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**The in vitro Functional Analysis of Single Nucleotide Polymorphisms
Associated with Growth Hormone (GH) Response in Children with GH
Deficiency**

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interest.

31 **Abstract (150 words)**

32 Response to recombinant human growth hormone (r-hGH) in the first year of therapy has been
33 associated with single nucleotide polymorphisms (SNPs) in children with GH deficiency
34 (GHD). Associated SNPs were screened for regulatory function using a combination of *in silico*
35 techniques. Four SNPs in regulatory sequences were selected for the analysis of *in vitro*
36 transcriptional activity (TA). There was an additive effect of the alleles in the four genes
37 associated with good growth response. For rs3110697 within *IGFBP3*, rs1045992 in *CYP19A1*
38 and rs2888586 in *SOS1*, the variant associated with better growth response showed higher TA
39 with r-hGH treatment. For rs1024531 in *GRB10*, a negative regulator of IGF-I signalling and
40 growth, the variant associated with better growth response had a significantly lower TA on r-
41 hGH stimulation. These results indicate that specific SNP variants have effects on TA that
42 provide a rationale for their clinical impact on growth response to r-hGH therapy.

43

Introduction

Children with growth hormone (GH) deficiency (GHD) have significant inter-individual variation in response to recombinant human GH (r-hGH). Some of this variation can be ascribed to clinical or biochemical factors (e.g. age, GH peak response, weight and r-hGH dose at the start of treatment) identified in growth prediction models. These explain ~60% of r-hGH responsiveness over the first year of therapy¹⁻³. However, as human growth and response to r-hGH result from an interaction of both genetic and non-genetic factors, the identification of genetic markers in addition to the auxological/biochemical predictors could improve the precision and accuracy of growth prediction⁴⁻⁶.

To date, hundreds of genetic variants associated with adult height and human growth have been identified⁷⁻⁹. A number of pharmacogenomic studies have been undertaken to study genotype-phenotype correlation¹⁰⁻¹⁷. The GH receptor (GHR) gene lacking exon 3 (d3-GHR) has been the most extensively investigated; its carriage was shown to influence both growth response to r-hGH^{1, 2} and GH signal transduction using *in vitro* transfection experiments¹⁰. Subsequent studies reported variable results, with some confirming the role of the d3 allele in growth response and others showing no effect^{18, 19}. A meta-analysis conducted on 12 studies confirmed the heterogeneity of results with an overall modest effect of d3-GHR on increased growth response²⁰. More recently response to r-hGH has been shown to depend on an interaction between GHD severity and d3-GHR carriage²¹. Despite the heterogeneity of results, these studies have introduced the important concept that common variations in GH/IGF axis genes may play a role in predicting response to r-hGH therapy. Moreover, a gene-environment interaction has been shown to play an important role in determining growth response in GHD children⁶.

Further pharmacogenomic studies have identified, in a number of growth disorders, novel polymorphisms (SNPs) associated with GH response and located within genes involved in

growth pathways, including *IGFBP3*²² and *SOCS2*²³, or involved in metabolic processes, such as the leptin receptor gene²⁴. To address this issue, the PREDICT study (NCT00699855; Merck Serono S.A. – Geneva, Study 28614) was launched to explore prospectively the effect of these genes on responses to r-hGH in treatment-naïve pre-pubertal children with GHD and Turner syndrome^{25, 26}. The carriage of SNPs in eleven genes in GHD and ten in TS, with two overlapping between conditions, were significantly associated with first year growth response²⁵. A validation study supported the association of four of these genetic markers with first year response to r-hGH treatment in GHD and TS children after controlling for clinical/auxological covariates, although the contribution of these SNPs in a prediction model of first year response was not deemed sufficient for routine clinical use²⁷.

Demonstrating that the SNPs in regulatory regions of DNA associated with growth response have functional consequences is important to provide a rationale for their clinical impact. The aims of this study were to **1)** to identify SNPs associated with response to r-hGH therapy in GHD children that were likely to have a functional effect on transcription and **2)** assess the impact of a number of these polymorphisms on transcriptional activity (TA). We adopted a two-step approach: firstly, polymorphisms were selected using *in silico* methods and by literature search to assess their potential effect on transcription²⁸; secondly, the chosen polymorphisms were linked to clinical phenotype by carriage of SNP variants, their additive contribution to growth response was assessed and their TA was tested *in vitro* (**Figure 1**).

Methods

Selection of polymorphisms

The selection of the genetic markers was performed, calculating a total score of 8 using two approaches: *in silico* study (graded 0-5) and literature search (graded 0-3) [scoring system described below]. Only the SNPs with a total score of 3 or above were selected. Ten polymorphisms linked to seven different genes were analysed; all had been associated with good growth response on r-hGH (within the upper quartile of growth in the first year [cm]) in a continuous analysis in the PREDICT study²⁵. These included the gene coding for the major GH-dependent carrier of IGF-I in the circulation, *IGFBP3*; signalling molecules *GRB10* and *SOS1* (MAPK pathway); the growth factor *TGF-α*; the phosphatase *INPPL1*; the tumour suppressor *TP53*; and *CYP19A1*, a P450 cytochrome enzyme with aromatase activity (**Table 1**).

i) In silico analysis

An *in silico* database search was undertaken to identify the location of the SNP (and its proximity to regulatory elements within the gene – promoters and their transcription binding sites, and enhancers or repressors) using the UCSC Browser version (<https://www.genome.ucsc.edu/>) with the human hg18 build. These data were derived from many different cell lines in which transcription factors and their binding sites responsible for modulating gene transcription, as identified by ChIP-seq, are listed in the ENCODE (Encyclopaedia of DNA Elements) database (<http://genome.ucsc.edu/ENCODE/>)²⁹. Data were also available on the number of cell lines in which TA is predicted to be affected by the region in which the SNP is located, using markers of chromatin state (<http://genome.ucsc.edu/cgi-bin/hgTrackUi?hgsid=224899637&g=wgEncodeBroadHmm>)³⁰.

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Five markers of TA were considered: **1)** proximity to transcription factor binding sites; **2)** sites of DNase hypersensitivity - DNA hypersensitive sites are considered to be accessible to transcription factors; **3)** chromatin state segmentation using 9 different cell lines; **4)** nucleosome occupancy - dynamic changes in nucleosome occupancy are known to be associated with the presence of transcriptional and chromatin regulators³¹; and **5)** mammalian conservation^{29, 30, 32}. The presence/absence of each marker was calculated with a score of 1 and 0 respectively. A maximum score of 5 was indicative of a polymorphism predicted to have high TA, whereas a score of 0 predicted no TA.

ii) Literature search

The literature search assessed the strength of the evidence that the polymorphism was associated with clinical parameters, including adult height (AH) and growth response to r-hGH. Genes related to AH in genome wide association studies (GWAS), were assessed by “GWAS Central” (available on <http://www.gwascentral.org/>)³³, using the negative logarithmic P value (-log p). A score of 1 was given for a -log p ≥ 1.0 which was considered as a tentative association with the trait, with a score of 0 given for a -log p ≤ 1.0 .

The polymorphisms were further analysed in relation to the association with growth response to r-hGH, as previously reported in the PREDICT study²⁵. The un-adjusted P value was evaluated. A p value < 0.001 for carriage of a SNP variant, considered as strongly associated with response to r-hGH, was assigned a score of 2 with a score of 0 given if the p value > 0.001 . A maximum score of 3 was indicative of a polymorphism predicted to have clinical relevance, being associated to AH and growth response to r-hGH.

Analysis of growth response for the selected polymorphisms

Patients' data were used from the previously published PREDICT long-term follow-up (LTFU) prospective study²⁵. Data linking growth response after one year of r-hGH therapy to carriage of associated SNPs was presented as previously published in the per protocol set of GHD children (n=115)²⁵. These data were supported by linking patient auxology to carriage of SNPs in the intention to treat cohort (n=118). Children were recruited from fourteen countries²⁵.

All children were naïve to r-hGH therapy and pre-pubertal at the start of treatment. The diagnosis of GHD was based on two different stimulation tests with a peak GH <10 µg/L³⁴. The median peak GH value was 4.1 µg/L. Patients were selected for r-hGH treatment by their local units and received r-hGH at an average dose of 35 µg/kg/day.

Subjects were excluded with acquired GHD due to central nervous system disorders, such as tumour, trauma, infection, infiltration, irradiation and cranial surgery. Other hormone deficiencies (cortisol and thyroxine), if present, were appropriately treated.

Analysis was conducted using growth response defined as: **1)** annualized height velocity (HV, cm per year) at the first-year visit (12±3 months of first-year treatment anniversary) and **2)** 1 month-change in circulating IGF-I levels, as previously reported²⁶.

This study was conducted in compliance with ethical principles based on the Declaration of Helsinki, the International Conference on Harmonization Tripartite Guideline for Good Clinical Practice, and all applicable regulatory requirements. Written informed consent was obtained from all parents and oral consent from all children.

Analysis of transcriptional activity

For the chosen polymorphisms, TA was assessed using reporter constructs. Each SNP was centrally located within a 500 bp fragment of synthesized promoter sequence (Gene Art, ThermoFisher, UK) and inserted into the pSEAP2 vector (Clontech Laboratories). The vector

contained secreted alkaline phosphatase (SEAP) as a reporter gene, using restriction enzyme (MluI 5' and XhoI 3') mediated heterologous directional cloning and antibiotic selection (Ampicillin). The reporter constructs were transiently transfected into the human MCF-7 cell line (from early passage stocks tested mycoplasma free), known to be GH responsive^{35, 36}. Transfections were performed with Effectene® Transfection Reagent (Qiagen), as per the protocol. The experiments were performed using 24-well plate and seeding the day before of the transfections with $2-8 \times 10^4$ cells. Genotypes were mimicked using a 1:1 stoichiometric mix of the relevant reporter gene constructs.

TA of each construct was evaluated by relative SEAP induction, at baseline and after 24 hours of GH stimulation (range: 2 and 20 ng/ml), using a luminometer and Ready-To-Glow Dual Secreted Reporter Vector Kit (Clontech)^{10, 37}. This assay system measures SEAP as the reporter of TA and luciferase on the plasmid backbone as co-transfection control. The experiments were performed using empty vectors as controls. Three-independent sets of experiments were performed at baseline and after GH stimulation, each experiment was performed in triplicate. The experiments were performed in triplicate using 24-well plates with cell seeding the day before transfection at a density of $2-8 \times 10^4$ cells in DMEM supplemented with 10% foetal bovine serum (Invitrogen, Paisley, UK). Cells were cultured in stable conditions at 37°C, 5% CO₂ in 75 cm² cell culture flasks.

Statistical analysis

All data were expressed as mean $\pm$ standard deviation (SD). Differences in continuous variables were examined for unpaired samples by the Kruskal Wallis test due to the ranked nature of the data and the small sample size. Differences in categorical variables were assessed by χ^2 test. Unpaired Student's t-tests were used to compare the results of transfection with different genotypes for each SNP. A p-value <0.05 was considered statistically significant.

Correlation and regression analysis to link the additive effect of SNPs to growth response was undertaken using a scoring system for carriage. Carriage of SNP variants associated with growth response after one year of r-hGH therapy was defined as previously published²⁵. For allele carriage, the best growth response was assigned a score of 1 and the poorer response was scored 0. The scores for carriage were added across the four SNPs and the total scores were generated for each individual patient. These data were then plotted against growth response after one year of r-hGH therapy as a scatter plot and a least squares regression line was fitted. Pearson's product-moment correlation was then performed to determine confidence in the findings.

Statistical analysis was performed using R³⁸ and the Statistical Package for Social Science (SPSS) program, version 20.0 software for Windows (SPSS, Chicago, IL, USA).

Results

Selection of polymorphisms to study in vitro: Four polymorphisms in four different genes were selected as they were predicted *in silico* to be functional and more strongly associated with clinical outcome, with a total score of 3 or above. These included rs3110697 (A/G) within *IGFBP3*, rs1024531 (A/G) within *GRB10*, rs10459592 (G/T) within *CYP19A1* and rs2888586 (C/T) within *SOS1*. The polymorphisms within *TGF- α* , *INPPL1* and *TP53* were not selected as they showed lower prediction of TA and a weaker relationship to growth response (**Table 2**).

The polymorphism rs1024531 (*GRB10*) was predicted to have weak TA, however it was located within 100 bp of the binding site for REST (RE1-Silencing Transcription Factor). This SNP was also associated with better growth response to r-hGH, defined by first year height velocity in cm/year (cm y^{-1}) with the lowest p-value (<0.001)²⁵. All the other polymorphisms within *GRB10* were predicted to have weak TA and to be variably associated with clinical

outcomes. However, the rs1024531 polymorphism was chosen for its highest score based on prediction *in silico* and on literature searches (5/8 vs 4/8 and 3/8 for rs12536500 and rs933360 respectively).

The polymorphisms rs3110697 (*IGFBP3*) and rs10459592 (*CYP19A1*) were situated in a genomic region of high DNase activity and in proximity to transcription factor binding sites. Moreover, they showed a possible association with adult height as indicated by genome wide association studies³³. Specifically, rs3110697 *IGFBP3* was located 50 bp upstream of the EGR-1 (Early Growth Response 1) binding site and 100 bp downstream of a STAT3 (Signal Transducer and Activator of Transcription 3) binding site³⁰. Multiple transcription factors bind to the DNA site containing rs10459592 including c-FOS, JunD, c-JUN, FOSL2, and BATF, all components of the JUN/FOS/ATF complex³⁰.

The polymorphism rs2888586 in *SOS1* fulfilled three out of the five markers of TA and was predicted to be weakly transcriptionally active in almost all cell lines analysed (8 out of 9). Moreover, it was located in a genomic region with moderate nucleosome occupancy and with mammalian conservation.

The proximity of the SNPs to control elements and the predicted TA of the region in which the SNP was located, is shown in **Table 2**.

Analysis of growth response for the selected polymorphisms

Clinical and biochemical variables were analysed by carriage for the four selected polymorphisms [rs3110697 (A/G), rs1024531 (A/G), rs10459592 (G/T) and rs2888586 (C/T)], carriage of SNP variants were defined as previously reported²⁵. These included parameters at baseline (age, gender, height SDS and peak GH concentration in response to stimulation testing) and markers of response to r-hGH [height velocity after the first year of r-hGH therapy [cm] (as previously reported) and 1 month-change in circulating IGF-I levels]²⁶ (**Table 3**).

When polymorphisms were divided by carriage, there were no significant differences in the parameters at baseline [age, gender, height SDS and GH peak (all $p > 0.05$)] with the exception of rs10459592 in CYP19A which had a significantly higher GH Peak associated with carriage of T compared to the GG genotype. Significant differences by carriage for first year height velocity were presented as previously reported²⁵ (in cm, $p < 0.05$) (**Table 3**).

The additive effect of associated SNPs on response to r-hGH

Scores representing the number of growth promoting variants of the four SNPs studied were derived for each GHD patient. Carriage of multiple growth promoting SNPs was correlated with height velocity after the first year of r-hGH treatment ($p = 6.2 \times 10^{-9}$, $r = 0.50$ [95% confidence interval: 0.36-0.63]) (**Figure 2**).

Analysis of transcriptional activity of the selected SNPs

We investigated *in vitro* whether a given genotype within the rs3110697, rs1024531, rs10459592 and rs2888586 polymorphisms affected TA after GH stimulation. We transiently co-transfected MCF-7 cells, known to be GH responsive^{33, 35, 39} using the pSEAP2-Control Vector reporter gene plasmid, with vectors expressing either the genotype associated with the highest or the lowest HV (**Figure 3**).

When the cells were exposed to GH concentrations of 2 and 20 ng/ml, the genotype associated with better growth response induced higher transcription of SEAP at both GH concentrations ($p < 0.05$). This was observed for the polymorphisms rs3110697 (*IGFBP3*, genotype GG), rs10459592 (*CYP19A1*, genotype GG) and rs2888586 (*SOS1*, genotype TT) (**Figure 3 B-D**).

For the SNP rs1024531 within *GRB10*, the genotype associated with better growth (AA) was associated with significantly lower SEAP induction ($p < 0.05$) at 2 and 20 ng/ml (**Figure 3A**).

Of note, higher GH concentrations (20 ng/ml) did not further increase SEAP induction for any of the SNPs tested. This might be attributed to a partial response at higher doses due to a limited number of cell surface GH receptors. Moreover, the tissue culture flask represents an artificial system *in vitro*, in which cellular response may reach saturation at GH concentrations much lower than those used *in vivo* for therapy (**Figure 3**).

Discussion

In this study we investigated *in vitro* whether polymorphisms known to be associated with clinical responses to r-hGH therapy²⁵ could affect TA. To achieve this aim we identified four SNPs using a combination of *in silico* and literature based findings that were likely to have an effect on the TA of their associated gene. We developed a model to test TA using a GH responsive cell line transfected with a construct consisting of the regulatory region of interest containing the SNP variants upstream of a reporter gene. This model identified gene-dependent patterns of TA, providing a platform to test the functional mechanisms that underlie clinical and genetic observations from PREDICT and other GH pharmacogenomic studies.

The GHD patients potentially carried more than one growth response associated allele from the four SNPs studied. An additive association of these SNPs with growth response was suggested by correlation with both carriage and genotype. This observation was consistent with the existence of a polygenic component to responsiveness to r-hGH⁹.

For all the SNPs situated in genes known to promote somatic growth²⁵ (rs3110697 within *IGFBP3*, rs2888586 within *SOS1* and rs10459592 within *CYP19A1*), we have linked the carriage of variants previously associated with better growth response²⁵ with higher TA on GH stimulation. These SNPs were also found to be located in genomic regions near to transcriptional regulators involved in cell growth and response to GH, including STAT3 (Signal Transducer and Activator of Transcription 3). On the converse, for the SNP rs1024531

within *GRB10*, the carriage of the variant associated with better growth response was associated with lower TA after GH stimulation. Interestingly, *GRB10* is a negative regulator of growth and of signalling through the IGF-I receptor⁴⁰. Specifically the transfection modelling the genotype AA had reduced TA after GH stimulation, consistent with better growth on GH in GHD children⁴⁰. Importantly, the analysis was conducted using growth hormone stimulation between 2 and 20 ng/ml - a range of growth hormone concentrations similar to physiological serum levels, supporting the *in vivo* relevance of these findings. SNPs that scored poorly on selection by *in silico* analysis and literature search were not analysed for TA. All the polymorphisms tested had a higher TA compared to transfection controls.

In this study we have compared the *in vitro* bioactivity of different alleles in genes related to GH response. These findings are in alignment with Dos Santos *et al*¹⁰ whose method to assess genotype was the transfection of equivalent stoichiometric ratios of SNP variants. This approach gives an indication of what would occur with a co-dominant effect but cannot account for chromatin mediated control of variant expression as would be found in dominant or recessive genetic associations. The associations with growth response previously reported were for carriage of all SNPs along with genotype for *GRB10* (rs1024531) and *IGFBP3* (rs3110697)²⁵. Here, however, TA analysis demonstrates clear effects of each allele in alignment with the growth response linked to SNP variant carriage²⁵.

A validation study of the SNPs associated with growth response to r-hGH after one year of therapy identified a number of genes common to both the validation and LTFU studies. The impact of the SNPs on growth varied between the studies, at least in part due to differences in the study cohorts²⁷. However, by adjusting for co-variant phenotype, an additional weight of evidence was provided that these SNPs do have a role to play in growth response. When GHD severity, as measured by response in two stimulation tests, was used as a covariate the rs2888586 SNP within *SOS1* was found to be associated with change in height (cm) by

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regression analysis. Additionally, the rs1024531 SNP within *GRB10* was shown to be predictive of growth response in severe GHD patients using random forest analysis, whilst the rs10459592 SNP within *CYP19A1* and the rs3110697 SNP within *IGFBP3* were also shown to be associated with growth response although the influence of genotype was inconsistent between studies. The association of the *CYP19A1* rs10459592 SNP with GH peak, as a marker of GH severity, found in this current study was not seen in the validation study²¹. The demonstration provided here of GH dependent TA associated with all four SNPs gives additional support for their relevance in mediating differential response to r-hGH therapy in GHD patients.

This study provides a reliable system for studying whether clinically relevant polymorphisms have also a functional correlate and it can be used as a model for further studies to test new regulatory polymorphisms that may be identified. The strength of this report is to have shown that, in response to a given dose of exogenous growth hormone, individual differences in carriage of these polymorphisms have an effect on the TA of their associated genes linked to clinically relevant changes in GHD patient growth rate. This may help in contributing to the progressive switch in growth hormone prescription from 'fixed dosage' to a more personalized adjustment of dose tailored on the genetic constitution of each child with GHD.

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Figure Legends

Figure 1. Algorithm on the methods used for the assessment of the functional impact of polymorphisms. Two steps were undertaken: step 1) *in silico* modelling and literature search to select a number of polymorphisms; step 2) the chosen polymorphisms were analysed by genotype in terms of growth response and tested *in vitro* cell models.

Figure 2. The additive effect of growth response associated SNPs. Scatter plots of genetic score against growth response with a least squares regression line. Carriage of SNP variants associated with growth response after one year of r-hGH therapy in GHD patients (n=115) as previously published²⁵. For carriage of SNPs the best growth response was assigned a score of 1 and the group associated with poorer response was scored 0. The scores were added over the four SNPs for each individual patient.

Figure 3. Transcriptional activity assessed using Alkaline Phosphatase reporter for four SNPs associated with response to GH therapy. Higher transcriptional activity across all concentrations of GH is seen for the genotype associated with better growth response for all SNPs assessed except GRB10, where lower transcriptional activity was seen with higher growth. Data presented as fold change of secreted alkaline phosphatase (SEAP) induction over empty vector control at 2ng/ml and 20ng/ml concentrations of GH. Genotypes were modelled by the transfection of equivalent stoichiometric ratios of variants.

Table Legends

Table 1. The single nucleotide polymorphisms previously associated with growth response²⁵.

The table shows in order:

- 1) The gene in which the SNP is located, the gene ID and the RefSeqGene.
- 2) Position: identifies the location of the SNP in the genome (hg18 build). Most polymorphisms were located within intronic regions of the gene. 5'UTR = 5 prime untranslated region.
- 3) Allele frequency of both the alleles of the polymorphisms identified by "1000 Genomes Project" (<http://www.internationalgenome.org>).
- 4) Primary assembly mapping: defines the DNA sequence where the SNP is located.
- 5) Transcript ID: identifies the transcript where the SNP is located.

Table 2. *In silico* modelling data of the single nucleotide polymorphisms previously associated with growth response²⁵.

Analysis was performed on the described tracks below within the UCSC genome browser using the human hg 18 build⁴¹.

The table shows in silico data, in order:

- 1) **ENCODE Transcription Factor ChIP Seq**, identifies the proximity to transcription factor ChIP-seq findings listed in the ENCODE database³². The presence or absence of transcription factors was identified with a score of 1 or 0 respectively.
- 2) **DNase Clusters**, identifies sites of DNase hypersensitivity. This is identified by "Items in Clusters" (0 to 148) and "Cluster Score" (100 to 1000). The presence of a DNase cluster is detected when Items in Clusters is > 1 and Cluster Score is > 100. The presence or absence of DNase hypersensitivity was identified with a score of 1 or 0 respectively.

- 3) **Chromatin State Segmentation (CSS)**, identified in 9 cell lines. Above is presented the CSS in the majority of cell lines. H: Heterochromatin; LS: Low Signal; WT: Weakly Transcribed; R: Repressed; TE: Transcriptional Elongation; WE: Weak Enhancer; TT: Transcriptional Transition; E: Enhancer; WE: Weak Enhancer. The presence or absence of WT or TE in the majority of cell lines was identified with a score of 1 or 0 respectively.
- 4) **Nucleosome Occupancy**, this is identified via the signals within K562 and GM12878 cell lines. It has a score of 0 to 8. L: Low occupancy (<2); M: Moderate occupancy (2-6). H: High occupancy (>8). The score of 0 was given for low occupancy, the score of 1 for moderate to high occupancy in at least one cell line.
- 5) **Mammalian conservation**, it has a score of 0-4. Not conservative (0); Conservative (1-2); Highly Conservative (3-4). The score of 0 was given for not conservative region, the score of 1 for conservative or highly conservative regions.
- 6) **Adult height GWAS**, a score of 1 was given for a $-\log p \geq 1.0$ which was considered as a tentative association with the trait, with a score of 0 given for a $-\log p \leq 1.0$.
- 7) **PREDICT study**, a p-value <0.001 for carriage of a SNP variant, considered as strongly associated with response to r-hGH, was assigned a score of 2 with a score of 0 given if the p value >0.001.

Table 3. Main clinical characteristics of children with GHD by carriage of the polymorphisms rs1024531 in *GRB10*, rs10459592 in *CYP19A1*, rs2888586 in *SOS1* and rs3110697 in *IGFBP3*. Data are mean $\pm$ standard deviation. (n), number of patients; (M/F): Males/Females; SDS, standard deviation score; IGF-I, insulin-like growth factor I. Change in IGF-I SDS = Change in IGF-I over 1 month of r-hGH therapy; GH Peak = average of two different stimulation tests with a peak GH <10 $\mu\text{g/L}$, measure of GHD severity (low GH Peak = severe GHD). Allele**

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593 = Major allele Dominant, Allele* = Major Allele Recessive. Clinical parameters reported for
594 118 patients, first year growth response [1st Year HV (cm)] reported for the per protocol group
595 (n=115) as previously reported²⁵.

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1 **Table 1.** The single nucleotide polymorphisms previously associated with growth response.

Gene	IGFBP3 rs3110697	IGFBP3 rs10255707	GRB10 rs1024531	GRB10 rs12536500	GRB10 rs933360	TGF- α rs958686	INPPL1 rs2276048	TP53 rs2909430	CYP19A1 rs10459592	SOS1 rs2888586
Polymorphism	A/G	C/T	A/G	C/T	C/T	C/G	A/G	C/T	G/T	C/T
Gene (ID)	IGFBP3 (3486)		GRB10 (2887)			TGFA (7039)	INPPL1 (3636)	TP53 (7157)	CYP19A1 (1588)	SOS1 (6654)
RefSeqGene	NG_011508.1		NG_012305.1			NG_029975.1	NG_023253.1	NG_017013.2	NG_007982.1	NG_007530.1
Position (hg18)	chr7:45921554 Intronic 7p13	chr7:45921217 Intronic 7p13	chr7:50781450 Intronic 7p12.2	chr7:50775527 Intronic 7p12.2	chr7:50725739 Intronic 7p12.2	chr2:70557538 Intronic 2p13.3	chr11:71618860 synonymous 11q13.4	chr17:7519370 intronic/5'UTR 17p13.1	chr15:49323433 intronic 15q21.2	chr2:39100793 intronic 2p22.1
Allele Frequency (1000 Genomes)	A: 0.4165 G: 0.5835	T: 0.1797 C: 0.8203	G: 0.4301 A: 0.5699	T: 0.3814 C: 0.6186	C: 0.4060 T: 0.594	C: 0.4834 G: 0.5166	G: 0.3622 A: 0.6378	C: 0.1571 T: 0.8429	G: 0.4433 T: 0.5567	C: 0.4649 T: 0.5351
Primary Assembly Mapping	CTTAGGCTTTCT GACTGTGGCCAC T[A/G]CTATGAAT CCTAGTATATGG TCACC	TTCAGCTAAGG CAACACAAGA GCCA[C/T]GCG TGCCTAGGCC GCTGAGTGTGC	TTATAGTTTCTA CTGAACACTTGT C[A/G]ATTTCACA CCATCCAAAAGC CAAAA	TTTTGAGACAGT CACAAACCCCAT C[C/T]TGTTC AAG AACTCAGTCTTG TTCCC	TTAATGATCC ACAACATATG GACC[A/G]TTT CCGTCCAAGG AAGTGCAAAT CC	TCTGAGTTTTG TACAGATGATT TTG[C/G]TTGTC CTTAGATAAA AGCCCTTCAA	CATCCACGCTCA GCGTGAACCTCT G[C/T]GACTTCCC AATCTTGGTCAG GTCAC	CTAGTGGGTTG CAGGAGGTGC TTAC[A/G]CAT GTTTGTTCCTT TGCTGCCGTCT	GAGGTGTAGGTT GAACAAACTTGT A[G/T]AGTGAGG AAAGAGTGAGTC ATCATT	TCGTAATAGA ACTTCTGGGAA ATCA[C/T]ATA ATCTTCAGGGG CCTTGGGATTT
Transcript ID	NM_000598.4. NM_001013398.1.	NM_000598.4. NM_001013398.1.	NM_001001555.2.	NM_001001555.2.	NM_001001549.2. NM_001001550.2. NM_001001555.2. NM_005311.4.	NM_001099691.2. NM_001308158.1. NM_001308159.1. NM_003236.3.	NM_001567.3.	NM_001126115.1. NM_001126116.1. NM_001126117.1. NM_001276697.1. NM_001276698.1. NM_001276699.1.	NM_001347254.1. NM_001347255.1. NM_001347256.1.	NM_005633.3.

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1 **Table 2.** *In silico* modelling data of the single nucleotide polymorphisms previously associated with growth response.

Gene	IGFBP3 rs3110697	IGFBP3 rs10255707	GRB10 rs1024531	GRB10 rs12536500	GRB10 rs933360	TGF- α rs958686	INPPL1 rs2276048	TP53 rs2909430	CYP19A1 rs10459592	SOS1 rs2888586
Polymorphism	A/G	C/T	A/G	C/T	C/T	C/G	A/G	C/T	G/T	C/T
<i>In Silico</i> Modelling										
ENCODE Transcription Factor ChIP Seq	Within 50bp 5' from Egr-1; Within 100 bp 3' from STAT3	Within Egr-1	Within 100 bp 5' from REST	Within 100 bp 5' from USF1 and USF2	-	-	-	-	Within c-FOS, eGFP-FOS, JunD, c-JUN, FOSL2, BATF, eGFP-JunD	-
Hypersensitive Site DNase Clusters Items in Clusters (out of 148) Cluster Score (out of 1000)	24 of 148 156	Not shown Score <100	Not shown Score <100	19 of 148 1000	28 of 148 439	Not shown Score <100	Not shown Score <100	Not shown Score <100	52 of 148 604	Not shown Score <100
Chromatin State Segmentation	H/LS (5/9)	H/LS (5/9)	WT (5/9)	WT (5/9)	WT (4/9)	R (4/9)	TE (9/9)	TE (7/9)	WE/R/H	WT (8/9)
Cell lines (9/9)	5/9 (H/LS) 2/9 (WT) 1/9 (R) 1/9 (TE)	5/9 (H/LS) 2/9 (WT) 1/9 (R) 1/9 (TE)	5/9 (WT) 2/9 (H/LS) 1/9 (TT) 1/9 (TE)	5/9 (WT) 2/9 (E) 2/9 (H)	4/9 (WT) 2/9 (TE) 3/9 (H)	4/9 (R) 3/9 (WT) 1/9 (TE) 1/9 (H)	9/9 (TE)	7/9 (TE) 1/9 (WT) 1/9 (WE)	3/9 (WE) 3/9 (R) 3/9 (H)	8/9 (WT) 1/9 (TE)
Nucleosome occupancy (score 0-8) K562 Sig GM12878 Sig	L L	L M	L L	L L	L L	M H	L L	L L	L L	M M
Mammalian Conservation (score 0-4)	Not Conservative	Not Conservative	Conservative	Highly Conservative	Not Conservative	Not Conservative	Not Conservative	Conservative	Not Conservative	Conservative
Prediction Score	2/5	2/5	3/5	4/5	2/5	1/5	1/5	2/5	2/5	3/5
Literature Search										
GWAS Height Related -log p >1.0	1.182	0.452	0.727	0.847	1.138	0.259	0.888	0.201	1.420	0.103
PREDICT STUDY (<0.001)	0.002	0.006	0.0006	0.001	0.002	0.002	0.025	0.014	0.003	0.0095
Clinical Score	1/3	0/3	2/3	0/3	1/3	0/3	0/3	0/3	1/3	0/3
Total Score	IGFBP3 <u>3/8</u>	2/8	<u>5/8</u>	GRB10 4/8	3/8	TGF- α 1/8	INPPL1 1/8	TP53 2/8	CYP19A1 <u>3/8</u>	SOS1 <u>3/8</u>

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GRB10(rs1024531)

Table 3. Main clinical and biochemical characteristics of children with GHD by carriage of the polymorphisms rs1024531 in *GRB10*, rs10459592 in *CYP19A1*, rs2888586 in *SOS1* and rs3110697 in *IGFBP3*.

<i>Clinical Parameters</i>				<i>IGFBP3(rs3110697)**</i>		
<i>GRB10 (rs1024531)*</i>						
<i>Genotypes</i>	<i>Carriage of G</i>	<i>AA</i>	<i>P</i>	<i>Carriage of G</i>	<i>AA</i>	<i>P</i>
Number of patients (n)	57	61		99	19	
Age (years)	9.0 ± 3.3	8.7±3.2	0.579	8.7 ± 3.2	9.7 ± 3.5	0.121
Gender (M/F)	(36/21)	(38/23)	0.923	(62/37)	(12/7)	0.965
GH Peak (µg/l)	4.1±2.0	3.9±2.3	0.548	4.0 ± 2.1	4.0 ± 2.3	0.884
Change in IGF-I SDS	1.5±1.2	1.3±0.8	0.950	1.4 ± 0.9	1.6 ± 1.4	0.437
Height SDS at Baseline	-2.5±1.1	-2.2±0.8	0.291	-2.3±0.9	-2.6±1.3	0.312
1 st Year HV (cm)	7.9±2.0	9.4±1.9	0.0006	9.0 ± 2.1	7.5 ± 2.0	0.002
<i>CYP19A(rs10459592)*</i>				<i>SOS1(rs2888586)*</i>		
<i>Genotypes</i>	<i>Carriage of T</i>	<i>GG</i>	<i>P</i>	<i>Carriage of T</i>	<i>CC</i>	<i>P</i>
Number of patients (n)	89	29		84	34	
Age (years)	8.8 ± 3.2	8.9±3.6	0.837	9.0 ± 3.2	8.6 ± 3.3	0.431
Gender (M/F)	(56/33)	(18/11)	0.934	(57/27)	(17/17)	0.069
GH Peak (µg/l)	4.5±2.0	2.5±1.9	<0.001	3.9 ± 2.2	4.2 ± 2.0	0.564
Change in IGF-I SDS	1.4±1.0	1.2±1.1	0.167	1.4 ± 1.1	1.4 ± 0.7	0.871
Height SDS at Baseline	-2.4±1.0	-2.2±1.0	0.595	-2.3±0.9	-2.5±1.2	0.433
1 st Year HV (cm)	8.0±1.9	10.0±2.2	0.003	9.0 ± 2.0	7.8 ± 1.8	0.0095

Data are mean±SDS. (n), number of patients; M/F: Males/Females; SDS, standard deviation score; Δ, delta; IGF-I, insulin-like growth factor I. Allele** = Major allele Dominant, Allele* = Major Allele Recessive. Clinical parameters reported for 118 patients, first year growth response [1st Year HV (cm)] reported for the per protocol group (n=115) as previously reported²⁵ (unmodified p-value).

First Step:

Selection of SNPs associated with response to Growth Hormone

10 SNPs

- 1) *In silico* analysis
- 2) Literature search

4 SNPs Selected

Second Step:

Analysis of the chosen SNPs

- SNP carriage vs growth response → Auxology & Height Velocity
- Additive effect of SNPs on growth response → Correlation of carriage with Height Velocity
- *in vitro* transcriptional activity of the SNPs → MCF-7 + GH stimulation

Future Perspectives - Targeted Genotyping in clinical management

Figure 1.

Carriage of alleles in the four genes

Figure 2.

A**rs1024531 *GRB10*** **B****rs3110697 *IGFBP3*** **C****rs10459592 *CYP19A1*** **D****rs2888586 *SOS1*** **Figure 3.**