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Original Article

Growth hormone and omic systems immunology: the prominent role of interferon signaling

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ABSTRACT

Growth hormone (GH) imposes pleiotropic effects on human tissues by coordinating growth, facilitating metabolic, cardiovascular, immune, and neuroendocrine systems, and governing the aging process. Growth hormone deficiency (GHD) is estimated to occur in 2.5% of children and may present with age-dependent symptoms: hypoglycemia, jaundice in infancy, and poor growth in childhood. This study attempts to define the GH-immune landscape and its complex molecular drivers at the systems level. Integrative statistical analysis of multi-omic data from blood of 52 children with short stature before and after a GH stimulation test showed significant, concordant changes imposed by GH across the whole genome, metabolome, and inflammatory proteome. These changes involved signaling molecules along the GH/IGF axis, the JAK-STAT pathway, cytokines, and GH-induced nitrogen metabolism. Interferon signaling network drivers were correlated significantly with GHD demonstrating the prominent role of interferon at the nexus of the endocrine, immune and metabolic systems.

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KEYWORDS: growth hormone, multi-omics data, network, interferon, data integration, molecular landscape, transcriptomics, metabolomics, immune system, stimulation test.

INTRODUCTION

Growth hormone (GH) is a peptide hormone produced by the anterior pituitary gland in a pulsatile manner, and it is primarily regulated by the stimulating actions of the hypothalamic GH-releasing hormone (GHRH) and the inhibiting effects of somatostatin. GH is also coordinated by the signaling cascades of the GH-insulin-like growth factor axis (GH-IGF axis) and the stomach-produced peptide, ghrelin [1, 2]. Being very dynamic and versatile, GH imposes pleiotropic effects on human tissues primarily by coordinating growth [2] but also by facilitating the function of many diverse systems such as the metabolic [3, 4], cardiovascular [5, 6], immune [7, 8] and neuroendocrine systems [9, 10], and by governing the aging process [11, 12].

Growth hormone deficiency (GHD) can be isolated or combined with other pituitary hormone deficiencies and may be total (no growth hormone is produced) or partial (growth hormone production is insufficient to support normal growth). GHD may be congenital, resulting from abnormal development of the pituitary or hypothalamus *in utero*, or acquired, stemming from damage to the pituitary or hypothalamus postnatally, or most often idiopathic. Clinical features of GHD depend on the age of onset, severity of deficiency and

association with other pituitary hormone deficits, and may present with hypoglycemia and jaundice in the neonatal period, while later in childhood, short stature and poor growth [13]. Adults with GHD present with low muscle mass and poor exercise tolerance, muscle weakness and increase in visceral adipose tissue. In children without organic or genetic causes, the diagnosis of GHD requires a GH stimulation test to be performed [14]. Although variable thresholds of peak growth hormone response during the GH stimulation test are utilized to distinguish GHS (Growth Hormone Sufficient) from GHD (Growth Hormone Deficient) children, the cut-off of 10 ng/mL is accepted by the Growth Hormone Research Society, the Pediatric Endocrine Society, and the European Society for Paediatric Endocrinology [15, 16]. Given the potential poor reproducibility of a single stimulation test, GH stimulation with two provocative agents is recommended [14]. The poor reproducibility of the GH stimulation test (due to probably interfering parameters such as body composition or pubertal status) [17] along with the current incomplete understanding of the effects of GH at the systems level [18, 19] makes the diagnosis of GHD challenging [17, 20-22] GH stimulation test contributes and is essential for the diagnosis but only within the overall appropriate clinical picture.

Tight coupling exists between the endocrine and immune systems, yet the complex molecular interactions that underpin immune-metabolic adaptation in growth disorders are not well understood. We along with others have demonstrated GH receptors on the cell surface of human thymus, spleen, lymph nodes, peripheral blood mononuclear cells (lymphocytes, NK cells and monocytes) and human IM-9 cultured lymphocytes [18-22]. Administration of GH to growth hormone deficient (GHD) children has shown a transient decrease in percent B cells and T cells, interleukin-2 receptor levels and lymphocyte mitogenic stimulation response [20, 22]. While we have previously demonstrated *in vitro* and *in vivo* effects of chronic GH stimulation on immune function, data are lacking about acute effects of GH [20-22]. This study attempts to define the GH-immune landscape and its complex molecular drivers at the systems level.

MATERIALS AND METHODS

In this study, we collected and integrated multiple, high-throughput, next-generation omics data (transcriptomics (RNA-Seq), proteomics (Olink® Inflammation Panel) and metabolomics from paired blood samples of 52 children (ages 5-18), who underwent a GH stimulation test as part of their growth failure evaluation. This evaluation included a prolonged period of monitoring of slow growth patterns as well as the exclusion of non-growth hormone related factors that may affect growth. Short stature is defined as height less than -1.5 standard deviation score (SDS) below the mean and less than -2 SDS below mid-parental height, and growth failure is defined as height velocity less than -2 SDS below mean over 1 year. Following initial clinical evaluation, the GH stimulation test was performed. In this study, we focused on the GH multi-omic interactions in children with potential growth failure serving as their own controls comparing pre and post stimulation data and thus, no negative controls were recruited (i.e., control children without potential growth failure) [17, 18]. The paired blood samples were collected at baseline (at T0) and at the end of the 3 hr GH stimulation test (T3). GHS and GHD children were identified based on a peak GH level of greater or less than 10 ng/ml respectively [14] (study schematic, Figure 1).

A total of 52 children evaluated for growth failure underwent a 3 h GH stimulation test as part of their clinical assessment. GHS and GHD states were determined based on their peak GH response above and below the diagnostic cutoff of 10 ng/mL. Demographics and clinical traits of the children enrolled in the study are shown in Figure 2. Blood was collected at half-hour intervals for specific blood markers and omics experiments as follows:

Blood markers at t0: GH, cortisol, insulin, glucose, hemoglobin_a1c, luteinizing hormone, estradiol, testosterone, IGF1

Blood marker at t0.5, t1, t1.5, t2, t2.5: GH

Blood markers at t3: GH, cortisol

Omics at t0 and t3: Transcriptomics (RNASeq), Proteomics (Olink® Inflammation Panel), Metabolomics (Metabolon, untargeted panel)

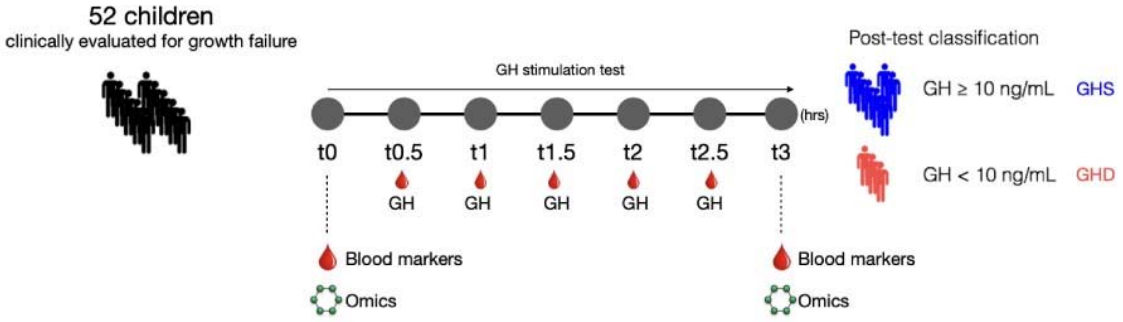


Figure 1. Study schematic and measurements.

Integrative statistical and network analyses of the omics data showed significant, concordant changes (in expression directionality) imposed by GH across the expression landscapes of the whole transcriptome, metabolome, and inflammatory proteome both at the molecular and functional levels. Although no significant changes were observed between GHS and GHD children at all three molecular landscapes before or after the GH stimulation test, further analysis of gene co-expression networks revealed a gene module (cluster) of interferon genes positively correlated with GHD across the entire cohort of samples (T0 and T3). Drivers of that cluster as well as molecular and functional associations at the metabolic level demonstrate the prominent role of interferon signaling at the nexus of the endocrine, immune and metabolic systems and serve as guides for uncovering the GH effects in the human body at the systems level.

RESULTS

The GH-induced transcriptome spans across the immune, metabolic, endocrine, and neural systems

Initial unsupervised analysis of the GH-induced transcriptome showed some overlap but also distinct separation of the expression profiles of samples at T0 vs. T3 (Figure 3A). To identify significant genes that changed expression between T0 and T3, we used a linear model adjusted for paired samples, sex, age, race/ethnicity, and BMI. At FDR < 0.05, we identified 3,817 significant differentially expressed genes (DEGs) activated (up-regulated) or suppressed (down-regulated) by GH. Across the GH/IGF axis, significant genes with central roles in cell signaling, proliferation and differentiation, such as the potent transcription factor, *JUN*, and the mTORC1 subunit, *AKT1S1* were significantly up-regulated at T3. Interestingly, some key molecules of the PI3-K and Ras-MAPK pathways, more downstream of the GH/IGF axis, were up-regulated (*IRS-2, AKT1S1, MAPK3, MAPK13*), while others were down-regulated (*IRS-1, PIK3R3, HRAS, MAPK11, MAPK12*) at T3. This expression pattern could be representative of more downstream GH effects rather than immediate GH responses because the GH levels of most participants peaked earlier, at 2 or 2.5 hrs post GH stimulation, and not at T3 (Figure 4).

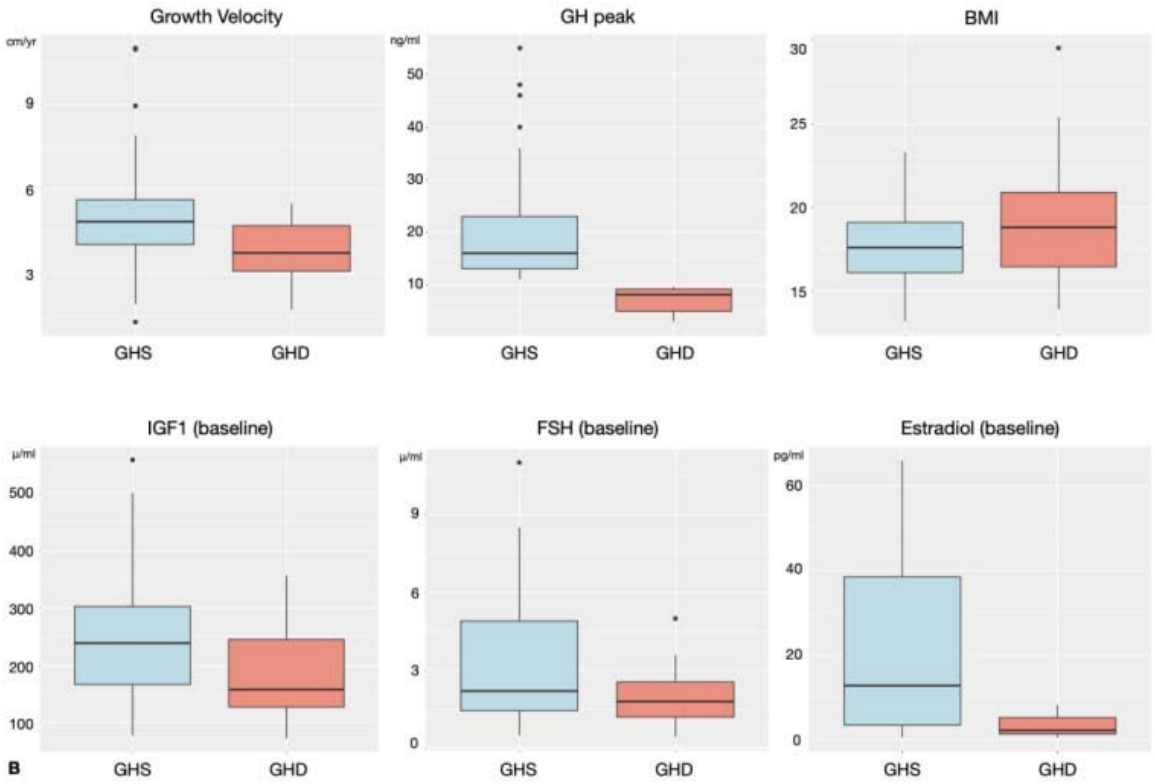
Investigating further the gene expression changes at T3, we observed a host of gene alterations more downstream of the GH/IGF axis. Among the most up-regulated, were genes linked to inflammatory and metabolic processes. Some of these genes included pro-inflammatory modulators such as the Stat signaling activator tyrosine kinase *BMX* [23]; Stat5b-activated *BCL2A1* [24-27]; the interleukin activator and matrix modulator *MMP9* [28]; the neutrophil regulator *CD177* [29]; and the orosomucoid, *ORM1*, which has been shown to function pleiotropically across the immune and the metabolic systems [30]. Specific genes related to metabolism were also highly up-regulated, such as the metabolic reprogramming genes *G0S2* and *TGM3*. The former induces cell proliferation, differentiation, and metabolism [31], and the latter, is a transglutaminase shown to be essential for epidermal terminal differentiation and formation of the cornified cell envelope [32, 33] but not previously reported as a GH-induced gene in the blood (Figure 3B).

The suppressive activity of GH [34] was shown in the anti-inflammatory *ALOX15*, which was among

Clinical characteristics of participants

	all patients (n = 52)	GHD (GH<10 ng/ml at T3)	GHS (GH>10 ng/ml at T3)
Age, yrs	12.2 ± 2.4	11.9 ± 2.3	12.4 ± 2.4
Male/Female, n	38/14	16/3	22/11
GHD/DHS, n	19/33	19/0	0/33
Growth hormone velocity, cm/yr	4.6 ± 2	3.8 ± 1	5.1 ± 2.3
GH peak, ng/ml SD	15.9 ± 11.6	7.1 ± 2.3	20.9 ± 11.8
BMI SD	18.4 ± 3.1	19.2 ± 3.8	17.9 ± 2.5
BMI classification SD (1=< 25%, 2=25-50%, 3=50-85%, 85%<4<94%, 5>=95%)	2.4 ± 1	2.7 ± 1.1	2.1 ± 0.9
IGF1 baseline SD, μ/ml	222.7 ± 106.9	186 ± 79.4	244.5 ± 116
FSH baseline SD, μ/ml	2.9 ± 2.5	1.9 ± 1.1	3.5 ± 2.9
Estradiol SD, pg/ml	18.9 ± 23.3	3.6 ± 4.1	23.5 ± 24.9

A



B

Figure 2. Clinical information and statistically significant clinical traits of the children enrolled in the study. **A.** Demographics of the children enrolled in the study **B.** Clinical traits that showed statistically significant differences (FDR ≤ 0.05) between the GH sufficient (GH peak >10 ng/μl) and GH deficient (GH peak <10 ng/μl) children post GH-stimulation test.

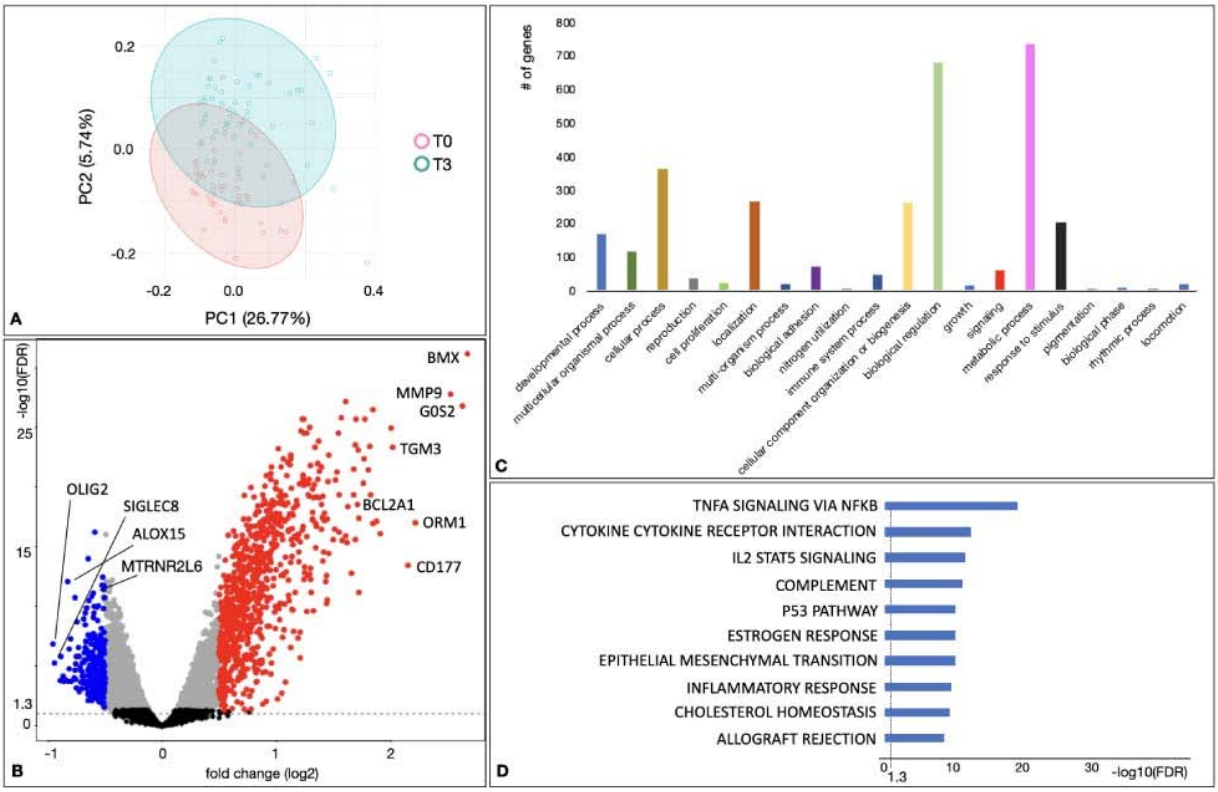


Figure 3. GH-induced changes across the entire transcriptomic landscape.

the most down-regulated genes, as well as the co-repressed genes *SIGLEC8* and *OLIG2* [35, 36]. Interestingly, *OLIG2* is a potent regulator of neuroectodermal progenitor cell fate located in a chromosomal region that mediates learning deficits in Down syndrome and early Alzheimer's disease [37, 38]. Also linked to neural diseases, age, metabolism, inflammation, and oxidative stress was the down-regulated *MTRNR2L6* (Human-like 6) gene, a mitochondrial polypeptide recently associated to the GH/IGF axis [39, 40]. No significant expression changes were found between the GHS and GHD cohorts (either at T0 or T3), and most of the highly altered genes at T3 seemed to settle at similar expression states for both cohorts (Figure 5). Similar response to GH therapy regardless of the GH status has been shown before [41].

The key functional properties of the most highly altered genes at T3 were shown in the classification and enrichment analysis of the entire GH-induced transcriptome (3,817 significant genes). Most of these genes were classified within biological and cellular processes, response to stimulus, and metabolic disruptions (Panther classification system querying GO Biological Processes, Figure 3C). Additionally, enrichment analysis revealed more specific inflammatory signaling pathways associated with well-studied genes in the GH/IGF axis such as TNFA, IL2-STAT5 [42-44], as well as cytokine interactions [44], the complement system [45], and pathways implicated in cancer such as EMT and P53 [46-48]. The estrogen response pathway was also enriched, demonstrating the known interrelationship between sex-hormone signaling and GH action [49, 50]. Lastly, cholesterol homeostasis represented the lipolytic metabolic effect of GH [51-54], whereas the allograft rejection pathway showed the possible transcriptomic changes of GH to organ transplant [55, 56] (GSEA querying Hallmark and KEGG databases, Figure 3D).

Comparing the 3,817 DEGs to the most recent RNA-Seq studies on GH stimulation (deposited in GEO database), we found striking similarities with GH-induced experiments in mouse liver cells

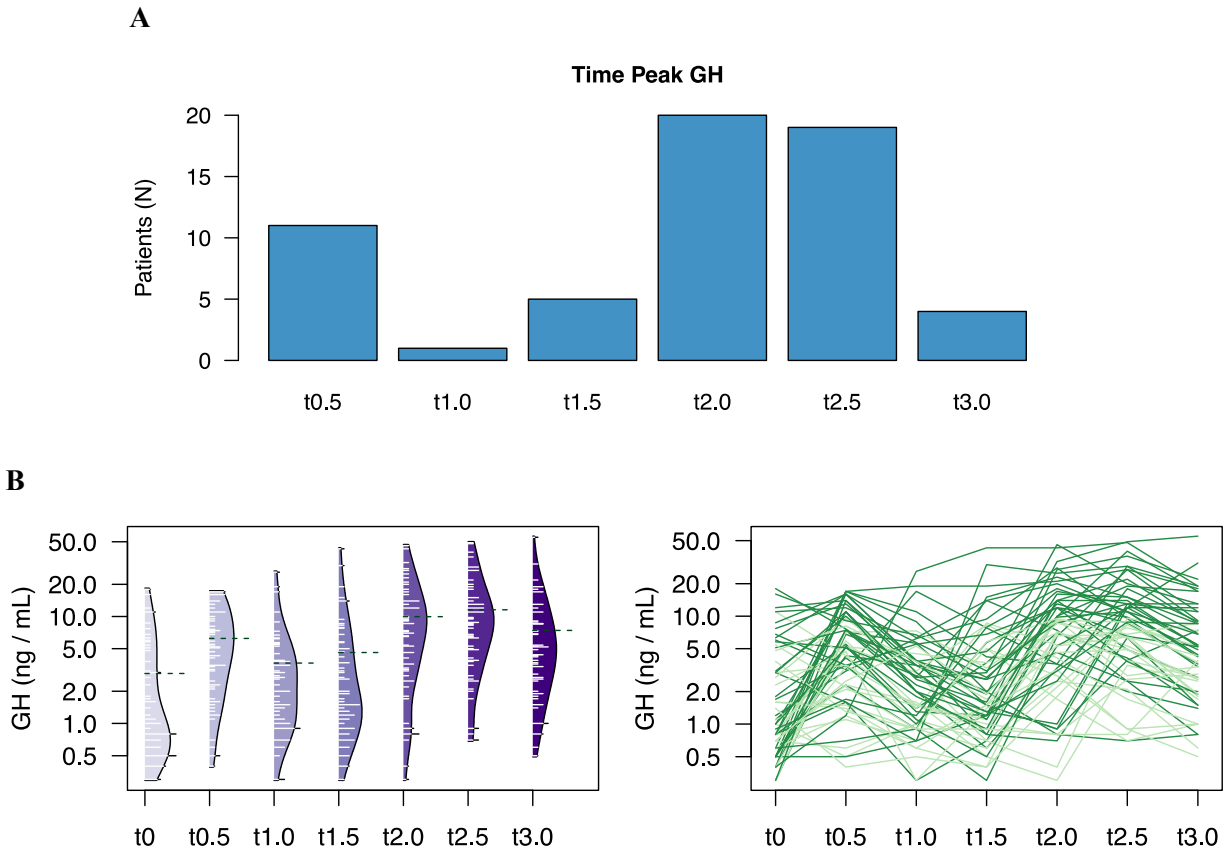


Figure 4. A. Time of peak of the growth hormone levels of all patients at 30 min, 60 min, 90 min, 120 min, 150 min and 180 min. Most of the participants recorded their highest levels of GH at 120 min (2hrs) or at 150 min (2 ½ hrs) during GH stimulation testing. **B.** Growth hormone levels of all samples at different time points measured (0, 0.5 hrs, 1 hr, 1.5 hrs, 2 hrs, 2.5 hrs and 3 hrs) represented as distributions (left panel) or line plots (right panel).

and some overlap in human adipocyte cells. In a mouse study on liver cells, before and after 24 hrs of GH treatment, 172 genes were significantly altered at FDR < 0.05 and 30 of those were significantly enriched (enrichment FDR<6.93E-14) in our 3,817 DEGs [57] (GEO number: GSE114610). More importantly, enriched pathways of those common 30 genes were the JAK-STAT signaling, estrogen response and xenobiotic metabolism, all of which, were included in our DEG enrichment (Figure 3D). This demonstrates great similarity in major GH-induced functions between the human blood and the mouse liver transcriptomes possibly due to similar inflammatory and hormonal signaling between the two species [58]. In another mouse study, it was also found that IL2-STAT5 and xenobiotic metabolism pathways were enriched for 26 common genes (10 hr post GH administration) with our 3,817 DEGs (enrichment FDR<3.93E-12) [59] (GEO number: GSE98585). Lastly, in a human adipocyte study of acromegaly, where endogenous GH is at significantly higher levels compared to controls, only 24 genes changed expression at FDR < 0.05. In comparison to our 3,817 GH-induced DEGs, there were 3 common genes (enrichment FDR<0.048): *DIRC3* (non-coding), *LIN9* (tumor suppressor, down-regulated in GH induction), and *VSTM4* (calcium-channel mediator, up-regulated) ([60], GEO number: GSE57803). This small overlap shows the different GH response that adipocytes may exhibit compared to blood cells and the different transcriptomic changes that GH imposes when it is at systemic higher levels (acromegaly) vs. short-term stimulatory levels (GH stimulation test).

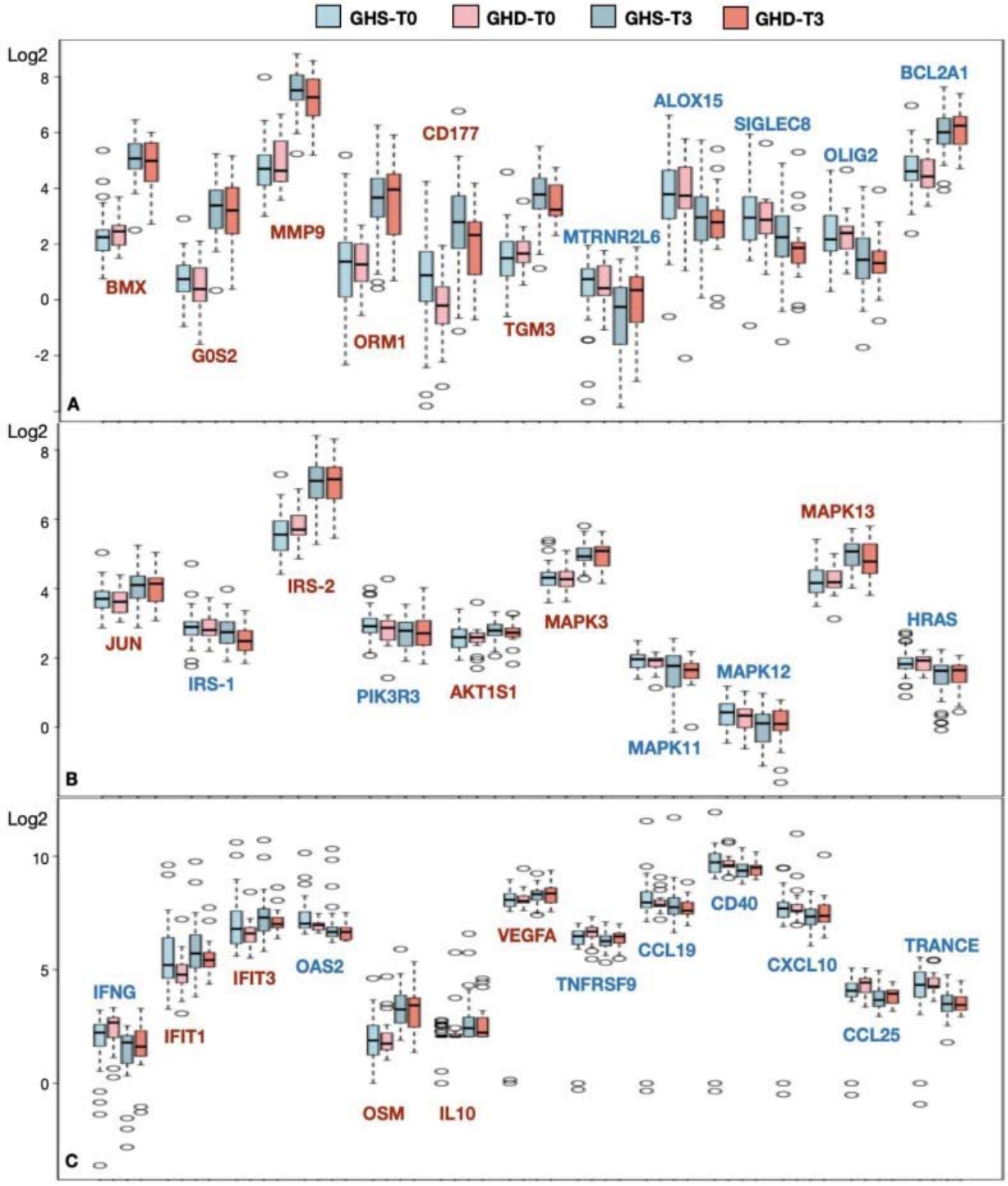


Figure 5. Boxplots of gene expression in GHS and GHD children at T0 and T3.
Expression levels of genes between T0 and T3 visualized separately for GHS and GHD patients (red-labeled genes indicate up-regulation in T0 vs. T3 and blue-labeled genes indicate down-regulation in T0 vs. T3) **A.** Expression levels of top significant DEGs in terms of fold-change between T0 and T3. **B.** Expression levels of major genes in the GH/IGF signaling cascade **C.** Expression levels of interferon gamma hub genes, and top inflammatory proteins

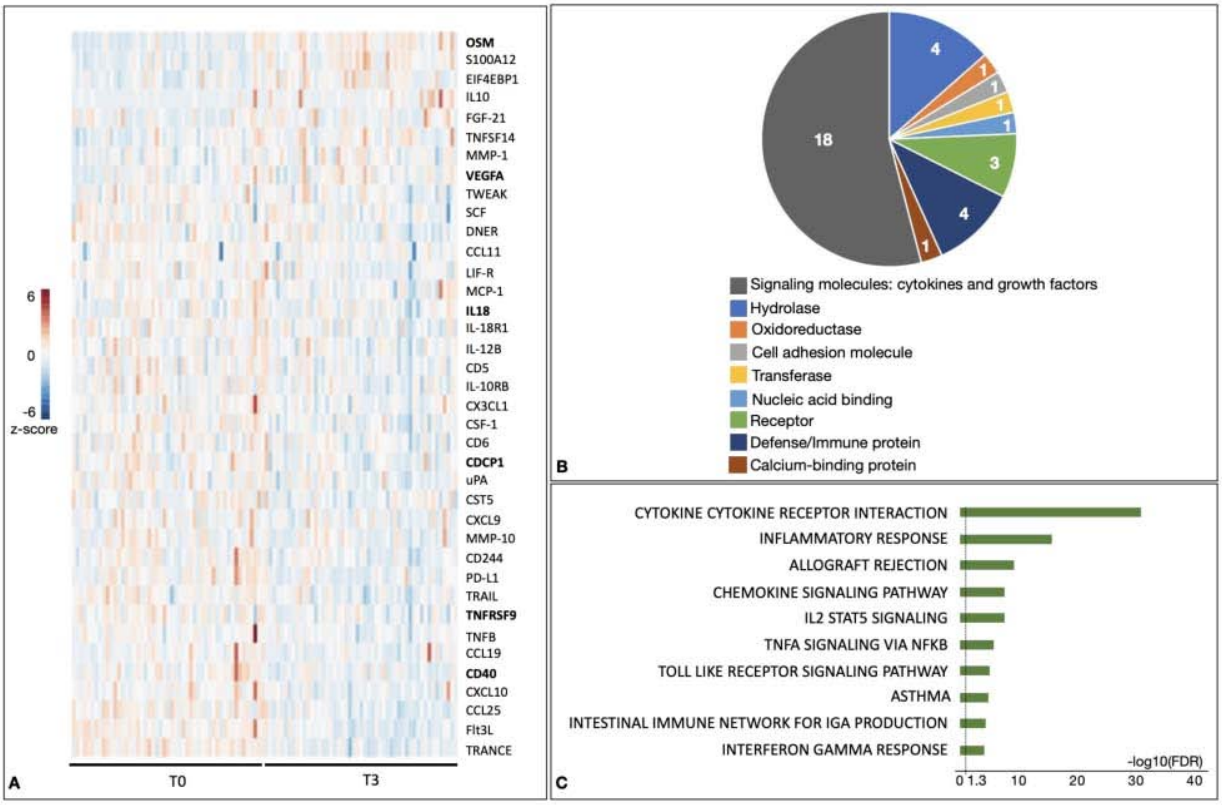


Figure 6. GH-induced changes across the inflammatory protein landscape.
A. Expression heatmap of the 38 significant inflammatory proteins in T3 vs. T0 blood samples measured by Olink®. The significance cutoff value was set at FDR < 0.05. Proteins shown in bold are those that were common and displayed the same direction of significant expression (up- or down-regulated) at the gene level. **B.** The classification of the 38 significant inflammatory proteins generated by Panther classification system using the protein class database. **C.** The 10 most enriched pathways of the 38 significant inflammatory proteins generated by GSEA using Hallmark and GO databases. The FDR cutoff was set at 0.05 or 1.3 at the -log10(FDR) scale (dashed line).

The GH-induced inflammatory proteome echoes the GH-induced transcriptome and provides additional insights into the pro- and anti-inflammatory GH-induced mechanisms

Employing a targeted protein panel (Olink® Inflammation Panel) and using the same linear model as the one used for the transcriptome analysis (i.e., adjusted for paired samples, sex, age, race, and BMI), we identified a total of 38 inflammation proteins whose expression was significantly modified post-GH stimulation at T3 (FDR<0.05, Figure 6A). Six of the 38 proteins changed expression similarly to their associated genes but most of the inflammatory proteins (32 total) were not detected at the gene expression level. More specifically, oncostatin (OSM), the GH-activated JAK-STAT pathway protein [47], was the most up-regulated protein and also among the significant up-regulated genes. OSM is a potent cytokine and growth regulator implicated in bone metabolism, cartilage catabolism, cancer, inflammation, skin conditions, atherosclerosis and cardiovascular diseases [61] but, to our knowledge, OSM has not been reported as a GH-stimulated inflammatory protein yet. Another highly up-regulated protein linked to JAK-STAT pathway activation is the endothelial homeostasis gatekeeper and major angiogenesis factor, VEGFA. Interestingly, both pro- and anti-inflammatory proteins were not detected at the gene expression level. These included the pro-inflammatory and immune response mediator S100A12 [62], the substrate of mechanistic target

of rapamycin (mTOR) signaling, EIF4EBP1 [63], and the immunoregulatory protein for the JAK-STAT pathway, interleukin IL10 [64]. These highly GH-activated proteins lie downstream of the GH/IGF axis and JAK-STAT pathways and while some displayed similar expression profile with the GH-activated transcriptome, others were uniquely detected by the GH-activated proteome.

An analogous pattern was observed for the suppressed proteins. Both at the gene and protein level, the anti-microbial and anti-inflammatory chemokine ligands, CXCL10, CCL25, and CCL19 were down-regulated. However, the most down-regulated proteins were not detected at the gene expression level. Among those, TRANCE (or RANKL), the most down-regulated protein, is a member of the tumor necrosis factor cytokine family, a receptor of NF-κB ligand, and a key factor for osteoclast differentiation and activation [65, 66]. Interestingly, although GH enhances osteoblast proliferation and osteoclast differentiation, it has previously been shown that RANKL concentrations (hereby suppressed) appear to be independent of GH secretory status at least in children with short stature [67]. Other proteins suppressed by GH stimulation but not detected at the gene expression level include suppressors of TNFB and other proteins of the TNF family, such as TNFRSF9, TRAIL (or TNFSF10) and TWEAK (or TNFSF12). This is particularly interesting in children with GHD, whose TNF-α levels were previously shown to be significantly higher compared to controls [43], suggesting that GH may attenuate this effect (Figures 3A and 5C).

From the classification and enrichment standpoint, most significant proteins were signaling molecules (cytokines and growth factors, Panther classification system querying Panther protein class database, Figure 6B). Half of the 10 most significant protein pathways overlapped with the 10 most significant gene pathways related to inflammation showing that inflammatory processes govern a large part of the GH stimulation molecular landscape (Figures 3D and 6C, GSEA querying Hallmark and KEGG databases).

The GH-induced metabolome mediates amino acid and lipid metabolism with neuro-modulatory implications

Although the metabolic effects of GH *via* complex interactions with insulin have been well established at the signaling and gene expression levels [3, 68], whole metabolome extensive studies are still lacking. Clinically, it is known that GH stimulates cartilage and bone growth (*via* prechondrocytes, osteoblast hypertrophy and increase in bone mineralization) and increases body mass. GH-mediated metabolic changes include rise in total body protein content, reduction in total body fat content and favorable changes in the plasma lipid profile such as increase in serum high density lipoprotein and reduction in low density lipoprotein. GH has also been established as a counter regulatory hormone against insulin and prevents hypoglycemia by increasing glucose production through gluconeogenesis and glycogenolysis. Here, we utilized Metabolon®, a global metabolomics platform, to assess expression changes of over 1,000 metabolites that span all known amino acids, carbohydrates, lipids, nucleotides, energy molecules, cofactors and vitamins, and xenobiotics as well as novel metabolic chemicals.

We employed the same statistical approach we used for the transcriptomic and proteomic analyses to identify significant metabolites that changed after the 3 hr GH stimulation test. Out of the 820 known metabolites that passed the platform's quality standards, over 500 changed expression significantly (575/820 or ~70% at FDR < 0.05). Compared to the baseline (T0), very few of those significant metabolites changed by more than 2-fold after the 3 hr GH stimulation test (T3) (41 metabolites, Figures 7A (blue and red dots) and 4C). Among those 41 metabolites, there were 22 up-regulated amino acids and lipids involved in urea cycle and fatty acid metabolism. A total of 13 metabolites were down-regulated and these were glycerol, cholate-associated lipids, and a handful of amino acids and peptides related to lysine and methionine metabolism (Figure 7C).

Pathway enrichment of all 575 significant metabolites provided a more specific view of the GH-induced metabolome (Figure 7B). The most significantly enriched pathways were nitrogen

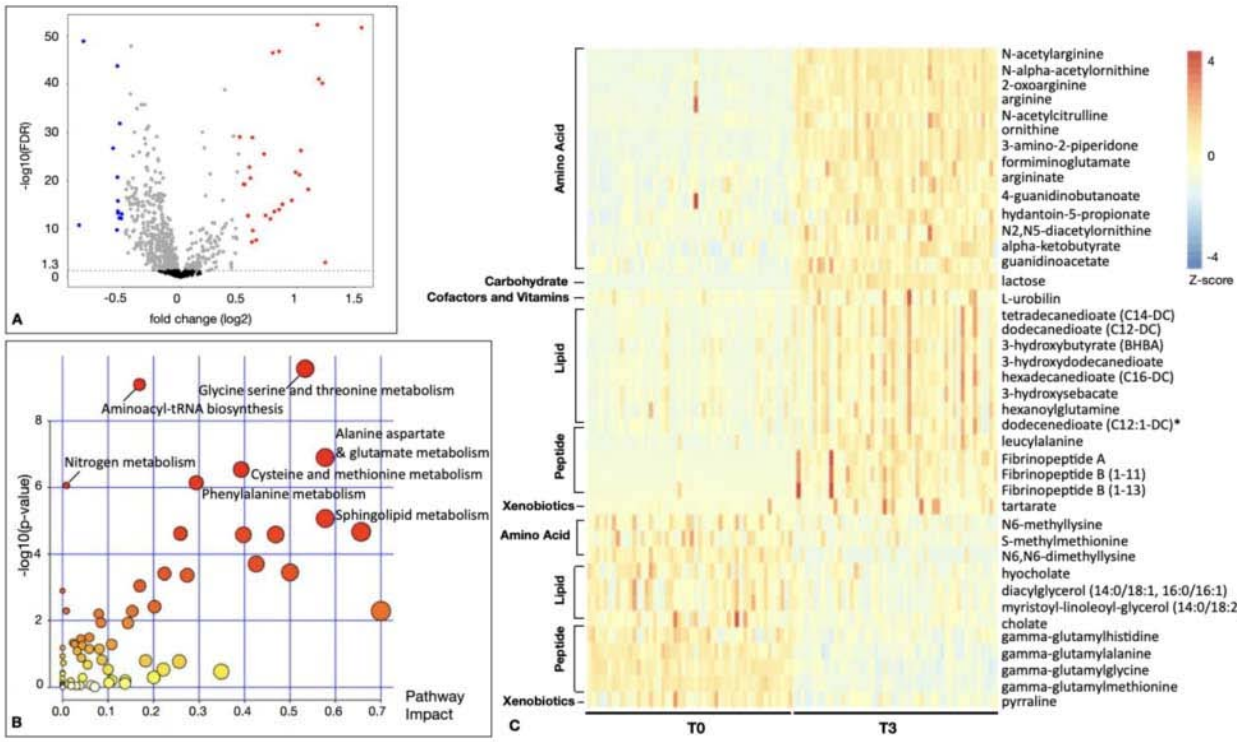


Figure 7. GH-induced changes across the metabolic landscape.
A. Volcano plot representing the fold-change of expression (in log2 scale) and the significance of the fold change (-log10 scale of FDR) of the 820 metabolites in T3 vs. T0 blood samples. The significance cutoff value was set at FDR < 0.05 or –log10(FDR) of approximately 1.3 (dashed line). There were 575 significant metabolites at FDR < 0.05 (grey dots). From those, 145 were up-regulated and 145 down-regulated, of which, 28 (red dots) and 13 (blue dots) had an equal or more than 2-fold expression change. **B.** The most enriched pathways of the 575 significant metabolites (generated by MetaboAnalyst). **C.** Expression heatmap of the 41 metabolites that changed expression significantly and by more than 2fold between T0 and T3 along with their categories generated by Metabolon®.

metabolism; the aminoacyl-tRNA biosynthesis; the glycine-serine-threonine metabolism; the sphingolipid metabolism; and a few essential and non-essential amino acid metabolic pathways such as methionine, alanine, cysteine, phenylalanine, and glutamate. Nitrogen retention has been studied as an effect of accelerated fat utilization after GH administration in mice [69], healthy adult subjects [68, 70] and children with GHD [71]. Moreover, enhanced protein synthesis (metabolism of alanine, cysteine, methionine, phenylalanine, etc.), and lipolysis are well-known properties of GH [4, 68, 72-74].

Sphingolipids have been recently added to the list of skeletal muscle regulatory elements turning them into important elements of the metabolic effect of GH on the musculoskeletal system [75, 76]. In addition, although earlier studies have looked at relating levels of GH and glycine in normal subjects [77, 78], the metabolic link of glycine to GH stimulation in children with short stature, is first seen in this study. L-serine has been shown to suppress ghrelin, which, apart from its orexigenic role, is also found in the hypothalamus affecting specific neurons in midbrain, hindbrain, hippocampus, and spinal cord [79, 80]. Ghrelin is one of the pivotal hormones within the GH/IGF axis, the immune, metabolic, and neuro-endocrine systems [81]. Lastly, glycine and threonine are potent neurotransmitters supporting the cardiovascular, liver, and immune system functions [82, 83]. In conclusion, the short-term 3 hr GH stimulation led to coordinated changes within the whole transcriptome, inflammatory proteome, and global metabolome that spanned across the immune, metabolic, and neural/neuro-endocrine systems.

Integrative "omics" network analysis reveals key drivers of the GH-induced transcriptomic and metabolomic landscapes

A gene-metabolite interaction network enables exploration and visualization of interactions between functionally related metabolites and genes. For this study, the chemical and human gene associations were extracted from STITCH, such that only highly confident interactions are used *via* the MetaboAnalyst Network Explorer webtool. Most of the associations in STITCH are based on co-mentions highlighted in PubMed Abstracts including reactions from similar chemical structures and similar molecular activities. Here, we used the list of genes (3,817 DEGs) and the HMDB annotated metabolites (400 out of the significant 575) that changed expression significantly post GH stimulation (FDR < 0.05) and performed a gene-metabolite interaction network analysis with the MetaboAnalyst Network Explorer webtool (Figure 8) [84].

The network analysis identified the most inter-connected genes and metabolite hubs and the top 15 nodes with the highest degree are denoted in Figure 8. The gene-hubs included the down-regulated *CYP3A4, SLC16A10,* and *IFNG*. *CYP3A4* is the predominant cytochrome P450-expressed gene in human liver, and it is responsible for the metabolism of endogenous steroids and many other drugs. The GH modulatory effects on hepatic CYP enzyme activity have been seen before in a randomized placebo study in GH-deficient adults [85] and in cultured hepatocytes [86]. However, *SLC16A10*, a proton-linked monocarboxylate transporter that catalyzes the movement of aromatic amino acids across the plasma membrane has not been reported to exhibit significant suppression and metabolic influence after GH administration. Interestingly, *SLC16A10* (along with *MCT8*) is a thyroid hormone transporter with fundamental roles in skeletal muscle physiology [87, 88]. *MCT8* knockout mice in particular, have been shown to be leaner, have increased energy expenditure and food and water intake with normal total activity indicating hypermetabolism [89]. To what extend the observed *SLC16A10* suppression in blood affects muscle metabolism and energy expenditure has yet to be studied in GH-deficient children. Lastly, the GH suppressive effect on *IFNγ* expression has been shown in rat anterior pituitary cell cultures [90, 91] but in human peripheral mononuclear cells or in a mixed leukocyte culture, GH has been reported to enhance *IFNγ* production [92, 93]. These dual roles of *IFNγ* have been studied in various systems like cancer [94, 95], the nervous system [96], or infectious diseases [97]. More importantly, *IFNγ* has emerged as a particularly sensitive molecule to cellular metabolic states [98].

From the metabolic perspective, the most inter-connected metabolite-hubs included L-Arginine, hydroquinone, and cholic acid. L-Arginine is a GH releaser and by study design it was anticipated to be found elevated because the GH stimulation test took place with glucagon and arginine. Cholic acid is one of two major bile acids produced by the liver and its reduced levels may reflect increased cholesterol breakdown [52, 99]. However, hydroquinone, an endogenous skin-lightening agent that inhibits melanin production, has not been studied in the context of GH stimulation before.

Weighted gene co-expression network analysis reveals significant correlation of GHD and interferon activity

From the 52 children that participated in the study and provided samples for the "omics" analyses (104 samples), a total of 19 were diagnosed as GHD (GHD was determined with a GH peak level of less than 10 ng/ml). We utilized the 6,668 annotated genes in all 104 paired samples across the two time points, T0 and T3, to perform weighted gene co-expression network analysis (WGCNA implemented with R) [100] and to infer co-expressed gene modules and central hub genes that correlated with GHD.

A total of six modules with co-expressed genes were identified. The size of the modules varied between 30 and 2,455 genes (Figures 9A, B). Further analysis identified modules significantly correlated with time (modules 1, 3, 4, and 5), sex (module 1), sex hormonal levels (testosterone: module 1, estradiol: modules 3 and 4), FSH levels (module 4), fasting glucose and insulin levels (modules 1 and 2), and cortisol levels at T0 and T3 (modules 1, 2, 4, 5, and 6) (Figure 9B).

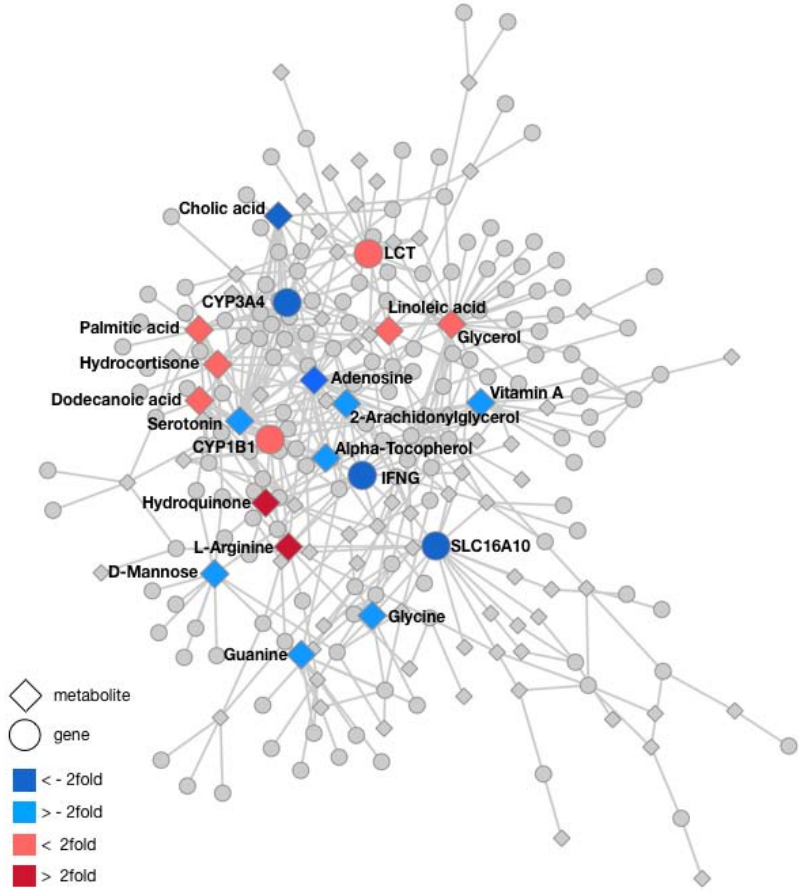


Figure 8

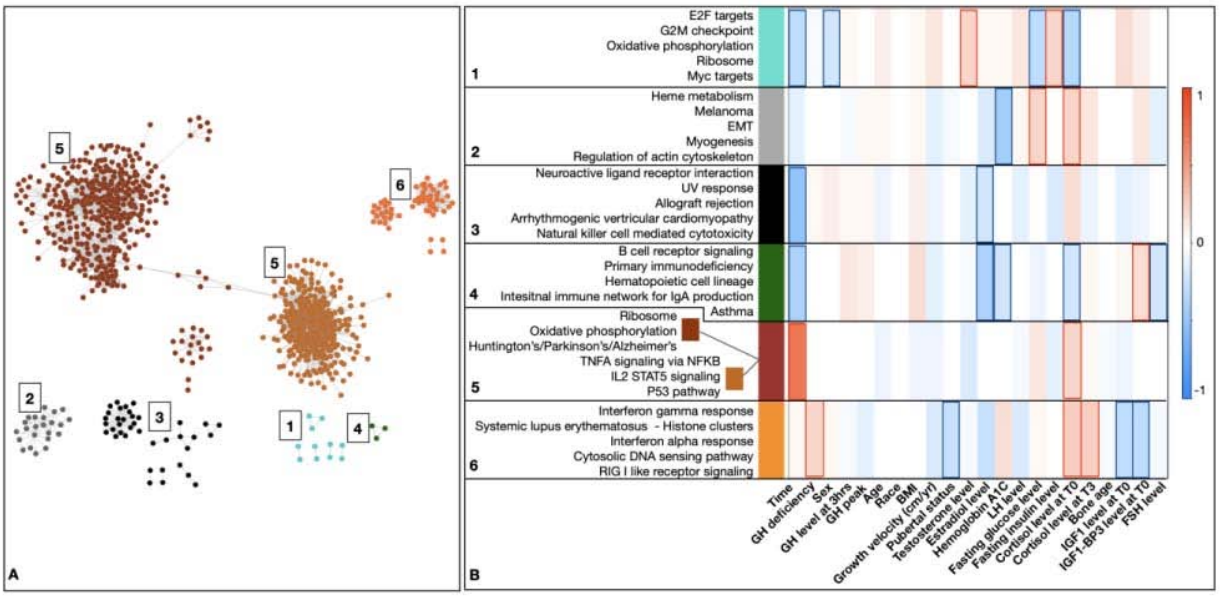


Figure 9

In particular, module 6 was significantly positively correlated with GHD and it was one of the two modules with the highest number of correlated clinical features (six) including cortisol levels at T0 and T3, IGF1, and IGF1-BP3 levels. Positive correlation of this module 6 with cortisol levels suggested that up-regulated genes in GHD children were positively impacted by higher cortisol levels. GH and cortisol have a complex, dynamic relationship showing significant correlations in children [101]. On the other hand, negative correlation with IGF1 and IGF1-BP3 levels suggested that up-regulated genes in GHD children may be positively impacted by lower IGF1 and IGF1-BP3, which indicates that GH and IGF1/IGF1-BP3 act in a synergistic manner at the transcriptional level. This provides additional evidence to the mechanisms of IGF-1 acting as a negative feedback regulator of GH production. This is consistent with the synergistic roles of these three hormones in growth, and although it has been suggested that IGF-1 or IGF1-BP3 are surrogate markers and not an adequate substitute for the stimulation test in the diagnosis of GHD in children of short stature [102, 103], they both have been considered valuable as auxiliary indices for detection of GHD [104, 105].

The representative co-expressed genes and modules (1-6) are shown in Figure 9A. The functional enrichment of the modules along with the correlation matrix of the clinical features are shown in Figure 9B. Module 6 was the only module significantly positively correlated with GHD. We compared all genes in module 6 (113 genes) with the 347 probe sets (270 annotated genes) identified as GHD classifiers from a published study [106] but there was no overlap. This is possibly because the 270 genes from that study were ranked by significance in GHD severity and not by significance in differentiating GHS vs. GHD children used for the genes in our study.

To identify the key drivers and obtain a more detailed view of the important genes that affected pathways related to specific clinical features, in each module, we selected the most interconnected genes that were also DEGs (hub genes). In particular, the genes expressed at higher levels in GH-deficient children (113 genes in module 6) were strongly inter-connected with three interferon induced proteins: IFIT1, IFIT3 and OAS2. The stimulatory effect of GH on interferon has been shown before [92, 107]; however interferon has not yet been linked as a potential multi-scale, immune-endocrine-metabolic mediator for GHD.

DISCUSSION

Despite the exploration of signaling cascade of the GH-IGF1 axis to define the spectrum of growth disorders from GHD to GH excess and despite the extensive research with advanced molecular technologies of “omics” data, our understanding of the GH influence at the systems level is limited. In the current study, we utilized cutting-edge multi-omic technology and state-of-the-art data analytics to investigate in-depth the GH effect on the human molecular system and identify more reliable associations with GH status in children with growth failure.

Comparative and integrative analyses at the molecular and functional landscapes of whole transcriptome, metabolome and inflammatory

Legend to Figure 8. The GH-induced integrative gene-metabolite network.
The integrative gene-metabolite network was based on highly confident interactions utilizing the Network Explorer of MetaboAnalyst webtool. The 20 nodes with the highest degree of connectivity are noted along with their fold change magnitude after the 3 h GH stimulation test. Highly up-regulated metabolites were L-Arginine and hydroquinone whereas highly down-regulated were adenosine and cholic acid. Top down-regulated genes were *IFNG*, *SLC16A10* and *CYP3A4*.

Legend to Figure 9. Co-expressed gene modules and significant correlations with clinical traits.
A. Representative modules of co-expressed genes in the transcriptome changes from T0 to T3 generated by WGCNA and Cytoscape. **B**. The top 5 enriched pathways of each of the six modules and the correlation matrix of each module with clinical traits. The highlighted red blocks represent the significant positive correlations, whereas the highlighted blue blocks represent the significant negative correlations (p-value < 0.05 and correlation coefficient > 0.2 or < -0.2). Module 6 is the only module with significant positive correlation with GHD status.

proteome unveiled both known and unknown GH-induced molecules and functional mechanisms. The largest expression differences were displayed at the transcriptome landscape compared to the metabolome and proteome. This high-volume gene and low-volume metabolite/protein expression change pattern may reflect different temporal GH responses in the three landscapes. It may also reflect a molecular state past the GH peak level because in most children of this study, GH peaked at 2 or 2.5 hrs post GH stimulation (Figure 5) and not at the 3 hr sampling point (see STAR Methods and [108]). This was also shown in our prior study [17].

At the transcriptomic level, changes presented along the GH/IGF signaling axis. These included the JAK-STAT-activated *JUN, BMX* and *BCL2A1*, the IGF-activated *IRS-2* as well as the metabolic reprogramming genes, *G0S2* and *TGM3*; the latter were discussed for the first time within the GH context in this work. Suppressed genes included anti-inflammatory, neuro-protective and metabolic or aging-related genes, such as the IGF signaling molecules, *IRS-1* and *PIK3R3*, and the repressed genes *ALOX15, OLIG2* and *MTRNR2L6*; the latter were associated with GH for the first time in this study.

The metabolite and protein changes happened at a smaller extent compared to the gene expression changes, but they were centralized within similar immune, metabolic and neural pathways. These pathways consisted of cytokine signaling, various amino acid and lipid metabolic processes, and the potent neuro-transmitting pathways of serine (which suppresses ghrelin), glycine and threonine. More specifically, activated proteins included the JAK-STAT-induced OSM and IL10 as well as the mTOR target, EIF4EBP1, and the major angiogenesis factor, VEGFA. In accordance with the suppressed genes, suppressed proteins included a host of anti-inflammatory chemokines (CXCL10, CCL25, and CCL19) and TNF-family proteins (TNFRSF9, TNFSF10, and TNFSF12). At the metabolite level, there was pronounced activity of the GH-induced nitrogen metabolic pathway and a plethora of amino acid (myogenic), lipid (fat activation) and neuro-modulatory metabolic processes. These combined results illustrate the first comprehensive multi-omic study of the GH stimulation effects on the blood molecular panorama and demonstrate the concordant response of the neuro-endocrine, the immune, and the metabolic systems at the functional level.

Interestingly, comparing the molecular systems of GHD and GHS children, we did not observe any significant differences. This could be partly because most of the children in this study displayed GH peak levels before the 3 hr collection point, T3 (Figure 4). Another explanation is that the observed GH levels occurred in a continuous spectrum. With poor reproducibility, prepubertal children or children with obesity may have been falsely classified as GHD. Therefore, at the molecular level, the GH differential response of the GHD and GHS children may not have been fully captured at the T3 timepoint.

However, gene co-expression network analysis revealed a significant positive correlation between GH status and a cluster of genes with central hubs involving genes induced *via* interferon. Interferon gamma response was among the topmost altered protein pathways, and it was also one of the three hub genes (down-regulated) in the gene-metabolite interaction network. There, *INFγ* was linked to repressed amino acids (glycine, guanine) and over-expressed lipids (palmitic acid, linoleic acid and dodecanoic acid) consistent with the phenotype of children with growth failure. *IFNγ* seems to phosphorylate *JAK2* additively but independently to GH, and uniquely phosphorylates *STAT1* in IM-9 human blood cells [109]. Therefore collectively, we showed here that *IFNγ* is repressed upon GH stimulation (at gene level), it is highly interconnected with the metabolic system (at the gene-metabolite level connecting to repressed amino acids and over-expressed lipids), it is linked to the JAK-STAT pathways (at the gene and protein level), and it modulates critical hub-genes associated with GHD.

CONCLUSION

A strong link exists between the endocrine and immune systems. With this study, we define the GH-immune landscape and its complex molecular drivers at the systems level. Integrative statistical analysis of multi-omic data from 52 children with short stature before and after a GH stimulation test showed significant, concordant changes

imposed by GH. Both known and unknown GH-induced molecules and functional mechanisms were unveiled by comparative and integrative analyses at the molecular and functional landscapes of whole transcriptome, metabolome and inflammatory proteome. These changes involved signaling molecules along the GH/IGF axis, the JAK-STAT pathway, cytokines, and GH-induced nitrogen metabolism. Interferon signaling network drivers were correlated significantly with GH status demonstrating the prominent role of interferon at the nexus of the endocrine, immune and metabolic systems. In conclusion, this prominent role of the interferon signaling cytokines upon short term GH response to stimulation sheds a new light at the nexus of the immune, endocrine, and metabolic systems, and centralizes new efforts towards uncovering the GH effects on the human body at the systems level. Our findings utilizing state of the art omic techniques confirm and expand on the previously reported close interaction between GH and the immune system with interferon network playing a major role.

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AUTHOR CONTRIBUTIONS

The research study was conceptualized and supervised by J.T.D., R.R and B.A.K. Patient clinical evaluation and supervision was done by J.G. and R.R. E.G., M.Y. and J.G. facilitated the resources. Formal analysis, data curation and original writing – draft and editing was done by L.L. Every author listed contributed to writing – review and editing.

CONFLICT OF INTEREST STATEMENT

Loukia Lili and Joel T. Dudley are employees and shareholders of Thorne HealthTech outside the scope of this work. Christine Becker is a consultant for Thorne HealthTech outside the scope of this work. Brian A. Kidd is an employee and shareholder of Bristol Myers Squibb outside the scope of this work.

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