doi: 10.1111/cen.13103

ORIGINAL ARTICLE

Dynamic response of C-type natriuretic peptide and its aminoterminal propeptide (NTproCNP) to growth hormone treatment in children with short stature

Robert C. Olney*, Parisa Salehi†,[1], Timothy C.R. Prickett‡, John J. Lima*, Eric A. Espiner‡, Kaitlin M. Sikes* and Mitchell E. Geffner†

*Nemours Children's Health System, Jacksonville, FL, †Children's Hospital Los Angeles, Keck School of Medicine of USC, Los Angeles, CA, USA and ‡University of Otago, Christchurch, New Zealand

Summary

Objective C-type natriuretic peptide (CNP) and its aminoterminal propeptide (NTproCNP) are potential biomarkers of recombinant human growth hormone (rhGH) efficacy. The objective of this study was to describe the pharmacodynamics of plasma CNP and NTproCNP levels in response to rhGH treatment and to identify the optimal time of sampling after starting rhGH.

Design This was a prospective, observational study. Subjects were treated with rhGH for 1 year, with blood sampled at regular intervals.

Patients Eighteen prepubertal children, eight with low levels of GH on biochemical testing and ten with idiopathic short stature, completed the study.

Measurements Blood levels of CNP, NTproCNP, GH, insulin-like growth factor-I, leptin and bone-specific alkaline phosphatase were measured. Anthropometrics were obtained.

Results Plasma levels of both CNP and NTproCNP reached peak levels 7–28 days after starting rhGH treatment and then declined to intermediate levels through the first year. Plasma NTproCNP levels after 14 days trended towards a correlation with height velocity after 6 and 12 months of treatment. Unexpectedly, serum GH levels measured 2 and 28 days after starting rhGH correlated strongly with height velocity after 6 and 12 months of treatment.

Conclusions This study identified 14 days after starting rhGH treatment as the optimal time for assessing CNP and NTproCNP levels as biomarkers of rhGH efficacy. Additionally, we identified GH levels as a potential biomarker. Larger, prospective studies are now needed to test the clinical utility of these biomarkers.

Correspondence: Robert C. Olney, Nemours Children's Health System, Jacksonville, FL 32207, USA. Tel.: (904) 697-3674; Fax: (904) 697-3948; E-mail: rolney@nemours.org

[1]Present address: Seattle Children's Hospital, University of Washington, Seattle, WA, USA

(Received 12 January 2016; returned for revision 6 May 2016; finally revised 11 May 2016; accepted 11 May 2016)

Introduction

Treatment of short-statured children with recombinant human growth hormone (rhGH) is now widespread practice. However, the growth response to rhGH therapy shows high interindividual variability. This is true in children with classic growth hormone deficiency[1,2] and even more so in children without growth hormone deficiency, such as those with Turner syndrome[3] and idiopathic short stature.[4] Within every diagnostic category, there are some children who do not show a significant growth response to rhGH therapy. Growth hormone therapy imposes a burden on treated children and their families, not the least of which is the need for a daily injection. It is also costly both to the family and the healthcare system. Hence, it would be of significant benefit to the child/family and to the healthcare system to identify those children who respond poorly to rhGH therapy as quickly as possible, either to allow for dose adjustment, discontinuation or for alternate therapy.[5,6]

Efforts to predict growth hormone responsiveness date back to shortly after rhGH became available [reviewed in ref.[5] and[6]]. A variety of auxological parameters (birth weight SD score, height SD score, pretreatment height velocity, parental heights, *etc.*) have been studied, but none alone or in combination provided a model with sufficient accuracy and precision to be clinically useful. The addition of pretreatment biochemical parameters, such as serum insulin-like growth factor-I (IGF-I) and peak serum GH level after stimulation, improved the models, as did the addition of rhGH dose. A variety of other biomarkers of growth response have shown promise[7–9] but none has been shown to correlate with height velocity in normal statured children. Moreover, none is specific to events in the endochondral growth plate, and hence linear growth.

C-type natriuretic peptide (CNP) is a small peptide hormone that plays a critical role in linear growth. It is produced in the growth plate and signals through a paracrine

mechanism. Its bio-inactive aminoterminal propeptide (NTproCNP) is easily measurable in plasma, and levels reflect the rate of CNP biosynthesis. Previous studies in lambs[10] and children[11] have shown that the plasma concentrations of NTproCNP correlate with linear growth velocity at all phases of skeletal growth and increase during rhGH therapy.[12–14] In GH-deficient rats, plasma NTproCNP levels correlate highly with length velocity and growth plate expansion during growth hormone treatment.[15] Collectively these findings suggest that changes in the plasma concentration of CNP peptides may serve as biomarkers of growth plate responsivity in children commencing rhGH treatment. However, before such clinical utility can be determined, the pharmacodynamic responses of blood levels of CNP and NTproCNP to rhGH need to be defined. The objective of this study was to describe the pharmacodynamics of CNP and NTproCNP in response to rhGH in two cohorts of children with short stature, one with low levels of GH on biochemical testing and another with idiopathic short stature. Based on previous research, we hypothesized that plasma NTproCNP would increase within days of starting rhGH treatment and fall to pretreatment levels within days of stopping treatment. Further, we postulated that the response profiles of NTproCNP (sourced from responsive growth plates) would differ from IGF-I (sourced from the liver) and from bone-specific alkaline phosphatase (BSAP, sourced from new bone formation). Because decreases in leptin is reported to reflect rhGH-stimulated height velocity,[16] the pattern of change in plasma leptin was also studied.

Material and methods

Subjects

Subjects were prepubertal children older than 3 years of age with short stature (height SDS $<-2{\cdot}0$) and classified as either having low levels of GH on biochemical testing (peak growth hormone level after stimulation of <7 ng/ml) or as idiopathic short stature (stimulated growth hormone level ≥7 ng/ml). Growth hormone stimulation tests were sequential arginine and insulin-induced hypoglycaemia and were not sex steroid-primed. All subjects were free of any other conditions that might impact growth. Subjects were naïve to rhGH treatment, and children on oral glucocorticoids or medications to treat attention deficit disorder were excluded. Children were free of any acute illnesses or fractures at the start of rhGH treatment.

Subjects were recruited and treated at the endocrinology clinics at Nemours Children's Specialty Care, Jacksonville, FL, or at Children's Hospital Los Angeles, Los Angeles, CA. This study was approved by the Institutional Review Boards of Nemours Children's Clinic and the Children's Hospital Los Angeles. Signed parental permission and child assent (subjects 7 years or older) were obtained for all subjects. This study was registered on ClinicalTrials.gov, trial number NCT01504802.

Study procedures

Subjects were treated with Norditropin rhGH (Novo Nordisk, Plainsboro, NJ, USA) at a dose of 0·05 mg/kg/day. Families were instructed to give the injection at 9 pm. Dose adjustments were made based on weight during the study. The rhGH was stopped for 7 days after the 6-month visit to assess biomarker clearance. Patients were seen at baseline (the day of starting rhGH, before the first injection was given), and at 3, 6 and 12 months after starting. Anthropometric measurements were performed at each visit, along with an assessment for adverse effects. Blood for biomarkers was drawn at baseline and at days 2, 4, 7, 14 and 28, and at 3 months. At the 6-month visit, the rhGH was stopped and blood was drawn that day, and then 2, 4 and 7 days later while the subject was off the rhGH. The rhGH was then restarted. A final blood sample was taken at the 12-month visit. Blood was drawn at 9 am, about 12 h after the previous rhGH injection. Blood samples were assayed for CNP, NTproCNP, GH, IGF-I, BSAP and leptin.

Assays

Blood was drawn into plain top (serum) and EDTA (plasma) collection tubes and stored at 4 °C until processed. Blood was centrifuged at 4 °C, and serum and plasma aliquoted and frozen at −80 °C until assayed. The radioimmunoassays used for plasma CNP and NTproCNP were as previously described[11,17]. Other assays (GH, IGF-I, BSAP and leptin) were run by Quest Diagnostics, Inc. (Madison, NJ, USA), using commercial assays. The GH assay used by Quest was the Siemens (Tarrytown, NY, USA) Immulite 2000 GH assay, which detects both the 20k and 22k isoforms.

Statistical analysis

Standard deviation scores (SDS) were calculated using the LMS method.[18] Height SDS were calculated using Center for Disease Control 2000 LMS data.[19] Standard deviation scores for NTproCNP were calculated using reference LMS data from our previous study of healthy children.[11] For IGF-I, SDS were calculated using the mean and SD data of Brabant, *et al.*[20]. Bone ages were determined using the method of Greulich and Pyle[21] and SDS calculated using the mean and SD data of the Brush Foundation Study.[22]

Data are summarized as median and interquartile range (25–75th percentiles). Anthropometric data of the subject groups were compared using Mann–Whitney rank-Sum tests. Pharmacokinetic and pharmacodynamic data were analysed using ANOVA with repeated measures with Holm-adjusted *t*-tests for *post hoc* pairwise analysis. Correlations between NTproCNP level and height velocity were performed by fitting a line by least squares and performing linear regression analysis. Pearson product–moment correlation coefficients (r) are reported. Statistics were calculated using PRIMER of Biostatistics software (version 7; The McGraw-Hill Companies, Inc., New York, NY, USA). Significance was assumed for P values less than 0·05.

Results

Subjects

Twenty-two subjects were enrolled in the study, ten with low levels of GH on biochemical testing and twelve with idiopathic short stature. Each of the ten subjects classified as having low levels of GH had head MRIs that showed a normal hypothalamus and pituitary gland. One subject was noted to have Chiari 1 malformation and another had a nonspecific finding of 'multiple tiny loci of T2 and flair hyperintensity'. One subject (with low levels of GH) was a screening failure and another (idiopathic short stature) withdrew consent before starting treatment. One subject (with low levels of GH) was lost to follow-up after the 3-month visit and not included in the analysis. One subject (idiopathic short stature) discontinued rhGH after the 6-month visit due to behavioural changes felt by the family to be medication-related. Pharmacokinetic and pharmacodynamic data on this subject were included in the analysis, but not in the correlation analysis involving height velocity. Hence, for the pharmacokinetic and pharmacodynamic analysis, 19 subjects (eight with low GH levels and 11 with idiopathic short stature) were studied. For the height velocity correlation analysis, 18 subjects (eight with low GH levels and 10 with idiopathic short stature) were studied. Subject characteristics are presented in Table 1. All subjects were prepubertal at baseline. By the 6-month study visit, two subjects (one boy, one girl), and by the 12-month study, an additional four subjects (two boys, two girls) had reached genitalia Tanner stage II. All subjects demonstrated an increase in height velocity during the year of treatment with a range of *change* in height velocity of 2·1–8·2 cm/year and a range of *change* in height SDS of 0·3–1·2. No serious adverse effects occurred during the study, although one family discontinued treatment due to behaviour changes perceived to be due the rhGH, but which were felt by the investigator to be unrelated. The only significant differences between the subjects with low levels of GH and those with idiopathic short stature were the peak growth hormone levels after stimulation and a higher height SDS in the low GH level group after 1 year of treatment. Height velocity (both in cm/year and change in height SDS) did not differ between the groups either before or after treatment.

Table 1. Subject Characteristics

	Low GH level	ISS
Number enrolled	10	12
Number completed	8	10
Age (year)	8·1 (7·2 to 9·4)	8·0 (6·6 to 10·2)
Sex (M:F)	5:4	7:4
Peak GH on testing (ng/ml)	4·1 (3·1 to 5·3)	14·0 (11·0 to 14·6)*
Baseline ht SDS	−2·6 (−2·8 to −2·2)	−3·0 (−3·4 to −2·7)
Height SDS – midparental ht SDS	−1·3 (−1·6 to −0·9)	−1·8 (−2·2 to −1·5)
Bone age SDS	−2·7 (−3·0 to −2·5)	−2·2 (−2·5 to −1·5)
Pretreatment HV (cm/year)	6·0 (5·1 to 6·2)	4·1 (3·8 to 5·1)
Baseline IGF-I SDS	−1·5 (−1·9 to −1·3)	−0·9 (−1·9 to −0·6)
Baseline NTproCNP SDS	−0·4 (−0·7 to 0·8)	−0·6 (−1·0 to 0·7)
Post-treatment ht SDS	−1·6 (−1·9 to −1·2)	−2·2 (−2·7 to −1·9)*
Treatment HV (cm/year)	10·7 (10·1 to 12·2)	9·6 (8·7 to 10·6)
Treatment HV (Δ SDS)	1·0 (0·8 to 1·1)	0·8 (0·7 to 1·0)
12-month IGF-I SDS	1·0 (0·7 to 1·6)	0·7 (0·2 to 2·0)
12-month NTproCNP SDS	0·8 (0·4 to 1·3)	0·6 (0·2 to 1·8)

Data are expressed as median (25 to 75th percentile).
Low GH level, short-statured children with low GH levels to biochemical testing; ISS, children with idiopathic short stature; IGF-I, insulin-like growth factor-I; NTproCNP, aminoterminal propeptide of C-type natriuretic peptide; ht, height; HV, height velocity.
*$P < 0·05$ between the treatment groups by Mann–Whitney rank-sum test.

Pharmacokinetics

Serum GH levels were measured at 9 am, about 12 h after the most recent rhGH dose. Comparison of the subjects with low levels of GH and the idiopathic short stature subjects showed no differences in GH level at any time point, nor in timing of maximum level of GH. The groups were then combined for subsequent analysis. Figure 1 (upper panel) shows the pharmacokinetics of GH for the 12 months of treatment. Summary data are shown in Table 2. There is considerable variability in the levels at each time point. The pharmacokinetic pattern is one of a very rapid rise, levels having already reached a peak 2 days after starting daily dosing. Levels then fell to a steady state by 14 days and stayed at this level for the remainder of the first year of treatment.

Pharmacodynamics

The response of plasma levels of CNP to rhGH is shown in Fig. 2 (upper panel). The pattern shows a rapid initial rise, reaching peak values after 7 days and plateauing through 28 days (Table 2). Levels then remain at this level or fall slightly through the rest of the year of treatment. Although ANOVA with repeat measures was significant ($P < 0·0005$), no pairwise comparisons between any time points reached significance. Median time interval to reach peak CNP concentration (T_{max}) within individuals was 28 days (Table 2).

Compared to levels of GH, IGF-I and CNP, there is less variability in NTproCNP levels (Fig. 2, lower panel). As with CNP, plasma NTproCNP levels rise rapidly through 7 days. The median time interval to reach peak NTproCNP concentration (T_{max}) within individuals was 14 days (Table 2), although levels at days 7, 14 and 28 were not statistically different. Levels measured at 4, 7, 14 and 28 days underestimated the actual NTproCNP peak by a mean of 22·3%, 11·6%, 8·9% and 10·2%, respectively. This is followed by a gradual, but significant, decline through the first

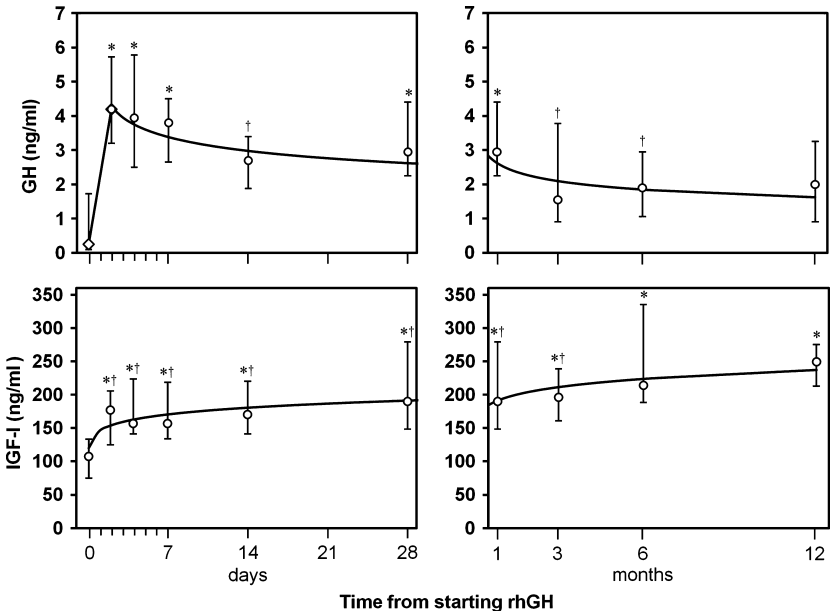

Fig. 1 Pharmacokinetics of rhGH and pharmacodynamics of IGF-I. Recombinant human growth hormone was given at about 9 pm nightly, and serum growth hormone levels were drawn at about 9 am. The low growth hormone level and idiopathic short stature groups were combined; $n = 19$ for all points except for month 12 ($n = 18$). *Points* show the median and *error bars* the 25th and 75th percentiles. *Upper panels,* pharmacokinetics of rhGH. The fitted curve is a power equation (excluding the baseline value). *, levels differed from baseline ($P < 0{\cdot}05$); †, levels differed from the peak value at treatment day two ($P < 0{\cdot}05$, ANOVA with repeated measures with Holm adjust *t*-test pairwise comparison). After treatment day 14, the levels were not statistically different. *Lower panels*, pharmacodynamics of IGF-I. The fitted curve is a power equation. *, levels differed from baseline ($P < 0{\cdot}05$); †, levels differed from the peak value at treatment month 12 ($P < 0{\cdot}05$).

Table 2. Pharmacokinetics and pharmacodynamics

	$Conc_{baseline}$	$Conc_{max}$	T_{max} (days)
GH (ng/ml)	0·3 (0·1–1·7)	6·8 (4·3–9·3)	5·5 (2–28)
CNP (pM)	1·4 (1·2–1·8)	2·6 (2·3–2·8)	28 (7–123)
NTproCNP (pM)	33·4 (29·9–36·4)	47·8 (43·1–60·6)	14 (14–28)
IGF-I (ng/ml)	107·5 (75·3–133·5)	249·5 (219·3–327·8)	203 (182–366)
BSAP (ng/ml)	63·1 (46·5–73·8)	97·8 (82·5–116·6)	182 (98–196)
Leptin (ng/ml)	2·1 (1·4–2·8)	3·2 (2·3–3·8)	4 (2–7)

Data are expressed as median (25–75th percentile).
$Conc_{baseline}$, analyte level measured before starting rhGH; $Conc_{max}$, maximum blood concentration measured during the first year of rhGH treatment; T_{max}, time after starting rhGH that analyte reached its maximum measured concentration; GH, growth hormone; IGF-I, insulin-like growth factor-I; CNP, C-type natriuretic peptide; NTproCNP, aminoterminal propeptide of CNP; BSAP, bone-specific alkaline phosphatase.

year. Levels at all treatment time points were higher than pretreatment levels.

When rhGH was stopped for 7 days at the 6-month treatment visit, levels of CNP and NTproCNP decreased (data not shown). For NTproCNP, the levels dropped in a linear manner, and at 4 and 7 days after stopping rhGH, levels were not different from baseline (pretreatment) levels.

The NTproCNP-to-CNP ratio is a measure of CNP clearance and did not change in response to rhGH treatment (ANOVA with repeated measures, $P = 0{\cdot}2$).

The pharmacodynamic response of serum IGF-I levels to rhGH is shown in Fig. 1 (lower panel). The pattern is one of a rapid rise in levels after 2 days, followed by continued slow increases through the first year of treatment. The peak level was at the 12-month time point (Table 2). All points on treatment were higher than baseline levels and all, except the 6-month time points, were less than the peak at 12 months.

For BSAP, serum levels did not start to increase until 14 days after starting treatment and peaked after 6 months (Fig. 3, upper panel). Leptin levels (Fig. 3, lower panel) showed a great deal of variability and no pairwise comparisons reached significance. The trend was for an increase already by treatment day 2, with levels falling back to baseline by day 28.

Correlations

Although this study was not powered to identify correlations between biomarker level and height velocity during growth hormone treatment, exploratory analysis identified an unexpected correlation between growth hormone levels after 2 days of rhGH treatment and height velocity after 6 months ($n = 19$, $r = 0{\cdot}575$, $P < 0{\cdot}05$) and after 12 months ($n = 18$, $r = 0{\cdot}755$, $P < 0{\cdot}0005$) (Fig. 4). Similarly, the GH level after 28 days of treatment correlated with height velocity at 6 months ($n = 19$, $r = 0{\cdot}589$, $P < 0{\cdot}01$) and 12 months ($n = 18$, $r = 0{\cdot}771$, $P < 0{\cdot}0005$).

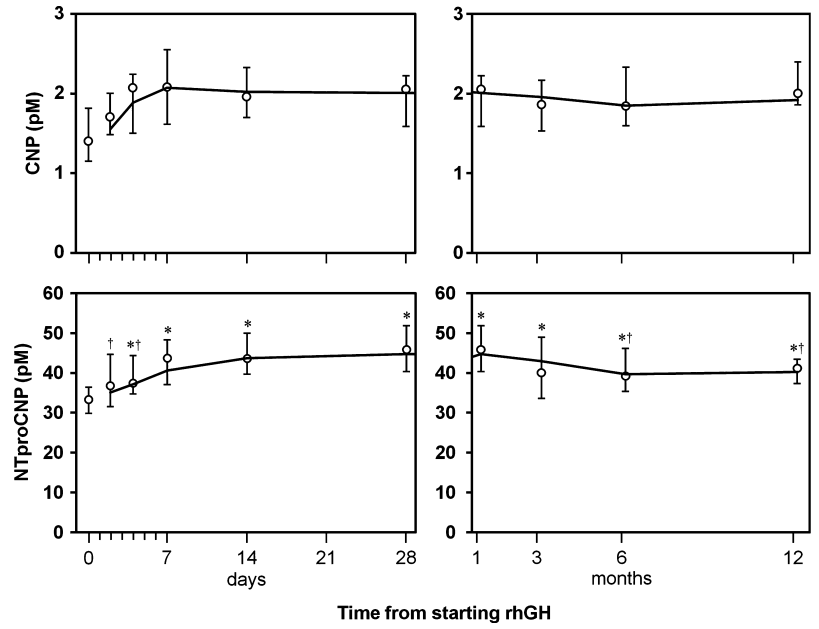

Fig. 2 Pharmacodynamics of CNP and NTproCNP to rhGH. *Points* show the median and *error bars* the 25th and 75th percentiles. The curves are smoothed, two-point running average; reasonable fits could not be obtained with traditional models. *Upper panels,* pharmacodynamics of CNP. ANOVA with repeated measures was significant ($P < 0{\cdot}0005$), but no pairwise comparisons reached significance. *Lower panels*, pharmacodynamics of NTproCNP. *, levels differed from baseline ($P < 0{\cdot}05$); †, levels differed from the peak value at treatment day 14 ($P < 0{\cdot}05$).

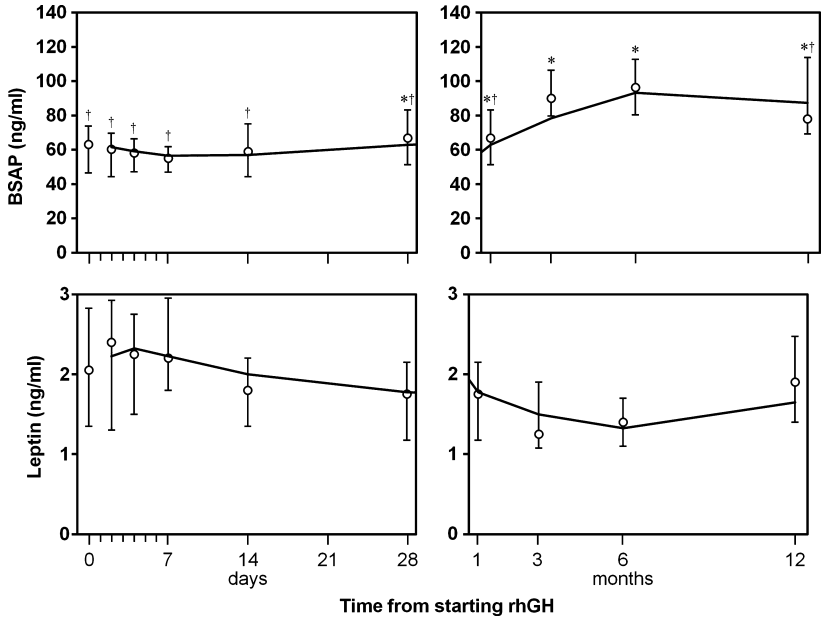

Fig. 3 Pharmacodynamics of bone-specific alkaline phosphatase and leptin to rhGH. *Points* show the median and *error bars* the 25th and 75th percentiles. The curves are smoothed, two-point running average; reasonable fits could not be obtained with traditional models. *Upper panel,* pharmacodynamics of bone-specific alkaline phosphatase (BSAP). *, levels differed from baseline ($P < 0{\cdot}05$); †, levels differed from the peak value at 6 months ($P < 0{\cdot}05$). *Lower panel*, pharmacodynamics of leptin. ANOVA with repeated measures was significant ($P < 0{\cdot}0005$), but no pairwise comparisons reached significance.

Plasma levels of NTproCNP after 14 days of treatment trended towards a correlation with height velocity after 6 months ($n = 19$, $r = 0{\cdot}443$, $P = 0{\cdot}06$) and after 12 months ($n = 18$, $r = 0{\cdot}446$, $P = 0{\cdot}06$).

Discussion

This was a small study, powered to evaluate the pharmacodynamic responses of plasma levels of CNP and NTproCNP to rhGH. This is the initial step in determining appropriate time points at which to measure these biomarkers in relation to rhGH administration. In this regard, we were able to ascertain that CNP and NTproCNP rise to peak levels 14 days after growth hormone initiation, suggesting that this is the best time to obtain these biomarkers. Levels were not statistically different between 7 and 28 days. However, levels drawn at 7 or 28 days were associated with an increased likelihood of underestimating that the actual NTproCNP peak level. Levels subsequently fall to intermediate levels through the first year of treatment. In contrast, after an initial rapid rise, IGF-I slowly increased to attain peak levels at 12 months. Blood BSAP levels were later to rise than those of NTproCNP and increased to peak levels at 6 months. While previous studies identified a decrease in leptin levels is response to rhGH, we found that leptin levels initially rose and then returned to pretreatment levels by 28 days.

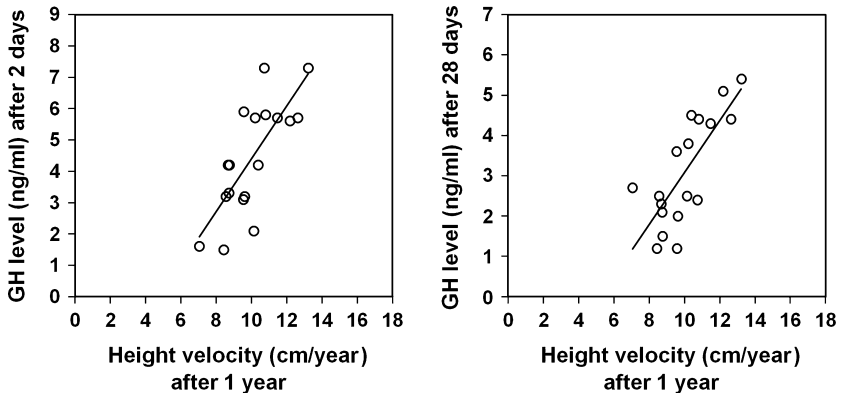

Fig. 4 Correlation between measured GH level and height velocity. Growth hormone levels were measured 2 days (*left panel*) and 28 days (*right panel*) after starting rhGH treatment and compared to height velocity after 1 year. *Lines* are the least squares best fit lines. The correlation is significant for the 2-day measurements ($n = 18$, $r = 0{\cdot}755$, $P < 0{\cdot}0005$) and the 28-day measurements ($r = 0{\cdot}771$, $P < 0{\cdot}0005$).

We attempted to compare children with true growth hormone deficiency with those with idiopathic short stature. However, other than the peak GH response to biochemical testing, the two groups were comparable in all other respects; the group with low GH levels had few other features of true growth hormone deficiency, such as low pretreatment height velocity or abnormalities on head MRI. Likely, many or most of the children in the low GH level group were children with idiopathic short stature and had 'false-positive' results on GH stimulation testing. For this reason, we have not designated this group as having growth hormone deficiency, but rather as having low GH levels on biochemical testing.

Previous work has clearly established a close link between plasma NTproCNP concentrations and concurrent linear growth velocity in rats,[15] sheep[10] and children.[10–14] This is the first report of the dynamic response in CNP peptides to rhGH initiation in children with short stature, the effect of rhGH withdrawal after 6 months treatment, and the response of CNP peptides during a 1-year treatment period. In line with our hypotheses, plasma NTproCNP increased rapidly after starting rhGH and, after cessation of rhGH, returned to baseline values within 7 days.

There is compelling evidence that CNP production within mammalian growth plates (or closely related tissue) acts as a driver of postnatal linear growth.[23,24] As postnatal endochondral bone growth is largely driven by GH, study of the time course of changing CNP production should preferably be undertaken in children with subnormal height velocity. We used conventional criteria for selecting children warranting GH treatment. Overall, the response in height velocity to rhGH in the combined group was within the expected range of response for this age group[25] using the dose employed in this study (0·05 mg/k/day). Earlier observations in rodent pups with severe GH deficiency noted an increase in plasma and growth plate tissue concentrations of CNP peptides occurred within 24 h of starting daily GH (0·3 mg/k/day).[15] We describe here a similar rapid response in children.

The molecular events linking GH actions to CNP production in growth plate chondrocytes remain to be clarified but likely involve IGF-I-mediated up-regulation of the CNP gene (*NPPC)* within growth plate chondrocytes, with the consequent increase in abundance of proCNP, CNP and NTproCNP. Increases in CNP peptides in plasma likely reflect growth plate tissue expression of these secreted peptides. Within the growth plate, the action of CNP is mediated through its receptor, natriuretic peptide receptor B (NPR-B), which generates cGMP as the intracellular second messenger. This signalling enhances extracellular matrix formation and stimulates chondrocyte differentiation into larger hypertrophic cells.[26] The dynamic responses as observed here are consistent with these cellular events. The present findings clearly show the quite different blood profiles of IGF-I (rapid increase, then increasing progressively during GH treatment) and NTproCNP, which is likely to reflect changes in growth plate activity. It is important to note that CNP is expressed across a wide range of tissues[27]; extraskeletal sources possibly responsive to GH also need to be considered. Similarly, skeletal sources other than growth plates may contribute as recent studies suggest that CNP participates in bone remodelling.[28,29] The pattern of BSAP (a marker of new bone formation) is also quite different. As expected, rise in BSAP occurs several days later than CNP and NTproCNP and then continues to increase progressively in the next 6 months. This profile represents contributions made by new bone formation in the growth plate, but also from skeletal remodelling. By comparison, CNP is a regulatory factor of growth plate chondrocytes; the two analytes are markers of distinct and separate processes within the skeleton, confirming previous findings.[30]

We took this opportunity to study the pharmacokinetics of rhGH with repeated daily dosing. We identified a complex pattern that could not be modelled using traditional approaches. Because IGF-I inhibits endogenous GH release, we explored models that included IGF-I levels, but again were unable to identify a reasonable fit for the data.

Although this study was not powered to look at correlations between biomarker levels and height velocity, exploratory analysis revealed some interesting findings. We observed a trend for a positive correlation between peak NTproCNP levels and 6- and 12-month height velocities. Although many factors have the potential to affect the linear growth response to exogenous GH, evidence that CNP is critical to endochondral bone growth and stimulates all zones of the growth plate[23] makes it a good candidate for possible clinical applications in assessing responsivity. Intuitively, a product of newly stimulated chondrocytes with multiple actions downstream which expand long bones would

be expected to correlate with sustained increases in height velocity.

An unexpected and novel observation of this study was the strong correlations seen between serum GH levels at 2 and 28 days and height velocities after 6 and 12 months of rhGH treatment. This was a *post hoc* finding and therefore needs to be interpreted with caution. Bozzola, *et al.*[31], also described a significant positive correlation between peak GH level (within 12 h after the first rhGH injection) and change in height velocity SDS after 1 year of treatment ($n = 8$, $r = 0{\cdot}89$, $P < 0{\cdot}01$) in prepubertal children with growth hormone deficiency. These were also *post hoc* findings in a small number of children but suggest that the absorption of the hormone from subcutaneous tissue affects efficacy. Our observations linking GH concentrations with height velocity are based on values at 12 h postinjection but may still be relevant to those of Bozzola as values at 12 h are likely to be related to peak values between 0 and 12 h of injection. Previous studies[32] show that the time interval to achieve maximal concentration (t_{max}) of rhGH after subcutaneous injection in prepubertal boys with growth hormone deficiency is $3{\cdot}8 \pm 0{\cdot}5$ h (mean±SD) and is followed by a disappearance half-life ($t_{1/2}$) of $2{\cdot}3 \pm 0{\cdot}4$ h ($n = 12$).[32] Hence, 12 h after an injection, GH levels are expected to be roughly between 5 and 15% of the peak GH level, depending on individual variation in the rate and extent of rhGH absorption and in rate of rhGH clearance. Once absorbed into the circulation, growth hormone is cleared primarily through renal filtration and through uptake by the growth hormone receptor.[33] In normal subjects, up to 40% of circulating GH is bound to a high affinity protein (growth hormone binding protein, GHBP) which is the soluble form of the extracellular domain of the GH receptor.[34] Growth hormone binding to GHBP reduces the renal filtration of GH, playing a significant role in GH clearance and rhGH half-life. Measurements of plasma GHBP itself have been shown to be positively associated with the height velocity response to rhGH in GH-deficient children,[35] although rhGH did not affect GHBP levels. Individual differences in GHBP levels and/or other factors that affect rhGH clearance might result in higher GH levels 12 h after rhGH administration and by prolonging rhGH exposure might increase its effectiveness. Collectively, these findings suggest that variability in the absorption and metabolism of rhGH may be playing a more significant role in growth responsiveness than previously thought.

In conclusion, these findings are encouraging and larger, prospective studies are now necessary to assess the value of using GH and NTproCNP levels as biomarkers at these identified time points to predict height velocity in children being treated with rhGH.

Acknowledgements

The authors would like to thank the subjects and their families for participating in this project. Norditropin rhGH and funding were provided through an investigator-initiated study grant from Novo Nordisk, Inc.

Conflict of interest statement

R.C.O had research contracts with Eli Lilly, Inc., Genentech, Novo Nordisk, and Versartis, was a member of advisory boards with Novo Nordisk and Alexion Pharmaceuticals, and received a research grant from Novo Nordisk. P.S. had research a contract with Zafgen, Inc. E.A.E. was a clinical trial consultant to BioMarin Pharmaceutical Inc. T.C.R.P. and E.A.E. have a patent filed entitled 'Assessment of skeletal growth using measurements of NT-CNP peptides'. M.E.G. was a clinical trial consultant to Daiichi-Sankyo, had research contracts with Eli Lilly, Inc., Endo Pharmaceuticals, Ipsen, Novo Nordisk, and Versartis, was a member of advisory boards with Endo Pharmaceuticals, Ipsen, Pfizer, and Sandoz, and received research grants from Genentech and Novo Nordisk and royalties from McGraw-Hill and UpToDate.

References

1 Ranke, M.B. & Guilbaud, O. (1991) Growth response in prepubertal children with idiopathic growth hormone deficiency during the first two years of treatment with human growth hormone. Analysis of the Kabi Pharmacia International Growth Study. *Acta Paediatrica Scandinavica. Supplement*, **379**, 109–115.

2 Sudfeld, H., Kiese, K., Heinecke, A. *et al.* (2000) Prediction of growth response in prepubertal children treated with growth hormone for idiopathic growth hormone deficiency. *Acta Paediatrica*, **89**, 34–37.

3 Pasquino, A.M., Pucarelli, I., Segni, M. *et al.* (2005) Adult height in sixty girls with Turner syndrome treated with growth hormone matched with an untreated group. *Journal of Endocrinological Investigation*, **28**, 350–356.

4 Cohen, P., Rogol, A.D., Deal, C.L. *et al.* (2008) Consensus statement on the diagnosis and treatment of children with idiopathic short stature: a summary of the Growth Hormone Research Society, the Lawson Wilkins Pediatric Endocrine Society, and the European Society for Paediatric Endocrinology Workshop. *The Journal of Clinical Endocrinology and Metabolism*, **93**, 4210–4217.

5 Ranke, M.B., Lindberg, A., Mullis, P.E. *et al.* (2013) Towards optimal treatment with growth hormone in short children and adolescents: evidence and theses. *Hormone Research in Paediatrics*, **79**, 51–67.

6 Wit, J.M., Ranke, M.B., Albertsson-Wikland, K. *et al.* (2013) Personalized approach to growth hormone treatment: clinical use of growth prediction models. *Hormone Research in Paediatrics*, **79**, 257–270.

7 Jorgensen, J.O., Hansen, T.K., Conceicao, F.L. *et al.* (2001) Short-term tools to measure responsiveness to growth hormone replacement. *Hormone Research*, **55**(Suppl 2), 40–43.

8 Bjarnason, R., Andersson, B., Kim, H.S. *et al.* (2004) Cartilage oligomeric matrix protein increases in serum after the start of growth hormone treatment in prepubertal children. *The Journal of Clinical Endocrinology and Metabolism*, **89**, 5156–5160.

9 Crofton, P.M., Stirling, H.F., Schonau, E. *et al.* (1996) Bone alkaline phosphatase and collagen markers as early predictors of height velocity response to growth-promoting treatments in short normal children. *Clinical Endocrinology*, **44**, 385–394.

10 Prickett, T.C., Lynn, A.M., Barrell, G.K. *et al.* (2005) Amino-terminal proCNP: a putative marker of cartilage activity in postnatal growth. *Pediatric Research*, **58**, 334–340.

11 Olney, R.C., Permuy, J.W., Prickett, T.C. *et al.* (2012) Amino-terminal propeptide of C-type natriuretic peptide (NTproCNP) predicts height velocity in healthy children. *Clinical Endocrinology*, **77**, 416–422.
12 Olney, R.C., Prickett, T.C., Yandle, T.G. *et al.* (2007) Amino-terminal propeptide of C-type natriuretic peptide and linear growth in children: effects of puberty, testosterone, and growth hormone. *The Journal of Clinical Endocrinology and Metabolism*, **92**, 4294–4298.
13 Xiao, Y., Dong, Z., Lu, W. *et al.* (2011) Measurement of amino-terminal propeptide of C-type natriuretic peptide in patients with idiopathic short stature or isolated growth hormone deficiency. *Journal of Pediatric Endocrinology & Metabolism: JPEM*, **24**, 989–994.
14 Kubota, T., Wang, W., Miura, K. *et al.* (2016) Serum NT-proCNP levels increased after initiation of GH treatment in patients with achondroplasia/hypochondroplasia. *Clinical Endocrinology*, **84**, 845–850.
15 Prickett, T.C., Bothwell, J.C., Yandle, T.G. *et al.* (2012) Pharmacodynamic responses of plasma and tissue C-type natriuretic peptide to GH: correlation with linear growth in GH-deficient rats. *The Journal of Endocrinology*, **212**, 217–225.
16 Kristrom, B., Carlsson, B., Rosberg, S. *et al.* (1998) Short-term changes in serum leptin levels provide a strong metabolic marker for the growth response to growth hormone treatment in children. Swedish Study Group for Growth Hormone Treatment. *The Journal of Clinical Endocrinology & Metabolism*, **83**, 2735–2741.
17 Prickett, T.C., Olney, R.C., Cameron, V.A. *et al.* (2013) Impact of age, phenotype and cardio-renal function on plasma C-type and B-type natriuretic peptide forms in an adult population. *Clinical Endocrinology*, **78**, 783–789.
18 Cole, T.J. (1990) The LMS method for constructing normalized growth standards. *European Journal of Clinical Nutrition*, **44**, 45–60.
19 National Center for Health Statistics (2002) 2000 CDC Growth Charts for the United States: methods and development. *Vital and Health Statistics*, **11**, 1–203.
20 Brabant, G., von zur Muhlen, A., Wuster, C. *et al.* (2003) Serum insulin-like growth factor I reference values for an automated chemiluminescence immunoassay system: results from a multicenter study. *Hormone Research*, **60**, 53–60.
21 Greulich, W.W. & Pyle, S.I. (1959) Radiographic Atlas of Skeletal Development of the Hand and Wrist, 2nd edn. Stanford University Press, Stanford, CA.
22 Simmons, K. & Greulich, W.W. (1944) The Brush Foundation Study of Child Growth and Development: II. Physical Growth and Development. *Monographs of the Society for Research in Child Development*, **9**, 1–87.
23 Olney, R.C. (2006) C-type natriuretic peptide in growth: a new paradigm. *Growth Hormone & IGF Research*, **16**(Suppl A), S6–S14.
24 Nakao, K., Osawa, K., Yasoda, A. *et al.* (2015) The Local CNP/GC-B system in growth plate is responsible for physiological endochondral bone growth. *Scientific Reports*, **5**, 10554.
25 Bakker, B., Frane, J., Anhalt, H. *et al.* (2008) Height velocity targets from the national cooperative growth study for first-year growth hormone responses in short children. *The Journal of Clinical Endocrinology & Metabolism*, **93**, 352–357.
26 Suda, M., Tanaka, K., Yasoda, A. *et al.* (2002) C-type natriuretic peptide/guanylate cyclase B system in ATDC5 cells, a chondrogenic cell line. *Journal of Bone and Mineral Metabolism*, **20**, 136–141.
27 Sellitti, D.F., Koles, N. & Mendonca, M.C. (2011) Regulation of C-type natriuretic peptide expression. *Peptides*, **32**, 1964–1971.
28 Prickett, T.C., Wellby, M., Barrell, G.K. *et al.* (2014) Differential response of C-type natriuretic peptide to estrogen and dexamethasone in adult bone. *Steroids*, **87**, 1–5.
29 Kondo, E., Yasoda, A., Fujii, T. *et al.* (2015) Increased bone turnover and possible accelerated fracture healing in a murine model with an increased circulating C-type natriuretic peptide. *Endocrinology*, **156**, 2518–2529.
30 Reh, C.S., Olney, R.C., Azen, C. *et al.* (2012) Plasma C-type natriuretic peptide forms and thyroid status in prepubertal children with acquired thyroid disease. *Clinical Endocrinology*, **76**, 228–235.
31 Bozzola, M., Radetti, G., Pagani, S. *et al.* (1999) The level of bioavailable growth hormone (GH) after the first GH injection predicts the first year's growth response in GH-deficient children. *Journal of Endocrinological Investigation*, **22**, 790–795.
32 Hou, L., Chen, Z.H., Liu, D. *et al.* (2016) Comparative pharmacokinetics and pharmacodynamics of a PEGylated recombinant human growth hormone and daily recombinant human growth hormone in growth hormone-deficient children. *Drug Design, Development and Therapy*, **10**, 13–21.
33 Haffner, D., Schaefer, F., Girard, J. *et al.* (1994) Metabolic clearance of recombinant human growth hormone in health and chronic renal failure. *Journal of Clinical Investigation*, **93**, 1163–1171.
34 Fisker, S. (2006) Physiology and pathophysiology of growth hormone-binding protein: methodological and clinical aspects. *Growth Hormone & IGF Research*, **16**, 1–28.
35 Martha, P.M. Jr, Reiter, E.O., Davila, N. *et al.* (1992) Serum growth hormone (GH)-binding protein/receptor: an important determinant of GH responsiveness. *The Journal of Clinical Endocrinology & Metabolism*, **75**, 1464–1469.