



ASSOCIATION STUDIES ARTICLE

# Protein QTL analysis of IGF-I and its binding proteins provides insights into growth biology

Eric Bartell<sup>1,2,†,‡</sup>, Masanobu Fujimoto<sup>3,4,†</sup>, Jane C. Khoury<sup>3,5</sup>, Philip R. Khoury<sup>6</sup>, Sailaja Vedantam<sup>2,7</sup>, Christina M. Astley<sup>1,2,7</sup>, Joel N. Hirschhorn<sup>1,2,7,‡</sup> and Andrew Dauber<sup>8,9,†,\*</sup>

<sup>1</sup>Department of Genetics, Harvard Medical School, Boston, MA 02115, USA, <sup>2</sup>Medical and Population Genetics, Broad Institute of MIT and Harvard, Cambridge, MA 02142, USA, <sup>3</sup>Division of Endocrinology, Cincinnati Children's Hospital Medical Center, University of Cincinnati College of Medicine, Cincinnati, OH 45229, USA, <sup>4</sup>Division of Pediatrics and Perinatology, Tottori University Faculty of Medicine, Yonago, Tottori 683-8504, Japan, <sup>5</sup>Division of Biostatistics and Epidemiology, Cincinnati Children's Hospital Medical Center, University of Cincinnati College of Medicine, Cincinnati, OH 45229, USA, <sup>6</sup>Heart Institute Research Core, Cincinnati Children's Hospital Medical Center, University of Cincinnati College of Medicine, Cincinnati, OH 45229, USA, <sup>7</sup>Division of Endocrinology, Boston Children's Hospital, Harvard Medical School, Boston, MA 02115, USA, <sup>8</sup>Division of Endocrinology, Children's National Hospital, Washington, DC 20010, USA and <sup>9</sup>Department of Pediatrics, George Washington University School of Medicine and Health Sciences, Washington, DC 20052, USA

\*To whom correspondence should be addressed. Tel: +1 2024762121; Email: [adauber@childrensnational.org](mailto:adauber@childrensnational.org)

## Abstract

The growth hormone and insulin-like growth factor (IGF) system is integral to human growth. Genome-wide association studies (GWAS) have identified variants associated with height and located near the genes in this pathway. However, mechanisms underlying these genetic associations are not understood. To investigate the regulation of the genes in this pathway and mechanisms by which regulation could affect growth, we performed GWAS of measured serum protein levels of IGF-I, IGF binding protein-3 (IGFBP-3), pregnancy-associated plasma protein A (PAPP-A2), IGF-II and IGFBP-5 in 838 children (3–18 years) from the Cincinnati Genomic Control Cohort. We identified variants associated with protein levels near *IGFBP3* and *IGFBP5* genes, which contain multiple signals of association with height and other skeletal growth phenotypes. Surprisingly, variants that associate with protein levels at these two loci do not colocalize with height associations, confirmed through conditional analysis. Rather, the *IGFBP3* signal (associated with total IGFBP-3 and IGF-II levels) colocalizes with an association with sitting height ratio (SHR); the *IGFBP5* signal (associated with IGFBP-5 levels) colocalizes

†Eric Bartell, <http://orcid.org/0000-0002-4380-1401>

†These authors contributed equally as co-first authors.

‡These authors contributed equally as co-senior authors.

§The authors wish it to be known that, in their opinion, the first 2 authors should be regarded as joint First Authors.

Received: March 25, 2020. Revised: May 21, 2020. Accepted: May 27, 2020

with birth weight. Indeed, height-associated single nucleotide polymorphisms near genes encoding other proteins in this pathway are not associated with serum levels, possibly excluding PAPP-A2. Mendelian randomization supports a stronger causal relationship of measured serum levels with SHR (for IGFBP-3) and birth weight (for IGFBP-5) than with height. In conclusion, we begin to characterize the genetic regulation of serum levels of IGF-related proteins in childhood. Furthermore, our data strongly suggest the existence of growth-regulating mechanisms acting through IGF-related genes in ways that are not reflected in measured serum levels of the corresponding proteins.

## Introduction

The insulin-like growth factor (IGF) system is known to play a role in growth (1) and disruption of the genes encoding growth hormone (GH1), insulin-like growth factor-1 (IGF1) or insulin-like growth factor-2 (IGF2) leads to a variety of growth disorders (2–4). In mice, knockout of *Igf1*, *Igf2*, or the gene encoding the IGF-I receptor (*Igf1r*) reduces birth weight and body size: birth weight is reduced to 40 and 60% for *Igf1* and *Igf2* knockouts, respectively (5–9). Furthermore, overexpression of *Igf2* results in overgrowth (8, 9). In humans, total IGF-I and total IGFBP-3 are used as clinical biomarkers in the assessment of children with short stature for potential growth hormone deficiency. IGF1 mutations lead to severe intrauterine growth restriction, postnatal growth failure and developmental delay (10), while IGF2 mutations have been shown to be associated with Silver-Russell Syndrome (11). For IGF signaling to occur, it has been shown that IGFs must be released from IGF binding proteins (IGFBPs), which, among other functions, sequester IGFs and prolong their half life (3, 12, 13). Some IGFBPs are also necessary for localization and transport of IGFs. Specifically, IGFBP-3 controls IGF-II concentration (14), binding between 90 and 96% of free IGF-I and IGF-II (15), and IGFBP-3 and IGF-II serum levels have been shown to be tightly correlated ( $r = 0.56$ ) in adults (16). Measurement of intact IGFBPs is complicated due to their cleavage by the proteases, pregnancy-associated plasma protein A (PAPP-A) and A2 (PAPP-A2). IGFBP cleavage allows IGF release, binding to their cell surface receptor, IGF-1R and downstream signaling. Multiple proteins in the IGF signaling pathway have been implicated by genome-wide association studies (GWAS) of both height and skeletal growth phenotypes (17–19); specifically, Wood *et al.* reported that height GWAS identifies loci at or near *IGF1*, *IGF2*, *IGFBP3*, *IGFBP5*, *PAPPA* and *PAPPA2*. GWAS of sitting height ratio (SHR), a measure of skeletal proportion, also implicate the *IGFBP3* locus. However, the mechanism by which the variants in these genes influence skeletal growth is still unknown. Further genetic study can offer insight into the interactions and shared underlying mechanisms of the GH system and human growth. Furthermore, genetic associations can enable causal inference and provide evidence regarding whether circulating levels of these proteins causally influence height and other skeletal growth phenotypes.

Previous work (20) described the distributions of serum concentrations for many of these proteins through childhood in a large cross-sectional cohort. In this report, we describe the distributions of IGF-II and IGFBP-5 levels and perform a GWAS of multiple circulating protein levels in participants from this same cohort to identify genetic variants that influence circulating protein levels during childhood (protein quantitative trait loci, pQTLs). We also explore the relationship between pQTLs for different growth-related proteins and skeletal growth phenotypes (including height and SHR), and identify surprising and distinct relationships between the genetic variants near genes encoding the growth-related proteins, the circulating protein levels and the skeletal growth phenotypes.

## Results

### IGF-II and IGFBP-5 levels throughout childhood

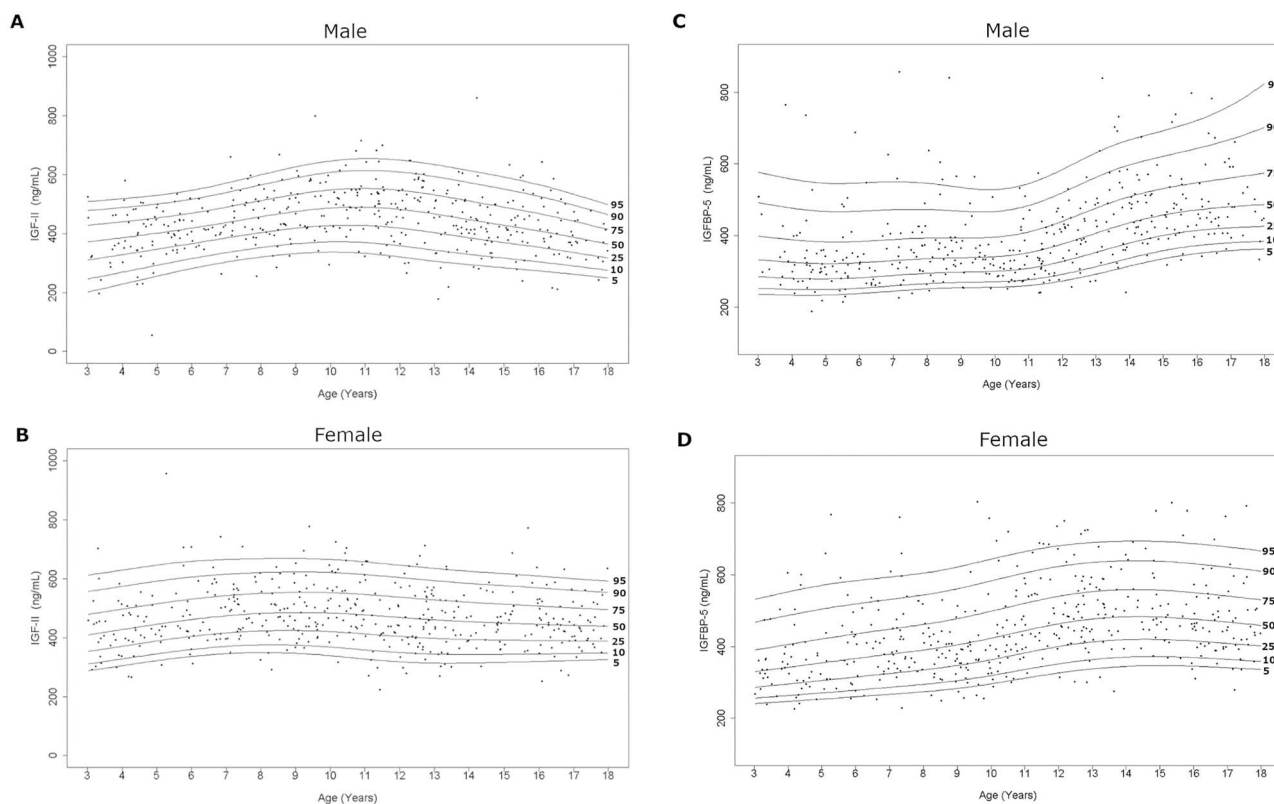
Our group has previously reported distributions of total and free IGF-I, PAPP-A2, total and intact IGFBP-3 throughout childhood as well as the correlation between these proteins (20). Herein, we present additional data describing IGF-II and IGFBP-5 levels in the same cohort. Serum IGF-II levels increased in childhood with a subsequent flattening of the curves (Fig. 1A and B, Supplementary Material, Table S3). Serum IGFBP-5 in females increased until approximately 13-years old and then flattened. Interestingly, serum IGFBP-5 levels in males remained flat until the age of 10–11 years and then progressively increased (Fig. 1C and D, Supplementary Material, Table S4).

Correlation analyses are presented in Table 1. IGF-II levels had a strong positive correlation with total IGFBP-3 in both sexes (M, F;  $r = 0.53, 0.51$ ;  $P < 0.0001$ ). However, IGF-II levels were weakly positively correlated with intact IGFBP-3 ( $r = 0.17$ ,  $P = 0.0006$ ) in males only. There were no significant correlations between IGF-II and height or BMI throughout childhood. There was no significant correlation between serum IGF-II and IGFBP-5 levels in either sex (Male, Female:  $r = -0.10$  ( $P = 0.05$ ),  $r = 0.07$  ( $P = 0.13$ ), respectively).

IGFBP-5 levels had a positive correlation with free and total IGF-I in both sexes (M, F; free IGF-I,  $r = 0.39, 0.41$ ; total IGF-I,  $r = 0.44, 0.51$ ; all  $P < 0.0001$ ). IGFBP-5 levels were significantly correlated with intact IGFBP-3 ( $r = 0.32$ ;  $P < 0.0001$ ) in both sexes. There were no significant correlations between IGFBP-5 and height or BMI throughout childhood.

### Genetic analyses

**Genome-wide association studies for protein levels.** GWAS was performed for each of the seven phenotypes (Free IGF-I, Total IGF-I, IGF-II, IGFBP-5, Total IGFBP-3, Intact IGFBP-3 and PAPP-A2) as well as two derived ratio phenotypes (Free/Total IGF-I, Intact IGFBP-3/Total IGFBP-3) and two conditional phenotypes (IGF-II conditioned on Intact IGFBP-3, IGF-II conditioned on Total IGFBP-3). This was done for two populations (African American and European American) for each sex strata (sex-combined, male, female), while adjusting for covariates described in Materials and Methods section. Directly genotyped or imputed single nucleotide polymorphisms (SNPs) were analyzed in the GWAS. Fixed-effects meta analysis was performed using METAL to combine GWAS results across the two populations, and likely independent SNPs were selected as having the best association at each 1 Mb genomic locus ('lead SNPs'). In total, we identified 17 independent lead SNPs with association  $P$  values below a commonly used genome-wide significance threshold of  $5 \times 10^{-8}$  (Supplementary Material, Table S1). Strongest associations were identified at the rs2854746 SNP within the *IGFBP3* locus, associated with both IGF-II and Total IGFBP-3 levels, and the rs11575194 SNP within the *IGFBP5* locus, associated with IGFBP-5 levels. The rs2854746 variant is a previously known expression QTL (eQTL) and protein QTL (pQTL) (21, 22) [also reported in



**Figure 1.** Serum IGF-II and IGFBP-5 curves in the study population. Age distribution of serum IGF-II and IGFBP-5 levels in males (A, C) and females (B, D) are represented respectively. The curves of the 5th, 10th, 25th, 50th, 75th, 90th and 95th percentiles calculated by the BCT method are displayed.

(23–27)], associated with levels of *IGFBP3* expression and *IGFBP3* in serum but has not to our knowledge been previously associated with IGF-II levels. By conditioning on *IGFBP3* levels, we show the novel rs2854746 association with IGF-II is independent of intact *IGFBP3* and is attenuated but remains significant when conditioning on total *IGFBP3* (Supplementary Material, Fig. S2). The rs11575194 variant is a known pQTL for *OSBPL11* (28, 29) but has not to our knowledge been reported to be associated with *IGFBP5* levels. 17 additional associations reached the genome-wide significance threshold, across a range of phenotypes as described in Supplementary Material, Table S1.

**Overlap with findings from GWAS of skeletal growth phenotypes.** GWAS was performed for standing height (referred to simply as ‘height’), SHR (sitting height to standing height ratio) and birth weight using data from the UKBiobank (see Materials and Methods). The rs2854746 SNP, which is associated with total *IGFBP3* and IGF-II levels, lies near the *IGFBP3* gene and is also significantly associated with both height ( $P = 5.0 \times 10^{-12}$ ) and SHR ( $P = 3.8 \times 10^{-69}$ ), but not with birth weight. However, although rs2854746 was also the strongest associated SNP for SHR in the region, it was only weakly correlated ( $R^2 = 0.0045$ ,  $0.0001$  respectively) with the two lead height SNPs in the region (rs11455214,  $P = 1.7 \times 10^{-25}$ , rs723149,  $P = 3.2 \times 10^{-50}$ ), consistent with earlier suggestions that these might reflect different signals at the same locus (19). The rs11575194 SNP, which is associated with *IGFBP5* levels, lies near the *IGFBP5* gene, and is associated with birth weight ( $P = 3.4 \times 10^{-9}$ ) but not with height or SHR, despite the fact that other SNPs near *IGFBP5* are strongly associated with height (lead SNP is rs2241192,  $P = 1.3 \times 10^{-14}$ ,  $R^2 = 0.0004$ , rs1478575,  $P = 8.9 \times 10^{-58}$ ,  $R^2 = 0$  in 1000 Genomes).

To look for other phenotypes that could be influenced by the levels of IGF-related proteins, we also performed an association study across many other phenotypes (pheWAS) using the T2Dportal (30, 31), the UKB pheWEB (<http://pheweb.sph.umich.edu/>) (Supplementary Material, Fig. S3) and the GWAS catalog (32) for the lead SNPs affecting total *IGFBP3* and *IGFBP5* levels. Although IGF-related proteins have been suggested as risk factors for cancer, neither lead SNP has been identified in the GWAS catalog as being strongly associated with colorectal, breast or prostate cancers. The only associations surviving multiple testing correction (besides skeletal growth phenotypes) were between rs2854746 in *IGFBP3* and multiple measures of bioelectrical impedance (legs, whole body and arms), as well as diastolic and systolic blood pressures.

**Conditional analyses to distinguish association signals at the *IGFBP3* and *IGFBP5* loci.** Although the weak correlations between lead SNPs for height and total *IGFBP3* levels suggest that these associations are distinct, the presence of multiple signals of association at a locus can complicate interpretation of simple correlation measures between lead SNPs. To more formally evaluate whether the associations at the *IGFBP3* locus with height and total *IGFBP3* levels actually reflect distinct associations, and hence distinct mechanisms, we performed serial conditional analyses. For each phenotype, we performed association analysis for the SNPs across the locus, conditioning on the lead SNP for that phenotype, and then continued to condition on the strongest remaining SNP associated with that phenotype until no genome-wide significant signal of association remained at the locus (see Materials and Methods). When conditioning in turn on each of two total *IGFBP3* associated independent SNPs

Table 1. Correlation analyses between IGF-II, IGFBP-5 and other variables and regression with height and BMI

Variable	IGFBP-5	Free IGF-I <sup>a</sup>	Total IGF-I <sup>a</sup>	Intact IGFBP-3	Total IGFBP-3	PAPP-A2 <sup>a</sup>	Free/total IGF-I <sup>a</sup>	Height z-score	BMI z-score
<b>Males</b>									
IGF-II (N = 405)	r P value	-0.15 (0.003)	0.07 (0.17)	0.17 (0.0006)	0.53 (<0.0001)	0.02 (0.65)	-0.19 (0.0002)	-0.015 (0.77)	-0.007 (0.89)
IGFBP-5 (N = 405)	r P value	0.39 (<0.0001)	0.44 (<0.0001)	0.32 (<0.0001)	0.008 (0.87)	-0.14 (0.004)	-0.009 (0.86)	0.12 (0.02)	-0.02 (0.71)
<b>Females</b>									
IGF-II (N = 431)	r P value	0.06 (0.22)	-0.05 (0.27)	0.04 (0.57)	0.51 (<0.0001)	0.12 (0.02)	0.10 (0.03)	-0.028 (0.56)	-0.045 (0.35)
IGFBP-5 (N = 431)	r P value	0.41 (<0.0001)	0.51 (<0.0001)	0.32 (<0.0001)	0.18 (0.0001)	-0.10 (0.04)	-0.04 (0.37)	0.04 (0.37)	-0.029 (0.55)

<sup>a</sup>The natural log-transformation was used for statistical analysis of free and total IGF-I, PAPP-A2 and free/total IGF-I ratio.

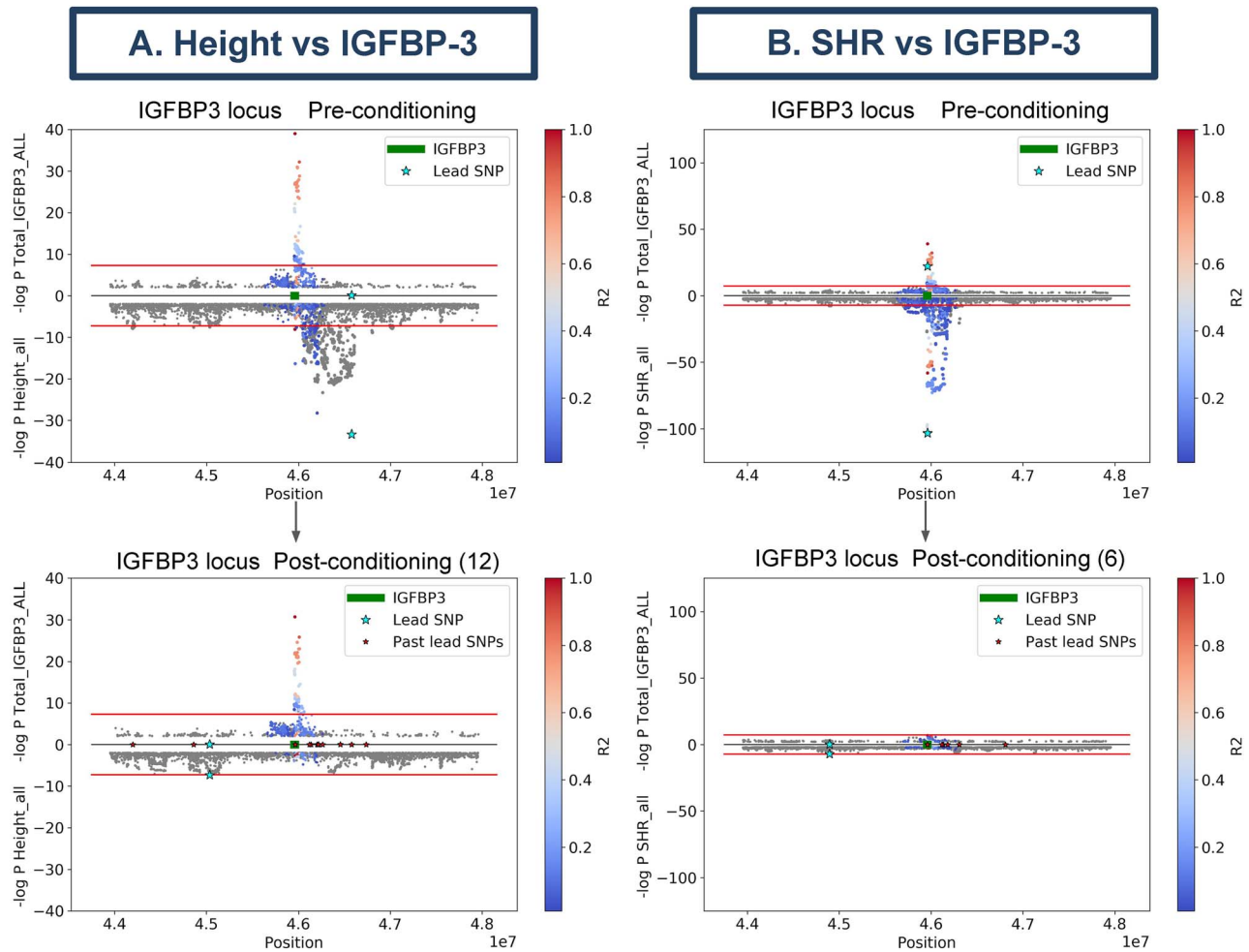
Note: Bivariate Pearson correlation analyses were performed. The *r* means the Pearson correlation coefficient. A *P*-value less than 0.05 was considered statistically significant.

in the *IGFBP3* locus, we observed minimal change in the strength of the height associations in the region. Similarly, when conditioning in turn on 13 height-associated SNPs, we observe that associations with total IGFBP-3 protein levels remained robust (Fig. 2A, Supplementary Material, Fig. S4A and B). In contrast, when we performed similar analyses comparing associations of total IGFBP-3 levels and SHR, the signals associated with SHR were dramatically attenuated when conditioning on the two total IGFBP-3 associated SNPs, and, comparably, total IGFBP-3 association dramatically attenuated when conditioning on the six SHR associated SNPs (Fig. 2B, Supplementary Material, Fig. S4C and D). Together, these analyses indicate that the associations with height at the *IGFBP3* locus are independent of associations with serum total IGFBP-3 protein levels but the effects on SHR and total IGFBP-3 levels likely represent the same signal.

We performed similar serial conditional analyses at the *IGFBP5* locus to examine the relationship between the associations with IGFBP-5 levels, height and birth weight. The signal of association with IGFBP-5 is vastly attenuated when conditioning birth weight signals in the region (and vice versa), suggesting that these reflect the same association signal (Fig. 2D and Supplementary Material, Fig. S4F). In contrast, when we serially conditioned on 18 height signals in the region, we observed minimal attenuation of the IGFBP-5 associations, and conditioning on IGFBP-5 associated SNPs did not substantially attenuate the height signals (Fig. 2C, Supplementary Material, Fig. S4E). Thus, similar to our observations at the *IGFBP3* locus, these results indicate that associations with height are distinct from the associations with serum levels of IGFBP-5, but the serum levels are associated with a distinct skeletal growth phenotype (in this case, birth weight).

Genetic studies of height have identified signals near other genes encoding IGF-related proteins (17, 18, 33). To further explore the relationship between serum levels of IGF-related proteins and height association signals near the genes encoding these proteins, we focused on the height-associated SNPs in the loci containing the *IGF1*, *IGFBP3*, *PAPPA2*, *IGF2* and *IGFBP5* genes. We tested nine common genome-wide significant height SNPs for association with protein levels. Results of each SNP association are presented in Supplementary Material, Table S2. The only significant association (after correction for multiple comparisons) was between the rs1044299 SNP at the *PAPPA2* locus and serum PAPP-A2 levels ( $P = 6.85 \times 10^{-5}$ ). Conditioning on one of the height signals near *PAPPA2* (rs1040457) vastly attenuated this association with PAPP-A2 serum levels; however, the most significant height lead SNP (rs10913200) is independent of the association with PAPP-A2 levels (Supplementary Material, Fig. S5). Thus, as with *IGFBP3* and *IGFBP5* the effects on height of variants at the other IGF-related genes are not mediated by serum levels, with the possible exception of one variant near *PAPPA2*.

**Mendelian randomization.** To evaluate causal relationships between identified associations at *IGFBP3* and *IGFBP5* and skeletal growth phenotypes, we performed Mendelian randomization (MR). In this method, a SNP associated with the proposed causal protein level (exposure) is used as an instrumental variable (IV) to test the exposure's effect on the anthropometric trait (outcome). We first used rs2854746 as an IV for total IGFBP-3 levels (before and after conditioning on other height signals in the region). After conditioning, we show increasing total IGFBP-3 levels by 1 standard deviation (SD) is associated with a 0.061 SD increase in SHR [95% CI: (0.053, 0.069)] and a much smaller



**Figure 2.** Conditional analysis. Serial conditional analyses were performed comparing (A) total IGFBP-3 association signal with height associations in the region. After conditioning on 12 nearby (2 MB window) height lead SNPs, SNPs in the region remain associated with total IGFBP-3 levels. (B) Total IGFBP-3 with SHR. After conditioning on six nearby SHR lead SNPs, SNP association with total IGFBP-3 levels is attenuated. (C) IGFBP-5 with height. After conditioning on 18 nearby height lead SNPs, SNPs in the region remain associated with IGFBP-5 levels. (D) IGFBP-5 with birth weight. After conditioning on the one nearby BW lead SNP, SNP association with IGFBP-5 levels is attenuated.

0.010 SD decrease in height [95% CI (−0.018, −0.0023)] (Fig. 3A). Similarly, we use rs11575194 as the IV for IGFBP-5 levels (before and after conditioning on other height signals in the region). After conditioning, we show increasing IGFBP-5 levels by 1 SD is associated with a 0.027 SD decrease in birth weight [95% CI (−0.036, −0.018)] and a smaller 0.010 SD decrease in height [95% CI (−0.017, −0.004)]; the IGFBP-5—birth weight relationship may act through correlated measures such as fetal serum levels. These results suggest that the main detectable causal effects of variation in serum levels of total IGFBP-3 and IGFBP-5 in childhood are on skeletal proportion (SHR) and birth weight, respectively (Fig. 4).

## Discussion

The GH and IGF regulatory system is critical for understanding biology driving human growth, height and related phenotypes. Recent work has explored IGF protein interactions and their regulation of height-relevant biology, across multiple age ranges (20, 34, 35). In this study, we performed GWAS of measured serum levels of IGF-related proteins throughout childhood, and identified 17 associated genetic loci. These included strong effects

at the IGFBP3 locus on total IGFBP-3 and IGF-II levels and at the IGFBP5 locus on IGFBP-5 levels. We leveraged genetic variation through MR to assess evidence of causality between measured serum levels with mechanisms driving height and related phenotypes. Here, we provide evidence that serum levels of IGF-II, total IGFBP-3 and IGFBP-5 do not appear to be causal for height but instead have evidence for causality for other skeletal growth phenotypes, notably total IGFBP-3 and/or IGF-II with SHR and IGFBP-5 with birth weight.

Normal levels of GH-IGF axis-related proteins are well described in adults and through childhood. Large population based studies have examined the concentrations of serum total IGF-I and total IGFBP-3 throughout the lifespan (36, 37). Our group has measured serum levels of PAPP-A2 and GH-IGF axis-related proteins (now including IGF-II and IGFBP-5) throughout childhood, and performed correlation analyses of all the analytes to investigate the interplay between the various proteins.

IGF-related proteins are known to play a role in regulation of growth and are canonically understood to affect human height; as such, changes in levels or function would logically be assumed to affect overall growth and therefore adult height. Because

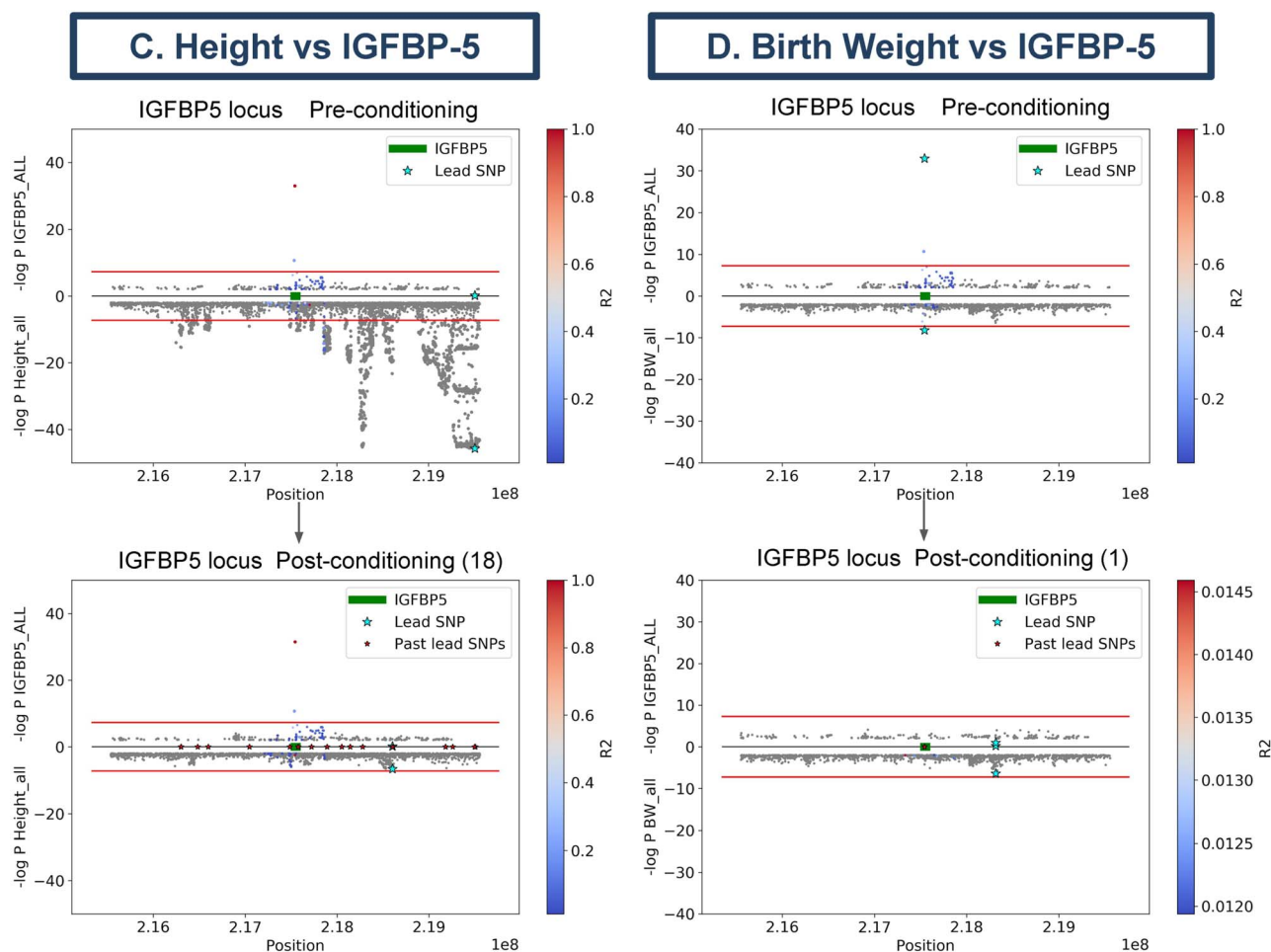


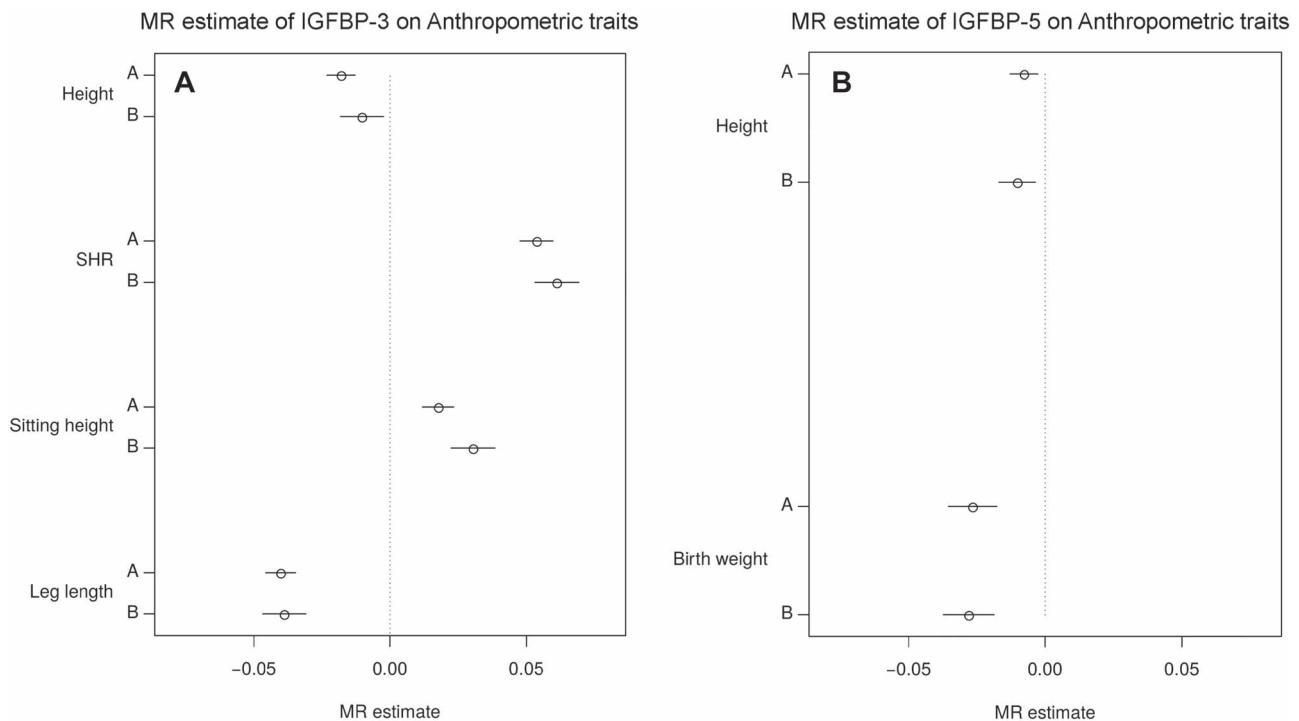
Figure 2. continued c,d.

genetic factors affect both protein levels and measures of human growth, genetic studies can be used to gain insights into the relationships between serum protein levels and skeletal growth. In the GWAS performed in this study, we confirm prior observations that there are signal(s) of association with height as well as with other skeletal growth phenotypes near the *IGFBP3* locus. Surprisingly, the variant associated with serum levels of total IGFBP-3 and IGF-II is far more strongly associated with adult SHR than with adult height; indeed, as suggested by earlier studies (19) our conditional analyses indicate that while SHR and protein levels share a common association at the *IGFBP3* locus, the associations with height and total IGFBP-3/IGF-II protein levels are independent of each other. These findings support a surprising conclusion, wherein serum levels of total IGFBP-3, IGF-II or other unmeasured correlated variables causally affect predominantly SHR, rather than height (shown graphically in Figure 4a). It follows that variation in human height is not caused by total IGFBP-3 nor IGF-II levels, as measured in the serum. Further, because there are at least 13 variants clustered near the *IGFBP3* gene that are independently associated with height but not with total IGFBP-3 levels, our results suggest that regulation of expression of *IGFBP3* somewhere in the body does influence overall height, but that *IGFBP3* expression in this as yet undefined place (or time) in the body does not correlate with measured serum levels of total IGFBP-3 in childhood. Future examination of the regulatory elements near the *IGFBP3* variants

associated with height should provide clues as to the time(s) and place(s), where total IGFBP-3 influences overall growth.

It is also curious that the variant at *IGFBP3* that is associated with serum levels affects SHR but not overall height—although many variants affect both SHR and height, some, like this *IGFBP3* variant, only have a detectable effect on SHR. Because overall height is composed of sitting height (head + spine) and leg length, the effect on SHR without an effect on overall height means that the variant must have symmetric and opposing effects on head + spine and leg length. We are not aware of an obvious mechanism for such coordinated and opposing effects on skeletal growth, although one could postulate a mechanism that somehow regulates overall height, meaning that an effect on one component of height (for example on leg length) could cause an indirect compensation reflected in the other component of height (for example, head + spine). Alternatively, total IGFBP-3 or IGF-II serum levels (or something correlated with these) could have two independent and opposing effects on head + spine and leg length that just happen to cancel each other out in their effect on total height.

Interestingly, the variant associated with total IGFBP-3 levels is also known to influence expression of *IGFBP3* mRNA in multiple tissues (28). However, these tissues (heart—left ventricle, thyroid, muscle—skeletal, lung, brain—cerebellum, artery—aorta, artery—tibial, esophagus—muscularis) (28) do not include the liver, which is presumed to be the main source of circulating



**Figure 3.** Mendelian randomization. MR was performed using the Wald method; rs2854746 was used as an instrumental variable (IV) for total IGFBP-3 levels and rs11575194 was used as an IV for IGFBP-5 levels. GWAS of both protein levels and anthropometric traits was performed before (A analyses) and after (B analyses) conditioning on nearby height SNPs as a sensitivity analysis for confounding LD. (A) MR analyses show change in total IGFBP-3 levels correlate to more SD changes in SHR than in height. (B) MR analyses show change in IGFBP-5 levels correlate to more SD changes in birth weight than in height.

IGFBP-3. Furthermore, this variant is associated only with total circulating IGFBP-3 but not with intact IGFBP-3 levels, which are thought to reflect the biologically active form of IGFBP-3. Because this variant influences SHR, these observations collectively raise the possibility that non-intact forms of IGFBP-3 may affect skeletal proportion either directly or indirectly (for example through IGF-II levels or other correlated measures) and that the relevant expression of IGFBP-3 is not mediated through changes in *IGFBP3* expression in the liver.

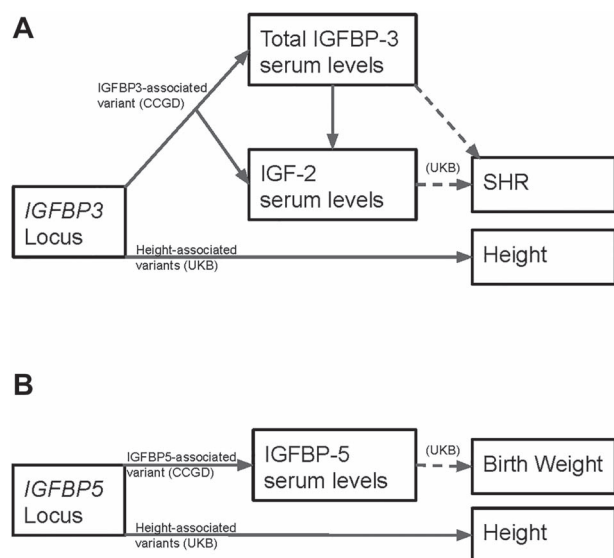
We also observed variation at the *IGFBP5* gene locus that is associated with IGFBP-5 levels. Like *IGFBP3*, the *IGFBP5* locus is known to be associated with human height (17, 18). However, as we observed for the *IGFBP3* locus, the associations with height and IGFBP-5 protein levels are independent, as shown by conditional analysis; this indicates circulating serum levels of IGFBP-5 are likely not mediating the effect of IGFBP-5 on height. In contrast to variation at the *IGFBP3* locus, the variation at *IGFBP5* that influences protein levels is not associated with SHR; however, it is associated with birth weight, suggesting that serum IGFBP-5 levels are important for prenatal growth through a mechanism that is distinct from the influence of IGFBP-5 on adult height. This is shown graphically in Figure 4b. As with *IGFBP3*, the influence of variation in *IGFBP5* expression on height must occur at a time and/or place that is not reflected in circulating protein levels in childhood.

We also performed an analysis of the lead height-associated SNPs near the remaining genes encoding IGF-related proteins in our study. None of the lead height SNPs was significantly associated with serum levels of the proteins, with the exception of one variant near *PAPPA2*. Again, this suggests that serum levels of these proteins during childhood are not necessarily determinative of adult height. These results do not mean that

these proteins cannot serve as clinical biomarkers but it does imply the existence of mechanisms for these genetic associations with height that are not reflected in measured serum proteins.

Beyond the signals at *IGFBP3* and *IGFBP5*, 17 additional genome-wide associations were identified with IGF-related protein levels. None had clear colocalization with either the genes encoding these proteins or other skeletal growth phenotypes, but may provide clues as to the regulation of these proteins. To allow further exploration of these results, we will provide access to a genome-wide set of summary association statistics.

This study has a number of limitations. First, the sample size for serum protein associations is small for a GWAS study, although the presence of highly significant associations with large effects on measured serum levels (as has been seen in other studies) shows that we had adequate power to successfully identify these strong associations. Larger sample sizes would also allow for additional analyses, such as estimates of heritabilities and genetic correlations for serum protein levels, as well as identification of variants with more modest effects. Second, we have not replicated these findings in a second independent study, so it is possible the associations we discover are a result of uncorrected confounders. The colocalization of associations with other, relevant phenotypes measured in independent studies supports the validity of these findings; however, replication in an independent study of the protein level associations would be valuable. Third, we provide evidence that some association signals overlap (such as total IGFBP-3 serum levels and SHR), but methods to rigorously evaluate colocalization (38) or genetic correlation (39) are limited in small samples and these analyses are further complicated by the presence of many independent



**Figure 4.** Schematic. (A) The *IGFBP3* locus is associated with both height and SHR. The total IGFBP-3 association, with lead SNP rs2854746, colocalizes with the SHR association but not the height association. As such, it is possible total IGFBP-3 serum levels and possibly IGF-II serum levels modulate SHR but not height, and other regulatory mechanisms of *IGFBP3* in other tissues or other time points are responsible for *IGFBP3*'s association with height. Dashed lines mark interactions for which there is most evidence in MR analyses. (B) Similarly, the *IGFBP5* locus is associated with both height and birth weight. The IGFBP-5 association, with lead SNP rs11575194, colocalizes with the birth weight association. Therefore, it is possible the *IGFBP5* locus affects birth weight through IGFBP-5 serum levels, and interacts with height not through serum levels, but rather in some other tissues or time points.

associations in each region. We also did not have parental genotypes, so could not directly analyze parent-of-origin effects, which decreases power at imprinted loci (for example, the *IGF2* gene is imprinted).

The GWAS analysis was corrected for population structure in the data using principal components (PC) of genetic ancestry, and our sample was stratified by ancestry before meta-analysis, but there may still be confounding from residual population stratification reflected in the summary statistics. In addition, data from this study were largely derived from European descent individuals (both in the UKB and Cincinnati cohorts), limiting the generalizability to non-European ancestry; it would be beneficial to expand these analyses of serum protein levels to other ancestries.

Because the rs2854746 in *IGFBP3* is a missense variant, it is theoretically possible that this variant affects binding of the ELISA probe, and is not actually associated with circulating serum protein levels. However, the amino acid altered by rs2854746 does not overlap the probe binding location, and the association with IGF-II levels is strongly indicative of a true biological effect, rather than a laboratory artifact. As with all GWAS associations, functional follow-up of this and the other loci we identified will be necessary to elucidate the specific mechanism of action of these findings.

In conclusion, this study identifies strong genetic associations with measured serum levels of IGF-related proteins, particularly between IGF-II and total IGFBP-3 serum protein levels and variation at the *IGFBP3* locus, and between IGFBP-5 protein levels and variation at the *IGFBP5* locus. These associations are distinct from and independent of the associations with adult height that have been observed at these loci. Rather, the *IGFBP3* variant rs2854746 is strongly associated with SHR, and

the *IGFBP5* variant rs11575194 is associated with birth weight. These findings suggest that genetically encoded variation in *IGFBP3* and *IGFBP5* expression affects height in ways that are as yet unknown but are independent of measured serum levels during childhood (for example, local paracrine effects in the growth plate or developing skeleton, or effects on serum levels in fetal life or infancy). In contrast, effects on skeletal proportion and birth weight may be more directly mediated through measured serum levels of total IGFBP-3 and IGFBP-5, respectively (and/or by correlated phenotypes, such as IGF-II levels). Further functional studies are needed to elucidate the mechanism of effect of variants in these genes on height, skeletal proportion and birth weight, and specifically whether and how their effects are mediated by local or systemic changes in the level or activity of growth factor-related proteins, possibly at specific times in development. Taken together, these findings challenge our understanding of how well-known growth factors affect skeletal growth and proportion in children.

## Materials and Methods

### Study population and design of the Cincinnati Genomic Control Cohort

Subject selection for this cohort has been previously described (20, 40–42). The study subjects and data were derived from the Cincinnati Genomic Control Cohort (CGCC), which is a community-based cross-sectional cohort comprised of 1020 children representative in terms of race, ethnicity, gender and socioeconomic status of the seven counties in Ohio and Kentucky surrounding Cincinnati. Recruitment and sample collection was completed in 2010. 143 children were excluded from the current study due to insufficient blood samples. 39 were excluded due to severe malnutrition (BMI Z-score < or > 3.0 SD), severe short stature (Height Z-score < -3.0 SD), overgrowth (Height Z-score > 3.0 SD), chronic diseases and pregnancy leaving a total of 838 children for whom blood analyte measurements were available. Of these, 815 individuals passed quality genotype quality filters: 415 (51%) were female and 400 (49%) were male; 691 (85%) were identified as ancestrally European and 124 (15%) were identified as ancestrally African American. The study was reviewed and approved by the institutional review board at Cincinnati Children's Hospital Medical Center (CCHMC).

### Anthropometric data and samples

All participants had their height and weight measured by a single investigator as previously described (20). The datasets of CDC 2000 growth charts were used to calculate age- and sex-adjusted Z-scores (43). Blood samples were taken from the participants to obtain serum and genomic DNA at once. Serum samples were isolated with a standard coagulation and centrifuge protocol and frozen at -80°C within 2 h of collection (20). The serum samples were stored at -80°C for more than 8 years without thawing.

### IGF-related protein measurements

Serum concentrations of IGF-II, IGFBP-5, free and total IGF-I, intact and total IGFBP-3, and PAPP-A2 were measured using commercially available enzyme-linked immunosorbent assays (ELISA) kits (AL-121, 122, 149, 120, 109, 131 and 127; Ansh labs, TX, USA) as previously published (20, 44). The limits of detection and limits of quantification, imprecision, linearity and recovery were determined for each assay. The IGF-II assay demonstrates no significant (<0.1%) cross-reactivity with IGFBP-2, -3, -4, -5 and IGF-I to 1000 ng/ml. The IGFBP-5 assay demonstrates no





rd.edu/cgi-bin/qtlassoc.cgi) (55) shown in Supplementary Material, Figure S6. Power estimates were generated for multiple true causal SNP effect sizes (percent variance explained, PVE) and sample sizes, and effect estimates of lead SNPs associated with total IGFBP-3 levels and IGFBP-5 levels were included.

### Statistical analysis for association between the dependent variables

As previously described (20), data were imported into SAS®, version 9.4 (SAS Institute, Cary NC), for data management and analysis except for GWAS analysis. Continuous data were examined for distributional properties and potential outlying values. After outliers removed, three of the dependent variables, free IGF-I, PAPP-A2 and total IGF-I were transformed using the natural logarithm for analysis. General linear models were used to examine changes over age for the dependent variables and differences by sex and the interaction of sex and age. Pearson correlation was used to examine the association between the dependent variables. A P-value of <0.05 was considered statistically significant.

### Supplementary Material

Supplementary Material is available at HMG online.

### Acknowledgements

The authors thank Lisa Martin and Susan Thompson and the entire Cincinnati Genomics Control Cohort team for providing the samples for the study. The authors also thank Melissa Andrew and Leah Tyzinski for their assistance with sample handling. Furthermore, the authors thank Joanne Cole for her help guiding GWAS analyses and Yu-han Hsu, Rebecca Fine and Tiffany Amariuta for many useful discussions.

*Conflict of Interest Statement:* Joel Hirschhorn is a Board Member for Camp4 Therapeutics. Besides this, no Conflicts of Interest to report.

### Funding

NIDDK (R01DK075787 to J.N.H.); NHGRI (5T32HG002295 to E.R.B.); NIDDK (K23DK120899 for C.M.A.).

### References

- Ranke, M.B. and Wit, J.M. (2018) Growth hormone-past, present and future. *Nat. Rev. Endocrinol.*, **14**, 285–300.
- Storr, H.L., Chatterjee, S., Metherell, L.A., Foley, C., Rosenfeld, R.G., Backeljauw, P.F., Dauber, A., Savage, M.O. and Hwa, V. (2019) Nonclassical GH insensitivity: characterization of mild abnormalities of GH action. *Endocr. Rev.*, **40**, 476–505.
- David, A., Hwa, V., Metherell, L.A., Netchine, I., Camacho-Hübner, C., Clark, A.J.L., Rosenfeld, R.G. and Savage, M.O. (2011) Evidence for a continuum of genetic, phenotypic, and biochemical abnormalities in children with growth hormone insensitivity. *Endocr. Rev.*, **32**, 472–497.
- Savage, M.O., Hwa, V., David, A., Rosenfeld, R.G. and Metherell, L.A. (2011) Genetic defects in the growth hormone-IGF-I axis causing growth hormone insensitivity and impaired linear growth. *Front. Endocrinol. (Lausanne)*, **2**, 95.
- Liu, J.P., Baker, J., Perkins, A.S., Robertson, E.J. and Efstratiadis, A. (1993) Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). *Cell*, **75**, 59–72.
- Powell-Braxton, L., Hollingshead, P., Warburton, C., Dowd, M., Pitts-Meek, S., Dalton, D., Gillett, N. and Stewart, T.A. (1993) IGF-I is required for normal embryonic growth in mice. *Genes Dev.*, **7**, 2609–2617.
- DeChiara, T.M., Efstratiadis, A. and Robertsen, E.J. (1990) A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting. *Nature*, **345**, 78–80.
- Baker, J., Liu, J.P., Robertson, E.J. and Efstratiadis, A. (1993) Role of insulin-like growth factors in embryonic and postnatal growth. *Cell*, **75**, 73–82.
- Sferruzzi-Perri, A.N., Owens, J.A., Pringle, K.G. and Roberts, C.T. (2011) The neglected role of insulin-like growth factors in the maternal circulation regulating fetal growth. *J. Physiol.*, **589**, 7–20.
- Woods, K.A., Camacho-Hübner, C., Savage, M.O. and Clark, A.J.L. (1996) Intrauterine growth retardation and postnatal growth failure associated with deletion of the insulin-like growth factor I gene. *N. Engl. J. Med.*, **335**, 1363–1367.
- Begemann, M., Zirn, B., Santen, G., Wirthgen, E., Soellner, L., Büttel, H.M., Schweizer, R., Van Workum, W., Binder, G. and Eggermann, T. (2019) Paternally inherited IGF2 mutation and growth restriction. *N. Engl. J. Med.*, **373**, 349–356.
- Bach, L.A. (2018) 40 years of IGF1: IGF-binding proteins. *J. Mol. Endocrinol.*, **61**, T11–T28.
- Baxter, R.C. (2000) Insulin-like growth factor (IGF)-binding proteins: interactions with IGFs and intrinsic bioactivities. *Am. J. Physiol. Endocrinol. Metab.*, **278**, E967–E976.
- Ismayilnadjatmeymurabadi, H. and Konukoglu, D. (2018) The relationship between IGF-2, IGFBP-2, and IGFBP-3 levels in patients suffering from pre-diabetes. *J. Biol. Regul. Homeost. Agents*, **32**, 63–68.
- Jones, J. I. and Clemmons, D. R. (1995) Insulin-like growth factors and their binding proteins: biological actions. *Endocr Rev.*, **16**, 3–34.
- Probst-Hensch, N.M., Yuan, J.M., Stanczyk, F.Z., Gao, Y.T., Ross, R.K. and Yu, M.C. (2001) IGF-1, igf-2 and IGFBP-3 in prediagnostic serum: association with colorectal cancer in a cohort of Chinese men in Shanghai. *Br. J. Cancer*, **85**, 1695–1699.
- Wood, A.R., Esko, T., Yang, J., Vedantam, S., Pers, T.H., Gustafsson, S., Chu, A.Y., Estrada, K., Luan, J., Kutalik, Z. et al. (2014) Defining the role of common variation in the genomic and biological architecture of adult human height. *Nat. Genet.*, **46**, 1173–1186.
- Yengo, L., Sidorenko, J., Kempner, K.E., Zheng, Z., Wood, A.R., Weedon, M.N., Frayling, T.M., Hirschhorn, J., Yang, J., Visscher, P.M. et al. (2018) Meta-analysis of genome-wide association studies for height and body mass index in ~700000 individuals of European ancestry. *Hum. Mol. Genet.*, **27**, 3641–3649.
- Chan, Y., Salem, R.M., Hsu, Y.H.H., McMahon, G., Pers, T.H., Vedantam, S., Esko, T., Guo, M.H., Lim, E.T., Franke, L. et al. (2015) Genome-wide analysis of body proportion classifies height-associated variants by mechanism of action and implicates genes important for skeletal development. *Am. J. Hum. Genet.*, **96**, 695–708.
- Fujimoto, M., Khoury, J.C., Khoury, P.R., Kalra, B., Kumar, A., Sluss, P., Oxvig, C., Hwa, V. and Dauber, A. (2020) Anthropometric and biochemical correlates of PAPP-A2, free IGF-I, and IGFBP-3 in childhood. *Eur. J. Endocrinol.*, **182**, 363–374.

21. Kaplan, R.C., Petersen, A.-K., Chen, M.-H., Teumer, A., Glazer, N.L., Dö Ring, A., Lam, C.S.P., Friedrich, N., Newman, A., Müller, M. et al. (2011) A genome-wide association study identifies novel loci associated with circulating IGF-I and IGFBP-3. *Hum. Mol. Genet.*, **20**, 1241–1251.
22. Machiela, M.J. and Chanock, S.J. (2015) Genetics and population analysis LDlink: a web-based application for exploring population-specific haplotype structure and linking correlated alleles of possible functional variants. *Bioinformatics*, **31**, 3555–3557.
23. Deal, C., Ma, J., Wilkin, F., Paquette, J., Rozen, F., Ge, B., Hudson, T., Stampfer, M. and Pollak, M. (2001) Novel promoter polymorphism in insulin-like growth factor-binding Protein-3: correlation with serum levels and interaction with known regulators 1. *J. Clin. Endocrinol. Metab.*, **86**, 1274–1280.
24. Canzian, F., McKay, J.D., Cleveland, R.J., Dossus, L., Biessy, C., Rinaldi, S., Landi, S., Boillot, C., Monnier, S., Chajès, V. et al. (2006) Polymorphisms of genes coding for insulin-like growth factor I and its major binding proteins, circulating levels of IGF-I and IGFBP-3 and breast cancer risk: results from the EPIC study. *Br. J. Cancer*, **94**, 299–307.
25. Schumacher, F.R., Cheng, I., Freedman, M.L., Mucci, L., Allen, N.E., NPollak, M., BHAYES, R., OStram, D., EHenderson, B., Hunter, D.J. et al. (2010) A comprehensive analysis of common IGF1, IGFBP1 and IGFBP3 genetic variation with prospective IGF-I and IGFBP-3 blood levels and prostate cancer risk among Caucasians. *Hum. Mol. Genet.*, **19**, 3089–3101.
26. Evans, D.S., Cailotto, F., Parimi, N., Valdes, A.M., Castañobetancourt, M.C., Liu, Y., Kaplan, R.C., Bidlingmaier, M., Vasan, R.S., Teumer, A. et al. (2015) Genome-wide association and functional studies identify a role for IGFBP3 in hip osteoarthritis. *Ann. Rheum. Dis.*, **74**, 1861–1867.
27. Teumer, A., Qi, Q., Nethander, M., Aschard, H., Bandinelli, S., Beekman, M., Berndt, S.I., Bidlingmaier, M., Broer, L., Cappola, A. et al. (2016) Genomewide meta-analysis identifies loci associated with IGF-I and IGFBP-3 levels with impact on age-related traits. *Aging Cell*, **15**, 811–824.
28. Aguet, F., Brown, A.A., Castel, S.E., Davis, J.R., He, Y., Jo, B., Mohammadi, P., Park, Y.S., Parsana, P., Segrè, A.V. et al. (2017) Genetic effects on gene expression across human tissues. *Nature*, **550**, 204–213.
29. Sun, B.B., Maranville, J.C., Peters, J.E., Stacey, D., Staley, J.R., Blackshaw, J., Burgess, S., Jiang, T., Paige, E., Surendran, P. et al. (2018) Genomic atlas of the human plasma proteome. *Nature*, **558**, 73–79.
30. Type 2 Diabetes Knowledge Portal. rs2854746. [type2diabetesgenetics.org](http://www.type2diabetesgenetics.org/variantInfo/variantInfo/rs2854746). (2020). <http://www.type2diabetesgenetics.org/variantInfo/variantInfo/rs2854746>.
31. Type 2 Diabetes Knowledge Portal. rs11575194. [type2diabetesgenetics.org](http://www.type2diabetesgenetics.org/variantInfo/variantInfo/rs11575194). (2020). <http://www.type2diabetesgenetics.org/variantInfo/variantInfo/rs11575194>.
32. Buniello, A., MacArthur, J.A.L., Cerezo, M., Harris, L.W., Hayhurst, J., Malangone, C., McMahon, A., Morales, J., Mountjoy, E., Sollis, E. et al. (2019) The NHGRI-EBI GWAS catalog of published genome-wide association studies, targeted arrays and summary statistics 2019. *Nucleic Acids Res.*, **47**, D1005–D1012.
33. Marouli, E., Graff, M., Medina-Gomez, C., Lo, K.S., Wood, A.R., Kjaer, T.R., Fine, R.S., Lu, Y., Schurmann, C., Highland, H.M. et al. (2017) Rare and low-frequency coding variants alter human adult height. *Nature*, **542**, 186–190.
34. Kløverpris, S., Gaidamauskas, E., Rasmussen, L.C.V., Overgaard, M.T., Kronborg, C., Knudsen, U.B., Christiansen, M., Kumar, A. and Oxvig, C. (2013) A robust immunoassay for pregnancy-associated plasma protein-A2 based on analysis of circulating antigen: establishment of normal ranges in pregnancy. *Mol. Hum. Reprod.*, **19**, 756–763.
35. Steinbrecher, A., Janke, J., Poy, M.N., Oxvig, C. and Pischon, T. (2017) Pregnancy-associated plasma protein-A2 and anthropometry, lifestyle, and biochemical factors in a human adult population. *Sci. Rep.*, **7**, 10455.
36. Friedrich, N., Wolthers, O.D., Arafat, A.M., Emeny, R.T., Spranger, J., Roswall, J., Kratzsch, J., Grabe, H.J., Hübener, C., Pfeiffer, A.F.H. et al. (2014) Age- and sex-specific reference intervals across life span for insulin-like growth factor binding protein 3 (IGFBP-3) and the IGF-I to IGFBP-3 ratio measured by new automated chemiluminescence assays. *J. Clin. Endocrinol. Metab.*, **99**, 1675–1686.
37. Bidlingmaier, M., Friedrich, N., Emeny, R.T., Spranger, J., Wolthers, O.D., Roswall, J., Körner, A., Obermayer-Pietsch, B., Hübener, C., Dahlgren, J. et al. (2014) Reference intervals for insulin-like growth factor-1 (IGF-I) from birth to senescence: results from a multicenter study using a new automated chemiluminescence IGF-I immunoassay conforming to recent international recommendations. *J. Clin. Endocrinol. Metab.*, **99**, 1712–1721.
38. Giambartolomei, C., Vukcevic, D., Schadt, E.E., Franke, L., Hingorani, A.D., Wallace, C. and Plagnol, V. (2014) Bayesian test for Colocalisation between pairs of genetic association studies using summary statistics. *PLoS Genet.*, **10**, e1004383.
39. Bulik-Sullivan, B., Loh, P.R., Finucane, H.K., Ripke, S., Yang, J., Patterson, N., Daly, M.J., Price, A.L., Neale, B.M., Corvin, A. et al. (2015) LD score regression distinguishes confounding from polygenicity in genome-wide association studies. *Nat. Genet.*, **47**, 291–295.
40. Baye, T.M., Butsch Kovacic, M., Biagini Myers, J.M., Martin, L.J., Lindsey, M., Patterson, T.L., He, H., Ericksen, M.B., Gupta, J., Tsoras, A.M. et al. (2011) Differences in candidate Gene Association between European ancestry and African American asthmatic children. *PLoS One*, **6**, e16522.
41. Butsch Kovacic, M., Biagini Myers, J.M., Wang, N., Martin, L.J., Lindsey, M., Ericksen, M.B., He, H., Patterson, T.L., Baye, T.M., Torgerson, D. et al. (2011) Identification of KIF3A as a novel candidate gene for childhood asthma using RNA expression and population allelic frequencies differences. *PLoS One*, **6**, e23714.
42. Mersha, T.B., Martin, L.J., Biagini Myers, J.M., Kovacic, M.B., He, H., Lindsey, M., Sivaprasad, U., Chen, W. and Khurana Hershey, G.K. (2015) Genomic architecture of asthma differs by sex. *Genomics*, **106**, 15–22.
43. Kuczmariski, R.J., Ogden, C.L., Guo, S.S., Grummer-Strawn, L.M., Flegal, K.M., Mei, Z., Wei, R., Curtin, L.R., Roche, A.F. and Johnson, C.L. (2002) 2000 CDC growth charts for the United States: methods and development. *Vital Heal. Stat.*, **11**, 1–190.
44. DiPrisco, B., Kumar, A., Kalra, B., Savjani, G.V., Michael, Z., Farr, O., Papatheanasiou, A.E., Christou, H. and Mantzoros, C. (2019) Placental proteases PAPP-A and PAPP-A2, the binding proteins they cleave (IGFBP-4 and -5), and IGF-I and IGF-II: levels in umbilical cord blood and associations with birth weight and length. *Metabolism*, **100**, 153959.
45. Auton, A., Abecasis, G.R., Altshuler, D.M., Durbin, R.M., Bentley, D.R., Chakravarti, A., Clark, A.G., Donnelly, P., Eichler, E.E., Flicek, P. et al. (2015) A global reference for human genetic variation. A global reference for human genetic variation. *Nature*, **526**, 68–74.
46. Chang, C.C., Chow, C.C., Tellier, L.C., Vattikuti, S., Purcell, S.M. and Lee, J.J. (2015) Second-generation PLINK: rising to the challenge of larger and richer datasets. *Gigascience*, **4**, 7.

47. Das, S., Forer, L., Schönherr, S., Sidore, C., Locke, A.E., Kwong, A., Vrieze, S.I., Chew, E.Y., Levy, S., McGue, M. et al. (2016) Next-generation genotype imputation service and methods. *Nat. Genet.*, **48**, 1284–1287.
48. Willer, C.J., Li, Y. and Abecasis, G.R. (2010) METAL: fast and efficient meta-analysis of genomewide association scans. *Bioinformatics*, **26**, 2190–2191.
49. Loh, P.R., Kichaev, G., Gazal, S., Schoech, A.P. and Price, A.L. (2018) Mixed-model association for biobank-scale datasets. *Nat. Genet.*, **50**, 906–908.
50. Pierce, B. L. and Burgess, S. Efficient design for Mendelian randomization studies: Subsample and 2-sample instrumental variable estimators. *Am J Epidemiol.*, **178**, 1177–1184.
51. Staiger, D. and Stock, J. (1997) Instrumental variables regression with weak instruments. *Econometrica*, *Econometric Society*, **65**, 557–586.
52. Burgess, S., Bowden, J., Fall, T., Ingelsson, E. and Thompson, S.G. (2017) Sensitivity analyses for robust causal inference from mendelian randomization analyses with multiple genetic variants. *Epidemiology*, **28**, 30–42.
53. Burgess, S., Small, D.S. and Thompson, S.G. (2017) A review of instrumental variable estimators for Mendelian randomization. *Stat. Methods Med. Res.*, **26**, 2333–2355.
54. Vanderweele, T.J., Tchetgen Tchetgen, E.J., Cornelis, M. and Kraft, P. (2014) Methodological challenges in Mendelian randomization. *Epidemiology*, **25**, 427–435.
55. Purcell, S., Cherny, S.S. and Sham, P.C. (2003) Genetic power calculator: design of linkage and association genetic mapping studies of complex traits. *Bioinformatics*, **19**, 149–150.