



# Growth hormone proteoformics atlas created to promote predictive, preventive, and personalized approach in overall management of pituitary neuroendocrine tumors

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## Abstract

Human growth hormone (GH) is the indispensable hormone for the maintenance of normal physiological functions of the human body, including the growth, development, metabolism, and even immunoregulation. The GH is synthesized, secreted, and stored by somatotroph cells in adenohypophysis. Abnormal GH is associated with various GH-related diseases, such as acromegaly, dwarfism, diabetes, and cancer. Currently, some studies found there are dozens or even hundreds of GH proteoforms in tissue and serum as well as a series of GH-binding protein (GHBP) proteoforms and GH receptor (GHR) proteoforms were also identified. The structure-function relationship of protein hormone proteoforms is significantly important to reveal their overall physiological and pathophysiological mechanisms. We propose the use of proteoformics to study the relationship between every GH proteoform and different physiological/pathophysiological states to clarify the pathogenic mechanism of GH-related disease such as pituitary neuroendocrine tumor and conduct precise molecular classification to promote predictive preventive personalized medicine (PPPM / 3P medicine). This article reviews GH proteoformics in GH-related disease such as pituitary neuroendocrine tumor, which has the potential role to provide novel insight into pathogenic mechanism, discover novel therapeutic targets, identify effective GH proteoform biomarker for patient stratification, predictive diagnosis, and prognostic assessment, improve therapy method, and further accelerate the development of 3P medicine.

**Keywords** Growth hormone proteoform · Growth hormone proteoformic pattern · Growth hormone receptor · Growth hormone receptor proteoform · Growth hormone-binding protein · Growth hormone-binding protein proteoform · Proteoform · Proteoformics · Growth hormone-related disease · Pituitary neuroendocrine tumor · Patient stratification · Predictive diagnosis · Prognostic assessment · Target therapy · Personalized medical service · Primary and secondary care · Healthcare efficacy · Individual outcomes · Predictive preventive personalized medicine (PPPM / 3P medicine)

## Introduction

### Brief description of growth hormone

The human growth hormone (GH), namely somatotropin, was first isolated in the 1930s, it is about eight decades since today [1]. A series of functions of GH were revealed, including promoting growth and development, regulating metabolism, and immunology [2, 3]. While if the GH in the body is disturbed, the body will suffer from various GH-related diseases, such as acromegaly, dwarfism, diabetes, and cancer [4]. Furthermore, an increasing number of GH proteoforms in pituitary tissue and serum were identified in plenty of studies [5–15], and the types and constitution of GH would dynamically vary from different physiological/pathological states of organism. However, the exact relationships between

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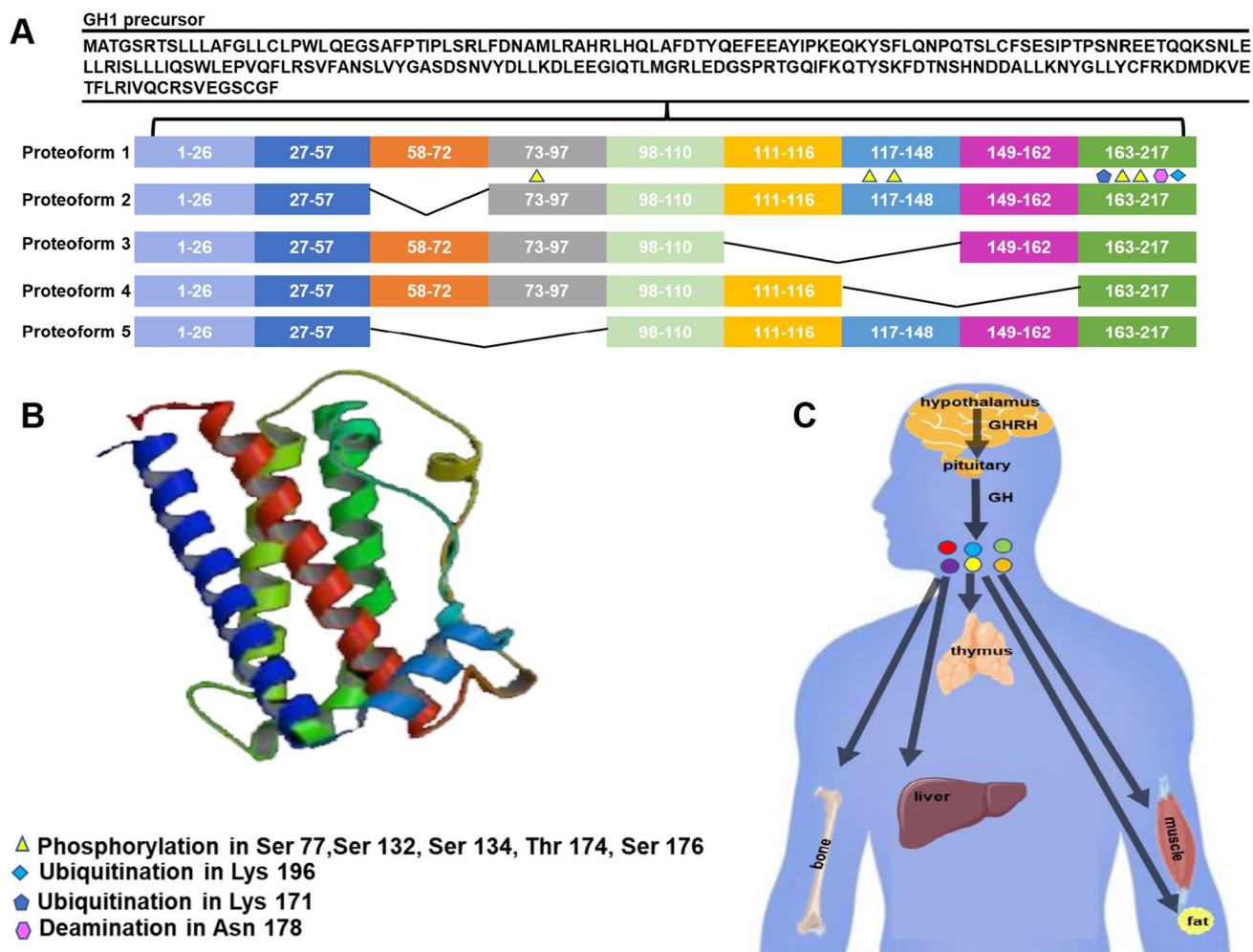
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GH proteoforms and different physiological/pathological states remain unclear. This review proposes that the use of “proteoformics” to systematically study the function of every GH proteoform and the relationship between every GH proteoform and different physiological/pathological states to promote the development of predictive preventive personalized medicine (PPPM; 3P medicine). This review conducted a systematic and comprehensive summary of the proteoforms of human GH, GH-binding protein (GHBp), and GH receptor (GHR) to find specific biomarkers for GH-related diseases towards the development of 3P medicine.

### Structure and functions of growth hormone

Human GH is a protein hormone with a single peptide chain, belonging to the somatotropin/prolactin (PRL) family, which is produced by GH-secreting cells, with eosinophilic granules, in the anterior pituitary [16, 17]. The human

GH genes locate on chromosome 17q23.3 in humans [18, 19]. They include five highly homologous and structurally similar genes GH-N (GH normal gene; GH1 gene), GH-V (GH variant gene; GH2 gene), CS-A (chorionic somatomammotropin A), CS-B (chorionic somatomammotropin B), and CS-L (chorionic somatomammotropin-like gene) [16, 19, 20]. GH-N is expressed in the pituitary gland and the other four genes are expressed in placenta paralogs [16, 19, 20]. The GH-N cDNA encodes GH precursor with 217 amino acids (sequence 1–217), which includes a signal peptide at amino acid sequence positions 1–26 (Fig. 1A and B). There are two main mature forms of GH—22 kD and 20 kD [16, 21, 22]. The 22 kD GH owns 191 amino acids, and is the main existence form in the pituitary, which is necessary for human growth and development. In addition, the 20 kD GH owns 176 amino acids, with a lack in amino acid sequence positions 32 to 46, accounting for about 5~10% of total serum GH, which is produced by alternative splicing of mRNA [16,



**Fig. 1** The structure and function of human growth hormone (hGH). **A** hGH amino acid sequence and its splicing variants. Data originates from Swiss-Prot No: P01241. The curve line represents the removed

sequence after splicing. **B** The 3D structure of hGH. Data originates from PROTEIN DATA BANK (PDB). **C** Hypothalamic-pituitary-GH axis and the main targeted organs of hGH

21, 22]. Besides the two monomeric forms in pituitary tissue and serum, there are some other kinds of forms, including their fragments, homo- and heteropolymers, and complexes with binding proteins as well as receptors [3]. Currently, it has been found that GH owns more than one hundred of forms in circulation. In fact, the expression products of GH gene make up a protein family, rather than a single protein [3].

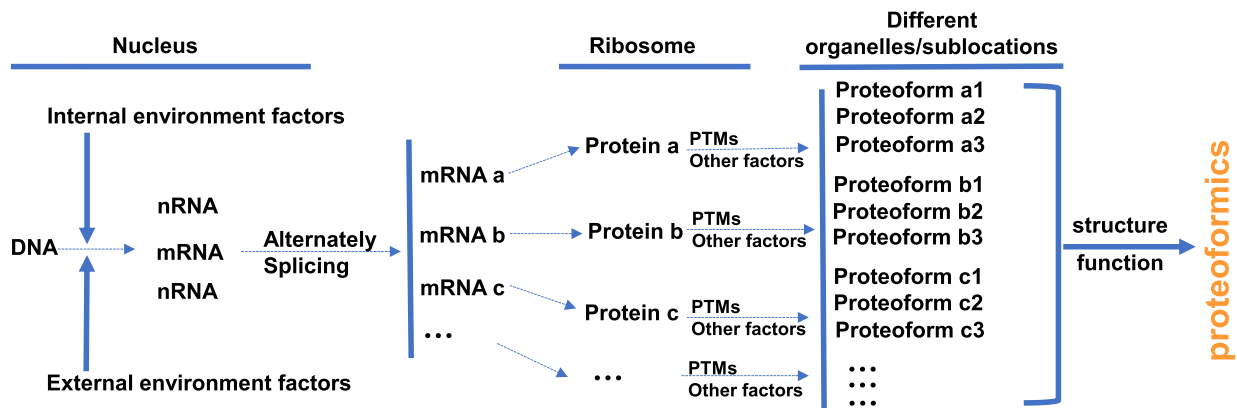
GHR is a member of the GH/PRL/cytokine receptor superfamily, and its signaling is mainly transduced through the nonreceptor tyrosine kinase pathway [23]. The initiation of the pathway is started with the binding of GH to GHRs, then the dimerization of GHRs to activate JAK2 (Janus kinase 2), one of nonreceptor tyrosine kinases. Once activated, JAK2 tyrosyl-phosphorylates both itself and the cytoplasmic domain of GHR. The GHR-JAK2 complex can recruit and activate the signaling molecules including Stat (signal transducers and activators of transcription factors) and IRSs (insulin receptor substrates) 1 and 2, and further modulate a series of cellular functions. GH has a wide variation of physiological functions, and the implementation of its function cannot be achieved without GHR. GH affects almost all tissues and cell types in human body, including bone, muscle, fat [24], hair follicle [25], reproductive [26] and immune systems [27, 28], and even the hematopoietic system [29] and central nervous system [16, 30, 31] (Fig. 1C). The two basic functions of hGH are to stimulate the growth of cells, tissues, and organs, and regulate metabolism of nutrients [16]. On the one hand, it promotes the growth and development of the body through increasing the size and number of somatic cells as well as protein synthesis. On the other hand, for the regulation of metabolism, the overall effect of GH on protein metabolism is to promote anabolism, mainly through promoting amino acid transport into cells to increase protein synthesis and inhibit protein

consumption [32]. Noteworthy, the effect of GH on protein synthesis is in harmony with its effect on growth. Furthermore, GH is a lipolysis hormone because it promotes fat degradation [24]. The effect of GH on glucose metabolism is secondary to its mobilization of fat. The increase of free fatty acids in the blood can inhibit glucose uptake by skeletal muscle and adipose tissues to reduce glucose consumption and increase blood glucose level, which is manifested as “anti-insulin” effect. GH also increases blood sugar by reducing the sensitivity of peripheral tissue to insulin.

Moreover, GH can promote the secretion of thymosin by thymic stromal cells, stimulate the production of antibodies by B lymphocytes, improve the activities of natural killer cells (NK cells) and macrophages, and thus participate in the function regulation of the immune system in the human body [28]. GH also has the effect of anti-aging [33, 34] and regulates emotional and behavioral activities [35]. GH is also one of the important stress hormones secreted by adenohypophysis.

### The concept of proteoform and proteoformics

With the development of molecular biology, the human genome project identified ~20,300 genes, rather than ~100,000 genes as initially predicted [36, 37]. This showed that the cause of the diversity of biological functions is not huge number of genes but protein variety [38]. The diversity can originate from allelic variations on the DNA level, alternative splicing of RNA transcripts, epigenetic modifications on DNA and RNA levels, and a series of post-translational modifications (PTMs) on the protein level (Fig. 2) [37, 39]. These events work together to produce different proteoforms originating from a single gene, which can involve in a wide range of biological processes, such as



**Fig. 2** The formation of proteoform and concept of proteoformics. PTM: post-translational modification. Modified from Zhan et al. [37] with copyright permission from MDPI publisher open access article,

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regulations of gene expression, energy metabolism, and cell signal transduction.

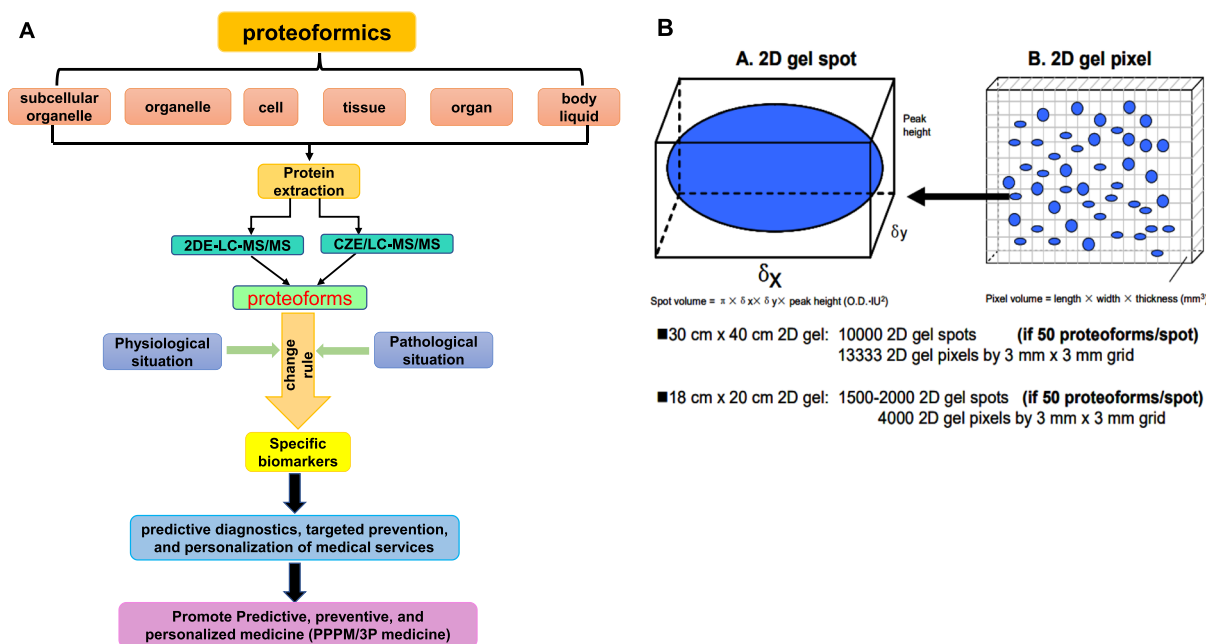
In 2012, Smith and Kelleher first proposed the term “proteoform” to designate all of the different molecular forms in which the protein products of a single gene, including changes due to genetic variations, alternatively spliced RNA transcripts, and post-translational modifications [40].

With the development of two-dimensional gel electrophoresis (2DGE) and mass spectrometry (MS), many proteoforms were identified, thus the concept of “proteoform” is enriched. The complete definition for a proteoform is that a proteoform is composed of its amino acid sequence, PTMs, spatial conformation, cofactors, binding partners, localization, and a function [37, 39, 41] (Fig. 2). A protein is an umbrella term for all proteoforms encoded by the same gene [41]. Proteoform is an objective substance and the term “proteoform” has been used for more than 10 years [40]. The term “proteomics” is commonly looked as the theories and methods to study “proteome” whose components are traditionally thought as the canonical “proteins” although currently ones look the “proteoforms” as the basic units of a proteome, and still use the term “proteomics” as the theories and methods to study proteoforms. In fact, to emphasize the important scientific merit of the term “proteoform,” it is necessary to propose a new term “proteoformics” that is the theories and methods to study proteoforms in a proteome (Fig. 2) [42]. The aim of “proteoformics” is to study the proteoform compositions and their change rules in a cell,

tissue, organ, and organism as well as the effects of proteoforms on physiological/pathological processes in human body (Fig. 3A).

## GH proteoforms

For human GH, currently, more than one hundred of its proteoforms have been identified in different tissues and sera [5, 9–15]. The reasons for the formation of GH proteoforms are currently described as gene variation, alternative splicing, and PTMs, and they are enriched with the development of proteoformics. The gene to code GH include five forms GH-N, GH-V, CS-A, CS-B, and CS-L, and they have expression tendency [3, 16, 18–20, 22]. GH-N is expressed in the pituitary gland, and the other four genes are expressed in the placenta. Due to alternative splicing, there are five forms of GH [3]. The GH isoform 1 is the most canonical form, with an amino acid sequence of 1–217. Differences from canonical form of GH, isoform 2 owns a missing of amino acid sequence 58–72, isoform 3 owns a missing of amino acid sequence 111–148, isoform 4 owns a missing of amino acid sequence 117–162, and isoform 5 owns a missing of amino acid sequence 58–97 (Fig. 1A). Furthermore, GH contains a signal peptide with the amino acid sequence 1–26. Every isoform of GH contains two forms - the removal and non-removal of signal peptide. In addition, a number of PTMs are also identified [5, 9]; for example, phosphorylation is characterized at residues Ser77, Ser132, Ser134, Thr174,



**Fig. 3** The methodology of proteoformics. **A** The general procedure and application of proteoformics. **B** The 2DE analysis of proteoforms. Modified from Zhan et al. [37] with copyright permission from MDPI

publisher open access article, copyright year 2019. 2-DE two-dimensional gel electrophoresis, LC liquid chromatography, MS/MS tandem mass spectrometry, PTM post-translational modification

and Ser176, ubiquitination at residue Lys196, acetylation at residue Lys171, and deamination at residue Asn178; and more kinds of PTMs and PTM-sites will be found. All of these PTMs together make up the diversity of GH but the specific physiological function of every GH proteoform has not been elucidated.

## GH proteoforms and diseases

The maintenance of homeostasis of GH levels is significantly essential to perform physiological functions of the human body. Whereas, once GH levels are disturbed, a series of GH-related disorders appears. During childhood, hyposecretion of GH will cause dwarfism, whose characteristics are slow growth, skeletal hypoplasia, and accompanying with sexual organ agenesis or lack of secondary sex characteristics [43–46]. While hypersecretion of GH can cause hyperplasia of bone and cartilage, metabolic disorders, and complications [47]. For the adult, the disorder of GH, which is mainly caused by PitNETs, can lead to acromegaly with a typical acromegaly facies, hypertrophy of hands and feet, skin hypertrophy, visceral enlargement, and various metabolic diseases and so on.

## Techniques to study GH proteoforms

It is estimated that the number of human proteoforms exceeds one million [37]. It is very urgent to find an effective method to achieve a high-throughput test of all proteoforms. Currently, there are two main strategies to study proteoforms: top-down mass spectrometry-based strategy (Top-down MS), and two-dimensional gel electrophoresis in combination with liquid chromatography-mass spectrometry strategy (2DE-LC/MS) (Fig. 3). Each strategy has its own advantages and disadvantages in the analysis of proteoforms [37].

- (i) Top-down MS: the general procedure of top-down MS is that, first, protein separation technology, such as capillary zone electrophoresis (CZE) and LC, is used to separate proteoforms; second, the separated proteoforms are analyzed with MS/MS; and third, MS/MS data are used to identify the amino acid sequence and PTMs against protein database (Fig. 3A) [48, 49]. The drawback of this method is that its analytical throughput is not high. Currently, the maximum analytical throughput is ~ 30,000 proteoforms derived from 1690 human genes [50]. Although this throughput is nearly 10 times more than previous studies, it is still much far from the number of all proteoforms in the human body.

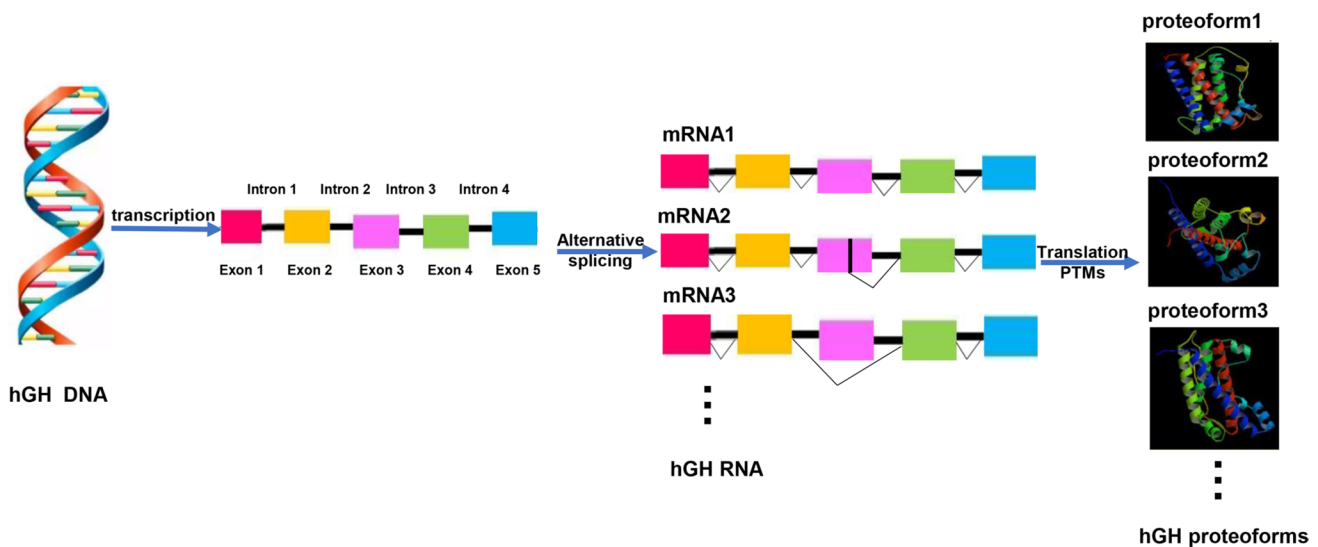
- (ii) 2DE-LC/MS: the general procedure of 2DE-LC/MS strategy is that, first, 2DE is used to separate proteoforms per their isoelectric point ( $pI$ ) and relative mass ( $M_r$ ); second, the separated proteoforms are digested with trypsin, followed by MS/MS identification; third, MS/MS data are used to identify the amino acid sequence and PTMs against protein database [37, 51]. Because each 2D gel spot includes over 50 to several hundreds of proteoforms, and 2DE can separate low-abundance proteoforms, 2DE-LC/MS has great potential for high-throughput analysis of proteoforms (Fig. 3B) [51].

## GH proteoforms in pituitary diseases

### GH proteoforms in pituitary tissues

GH presents a variety of proteoforms, including monomeric [52–54], an oligomeric series of dimeric to pentameric forms [55], and segments/fragments, with different modifications, such as glycosylation, phosphorylation, acetylation, ubiquitination, and deamination [9, 10, 52, 53]. All of these factors cause the human GH heterogeneity in structure and function, which might be significant to clarify various GH functions in various physiological/pathological conditions (Fig. 4).

Different GH proteoforms are identified in the pituitary gland, and their functions are also clarified partially [5–10, 56, 57]. In 1990s, the 17 kD GH fragment (amino acid sequence 44–191) and the 5 kD GH fragment (amino acid sequence 1–43) were identified in pituitary and plasma in several studies, and the 17kD GH was found to involve in glucose intolerance [6–8, 56]. Later with the development of 2DE and mass spectrometry, more GH proteoforms were identified. In 2009, 9 kD and 12 kD GH fragments, 20 kD GH, 22 kD GH, and 23 kD GH with glycosylation were identified in the pituitary, and different GH proteoforms show different abundance [10]. Furthermore, PitNET has a high prevalence and causes huge pain for patients, but its pathogenesis mechanism has not been clarified. In 2005 and 2021, two systematic studies on GH proteoforms were conducted [5, 9]. At first, a total of 24 human GH proteoforms [5] were identified in normal pituitary with 2DGE coupled with mass spectrometry, and those GH proteoforms showed significantly different abundance. Moreover, phosphorylation at residues Ser-77, Ser-132, and Ser-176, and deamination at residue Asn-178 were identified in GH. These findings not only enrich the GH proteoform atlas but also provide a new perspective to further explore the action mechanisms of GH in physiological/pathological conditions of the human body (Fig. 5A and B) [5]. Later in 2021 [9], a total of 46 GH proteoforms in GH-secreting PitNETs and 35 GH proteoforms



**Fig. 4** The formation of hGH proteoforms. The broken line represents the splicing methods of hGH exons. Data originates from Swiss-Prot No: P01241

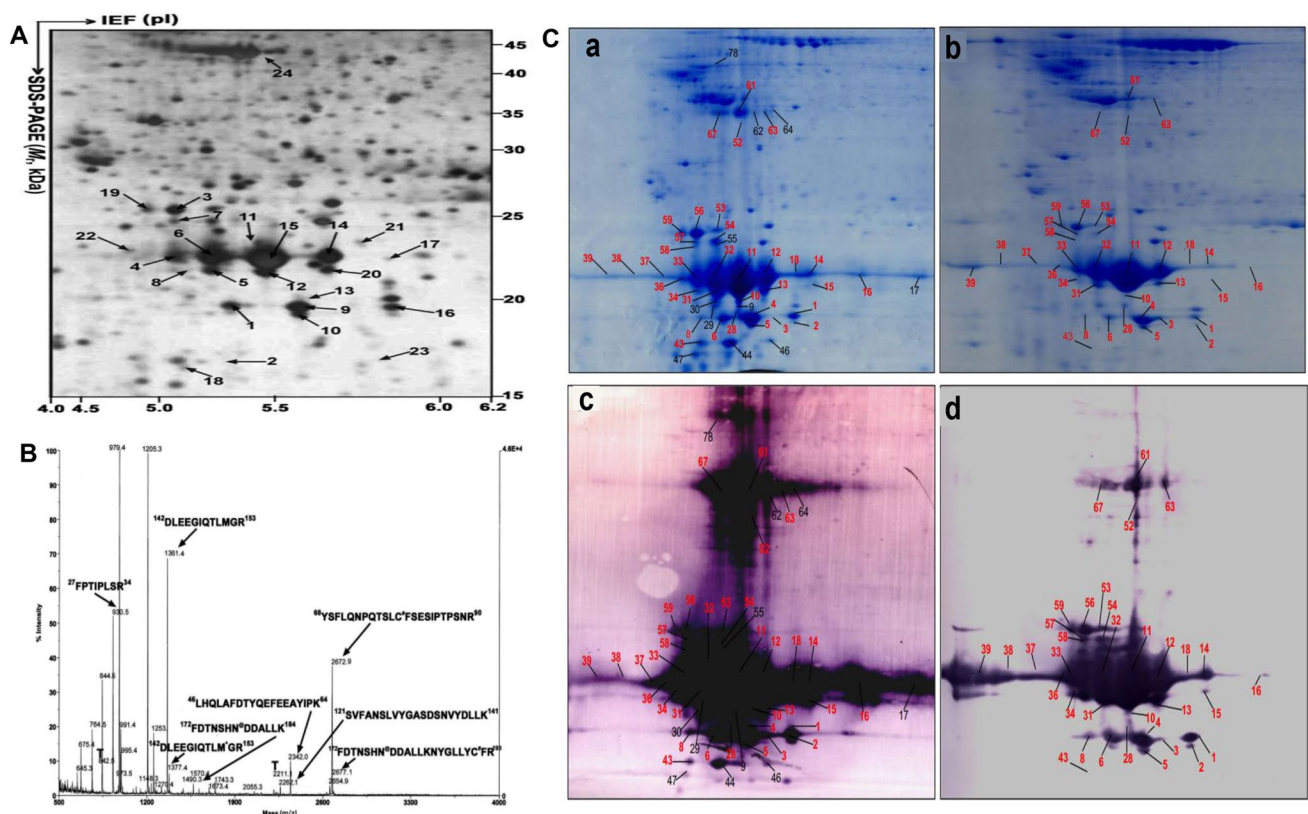
in control pituitaries were identified with 2DE in combination with LC-ESI-MS/MS, MALDI-TOF-MS, and MALDI-TOF-TOF-MS/MS (Fig. 5C). Further analysis of every GH proteoform found that 11 GH proteoforms only existed in GH-secreting PitNETs but not in control pituitaries, and the remaining 35 GH proteoforms existed in both GH-secreting PitNETs and control pituitaries and had different abundance differences in tumors and controls. Different PTMs (acetylation, ubiquitination, phosphorylation, and deamination) were also characterized in GH proteoforms between GH-secreting PitNETs and controls. For example, phosphorylation has been characterized in GH proteoforms with MS/MS analysis (Fig. 6), and MS/MS analysis can accurately discriminate phosphorylated peptide and non-phosphorylated peptide that were derived from GH proteoforms (Fig. 7). This study provides the first human GH proteoform atlas and their partial PTMs in GH-secreting PitNETs compared to normal control pituitary tissues, which provides novel perspective to predict, prevent, treat, and conduct prognostic assessment for effective medical service for GH-secreting PitNETs, especially insight into development of feasible GH proteoformic pattern biomarkers for prediction, prevention, diagnosis, patient stratification, and prognostic assessment of GH-secreting PitNETs and GH-related diseases in the context of PPPM practice [9].

### GH proteoforms in serum

The type and concentration of different GH proteoforms in sera are not invariable but change constantly with different physiological/pathological states [3]. Therefore, exploring GH proteoformic pattern in different diseases is of great

significance, and the specific GH proteoforms related to specific diseases have great potential for the development of pattern biomarkers. Currently, the number of GH proteoforms in plasma is more than one hundred [3], and the forms of GH proteoforms include monomers, fragments, homopolymers, heteropolymers, and their combinations with GHBPs and other binding proteins [11–13]. For GH monomers, the main forms include 22 kD GH proteoforms, 20 kD GH proteoforms, and acidic GH proteoforms, probably including GH proteoforms with deamidation and N-acylation, and their ratio is about 75%, 16%, and 9%, respectively [14, 15, 52–54]. In addition to monomeric GH proteoforms, oligomeric forms, with an oligomeric series of dimeric to pentameric GH, are also identified in sera [55]. Furthermore, it is significantly worth noting that serum GH is not consistent, but varies dynamically with time and physiological/pathophysiological states. Therefore, it is very reasonable to infer different patterns of GH proteoforms that can play various functions to fit a variety of situations that an organism needs to face.

Currently, the 22 kD GH proteoforms and 20 kD GH proteoforms are the most studied, and both of them bind to and activate GHR. However, they exhibit different signal transmission characteristics; the 22 kD GH proteoforms exhibit stronger receptor-binding ability and signaling transmission ability than the 20 kD GH proteoforms [57]. This finding showed that different serum proteoforms might exhibit various effects for the growth and development of the human body and the progression of GH-related diseases. Therefore, it is urgent to systematically study GH proteoforms in sera and draw GH proteoform atlas, further clarify the influence of every GH proteoform and every cluster



**Fig. 5** The hGH proteoforms identified in normal pituitary and GH-secreting PitNETs. **A** 2DE image of GH proteoforms in human pituitary tissues. A total of 24 hGH proteoforms were identified with 2DE and MS, and labeled in 2DE map. Modified from Zhan et al. [5] with copyright permission from Wiley publisher, copyright year 2005. **B**. The MALDI-TOF-MS PMF of GH proteoform in spot 6 in Fig. 5A. T means the autodigestion fragments of trypsin. M\* means oxidized Met; C# means carbamidomethyl-Cys; N@ means deamidated Asn. Modified from Zhan et al. [5] with copyright permission from Wiley publisher, copyright year 2005. **C** 2DE image of GH proteoforms in

GH-secreting PitNETs relative to control pituitary tissues. (a) 2DE image of GH proteoforms in GH-secreting PitNETs. (b) 2DE image of GH proteoforms in control pituitary tissues. (c) Western blot image of GH proteoforms in GH-secreting PitNETs. (d) Western blot image of GH proteoforms in control pituitary tissues. Modified from Li et al. [9] with copyright permission from Springer publisher open access article, copyright year 2021. IEF isoelectric focusing, GH growth hormone, 2DGE two-dimensional gel electrophoresis, m/z mass to charge

of different proportion of GH proteoforms to conduct an accurate molecular typing of different GH proteoforms corresponding to clinical characteristics of GH-related diseases to better clarify mechanisms of their occurrences and progression.

### GH proteoforms and GH-binding proteins in serum

GH-binding proteins (GHBPs) were initially characterized in two independent studies in 1986 [58, 59]. The researchers found that  $^{125}\text{I}$ -labeled GH can specifically bind to a protein in human plasma, whose  $pI$  is of about 5 and  $M_r$  is about 60–65 kD [3, 58].

GHBPs mainly include two forms, namely high-affinity GHBP (HGHBP) and low-affinity GHBP (LGHP) [60–67]. HGHBP is a heat-labile, high-affinity, low-capacity binding protein, which specifically binds to 22 kD GH, with  $pI$ =about 5.0 and  $M_r$ =about 61 kD [3, 56, 60]. Whereas,

LGHP is a heat-stabilized, low-affinity, high-capacity binding protein, which specifically binds to 20 kD GH, with  $pI$ =about 7.1 and  $M_r$ =about 100 kD [62, 63].

GHBP originates from the shed of ectodomain of GHR by specific proteolytic enzymes. Among these enzymes, TACE (tumor necrosis factor- $\alpha$  converting enzyme) is necessary for GHR proteolysis and GHBP generation [68, 69]. GHBP is widely distributed in body fluids, binding about averaged 50.1% (range, 39–59%) 22kD GH but averaged 28.5% (range, 26–31%) 20 kD GH [61, 65]. It can maintain relatively stable concentration and half-life of GH in plasma by regulating the proportion of free GH and binding GH by inhibiting GH binding to receptors. Furthermore, it can also regulate metabolism, transport, and biological function of GH [66, 67]. It significantly demonstrates the importance of GHBP to the implementation of GH function. The identification of many GH proteoforms [9, 52–55] provides a basis to speculate that there are many





## The relationship between GH proteoforms and the invasiveness of GH-secreted PitNETs

GH-secreted PitNETs are usually benign, but some of them are invasive and can cause serious outcomes similar to malignant tumors. The invasive tendency is accounted for 49.7% of all GH-secreted PitNETs [70, 71]. Not only the normal anterior pituitary gland can synthesize and secrete GH, but the tumor cells of GH-secreted PitNETs can also synthesize and secrete GH, and the GH synthesized and secreted by GH-secreted PitNETs is closely related to its invasive behavior [71–75]. Acromegaly is caused by excessive secretion of GH and is commonly caused by GH-secreted PitNETs [75]. A meta-analysis of surgical therapy of GH-secreted PitNETs found that preoperative GH level is an important outcome predictor of the remission rate of acromegaly [73]. GH-secreted PitNETs are divided into two subtypes, namely sparse granular type (SG) and dense granular type (DG) [73]. Studies showed that SG are more often associated with aggressive features, such as local invasion and cavernous sinus invasion, and when preoperative GH levels were correlated with histological subtypes of GH-secreted PitNETs, the tendency was found for SG tumors to be associated with lower basal GH levels. Although most studies have shown that SG adenomas are more often associated with invasive features, histological subtypes of SG and DG do not absolutely predict invasive behavior. There are various hypotheses about the reasons for the invasive behavior to grow under the saddle of GH-secreted PitNETs [74], including the effect of GH itself on sella thinning, GH proteoforms of anterior pituitary, and the promotion of bone degradation by GH [74]. All of these show GH proteoforms are highly related to the invasive behavior of GH-secreted PitNETs. Therefore, exploration of GH proteoform atlas between invasive and non-invasive GH-secreted PitNETs will further clarify the relationship between GH proteoforms and the invasive behavior of GH-secreted PitNETs.

## Future perspectives

### Serum GH proteoforms and GHR proteoforms in target organs

GH has a series of functions—mainly promote the growth and development, modulate metabolism, and regulate immunity [16, 24, 27–32]. However, serum GH cannot perform its functions unless it binds to human GHR in target organs. The main targeted organs of GH include the liver, bone, muscle, and immune system [16, 27–29, 32]. Thus, GHR is vitally crucial to transduction of downstream signals of GH and the implementation of different GH function. Therefore, it is very reasonable to infer that there are different GHR proteoforms in different targeted organs corresponding to various functions.

There are nine exons encoding GHR in chromosome 5 [76–78]. Among them, the exons 2 to 7 encode extracellular part of GHR, binding specifically to GH. Currently, two human GHR mRNAs are identified, one contains exon 3 and the other excludes exon 3, and their expressions are tissue-specific, thereby exon 3 might influence receptor signaling [76–78]. The dysfunction of GHR gene is found to relate to GH-related diseases. Laron-type dwarfism is an autosomal recessive inheritance, with the characteristics of high concentration GH and low concentration IGF-1 (insulin-like growth factor-1) in the circulation [78]. Characterization of GHR gene from nine patients with Laron-type dwarfism showed that two patients have a deletion of a large portion of the hormone-binding domain of GHR gene, showing the defect of GHR gene is tightly related to Laron-type dwarfism [78]. Another analysis of the GHR-gene RNA transcripts revealed that Laron-dwarfism is caused by GHR gene abnormality, and it may vary from family to family [79].

All of these studies indicated that there is not just one form of GHR, and different GHR proteoforms have different functions, and GHR proteoforms have a close relationship with GH-related diseases. However, currently, only several GHR proteoforms have been characterized, and their exact physiological functions and their relationships to GH-related disease have not been well clarified. Therefore, it should be laid emphasis on finding more GHR proteoforms and clarifying their physiological effects and pathogenesis of GH-related diseases to promote the development of 3P medicine in GH-related diseases.

### Serum GH proteoforms and GHR proteoforms in pituitary tissues for invasive behavior in GH-secreting PitNETs

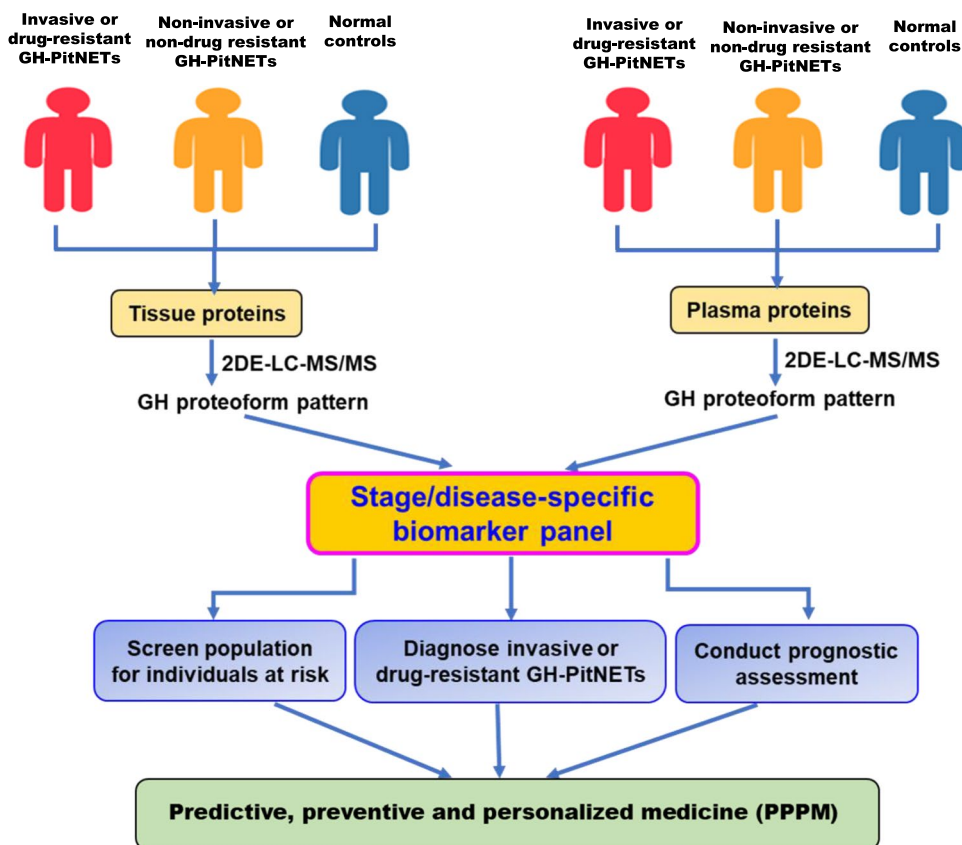
Currently, some studies have found GH concentration in serum is related to the invasive behavior and therapeutic effect of GH-secreted PitNETs [73, 74]. GH proteoforms in GH-secreting PitNETs show a significant difference, including species, abundance, and PTMs compared to normal pituitary glands [5, 9]. Moreover, different GHBP proteoforms and GHR proteoforms also show close relationship with GH signal transduction and GH-related diseases [3, 66, 67, 78, 79]. Based on these studies, we can reasonably infer that serum GH proteoforms and GHR proteoforms in pituitary tissues may play an essential role in tumorigenesis, development and its invasive behavior of GH-secreting PitNETs, and more studies are needed to explore the relationship among GH proteoforms, GHR proteoforms in pituitary tissues, and PitNET invasive behavior, which will reveal the mechanism of invasive behavior of GH-secreting PitNETs, develop targeted drugs, and find specific biomarkers to achieve precise patient stratification and proper therapy methods, promoting 3P medicine of GH-secreting PitNETs.

## Clinical problem-driven study design for GH proteoforms

The judgement of invasiveness and drug resistance of PitNETs is an intractable clinical problem. We emphasize the following studies on GH proteoforms: (i) it is a problem-driven urgent work to find key molecules that are involved in the invasive behaviors of PitNETs. It is a valid study to compare the differences of GH proteoforms among invasive PitNETs, non-invasive PitNETs, and normal control pituitary tissues or patient plasmas, which has huge potential for the development of targeted drugs to treat invasive PitNETs (Fig. 8). (ii) Currently, no specific drugs are used to treat PitNETs, especially invasive PitNETs; only several drugs are used to lower the secretion of hormones or relief clinical symptoms; however, a relatively high rate of drug resistance occurs in the actual clinical practice. Thereby, the comparison of GH proteoforms among drug-resistant PitNETs, drug-sensitive PitNETs, and normal control pituitary tissues or patient plasmas is necessary to reveal the molecular mechanism of drug resistance, and guide precise clinical medication (Fig. 8). (iii) The comparison of GH proteoforms between PitNET tissue and plasma from the same patient can identify the same proteoforms between tumor tissue and plasma, which might be developed as biomarkers to screen invasive and

drug-resistant patients for proper treatment, this screening can be achieved with non-invasive serological examination (Fig. 8). (iv) The previous publications demonstrate that many post-translation modifications, including glycosylation, acetylation, ubiquitination, phosphorylation, and deamination occur in GH proteoforms. However, the functions of these modifications remain unclear. It is necessary to study the functions and action mechanism of each specific modification with multiple methodologies such as knock out, knock down, and overexpression of specific GH gene. (v) GH is involved in cell signal transduction, which might participate in the occurrence and progression of GH-secreted PitNETs. It is of great significance to clarify the functions of every GH proteoform and its relationship with GH-related diseases. (vi) 2DE in combination with western blotting has a powerful capability to detect and separate different GH proteoforms according to their  $pI$  and  $M_r$ . However, how to purify and enrich each GH proteoform is still a technique bottleneck for top-down MS analysis. It is necessary to develop a new method to purify and enrich every GH proteoform. (vii) The structure of a biomolecule decides its specific function. It is worth studying the structures of every GH proteoform in future GH proteoformics to in-depth reveal the functions and mechanism of each GH proteoform in GH-related disease.

**Fig. 8** The experimental flow-chart of GH proteoformics analysis among invasive vs. non-invasive GH-secreted PitNETs compared to controls in tissues and plasma, or among drug-resistant vs. non-drug-resistant GH-secreted PitNETs compared to controls in tissue and plasma. Red shape means invasive or drug-resistant GH-secreted PitNETs. Orange shape means non-invasive or non-drug resistant GH-secreted PitNETs. Blue shape means normal controls. 2DE two-dimensional gel electrophoresis, LC liquid chromatography, MS/MS tandem mass spectrometry



## Conclusions and expert recommendation in the framework of 3P medicine

GH, known as one of the most indispensable hormones for the growth and metabolism of human body, is identified to have a variety of proteoforms, and different GH proteoforms might have different functions and roles, and might have different relationships with different GH-related diseases. It is a significantly urgent work to clarify the function of every GH proteoform and its relationship with GH-related diseases. The clarification of different function of GH proteoforms will help to find specific biomarkers to predict the risk of diseases, achieve early diagnosis and classification of diseases, and further conduct proper treatment for the best prognosis. Therefore, GH proteoformics offers great potential for insight into molecular mechanisms of GH-related diseases, and construction of effective biomarkers for predictive diagnosis, prognostic assessment, and even discovery of novel therapeutic targets in the framework of PPPM.

We strongly recommend GH proteoformics studies in GH-related diseases towards PPPM practice. GH is encoded by GH gene. However, many factors such as PTMs, alternative splicing, conformation, binding proteins, and others cause different GH proteoforms at the protein level under the different pathophysiological conditions. We hypothesize that GH proteoforms are different in GH-related diseases vs. normal controls, invasive vs. non-invasive PitNETs, and drug-resistant vs. drug-nonresistant PitNETs. The abnormal GH proteoforms or abnormal GH proteoform patterns are the important resource for insight into molecular mechanisms of GH-related diseases, invasive PitNETs, and drug-resistant PitNETs, discovery of effective therapeutic targets, and construction of GH proteoform biomarkers for patient stratification, and personalized medical services towards PPPM. The GH proteoformics offers the PPPM innovation in the following three aspects.

- (i) Predictive approach: GH proteoformics is a completely innovative concept, which enables to clarify the fine structure and function of GH in GH-related diseases, such as GH-secreting PitNETs. Invasive behavior is the challenging clinical problem. Currently, the GH concentration in plasma has been found to be related to the invasiveness of GH-secreted PitNETs [71–75]. The higher the plasma GH level, the more likely the GH-secreted PitNETs is to be invasive. Moreover, per-operative plasma GH half-life is also found to be related to the completeness of surgery in acromegaly [80]. However, previous studies were only conducted from the overall level of plasma GH. If more in-depth and detailed studies can be conducted from the level of GH proteoform and GH proteoform pattern unique to invasive

GH-secreted PitNETs are found, it would be very inspiring to use the unique GH proteoform pattern to predict the invasiveness of GH-secreted PitNETs, guide doctors to make clinical decisions based on the judgement of tumor invasiveness, and conduct the prognosis of patients as the follow-up indicator. 2DE-LC/MS is an effective method to array and identify GH proteoform pattern changes in invasive vs. non-invasive PitNETs. The changed GH proteoform pattern might be the effective predictive biomarkers for early-stage predictive diagnosis and prognostic assessment of invasive behaviors of PitNETs towards the innovative predictive approach.

- (ii) Targeted prevention: different GH proteoforms derived from the same GH gene are related to different GH-related diseases. For example, identification of any GH proteoform specific to invasive behaviors of PitNETs, and if this GH proteoform is involved in the molecular mechanism of PitNET invasive behaviors, then it is possible to develop the inhibitors or transform them, further reducing or eliminating the invasiveness and maximizing the benefits to the patients, which will be the innovative therapeutic target for targeted prevention and targeted therapy of invasive GH-secreting PitNETs. In addition, the GH must bind to its receptor in the targeted cells, targeted tissues and targeted organs to implement its function. If the tumor is aggressive, the GHR proteoforms may be changed. Therefore, if the unique GHR proteoform pattern of invasiveness is found, it is possible to develop the inhibitors or transform them, further delaying or reversing the patient's clinical symptoms. It is possible to develop the inhibitors or transform them, further reducing or eliminating the invasiveness and maximizing the patient's recovery.
- (iii) Personalization of medical services: the 2DE pattern of GH proteoforms can be developed as effective biomarkers for patient stratification, predictive diagnosis, prognostic assessment, and personalized treatment towards innovative personalization of medical services. If GH proteoform pattern unique to invasive GH-secreted PitNETs are found, it would be used to predict the invasiveness of GH-secreted PitNETs and guide doctors to the make clinical treatment accurately based on the judgement of tumor invasiveness, further providing personal medical services.

In summary, quantitative GH proteoformics can detect, identify, and quantify GH proteoforms, and GH proteoform pattern alterations, which is an innovative area for GH-related diseases, such as PitNETs. The abnormal GH proteoforms, or GH proteoform pattern will provide novel insight into molecular mechanisms, therapeutic targets, and

discovery of effective biomarkers of GH-related diseases towards predictive diagnosis, targeted prevention, and personalized medical service. This present GH proteoformics demonstrates an innovative, GH proteoform-based state of the art contributing to the paradigm shift from reactive medicine to PPPM in human GH-related diseases.

**Abbreviations** CZE: Capillary zone electrophoresis; GHBHP: Growth hormone-binding proteins; GHR: Growth hormone receptor; hGH: Human growth hormone; HGHBHP: High-affinity GHBHP; JAK2: Janus kinase 2; IRSs: Insulin receptor substrates; LC: Liquid chromatography; LGHBHP: Low-affinity GHBHP; MS: Mass spectrometry; MS/MS: Tandem mass spectrometry;  $M_r$ : Molecular weight; pI: Isoelectric point; PPPM/3PM: Predictive preventive personalized medicine; PTMs: Post-translational modifications; Stat: Activators of transcription factors; TACE: Tumor necrosis factor- $\alpha$  converting enzyme; 2DGE: Two-dimensional gel electrophoresis

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## Declarations

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