body tissue, especially body fluid, of a (human) subject, but preferably blood, plasma or serum. Certain methods disclosed herein involve collecting a biological sample from a subject. The collection of biological samples is performed by standard methods. Typically, once a sample is collected, the biomarkers are detected and measured. The disclosed biomarkers are detected using any suitable technique. Further, molecules that interact with or bind to the disclosed biomarkers, such as antibodies to a biomarker, are detected using known techniques. Many suitable techniques--such as techniques generally known for the detection of proteins, peptides and other analytes and antigens--are known, some of which are described below.

[0048] "Blood sample" as used herein refers to whole blood or any fraction thereof that may contain detectable levels of marker proteins therein (if marker proteins are present in the whole blood sample from which said fraction is obtained), and in particular embodiments refers to a blood sera or blood plasma sample.

[0049] While the following description focuses primarily on methods and assays for detecting or screening for GH exposure and or GH doping, it will be appreciated that the embodiments may also be utilized in connection with other conditions in which GH levels are altered. For example, if a specific biomarker concentration goes down following the administration of supraphysiological GH, one of skill in the art may expect the marker to go up following administration of an agent that depresses GH action (e.g., the GH receptor antagonists termed Pegvisomant).

[0050] Assay Procedures

[0051] The step of collecting a sample can be carried out either directly or indirectly by any suitable technique. For example, a blood sample from a subject can be carried out by phlebotomy or any other suitable technique, with the blood sample processed further to provide a serum sample or other suitable blood fraction.

[0052] The step of determining the presence of an altered level of a marker protein in the sample, and/or depressed level of a marker protein in the sample, can also be carried out either directly or indirectly in accordance with known techniques, including, but not limited to, mass spectrometry, chromatography, electrophoresis, sedimentation, isoelectric focusing, and antibody assay. See, e.g., U.S. Pat. No. 6,589,748; U.S. Pat. No. 6,027,896.

[0053] In various embodiments, marker proteins may be identified by two-dimensional electrophoresis (2-D electrophoresis). 2D-electrophoresis is a technique comprising denaturing electrophoresis, followed by isoelectric focusing; this generates a two-dimensional gel (2D gel) containing a plurality of separated proteins. For an example of a preferred means of carrying out 2D-electrophoresis to identify marker proteins, see, e.g. WO 98/23950; U.S. Pat. No. 6,064,654 and U.S. Pat. No. 6,278,794. Briefly, spots identified in a 2D gel are characterized by their isoelectric point (pI) and apparent molecular weight (MW) as determined by 2D gel electrophoresis. Altered levels of marker proteins in a first sample or sample set with respect to a second sample or sample set can be determined when 2D gel electrophoresis gives a different signal when applied to the first and second samples or sample sets. Altered levels of marker proteins may be present in first sample or sample sets at increased, elevated, depressed or reduced levels as compared to the second sample or sample sets. By "increased level" it is meant (a) any level of a marker protein when that marker

protein or a particular isoform of a marker protein is not present in a control and or comparative subject as well as (b) an elevated level (e.g., a two- or three-fold increase in detected quantity) of marker protein or a particular isoform of a marker protein when that protein or a particular isoform is present in a control and or comparative subject. By "depressed level" it is meant (a) an absence of a particular marker protein or isoform of a particular marker protein when that marker protein is present in a control and or comparative subject, as well as (b) a reduced level (e.g., a two- or three-fold reduction in detected quantity) of a marker protein or isoform of a marker protein when that protein or isoform is present in a control and or comparative subject.

[0054] In general, the steps of (a) assaying a sample for an elevated level of a marker protein and/or depressed level of a marker protein, and (b) correlating an elevated level of a marker protein and/or a depressed level of a marker protein in the sample with exposure to exogenous GH, can be carried out in accordance with known techniques or variations thereof that will be apparent to persons skilled in the art.

[0055] Signals obtained upon analyzing a biological sample or sample set from subjects receiving supraphysiological doses of GH (e.g., GH doping) relative to signals obtained upon analyzing a biological sample or sample set from normal subjects will depend upon the particular analytical protocol and detection technique that is used. Accordingly, the invention contemplates that each laboratory will establish a reference range for each marker protein identifier (e.g., pI and/or MW) in normal subjects according to the analytical protocol and detection technique in use, as is conventional in the diagnostic art.

[0056] Kits for detecting exogenous GH exposure are also provided, and in some embodiments include at least one biochemical material and/or reagent, such as buffers and/or binding partners that are capable of specifically binding with one or more marker proteins from Table 1. These can provide a means for determining binding between the biochemical material and one or more marker proteins, whereby at least one analysis to determine a presence of one or more marker proteins, analyte thereof, or a biochemical material specific thereto, is carried out on a biological sample. Optionally such analysis or analyses may be carried out with the additional use of detection devices for immunoassay, radioimmunoassay, immunoblotting, chromatography, spectrometry, electrophoresis, sedimentation, isoelectric focusing, colorimetric, laser, or any combination thereof. Analysis may be carried out on a single sample or multiple samples. In addition, the kit may optionally include instructions for performing the method or assay. Additionally the kit may optionally include depictions or

photographs that represent the appearance of positive and negative results. In some embodiments, the components of the kit may be packaged together in a common container.

[0057] Various embodiments include a kit for detecting GH doping in a subject, comprising ligands specific for two or more of apolipoproteinA-1, inter-alpha-trypsin inhibitor heavy chain H4, alpha-1 antitrypsin, transthyretin, and hemoglobin beta chain. In some embodiments, the ligands are isoform specific antibodies. Such a kit optionally comprises a labeling means and/or a therapeutic agent. Additionally, the kit may include instructional materials for performing various methods presented herein. These instructions may be printed and/or may be supplied, without limitation, as an electronic-readable medium, such as a floppy disc, a CD-ROM, a DVD, a Zip disc, a video cassette, an audiotape, and a flash memory device. Alternatively, instructions may be published on an internet web site or may be distributed to the user as an electronic mail. When a kit is supplied, the different components can be packaged in separate containers. Such packaging of the components separately can permit long term storage without losing the active components' functions.

[0058] Panel Tests

[0059] The marker proteins described herein can be detected individually or in panels with one another or other additional markers of exogenous GH exposure. Where used in a panel test, the levels of the various markers are optionally but preferably tested from the same biological sample obtained from the subject (e.g., by detecting the quantities or amounts of various proteins in the same blood sample obtained from the subject). When combined in a panel test, the panel test may include determining an altered level for each of 2, 3, 4, 5, 6, 7 or more different marker proteins (e.g., a panel of some or all proteins set forth in Table 1 below). The combination of multiple marker proteins in a panel test serves to reduce the number of false positives and false negatives should an aberrant value for one particular member of the panel be found.

[0060] Various immunoassays are described in U.S. Pat. No. 7,713,525, incorporated by reference herein. Immunodetection methods may be used for detecting, binding, purifying, removing and quantifying various molecules including the disclosed biomarkers. Further, antibodies and ligands to the disclosed biomarkers are detected. For example, the disclosed biomarkers are employed to detect antibodies having reactivity therewith.

[0061] Immunoassay methods are based on the reaction of an antibody to its corresponding target or analyte and can detect the analyte in a sample depending on the specific assay format. To improve specificity and sensitivity of an assay method based on immuno-reactivity, monoclonal antibodies are often used because of their specific epitope recognition.

Polyclonal antibodies have also been successfully used in various immunoassays because of their increased affinity for the target as compared to monoclonal antibodies. Immunoassays have been designed for use with a wide range of biological sample matrices. Immunoassay formats have been designed to provide qualitative, semi-quantitative, and quantitative results.

[0062] Quantitative results are generated through the use of a standard curve created with known concentrations of the specific analyte to be detected. The response or signal from an unknown sample is plotted onto the standard curve, and a quantity or value corresponding to the target in the unknown sample is established.

[0063] Numerous immunoassay formats have been designed. ELISA or EIA can be quantitative for the detection of an analyte. This method relies on attachment of a label to either the analyte or the antibody and the label component includes, either directly or indirectly, an enzyme. ELISA tests may be formatted for direct, indirect, competitive, or sandwich detection of the analyte. Other methods rely on labels such as, for example, radioisotopes (I^{125}) or fluorescence. Additional techniques include, for example, agglutination, nephelometry, turbidimetry, Western blot, immunoprecipitation, immunocytochemistry, immunohistochemistry, flow cytometry, Luminex assay, and others (see *ImmunoAssay: A Practical Guide*, edited by Brian Law, published by Taylor & Francis, Ltd., 2005 edition).

[0064] Exemplary assay formats include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay, fluorescent, chemiluminescence, and fluorescence resonance energy transfer (FRET) or time resolved-FRET (TR-FRET) immunoassays. Examples of procedures for detecting biomarkers include biomarker immunoprecipitation followed by quantitative methods that allow size and peptide level discrimination, such as gel electrophoresis, capillary electrophoresis, planar electrochromatography, and the like.

[0065] Methods of detecting and/or quantifying a detectable label or signal generating material depend on the nature of the label. The products of reactions catalyzed by appropriate enzymes (where the detectable label is an enzyme; see above) can be, without limitation, fluorescent, luminescent, or radioactive or they may absorb visible or ultraviolet light. Examples of detectors suitable for detecting such detectable labels include, without limitation, x-ray film, radioactivity counters, scintillation counters, spectrophotometers, colorimeters, fluorometers, luminometers, and densitometers.

[0066] Any of the methods for detection can be performed in any format that allows for any suitable preparation, processing, and analysis of the reactions. This can be, for example, in multi-well assay plates (e.g., 96 wells or 384 wells) or using any suitable array or microarray.

Stock solutions for various agents can be made manually or robotically, and all subsequent pipetting, diluting, mixing, distribution, washing, incubating, sample readout, data collection and analysis can be done robotically using commercially available analysis software, robotics, and detection instrumentation capable of detecting a detectable label.

[0067] One embodiment comprises performing a binding assay for the marker protein. Preferably, an isoform specific binding partner may be used. The binding partner may be labeled. Preferably the assay is an immunoassay, especially between the marker and an antibody that recognizes the protein, or more preferably, the relevant protein isoform, especially a labeled antibody. It can be an antibody raised against part or all of it, for example, a monoclonal antibody or a polyclonal anti-human antiserum of high specificity for the marker protein.

[0068] Thus, the marker proteins described above are useful for the purpose of raising antibodies thereto which can be used to detect the increased or decreased concentration of the marker proteins present in a diagnostic sample. Such antibodies can be raised by any of the methods well known in the immunodiagnostics field.

[0069] The antibodies may be isoform specific, i.e. they recognize a specific isoform of a given biomarker. Moreover, the antibodies may be anti- to any biologically relevant state of the protein. Thus, for example, they can be raised against the unglycosylated form of a protein which exists in the body in a glycosylated form, against a more mature form of a precursor protein, e.g. minus its signal sequence, or against a peptide carrying a relevant epitope of the marker protein.

[0070] Various immunoassays may be carried out by measuring the extent of the protein/antibody interaction. Any known method of immunoassay may be used. In one embodiment, a sandwich assay may be used. In this method, a first antibody to the marker protein is bound to the solid phase such as a well of a plastics microtitre plate, and incubated with the sample and with a labeled second antibody specific to the protein (or specific protein isoform) to be assayed. Alternatively, an antibody capture assay can be used. Here, the test sample is allowed to bind to a solid phase, and the anti-marker protein antibody is then added and allowed to bind. After washing away unbound material, the amount of antibody bound to the solid phase is determined using a labeled second antibody, anti- to the first.

[0071] The binding partner in the binding assay is preferably a labeled specific binding partner, but not necessarily an antibody. The binding partner will usually be labeled itself, but alternatively it may be detected by a secondary reaction in which a signal is generated, e.g. from another labeled substance.

[0072] It is helpful to use an amplified form of assay, whereby an enhanced "signal" is produced from a relatively low level of protein to be detected. One particular form of amplified immunoassay is enhanced chemiluminescent assay. Conveniently, the antibody is labeled with horseradish peroxidase, which participates in a chemiluminescent reaction with luminol, a peroxide substrate and a compound which enhances the intensity and duration of the emitted light, typically 4-iodophenol or 4-hydroxycinnamic acid.

[0073] EXAMPLES

[0074] The following examples are included to demonstrate embodiments. It should be appreciated by those skilled in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventors to function well in practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents that are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention.

[0075] A. Materials and Methods

[0076] **Subjects and design**

[0077] Serum samples were obtained from 8 healthy male subjects that underwent placebo and GH injections in a randomized cross-over design. Each subject underwent two periods of 8 days in a randomized, double-blind, placebo-controlled manner: (I) placebo injections, (II) daily GH treatment (Norditropin SimplexX; Novo Nordisk, Copenhagen, Denmark; 2 mg sc at 10 PM, last injection on day 7) . There was a 1-3 week washout period between the two periods. Compliance was evaluated by returned vials. Serum samples were obtained at day 0, 3, and 8 for both periods.

[0078] All subjects were gave a written informed consent before participating in the experiment, which was approved by The Central Denmark Region Committees on Biomedical Research Ethics (200401184) in adherence to the declaration of Helsinki. The protocol was also approved by the Ohio University Institutional Review Board.

[0079] **Total serum protein concentration measurement**

[0080] Serum protein concentration was measured by the Bradford method using protein essay reagent from Bio-Rad (Hercules, CA).

[0081] **Two-dimensional gel electrophoresis (2-DE)**

[0082] The method for 2-DE was described previously. Briefly, for each sample, 750 ug of serum proteins were treated for 2 hours at room temperature with 8M urea, 1.8M thiourea, 4% CHAPS, 5mM reducing agent tributylphosphine, and a protease inhibitor cocktail (50X) containing 2 mM AEBSF, 1 uM Phosphoramidon, 0.2 uM aprotinin, 1 uM leupeptin, 130 uM bestatin, 10 uM pepstatin A and 14 uM E-64 (Sigma-Aldrich, Inc., St. Louis, MO). Then 15mM iodoacetamide was added for alkylation of reduced sulfur side chains. The sample was loaded onto a 17cm IPG strip with a linear pI range of 3–10 (Bio-Rad, Hercules, CA). After actively rehydrated (50V) for 12 hours at 20 °C using a Protean IEF cell (Bio-Rad), the strips were subjected to first dimensional IEF at 10000V for 60000V-Hr, followed by incubation of the strips in a buffer containing 2% (w/v) SDS, 0.5M Tris/HCl (pH 6.8), 20% (v/v) glycerol for 25min. The middle section of the strip (pI 5-8) possessing the majority of plasma proteins was cut out and subjected to second dimension SDS-PAGE. Polyacrylamide (15%) gels (8 x 7cm) were used for the 2nd dimension electrophoresis at a current of 25mA/gel until a total of 250 V-Hr was reached. After electrophoresis, the gels were fixed overnight in 40% ethanol and 2% acetic acid followed by washing three times in 2% acetic acid. The gels were then stained with SYPRO Orange (1:5000) (Molecular Probes, Eugene, OR) for 2 hours before gel images were captured using a laser-scanner Pharos FX plus (Bio-Rad) with an excitation wavelength of 488nm and an emission wavelength of 604nm.

[0083] Protein quantification and statistical analysis

[0084] Protein spots were matched across all images using PDQuest (Bio-Rad) software and manually checked and corrected. For quantification, the intensity of each protein spot was determined according to the fluorescence signal strength, and then normalized by the total density of each image using PDQuest software. The results were then exported and analyzed using SPSS version 14.0 software (Chicago, IL). For each protein spot, the intensity difference between placebo and GH treatment phases [intensity (GH-placebo)] at each time point was subjected to one-way repeated measures ANOVA to evaluate the effect of time (day 0, 3, and 8), followed by Tukey Test as a post hoc test using SigmaPlot 11.0 (San Jose, CA), with $p < 0.05$ as a significance cutoff. Similarly, data for total serum protein concentration were normalized by subtraction of the placebo from GH phase and subjected to one-way repeated measures ANOVA for the effect of time, followed by Tukey Test as a post hoc test ($p < 0.05$ as significant). All data were presented as the mean $\pm$ SEM.

[0085] MS and MS/MS analysis

[0086] Proteins of interest were excised manually from the SDS-PAGE gels and shipped to Protea Biosciences, Inc. (Morgantown, WV) for MS and MS/MS analyses using matrix-

assisted laser desorption/ionization (MALDI)-time of flight (TOF) and MALDI-TOF-TOF. The methods were described in details previously. Gel plugs were treated with acetonitrile and 50mM ammonium bicarbonate, then reduced and alkylated with 250mM dithiothreitol (DTT, 60min / 55°C) and 650mM iodoacetamide (60min / room temperature / in the dark). Digestion was performed with 500ng trypsin in 50mM ammonium bicarbonate buffer overnight. Peptides were extracted using 5% formic acid in 50% acetonitrile (dehydration), followed by rehydration with 50mM ammonium bicarbonate. For each extraction step, the solution was aspirated, collected, and collated. Three extraction cycles (dehydration and rehydration) were performed per sample. The recovered peptides were lyophilized, reconstituted in 10mM acetic acid, and re-lyophilized to yield a purified protein digest.

[0087] After in-gel digestion by trypsin, proteins were analyzed by ABI 4800 MALDI TOF-TOF analyzer. A C18 ProteaTip was washed and then equilibrated in a 0.1% trifluoroacetic acid (TFA) / 50% acetonitrile solution followed by a 0.1% TFA / 2% acetonitrile solution. The remaining reconstituted protein digest solution in an auto sampler vial (~65% of sample) was loaded onto the C18 ProteaTip by aspirating and expelling the sample 5-10 times. The bound sample was washed twice with the 0.1% TFA / 2% acetonitrile solution by aspirating and expelling 20μL of the wash solution 5-10 times. The sample was spotted directly onto a MALDI target that was pre-spotted with 0.6μL MALDI matrix α -cyano-4-hydroxycinnamic acid (CHCA) using 1μL of an elution solution (0.1% TFA / 90% acetonitrile).

[0088] The MALDI MS parameters used for analyses were: MS acquisition in reflector mode; positive ion mode; mass range = mass/charge (m/z) = 850 – 4000; 400 laser shots per spectrum; minimum signal/noise (S/N) = 10 for MS acquisition; 15 strongest precursors chosen for MS/MS; minimum S/N = 30 for MS/MS precursors; MALDI spot interrogated until at least 4 peaks in the MSMS spectra achieved a S/N = 70.

[0089] Manual search in protein database for identification

[0090] MS and MS/MS data were manually submitted to MASCOT at <http://www.matrixscience.com/> for protein identification. For MS data, the searching criteria were as follows: *Swiss-Prot* as the database; *human* as the species; *trypsin* digestion; *maximum one missed cleavage*; fixed *carbamidomethylation of Cys*; variable modifications of *oxidation-M (methionine)*, *pyro-Glu*; *monoisotopic*; and *50 ppm* of peptide mass or parent tolerance. For MS/MS ion search, in addition to the above conditions, a peptide charge of *+1* and a fragment mass tolerance of *0.5 Da* were used.

[0091] Western blotting

[0092] A subset of human serum proteins were subjected to 1-D and 2-D Western blotting using primary antibodies (1:1000) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). For 1-D Western blotting, 50 ug protein was diluted in 2% (w/v) SDS, 0.5M Tris/HCl (pH 6.8), 20% (v/v) glycerol, 2% β -mercaptoethanol and a trace of Bromophenol blue, boiled at 100 °C for 5 min, and loaded for SDS-PAGE. The proteins on the gel were then transferred to a PVDF membrane in a buffer containing 19.2 mM glycine, 2.5 mM Tris and 20% (v/v) methanol at 70 V for 2 hours at 4 °C. Following blocking in Tris-buffered saline with 1% Tween-20 (TBS/T) containing 5% non-fat dry milk for 1 h, the membrane was incubated with a primary antibody, (rabbit anti-human AAT, goat anti-human ITIH4, or rabbit anti-human TTR) at 4 °C overnight. Following washing with TBS/T, the membrane was incubated in horseradish peroxidase (HRP)-linked secondary antibodies (1:5000) including donkey anti-goat (Santa Cruz Biotechnology), donkey anti-mouse (Santa Cruz Biotechnology) or goat anti-rabbit (Millipore, Temecula, CA) for 2 h at room temperature. The membrane was exposed to Pierce[®] ECL Western blotting substrate (Thermo Scientific, Rockford, IL) for 1 min; then exposed to HyBlot CLTM autoradiography film (Denville Scientific Inc., Metuchen, NJ) for 0.5-2 min depending on the signal strength. For 2-D Western blotting, each sample containing 750 ug protein was treated as described above for 2-DE, followed by the same immunoblotting procedure as described for 1-D Western blotting.

[0093] B. Results

[0094] Serum protein concentration

[0095] Total serum protein concentration showed a significant change during treatment ($p < 0.05$). While concentration differences between GH and placebo treatments were negligible at day 0 and 3, they were approximately -10 ug/ul at day 8 (Fig. 1).

[0096] Serum protein levels which were significantly altered by GH treatment

[0097] A total of 94 spots were analyzed for each gel. After adjusting protein intensity (intensity of GH phase – intensity of placebo phase), five spots showed significant differences as a function of time (day 0, day 3, and day 8; $p < 0.05$), indicated by circled spots in Fig. 2. General information concerning these proteins, including MS, MS/MS scores, molecular weight (Mw), pI and PTMs are listed in Table 1. Among these, one isoform of alpha-1 antitrypsin (AAT, spot 2502) and one isoform of transthyretin (TTR, spot 4001) did not change from day 0 to day 3 but increased at day 8 (Fig. 3). One isoform of apolipoprotein A-1 (APOA1, spot 2204) and one isoform of hemoglobin beta chain (HBB, spot 8004) showed a trend to decrease at day 3, then significantly decreased at day 8; and one isoform of inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4, spot 2304) showed a significant

decrease as early as day 3 (Fig. 4). As a way to visualize the protein intensity change, the 3-D view of spot intensity for AAT and TTR isoforms are shown in Fig. 3 C and D and the ITIH4 isoform visualization in Fig. 4 D. It was clear that the AAT and TTR isoforms did not change from day 0 to 8 in the placebo phase, but increased at day 8 in the GH phase. On the other hand, the ITIH4 isoform decreased from day 0 to day 8 in rhGH treated individuals when compared to placebo controls.

[0098] Western blotting confirmation of AAT, TTR and ITIH4

[0099] AAT, TTR and ITIH4 were confirmed by Western blotting using samples from the same subjects at day 0 and day 8 of rhGH treatment phase. The total levels of these proteins were not different after 7 days of rhGH treatment, as shown by 1-D Western blotting result (Fig. 5). However, the specific isoforms identified to be up or down-regulated by rhGH treatment via 2-DE showed the same changes by 2-D Western blotting (Fig. 6). Of the train of spots recognized as AAT isoforms on the x-ray film, the one corresponding to spot 2502 appeared more intense at day 8 compared to day 0 (Fig. 6 A). Among the seven TTR isoforms recognized by the antibody, the one corresponding to spot 4001 was increased in intensity at day 8 compared to day 0 (Fig. 6 B). Finally, among the seven isoforms of ITIH4 recognized by the antibody, the one corresponding to spot 2304 appeared less intense at day 8 than day 0 (Fig. 6 C).

[00100] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which these embodiments pertain. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of various embodiments, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety for all purposes. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

[00101] The section headings used herein are for organizational purposes only and are not to be construed as limiting the described subject matter in any way. It will be appreciated that there is an implied “about” prior to metrics such as temperatures, concentrations, and times discussed in the present teachings, such that slight and insubstantial deviations are within the scope of the present teachings herein. In this application, the use of the singular includes the plural unless specifically stated otherwise. Also, the use of “comprise”, “comprises”, “comprising”, “contain”, “contains”, “containing”, “include”, “includes”, and

“including” are not intended to be limiting. It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention. The articles “a” and “an” are used herein to refer to one or to more than one (i.e., to at least one) of the grammatical object of the article. By way of example, “an element” means one element or more than one element.

OTHER EMBODIMENTS

[00102] It is to be understood that while embodiments have been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the method and compositions described herein. Such equivalents are intended to be encompassed by the following claims.

Table 1. MS, MS/MS scores of proteins.

Protein name	ssp # ^a	Accessi on # ^b	MS Mascot score ^c	Sequenc e coverag e ^d	MS/MS Mascot score ^e	Sequences matched by MS/MS (ions score) ^f	Theoretica l Mw (kDa) and pI ^g	Observed Mw (kDa) and pI ^h	Known PTMs ⁱ
Apolipoprotein A-1	2204	P02647	139	40%	78	K.VQPYLDDDFQK.K (Ions score 34) K.VSFLSALEEYTK.K (Ions score 54)	28.1 kDa pI 5.27	28 kDa pI 5.7	Palmitoylation Glycosylation Glycation phosphorylation
Inter-alpha- trypsin inhibitor heavy chain H4 (70 kDa and 35 kDa subunits are known; this spot matched 35	2304	Q14624	68	19%	240	R.QGPVNLLSDPEQG VEVTGQYER.E (Ions score 71) K.NPLVWVHASPEH VVVTR.N (Ions score 78) R.RLDYQEGPPGVEI	For 35 kDa subunit: 26.9 kDa pI 6.26	For 35 kDa subunit: 35 kDa pI 5.8	O-linked glycosylation for 35 kDa subunit

kDa subunit)	2502	P01009	103			41%	212	SCWSVEL. (Ions score 36)	44.3 kDa pI 5.37	45 kDa pI 5.8	Glycosylation
Alpha-1 antitrypsin								K.ITPNLAEFASFSLYR .Q (Ions score 86) K.LYHSEAFIVNHGD TEEAKK.Q (Ions score 36) K.FNKPFVFLMIEQN TK.S (Ions score 57)			
Transthyretin	4001	P02766	170			69%	190	R.GSPAINVAHVFR .K (Ions score 81) K.ALGISPFHEHAEV VFTANDSGPR.R (Ions score 76)	13.8 kDa pI 5.35	14 kDa pI 6	Glycosylation

Hemoglobin	8004	P68871	126	89%	56	R.LLVVYPWTQR.F (Ions score 55)	15.9 kDa pI 6.81	12 kDa pI 7.2	S-nitrosylated Glycation Acetylation
beta chain						K.VLGAFSDGLAHL DNLK.G (Ions score 17) R.LLGNVLCVLAH HFGK.E (Ions score 17)			

a. ssp numbers are assigned to each unique protein spot on the 2-D gel by PDQuest software.

b. Accession number in UniProtKB/Swiss-Prot database.

c. The MOWSE score is calculated by the Mascot search engine for each protein matched from the MS peak list. This score is based on the probability that peptide mass matches are non-random events. If the protein score is equal to or greater than the Mascot® Significance Level calculated for the search, the protein match is considered to be statistically non-random at the 95% confidence interval.

d. Sequence coverage is calculated using matched peptides over full length protein without removing signal peptide

e. A score calculated by weighting ion scores for all individual peptides matched to a given protein.

f. Start to end position of the matched protein sequences, as well as individual ion scores are shown. In all cases, at least two significant peptides are reported, confirming the confident identification of proteins.

g. Theoretical Mw and pI are calculated using 'ScanSite pI/Mw' tool from ExPaSy website (http://scansite.mit.edu/calc_mw_pi.html), after removal of signal peptides, which are determined by Swiss-Prot/TrEMBL protein database (<http://ca.expasy.org>). In case of a protein with multiple subunits, Mw and pI of each subunit are also given.

h. Observed Mw and pI are estimated from 2-D gel

i. PTMs found in human proteins that affect Mw and/or pI are listed. The known PTMs of proteins were reported in Swiss-Prot/TrEMBL protein database (<http://ca.expasy.org>).

CLAIMS

1. A method for evidencing GH doping in a human subject, comprising:
 - (a) detecting one or more markers in a biological sample of the subject, said markers comprise a protein isoform selected from the group consisting of SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4, and SEQ ID NO. 5; and
 - (b) determining altered levels of said marker(s), said altered levels indicating a likelihood of GH doping.
2. The method of claim 1, wherein said one or more markers are included in a diagnostic panel including at least two of SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4, and SEQ ID NO. 5.
3. The method of claims 1 or 2, wherein said altered levels are compared to a control sample, the control sample taken from the same or a different subject(s).
4. The method of any one of claims 1-3, wherein the detecting step is carried out by immunoassay, chromatography, spectrometry, electrophoresis, sedimentation, isoelectric focusing, or any combination thereof.
5. The method according to any one of claims 1-4, wherein the detecting step further comprises the steps of subjecting the biological sample to two-dimensional gel electrophoresis to yield a stained gel and an increased or decreased concentration of the protein isoform is detected by an increased or decreased intensity of a protein-containing spot on the stained gel, compared with a corresponding control gel.
6. The method of any of claims 1, 2, or 5, wherein the protein isoform of apolipoproteinA-1 has a MW of about 28 and a pI of about 5.7; the protein isoform of inter-alpha-trypsin inhibitor heavy chain H4 has a MW of about 35 and a pI of about 5.8; the protein isoform of alpha-1 antitrypsin has a MW of about 45 and a pI of about 5.8; the protein isoform of transthyretin has a MW of about 14 and a pI of about 6.0; and the protein isoform of hemoglobin beta chain has a MW of about 12 and a pI of about 7.2.
7. The method of claims 5, wherein the intensity of spot 2502, 4001, 2204, 2304, and 8004 in FIG. 2 is detected.
8. The method of claims 7, wherein the increased intensity of spot 2502 or 4001 or the decreased intensity of spot 2204, 2304, and 8004 in FIG. 2 is detected.
9. The method according to any one of claims 1-8, wherein the sample is a blood sample.

10. The method according to any one of claims 1- 9, wherein the method further comprises the step of identifying a subject at risk for GH doping.
11. A method of screening for GH doping in a diagnostic sample of a valid body tissue taken from a human subject, which comprises detecting an increased concentration of a protein isoform in the diagnostic sample, compared with a control, normal human sample, the protein isoform being selected from the group consisting of:
 - SEQ ID NO. 3; and
 - SEQ ID NO. 4;or a decreased concentration of a protein isoform in the diagnostic sample, compared with a control, normal human sample, the protein isoform being selected from the group consisting of:
 - SEQ ID NO. 1;
 - SEQ ID NO. 2; and
 - SEQ ID NO. 5;wherein the protein isoform of apolipoproteinA-1 has a MW of about 28 and a pI of about 5.7; the protein isoform of inter-alpha-trypsin inhibitor heavy chain H4 has a MW of about 35 and a pI of about 5.8; the protein isoform of alpha-1 antitrypsin has a MW of about 45 and a pI of about 5.8; the protein isoform of transthyretin has a MW of about 14 and a pI of about 6.0; and the protein isoform of hemoglobin beta chain has a MW of about 12 and a pI of about 7.2.
12. The method of claim 11, wherein the valid body tissue is blood or a blood product such as serum or plasma.
13. A method according to claim 11 or 12, wherein the control sample is taken from healthy tissue of a different human subject than the one providing the diagnostic sample.
14. A method according to claim 13, wherein the body tissue is a body fluid and the body fluid is subjected to an immunoassay.
15. The method according to any of claims 11-14, wherein the detecting step further comprises the steps of subjecting the diagnostic sample to two-dimensional gel electrophoresis to yield a stained gel and an increased or decreased concentration of the protein isoform is detected by an increased or decreased intensity of a protein-containing spot on the stained gel, compared with a corresponding control gel.
16. The method of claim 15, wherein the increased intensity of spot 2502 or 4001 or the decreased intensity of spot 2204, 2304, and 8004 in FIG. 2 is detected.

17. A method of monitoring the efficacy of GH administration in a subject with a GH deficiency, comprising:
 - administering GH to the subject with a GH deficiency;
 - acquiring a diagnostic sample from the subject;
 - detecting an increased concentration of a protein in the diagnostic sample, compared with a control, normal human sample, the protein being selected from the group consisting of:
 - SEQ ID NO. 3; and
 - SEQ ID NO. 4;
 - or a decreased concentration of a protein in the diagnostic sample, compared with a control, normal human sample, the protein being selected from the group consisting of:
 - SEQ ID NO. 1;
 - SEQ ID NO. 2; and
 - SEQ ID NO. 5.
18. The method according to claim 17, wherein the diagnostic sample is subjected to two-dimensional gel electrophoresis to yield a stained gel and the increased or decreased concentration of the protein is detected by an increased or decreased intensity of a protein-containing spot on the stained gel, compared with a corresponding control gel.
19. The method according to claims 17 or 18, wherein the protein isoform of apolipoproteinA-1 has a MW of about 28 and a pI of about 5.7; the protein isoform of inter-alpha-trypsin inhibitor heavy chain H4 has a MW of about 35 and a pI of about 5.8; the protein isoform of alpha-1 antitrypsin has a MW of about 45 and a pI of about 5.8; the protein isoform of transthyretin has a MW of about 14 and a pI of about 6.0; and the protein isoform of hemoglobin beta chain has a MW of about 12 and a pI of about 7.2.
20. A kit for detecting GH doping in a subject, comprising ligands specific for two or more of SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4, and SEQ ID NO. 5.
21. The kit of claim 20, wherein the ligands are antibodies.
22. The kit of claim 21, wherein the antibodies are isoform specific monoclonal antibodies.

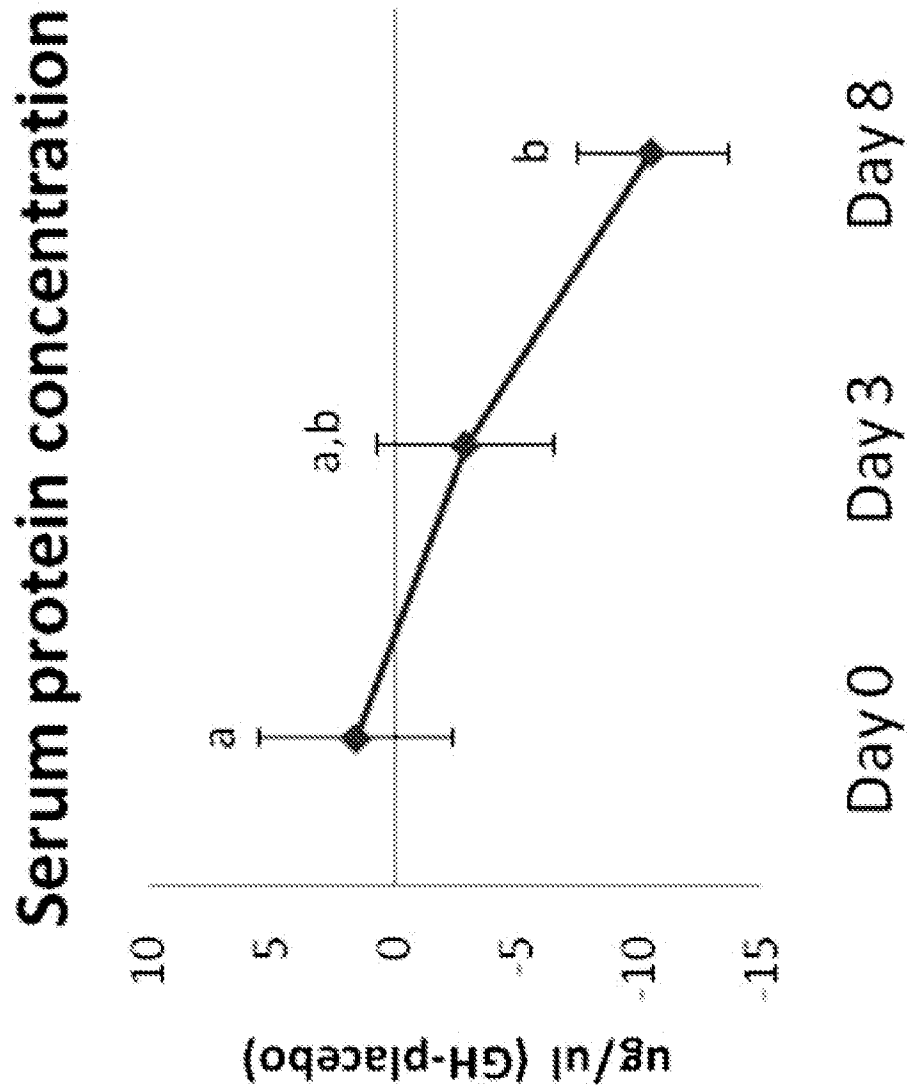


FIG – 1

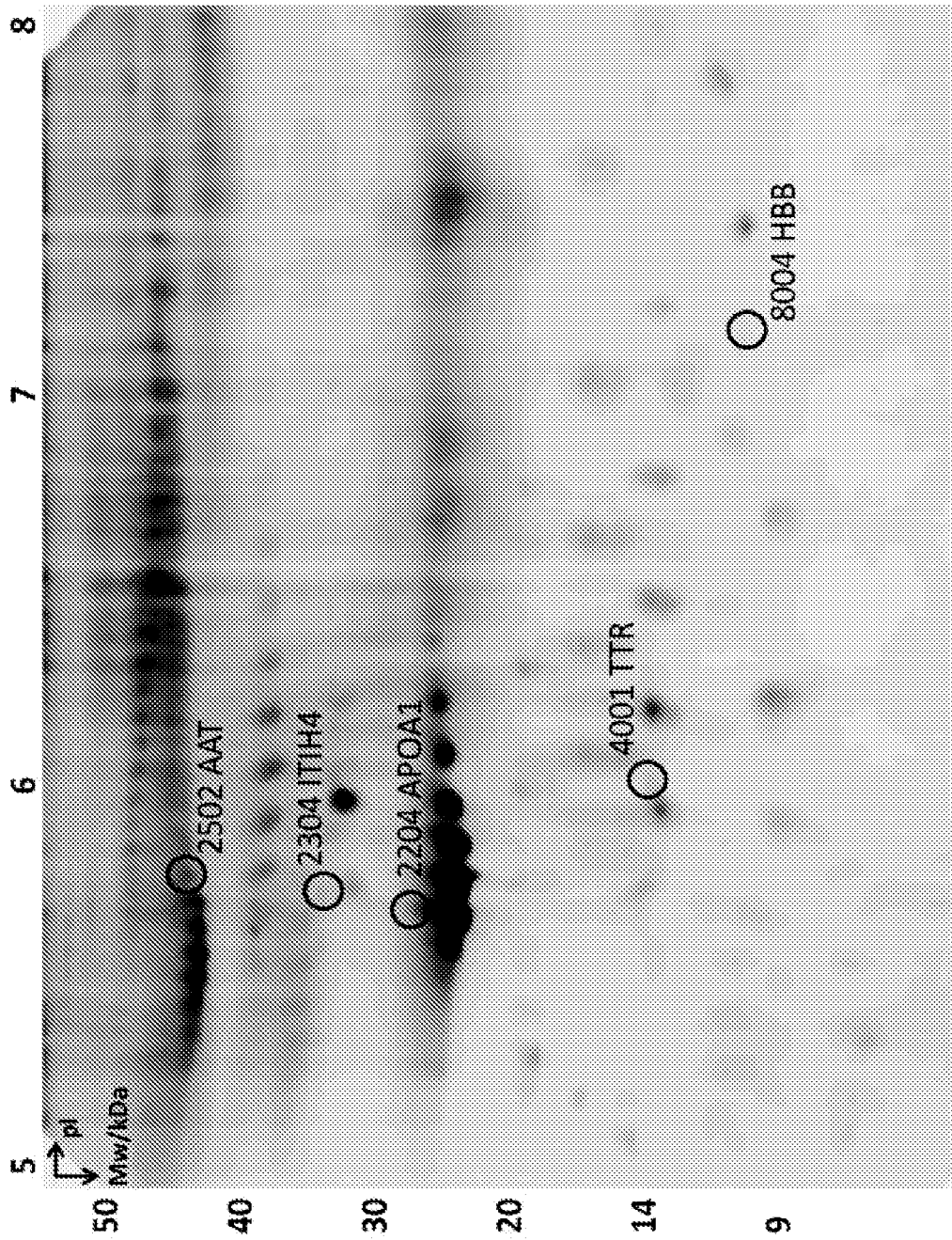


FIG - 2

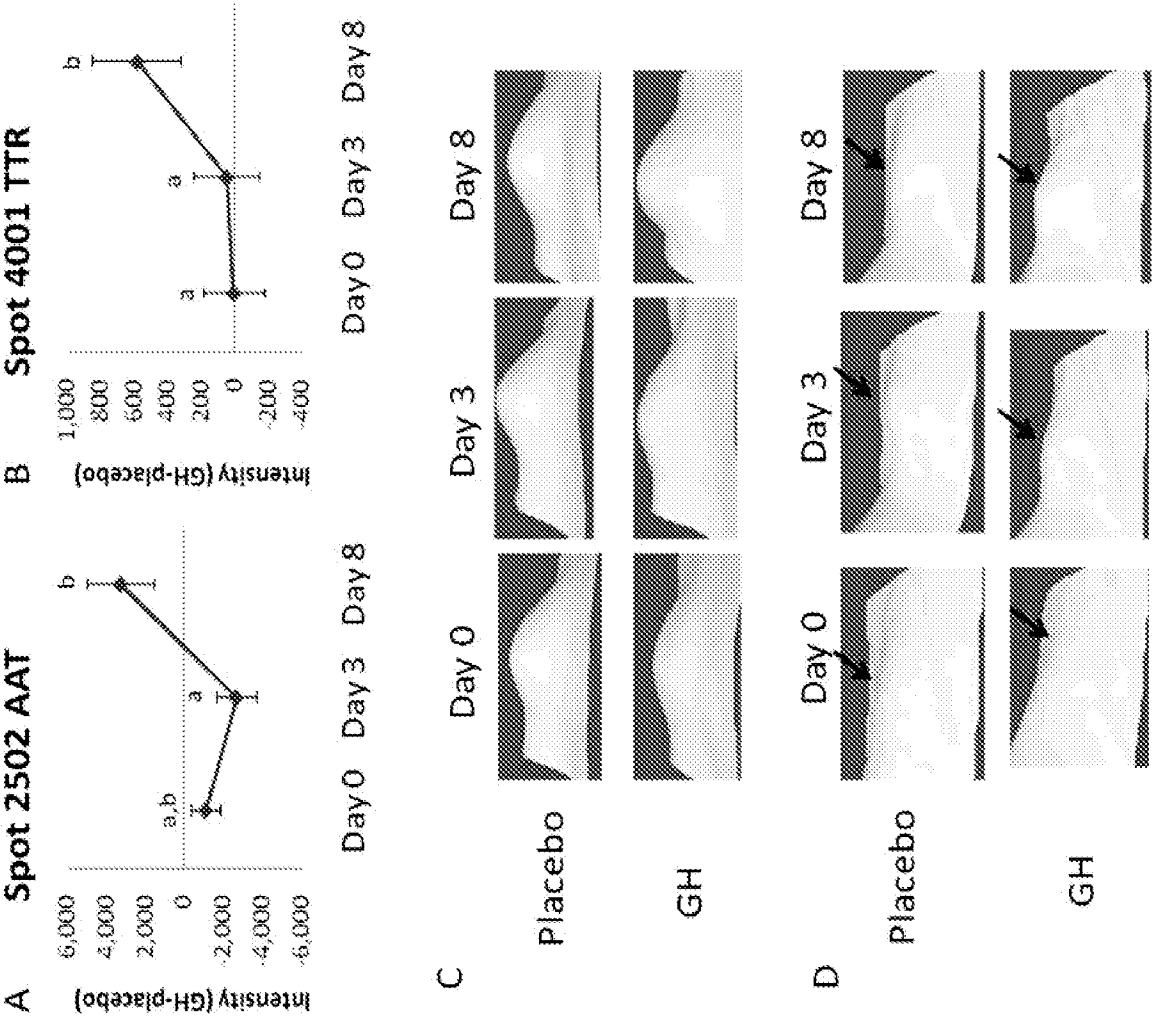


FIG - 3

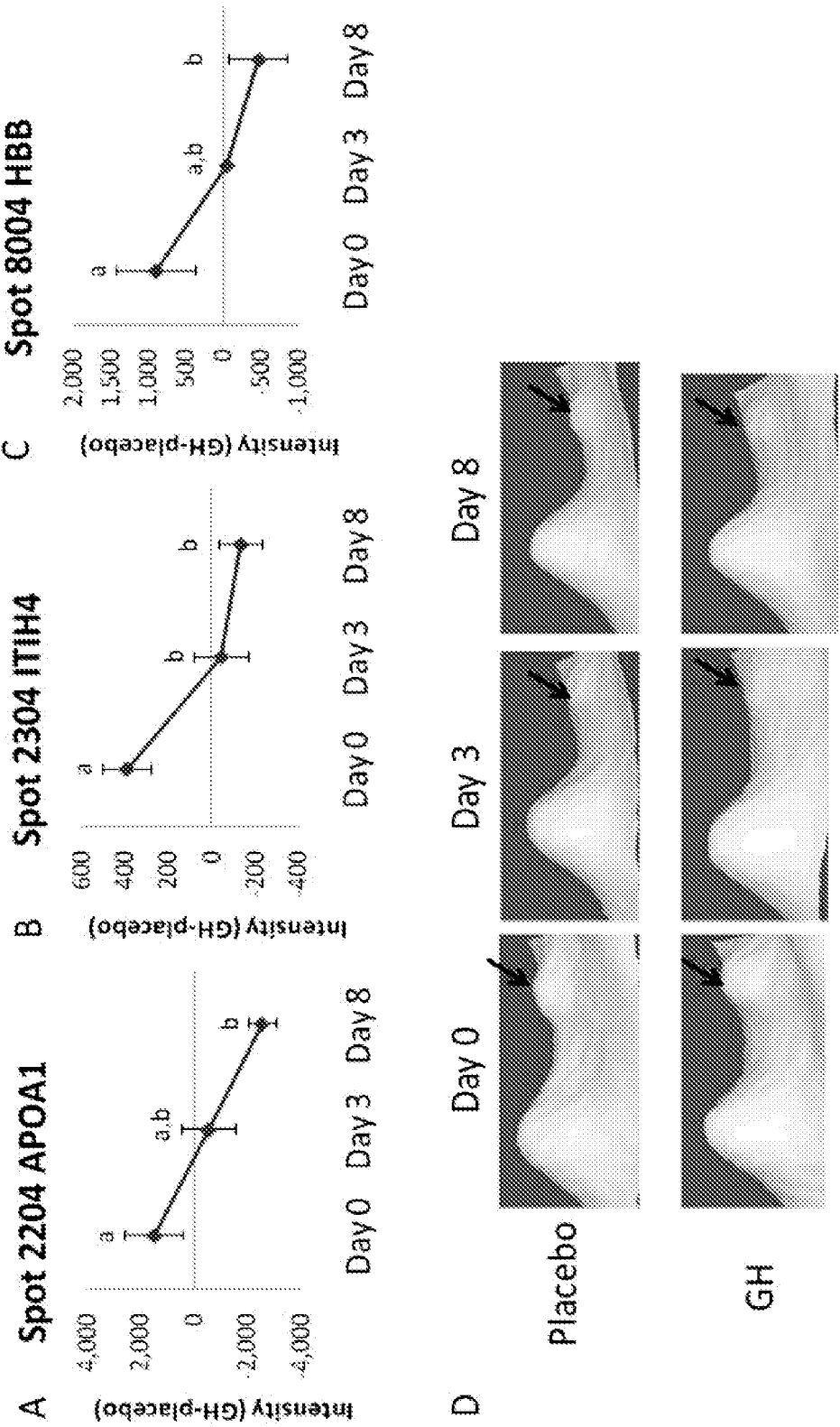


FIG - 4

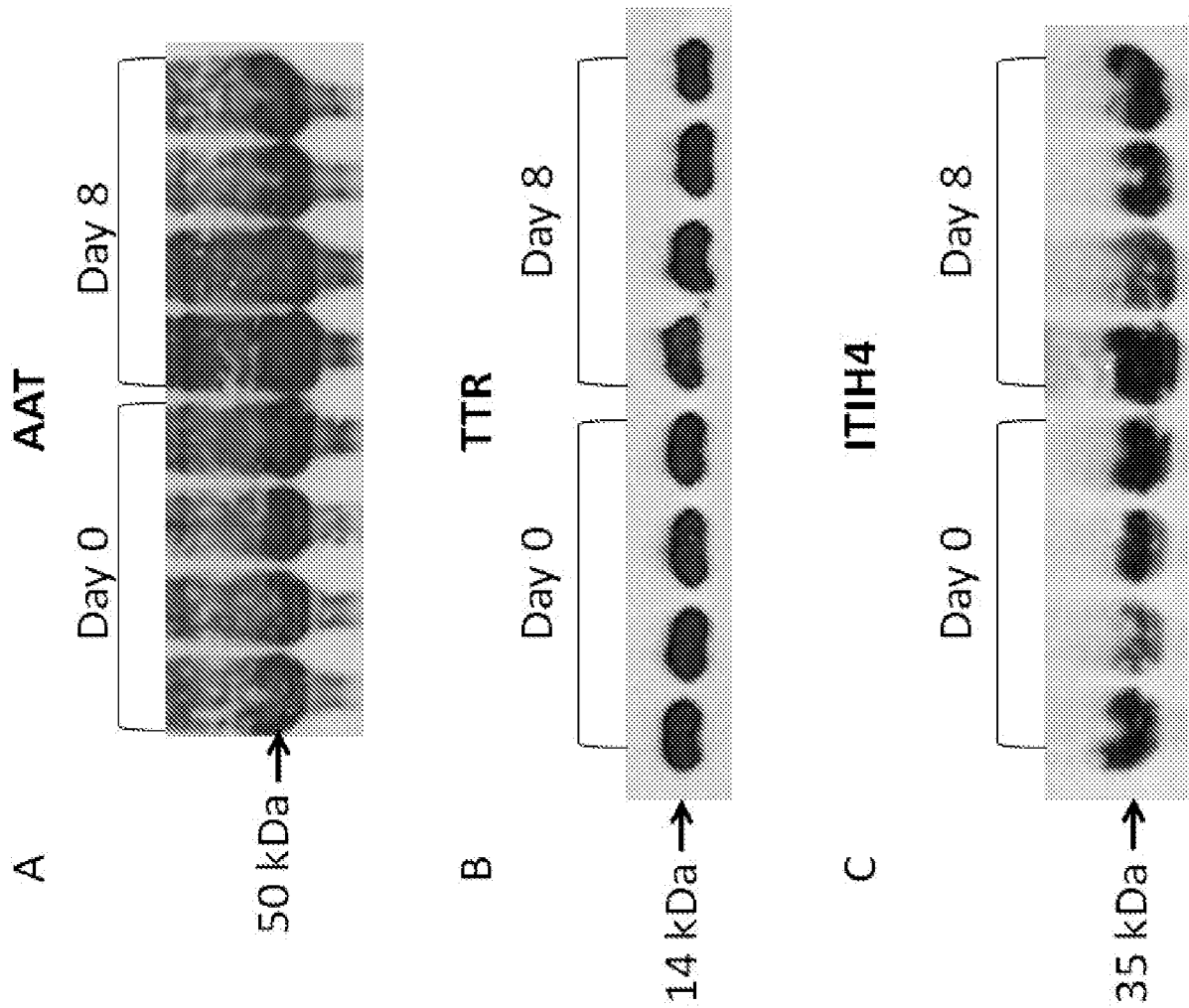


FIG - 5

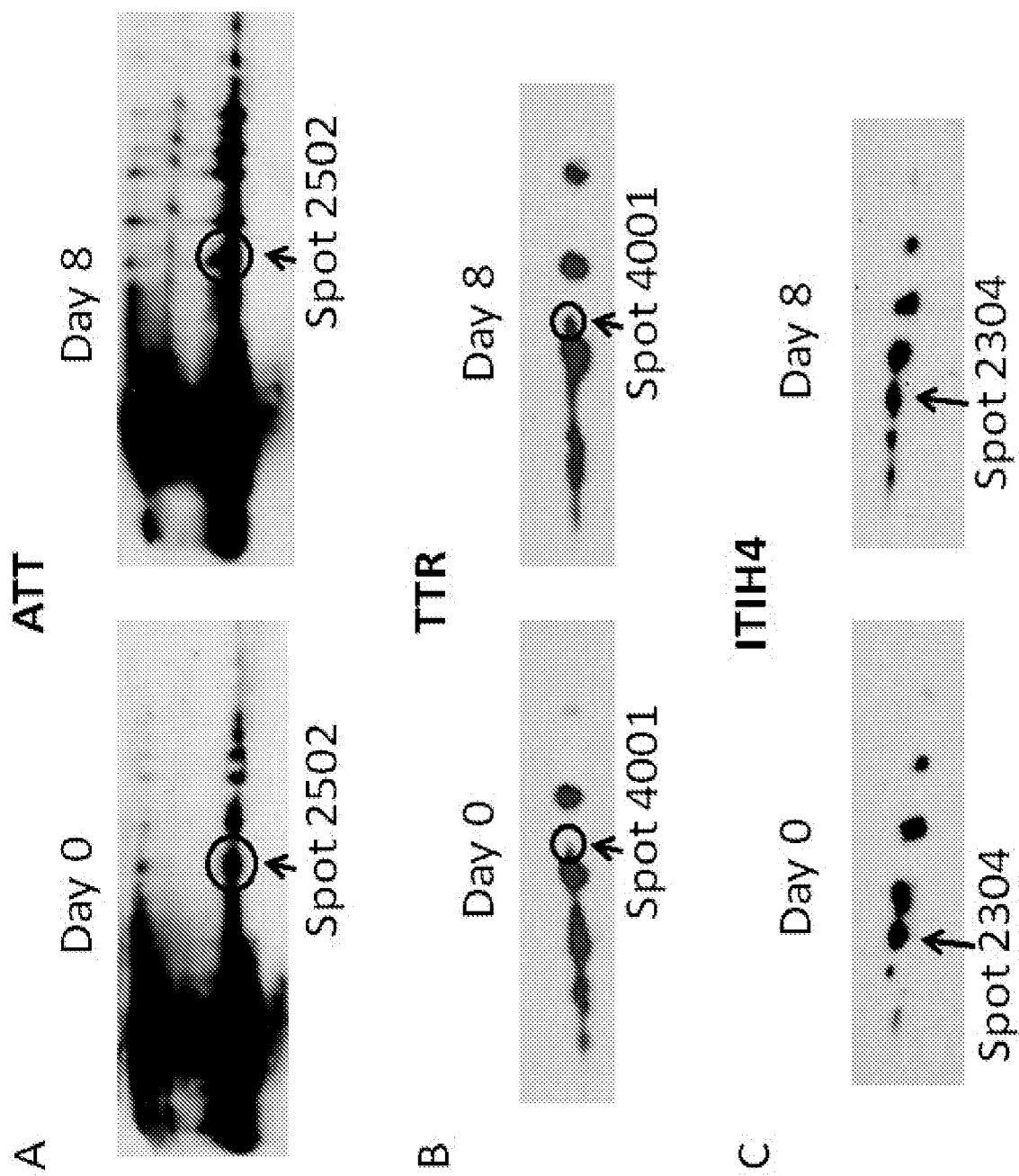


FIG - 6