Mini Review

HORMONE RESEARCH IN PÆDIATRICS

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Plasma C-Type Natriuretic Peptide: Emerging Applications in Disorders of Skeletal Growth

Eric Espiner[a] Tim Prickett[a] Robert Olney[b]

[a]Department of Medicine, University of Otago, Christchurch, Christchurch, New Zealand; [b]Division of Endocrinology, Nemours Children's Specialty Care, Jacksonville, FL, USA

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NTproCNP · Height velocity · Endochondral bone growth · Growth hormone · Skeletal dysplasia · Biomarker

Abstract
Although studies in experimental animals show that blood levels of C-type natriuretic peptide (CNP) and its bioinactive aminoterminal propeptide (NTproCNP) are potential biomarkers of long bone growth, a lack of suitable assays and appropriate reference ranges has limited the application of CNP measurements in clinical practice. Plasma concentrations of the processed product of proCNP, NTproCNP – and to a lesser extent CNP itself – correlate with concurrent height velocity throughout all phases of normal skeletal growth, as well as during interventions known to affect skeletal growth in children. Since a change in levels precedes a measurable change in height velocity during interventions, measuring NTproCNP may have predictive value in clinical practice. Findings from a variety of genetic disorders affecting CNP signaling suggest that plasma concentrations of both peptides may be helpful in diagnosis, provided factors such as concurrent height velocity, feedback regulation of CNP, and differential changes in peptide clearance are considered when interpreting values. An improved understanding of factors affecting plasma levels, and the availability of commercial kits enabling accurate measurement using small volumes of plasma, can be expected to facilitate potential applications in growth disorders including genetic causes affecting the CNP signaling pathway.

Introduction

After the discovery of the crucial role of C-type natriuretic peptide (CNP) in stimulating endochondral bone growth in genetically modified mice [1–3], measurements of circulating concentrations of CNP products in plasma have illuminated its role in regulating postnatal skeletal growth in mammals. Evidence that the growth plate is a major contributor to circulating CNP products in the immature skeleton, and that plasma concentrations reflect concurrent long bone growth in children, points to a range of possible clinical applications including screening for genetic disorders of skeletal growth due to aberrations in the CNP signaling pathway.

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www.karger.com/hrp

Tim Prickett
Department of Medicine, University of Otago, Christchurch
2 Riccarton Avenue, PO Box 4345
Christchurch 8140 (New Zealand)
E-Mail tim.prickett@otago.ac.nz

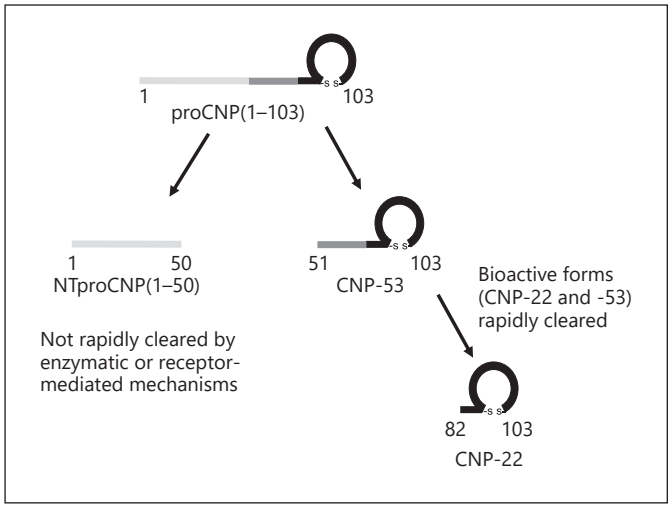

Fig. 1. Processing of proCNP. In humans, the CNP gene (*NPPC*) is located on chromosome 2 and codes for the 103-amino acid residue propeptide, proCNP. ProCNP is cleaved intracellularly (presumably by the enzyme furin), generating the bioactive 53-amino acid residue (C-terminal) peptide (CNP-53) and equimolar amounts of a presumably bioinactive N-terminal product, aminoterminal proCNP (NTproCNP). Not being subject to clearance by NPR3, or to enzyme degradation, NTproCNP enters the extracellular fluid and plasma, where it can be readily detected and measured. CNP-53 appears to be the main form in tissues but is further processed at unknown sites to the smaller bioactive peptide (CNP-22, sequence emboldened as shown above). CNP-22 is considered to be the main form present in the circulation, but the very low levels in plasma prohibit identifying specific molecular forms. All three bioactive CNP forms (proCNP, CNP-53, and CNP-22) appear to have similar activity in vitro, but differences in half-life (longer in larger peptides) are likely to materially affect their potency in vivo. Differential half-lives in the circulation (CNP-22: 2–3 min; NTproCNP: approx. 30–40 min) presumably account for the 15- to 30-fold higher levels of steady-state concentrations of the bioinactive peptide NTproCNP compared to the bioactive CNP-22. Thus, the ratio of NTproCNP to CNP largely reflects the clearance rate of CNP, provided renal function is normal. CNP, C-type natriuretic peptide.

"Natriuretic peptides" have been the subject of numerous reviews [4–6], including the role of CNP in regulating skeletal growth [7], translational use in skeletal dysplasias [8], and use as a putative biomarker in a range of clinical disorders [9]. However, applications of plasma CNP measurements in the assessment of disorders of skeletal growth have not been previously reviewed. The topic is timely for several reasons. CNP analogs have gained increasing attention as potential stimulants to skeletal growth – one of which is currently being assessed in children with achondroplasia (ClinicalTrials.gov identifier NCT02055157). Conceivably, products of endogenous proCNP secretion in plasma could be useful in monitoring responses to exogenous CNP treatments. Further, evidence of complex interactions between CNP and other molecular pathways, and evidence of feedback regulation of CNP, needs to be considered when interpreting plasma levels in the context of growth disorders. And, finally, the variety of genetic disorders affecting discrete nodes within the CNP pathway has increased the need for an improved understanding of CNP measurements and their interpretation.

In this mini-review, we summarize the evidence linking plasma CNP products with skeletal growth and provide guidelines for the use of these assays in elucidating disorders of growth in children. To achieve these objectives, the PubMed database was searched using the following terms: "plasma C type natriuretic peptide," "CNP," "proCNP," "amino terminal proCNP," "NTproCNP," "natriuretic peptides" AND "skeletal growth," "bone growth," "growth disorders," "children," "height," and "height velocity."

Plasma CNP Products and Bone Growth

Early findings of very low concentrations of CNP in plasma from rodents [10] or humans [11] made it unlikely that the dynamic (paracrine) role of CNP in endochondral bone growth could be readily captured in vivo. However, discovery of the 5-kDa aminoterminal propeptide NTproCNP (a cleaved product of the prohormone proCNP; Fig. 1), readily measurable in plasma [12], opened the possibility that its measurement could reflect changes in CNP gene (*NPPC*) expression within growth plate tissues and consequential changes in skeletal growth velocity.

This rationale was based on several assumptions. First, in response to upregulation of gene expression and increased proCNP production in tissues, equimolar secretion of CNP-53 and NTproCNP ensues [13]. Second, while bioactive CNP is subject to rapid clearance or degradation at sites of production and after entry into the circulation [14], NTproCNP is not [15]. Third, growth plate tissues are the predominant source of circulating levels. Underpinning these was the assumption that an increase in CNP concentration in growth plate tissues would continue to drive postnatal growth plate expansion (not just initiate growth at defined periods of development) and hence be related to height velocity at any age. The finding that plasma NTproCNP concentrations were

Table 1. Commercial CNP and NTproCNP assays

Supplier	Product code	Test method	Analytical range, pmol/L[a]	Detection limit, pmol/L[a]	Detection limit < expected endogenous plasma levels
CNP assays					
Cloud-Clone Corp	SEA721Hu	ELISA	28–1,820	12	no
MyBioSource	MBS2021247	ELISA	14–910	5.1	no
MyBioSource	MBS721233	ELISA	114–2,275		no
MyBioSource	MBS2024183	ELISA	28–1,820	11	no
Phoenix Pharmaceuticals	EKE-012-03	EIA	0–45,500		no
Phoenix Pharmaceuticals	RKU-012-03	RIA	0.6–72.8	0.5	yes
Kamoya Biomedical Company	KT-12470	ELISA	28–1,820	12	no
Novus Biologicals	NBP2-62165	ELISA	0–40	0.2	yes
NTproCNP assays					
BioMedica	BI-20812	ELISA	0–128	0.7	yes
MyBioSource	MBS2019321	ELISA	2.5–200	1.1	yes
PromoKine	PK-EL-KB20872	ELISA	0–128	0.7	yes

CNP, C-type natriuretic peptide; EIA, enzyme immunoassay; RIA, radioimmunoassay. [a] Manufacturer data expressed as pg/mL have been reexpressed in molar units for standardization.

up to 10-fold higher in children than in adults, and that NTproCNP levels were at least 10- to 20-fold higher than those of CNP [16], has been the stimulus for more focused study of relationships between CNP products in plasma and skeletal growth in mammals and throughout childhood.

Assays of CNP Products

Critical to any study of the role of CNP in vivo is the method used to accurately and reliably measure circulating products. Ensuring that the CNP moiety being measured is fully characterized by high-performance liquid chromatography or mass spectrometry and that it behaves as the authentic standard (CNP-22/-53 or proCNP[1–50]) are essential steps in validating an assay's performance. Table 1 lists currently available commercial assays of CNP products.

An assay of "CNP" (either CNP-22 or CNP-53; see Fig. 1) requires a concentration step (usually employing a Sep-Pak C18 column) and quantitation using a highly sensitive and specific antiserum directed at the CNP ring structure, which is commercially available (Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA; Cat. No. G-012-03). The level of detection using this assay is approximately 0.6 pmol/L (ED_{50} 7–10 pmol/L). Because of the low concentrations of CNP in blood from children [16, 17], larger volumes of plasma (>0.5 mL) are concentrated 4-fold prior to a radioimmunoassay (RIA) to maximize sensitivity. This requirement introduces the potential for loss of the labile peptide during collection and processing and limits the use of CNP measurements to specific settings – for example, in detecting an arteriovenous gradient across organs [18, 19] or where the ratio of NTproCNP to CNP has applications in clinical diagnosis. It should be noted that, unlike NTproCNP, interactions of atrial natriuretic peptide (ANP) or B-type natriuretic peptide (BNP) at high plasma concentrations will raise CNP concentrations by competitive displacement of the ligand from the clearance receptor natriuretic peptide receptor C (NPR3) [20–22], which may confound the interpretation of results in some pathological settings.

Assays of NTproCNP in research studies have employed RIA and polyclonal antisera [12] after a concentration step as used for CNP. As with CNP, the plasma volume required for accuracy was relatively high (>250 μL). The level of detection using this assay is approximately 1.5 pmol/L (ED_{50} 60–70 pmol/L). A commercial assay (Biomedica Medizinprodukte GmbH & Co KG, Vienna, Austria) employs a two-site polyclonal direct ELISA (detection limit 0.2 pmol/L) and is sufficiently sensitive to detect concentrations in adults and children using small plasma volumes (50 μL). When studied by head-to-

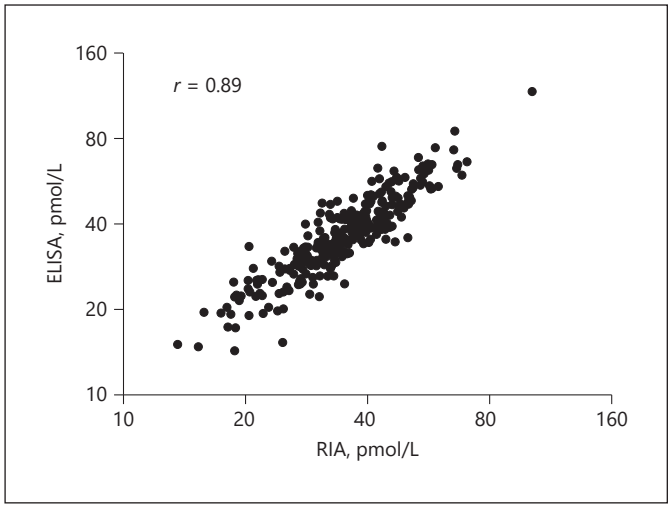

Fig. 2. Comparison of results obtained from samples assayed with the Biomedica second-generation ELISA and those obtained with the RIA (n = 310). RIA, radioimmunoassay.

head comparison with a fully validated RIA using plasma from healthy children, reasonable agreement (r = 0.78) was obtained between the two assays, although the readout of the ELISA was only 20% of that measured by the RIA [23]. However, values obtained using a second generation of ELISA (Biomedica Cat. No. BI-20812) show excellent agreement (Fig. 2) and closely approximate values obtained by RIA. The detection limit for the second-generation ELISA is 0.7 pmol/L. Within- and between-assay coefficients of variation are 6 and 7%, respectively, similar to those in the RIA. The close concordance between the research RIA and the second-generation commercial ELISA should facilitate clinical studies, but the development of monoclonal two-site assays of both CNP and NTproCNP – possibly simultaneously using multiplex technology – will be the ultimate objective.

Evidence that Plasma CNP Products Correlate with Skeletal Growth Velocity

Findings in Experimental Animals

In healthy lambs (aged 1–30 weeks), plasma NTproCNP and CNP levels [16] correlate with metacarpal growth velocity (r = 0.55 and r = 0.4, respectively). Values of plasma NTproCNP fall to stable levels (20–25 pmol/L) after 30 weeks of age, when linear growth is largely complete. Notably, in healthy, rapidly growing, 4-week-old lambs, administration of long-acting bovine growth hormone (GH) for 12 days increased plasma insulin-like growth factor 1 (IGF-1) levels more than 2-fold [24]; however, plasma CNP products and metacarpal growth velocity were unaffected. This finding suggests that neither GH nor IGF-1 directly stimulates CNP gene expression, and that any such increase is contingent on a demonstrable increase in bone growth.

However, in GH-deficient rat pups, daily administration of recombinant human GH (rhGH) for 1 week increased both plasma CNP and NTproCNP within 24 h of starting treatment, before any detectable change in linear growth or change in growth plate histology [25]. The increased levels in plasma were maintained at 1 week, at which time strongly positive associations were identified with growth plate width, hypertrophic zone expansion, and nose-tail growth velocity. That growth plate or closely related tissues likely make important contributions to plasma CNP products is evidenced by the 2- to 3-fold higher abundance of CNP products in proximal tibial and distal ulnar heads compared with what is found in muscles, the heart, or the liver both before and after rhGH stimulation [25].

The concentration of CNP products in these bone extracts closely correlated with plasma NTproCNP in time-matched studies. Together with evidence of a venoarterial CNP gradient across dense bone tissues in growing lambs [19], these findings suggest not only that growth plate tissues make a major contribution to circulating levels of NTproCNP, but also that plasma concentrations are in turn impacted by the activity of these tissues. This conclusion aligns with recent findings from studies using genetic modifications showing that the local (paracrine) CNP signaling pathway in growth plates is responsible for physiological endochondral bone growth [26].

Plasma CNP Products and Changing Growth during Interventions

In view of the close correlation observed between plasma CNP products and endochondral bone growth in spontaneous growth, equally strong associations could be expected between changes in plasma CNP products and changes in skeletal growth induced by interventions known to affect long bone growth in clinical practice (Table 2). Such studies have the potential to reveal the temporal links between changes in blood peptide values and corresponding changes in bone growth – as shown in GH-deficient rodent pups receiving GH [25]. Both glucocorticoids [16] and caloric restriction [24] in rapidly growing lambs reduce skeletal growth and promptly re-

Table 2. Effect of interventions on linear growth and plasma CNP products

Intervention [Ref.]	Species	Acute[a]			Chronic[a]		
		CNP	NTproCNP	growth	CNP	NTproCNP	growth
Growth hormone [24]	sheep		nil	nil			
Growth hormone [25]	rat	↑	↑	↑			
Growth hormone [36]	human	↑	↑	nil	ns	↑	↑
Testosterone [27]	sheep	nil	nil	nil			
Testosterone [34]	human					↑	↑
Estrogen [27]	sheep	↑	↑	nil			
Thyroxine [38]	human				ns	↑	↑
Glucocorticoids [16]	sheep	↓	↓	↓			
Glucocorticoids [37]	human	↓	↓	nil	↓	↓	↓
Caloric restriction [24]	sheep	↓	↓	↓			

Radioimmunoassay measurements using species-specific antisera. CNP, C-type natriuretic peptide; ↑, increase; ↓, decrease; nil, no change. [a] Stimulus duration: acute, <2 weeks; chronic, >2 weeks.

duce circulating levels of CNP products. Again, changes in peptide levels precede those detected in growth – both at initiation and cessation of the intervention. Actions of sex steroids on skeletal growth have received less attention, but they appear to differ from responses observed in children (see below). Estrogen administration to (presumably) prepubertal ewe lambs aged 15 weeks increased both plasma NTproCNP and CNP but had no effect on metacarpal bone growth [27]. On the other hand, exogenous testosterone in ram lambs affects neither bone growth nor plasma CNP products – in contrast to findings in human adolescents. Difficulties in establishing timing of puberty, and in accurately measuring changes in bone growth in experimental animals, may be factors affecting outcomes which clearly need closer study.

Collectively, these results from studies in experimental animals indicate that normal skeletal long bone growth – as well as changes induced by acute interventions – is associated with corresponding changes in plasma NTproCNP, and to a lesser extent CNP. The finding that changes in peptide concentrations precede detectable changes in skeletal growth suggests possible applications of the biomarker in clinical practice.

Findings in Children

Several studies have examined changes in height velocity and plasma CNP products during normal growth. In a cross-sectional study of 258 healthy US children aged 3 months to 20 years [23], NTproCNP was higher in males and inversely associated with age in both genders. In 139 of these children, height velocity was determined by the increase in height at 6 months after blood collection. Annualized height velocity was highly correlated ($r = 0.71$) with plasma NTproCNP level, explaining 51% of the latter's variability. Significant increases in plasma NTproCNP concentrations were observed in adolescents, with peak levels coinciding with Tanner stage IV in boys (genitalia) and stage III (breasts) in girls. Overall, the patterns of change in CNP concentration were similar between boys and girls.

As shown in Figure 3, changes in plasma NTproCNP closely follow changes in height velocity in normal children. In keeping with diminished production once growth has ceased, plasma NTproCNP and CNP levels decline in the 3rd decade to relatively stable levels until the 5th decade, when values start to increase somewhat [28]. These reference data on children have standard deviation scores (SDS) which correct for the subjects' age and gender. In a Japanese study of 23 children with idiopathic short stature [29], serum NTproCNP was inversely associated with age ($r = -0.55$). Similar findings of an inverse association of plasma NTproCNP with age were also reported in a Turkish study [30] of 146 healthy children aged 0–18 years, and in Italian children [31] when plasma CNP was measured at 3 discrete age bands (3–30 days, 1–12 months, and 1–12 years). In none of these studies was height velocity reported.

Over much shorter periods of rapid growth – as observed in neonates and infants – associations were found between plasma NTproCNP at 1 week of age and lower leg growth velocity over the subsequent month when measured by knemometry [32]. Very similar correlation

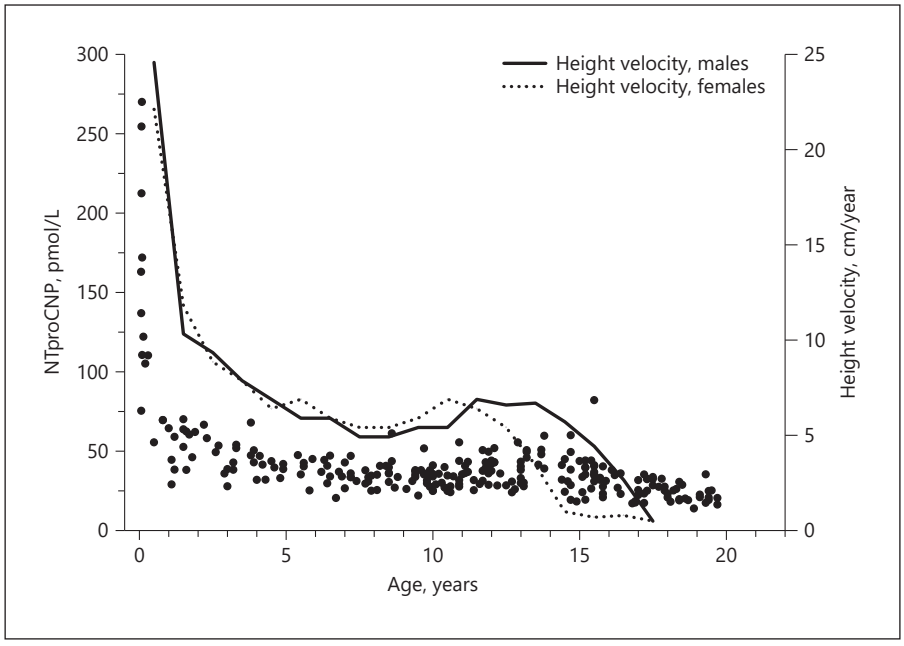

Fig. 3. Plot of plasma NTproCNP concentration against age in normal infants, children, and adolescents. Annualized height velocity curves for normal males and females are shown for comparison. The data are derived from 258 children studied by Olney et al. [23] and 24 children studied in Christchurch, New Zealand.

coefficients were reported in a Finnish study where plasma NTproCNP at the age of 3 months was linked to height velocity calculated across the subsequent year in 125 infants with normal or preterm birth [33].

Collectively, the above studies support the view that CNP production continues throughout postnatal life and reflects the rate of long bone growth of the immature skeleton. A number of products derived from cartilage and bone have been proposed and studied as potential biomarkers of linear growth. Of the many growth factors capable of driving growth plate activity, only plasma products of CNP correlate with height velocity in healthy children. However, it remains to be seen whether the relatively small difference from normal in plasma concentration (SDS) in an individual child exhibiting aberrant growth will prove useful in clinical practice. In this context, assay accuracy and replication (test retest) will be critical.

Changes during Interventions

Several studies report associations of changing height velocity with plasma CNP products during interventions known to affect height velocity. In 15 adolescent children with diminished growth due to idiopathic short stature or GH deficiency, a significant increase in plasma NTproCNP was found when first assessed after commencing rhGH or testosterone treatment [34]. More focused studies of the effect of GH on both height velocity and change in NTproCNP also show significant associations. For example, in a Chinese study [35] of 48 prepubertal children (25 with isolated GH deficiency, 23 with idiopathic short stature), the increase in plasma NTproCNP after 6 months of treatment was positively correlated with height velocity at 1 year in both groups ($r = 0.49$ and $r = 0.77$, respectively). Compared with changes in plasma IGF-1 SDS, the links with height velocity were similar in GH deficiency, but in idiopathic short stature, the change in IGF-1 SDS was not significant. In a small study of 13 young children (<5 years) with achondroplasia or hypochondroplasia [29], plasma NTproCNP levels were significantly increased 3 months after starting GH therapy, and the levels correlated ($r = 0.72$) with the change in height velocity SDS at 1 year.

Collectively, these studies suggest that the response of CNP products to GH could be useful in predicting the subsequent response in height velocity. This important question was addressed in a pilot study [36] of 18 children with short stature by examining the pharmacodynamic response of CNP products to rhGH. After commencing daily standard rhGH injections, plasma CNP and NTproCNP increased promptly to attain peak concentrations 7–28 days after starting therapy and remained significantly above pretreatment levels throughout the 1-year study. The pattern in the IGF-1 response was quite different from that of CNP and was not associated with change in height velocity. Although insufficiently powered to examine correlations with height velocity, promising correlations of plasma NTproCNP levels at day 14

with height velocity at 6 months ($r = 0.44$) and at 1 year ($r = 0.44$) ($p = 0.06$ for both) were identified. It remains to be seen whether early sampling of NTproCNP could be used to predict poor responders to rhGH and/or assist in dose monitoring in clinical settings.

The effect on CNP of other factors affecting skeletal growth is limited. Significant and concordant correlations of NTproCNP with height velocity have been identified in children with acute lymphoblastic leukemia receiving high-dose glucocorticoids and chemotherapy [37], and in children with thyroxine excess or deficiency undergoing corrective treatments [38]. To our knowledge, the response of plasma CNP products to estrogens – and possible links with height velocity – have not been studied in humans. On the other hand, exogenous testosterone in adolescent boys markedly increases plasma NTproCNP measured 4 weeks after commencing treatment [34] – a finding consistent with the timing of peak plasma values at Tanner stage IV in cross-sectional studies [23]. The temporal change in plasma NTproCNP in response to testosterone administration has not been studied but – as seen after initiation of GH – is likely to be prompt and may have clinical applications, for example, in the management of children with pubertal disorders and those with congenital adrenal hyperplasia. In all of the above studies, the number of children studied was small; thus, confirmation of the findings in larger groups – preferably over longer time periods – is clearly needed.

Notwithstanding the above findings, contributions to plasma CNP products by tissues other than growth plates need to be considered. For example, bone remodeling could contribute to plasma levels in children given that CNP is expressed by osteoblasts [39] and osteoclasts [40], and that in adults changes in bone turnover markers are associated with changes in plasma CNP products [41]. Although growth plate tissues in the premature skeleton appear to make a major contribution to plasma NTproCNP and CNP levels, their concentrations do not fall to undetectable levels once final height is attained [28]. The source(s) of this is/are still unclear, though the vascular endothelium likely makes an important contribution [42]. In adults, renal impairment [28] (raising NTproCNP), severe sepsis [43] (increasing CNP and NTproCNP), heart failure and hypertension [12, 28], and liver disease [44] (all raising CNP products) are likely to similarly affect levels in children. The obvious signs and symptoms of these diseases make their presence unlikely to confound interpretation. However, acute inflammation, which appears to lower NTproCNP in young children [45], could be overlooked in some settings. Reports of lower levels of CNP products in obese adolescents [46, 47] have not been supported by a more formal study where the crucial impact of age (and therefore height velocity) was taken into account [30]. While there is still much to be learned about the impact of other pathophysiological variables and drugs on circulating CNP products, current knowledge suggests that the time of day for sampling [48], posture, and diet (unless calorie restricted) [49] are unlikely to affect values in pediatric clinical practice.

Plasma CNP Products and Genetic Disorders of Growth

Over and above applications in monitoring height velocity in appropriate clinical settings, measuring peptide concentrations in plasma may be useful in clarifying the pathophysiology in skeletal dysplasia, as well as in screening or confirming several of the genetic disorders affecting CNP pathway activity within growth plates (Table 3). NTproCNP levels are highly elevated [50] in children with acromesomelic dysplasia, Maroteaux type (AMDM; MIM #602875), an autosomal recessive syndrome caused by disruptive mutations in *NPR2*, the receptor for CNP, also known as NPRB. Levels are also elevated in children with achondroplasia and hypochondroplasia [50], and levels are lower in children with osteogenesis imperfecta, even in those with normal height velocity (manuscript in preparation). The greatly elevated levels associated with a loss-of-function (LOF) mutation in *NPR2* suggest that production of CNP is sensitive to reduced CNP intracellular activity (Fig. 4), as discussed below. As well as gaining insight into interactions between growth factors in pathological disorders of bone growth, these findings have important implications when interpreting plasma CNP products in genetic disorders affecting CNP activity.

Accelerated Linear Growth

Increased production of CNP associated with chromosomal translocation – presumably due to loss of activity of a local restraining regulator of NPPC – causes extremely tall stature and a marfanoid body habitus [51–54]. Very tall stature has also recently been reported in a 6-year-old girl with duplication of the CNP gene (M. Karbonits, pers. commun.). In all cases where CNP products have been measured, plasma CNP is raised, though in many reports the reference range is inappropriate or not specified. Measured by RIA, levels of both CNP (SDS 8.0 and 3.4) and NTproCNP (SDS 7.1 and 3.2) were markedly elevat-

Table 3. Plasma CNP products in genetic disorders affecting the CNP signaling pathway in children

	Phenotype	CNP	NTproCNP	Ratio
Diminished growth				
NPR2 LOF$^{+/+}$	Severe SS	↑↑	↑↑	N
NPR2 LOF$^{+/-}$	ISS	N	N	N
FGFR3 GOF	Ach, Hch	↑	↑	N
NPPC[a] LOF$^{+/-}$	SS, small digits	?	?	?
Excessive growth				
NPPC gene translocation	Tall stature/marfanoid	↑↑	↑↑	N
NPR2 GOF$^{+/-}$	Tall stature/marfanoid	N or ↓	↓	? N[b]
NPR3[c] LOF$^{+/+}$	Tall stature, long digits, extra epiphyses in digits	↑	↓	↓

Directional changes as indicated by arrows denote changes outside the age- and gender-adjusted normal range. A change in ratio (NTproCNP/CNP) will reflect a change in CNP clearance, and the ratio therefore falls when clearance of CNP is reduced. CNP, C-type natriuretic peptide; *NPPC*, natriuretic peptide precursor C, CNP; *NPR2*, natriuretic peptide receptor 2, CNP receptor; *NPR3*, natriuretic peptide receptor 3, natriuretic peptide clearance receptor; *FGFR3*, fibroblast growth factor receptor 3; LOF, loss of function; SS, short stature; ISS, idiopathic short stature; GOF, gain of function; A(H)ch, a(hypo)chondroplasia; N, normal. $^{+/+}$ Homozygous; $^{+/-}$ heterozygous. [a] Dominant heterozygous mutations affecting the amino acid sequence within the CNP ring structure reduce bioactivity, but the impact on plasma CNP peptide concentrations is unknown. Biallelic mutations are not yet reported – they would be expected to cause severe SS and increases in plasma NTproCNP, and possibly in CNP if the mutated CNP form retains immunoreactivity in the assay used for its detection. [b] The ratio may be artifactually reduced as the fall in NTproCNP may exceed any detectable decrease in CNP. [c] LOF in the clearance receptor similarly affects plasma concentrations of atrial natriuretic peptide and B-type natriuretic peptide and ratios of bioactive to bioinactive peptide.

ed in the 2 boys (aged 12 and 15 years, respectively) reported on by Moncla et al. [52]. Similar skeletal overgrowth is observed in subjects with activating mutations in *NPR2*, but here plasma levels of NTproCNP are "normal" [55, 56] or reduced [57], in keeping with the assumed negative feedback loop whereby enhanced CNP activity reduces CNP production (Fig. 4).

The interpretation of results clearly depends on an appropriate age and gender reference range and knowledge of the concurrent height velocity at the time of sampling. In subjects exhibiting abnormal growth acceleration, a "normal" level (based on an age- and gender-adjusted reference range) should be seen as inappropriately low. The logic underpinning this is related to the fact that all of the drivers of endochondral bone growth studied to date (LOF mutations in *NPR3* excepted; see below) result in an increase in plasma NTproCNP. Notably, in an adult with an *NPR2* gain-of-function mutation [57], plasma NTproCNP was reduced, consistent with a systemic overdrive in CNP intracellular activity that was independent of active growth plates. Measurements of CNP itself are unlikely to be helpful in the diagnosis of these gain-of-function mutations, as concentrations of bioactive CNP are close to the level of detection of the assay in normal subjects, making the detection of low levels challenging. Recently, 4 children (from 3 families) with an LOF mutation in *NPR3* have been reported with phenotypes of tall stature and a distinctive pattern of extra epiphyses in their hands and feet [58]. NPR3 is a clearance receptor for all natriuretic peptides and plays an important role in CNP clearance. Affected subjects have increased plasma CNP concentrations but normal or low levels of NTproCNP, such that the ratio of NTproCNP to CNP is greatly reduced. This pattern of change differs from findings in most other states of accelerated growth, where the two peptides are raised concordantly. Similarly, the ratio of bioinactive to bioactive levels of ANP and BNP was also reduced.

Collectively, these results are consistent with a reduced rate of clearance of all three bioactive natriuretic peptides (increasing bioactive forms in plasma) as well as feedback inhibition of production [50], which lowers the plasma concentration of bioinactive propeptide forms. While data from larger numbers of cases are needed for confirmation, it is likely that an abnormally low NTproCNP-to-CNP ratio will be a diagnostic signature of this muta-

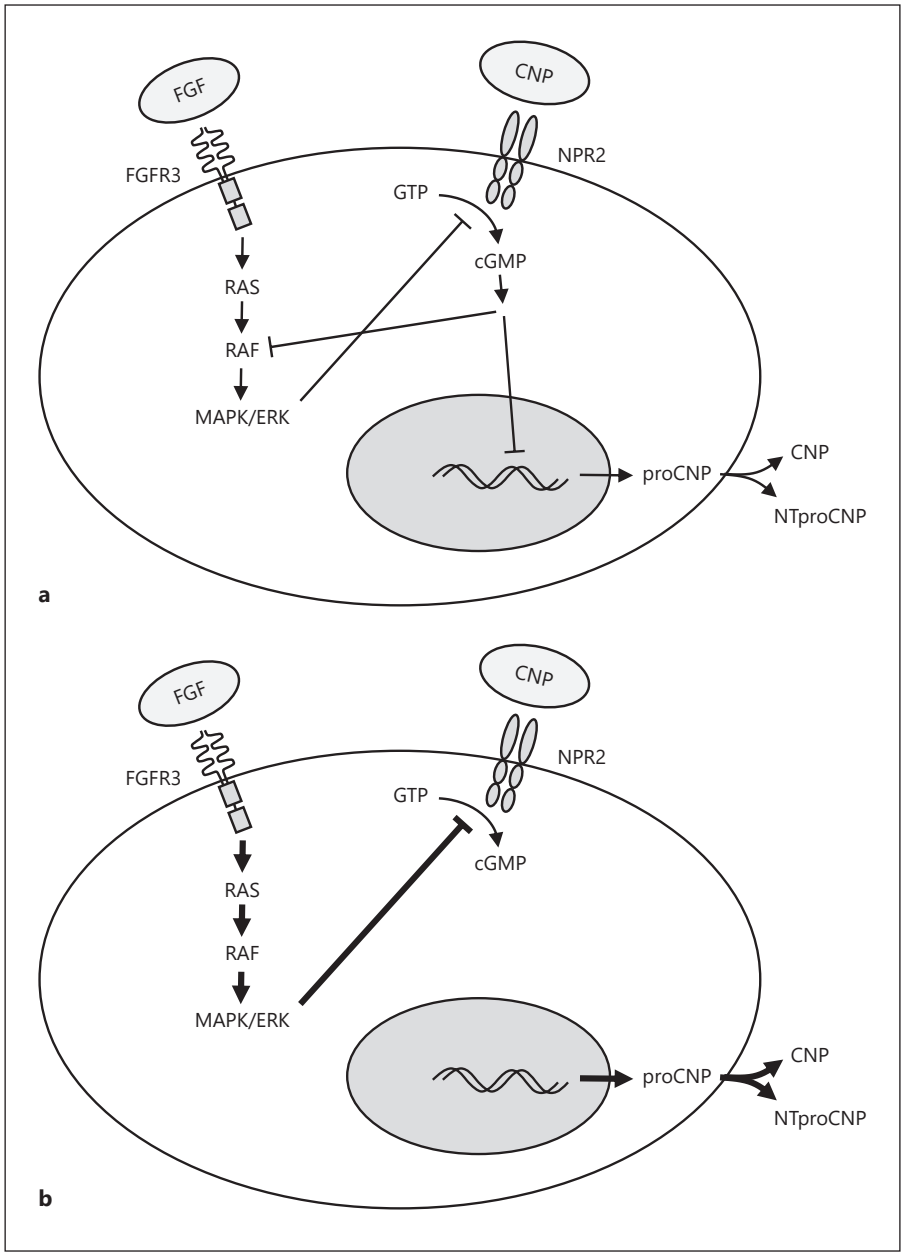

Fig. 4. Autocrine regulation of C-type natriuretic peptide (CNP) in growth plate chondrocytes showing inhibitory reciprocal interactions between FGF/FGFR3 and CNP/NPR2 pathway activity, and putative negative feedback of intracellular CNP activity on CNP production. This scheme depicts a single chondrocyte responsive to CNP as well as secreting CNP. Paracrine actions among cells may also be mediated by diffusible products of NPR2 activation such as cyclic guanosine monophosphate (cGMP). **a** Normal state showing a balanced and reversible interaction between FGFR3 and NPR2 activation. Activated MAPK and ERK1/2 inhibit cGMP generation by CNP/NPR2, whereas activated NPR2 inhibits RAF via cGMP/cGMP protein kinase (cGK). Also shown is the putative (and weaker) inhibitory impact of cGMP/cGK on CNP production. **b** Overactivation of FGFR3 – as in achondroplasia – by enhancing MAKP/ERK results in a functional inhibition of NPR2 activity, thereby reducing the brake of cGMP/cGK on CNP production and raising concentrations of plasma CNP products. Note that in the presence of nonfunctional (disrupted) NPR2, the inhibitory effect of FGFR3 is amplified and the consequential further reduction in cGMP/cGK markedly increases CNP production.

tion in subjects with skeletal overgrowth. It is interesting to note that boys with Klinefelter (XXY) syndrome – which is associated with tall stature – show a significantly elevated NTproCNP-to-CNP ratio [59], suggesting an increase in CNP clearance. The reasons for this finding and its clinical implications are unclear.

Diminished Skeletal Growth

The first indication that plasma CNP products were perturbed in any disorder of skeletal growth [60] arose from findings in AMDM, where the CNP receptor (NPR2) is nonfunctional. SDS of both CNP and NTproCNP are markedly raised in these children [50] in the face of a profoundly disproportional short stature and greatly reduced height velocity. Similar findings of elevated values of plasma NTproCNP (Biomedica assay) were reported for 3 boys with AMDM aged 10–13 years [61], although an age-related reference range for the assay was not reported.

Elevated circulating levels in the face of an inactive CNP receptor, and therefore reduced intracellular CNP

activity, points to hormone resistance and a regulatory mechanism whereby secretion is normally negatively modulated by a cellular component of CNP activation (Fig. 4), as discussed above. Importantly, subjects with one dysfunctional *NPR2* allele also have diminished height [62, 63] and are likely to present with idiopathic short stature [62, 64]. Although plasma NTproCNP appears to be normal in these subjects when measured in adulthood [63], the possibility that circulating levels during phases of active growth could be useful diagnostic aids deserves closer study in view of the relatively high prevalence (2–4%) of children with this mutation presenting with idiopathic short stature [62, 64].

Dominant heterozygous mutations in *NPPC* affecting the amino acid sequence within the ring structure of CNP have recently been described in 2 children presenting with mild short stature and small hands [65]. Both mutations affected highly conserved amino acids within the 17-residue ring and were associated with reduced cyclic guanosine monophosphate (cGMP) in response to NPR2 activation by the mutated peptide in vitro. Plasma concentrations of CNP products were not reported, but since the primary defect is likely based on reduced activation of the CNP receptor by the mutated peptide, it is anticipated that levels of CNP and NTproCNP will be relatively increased unless mutations also reduce CNP immunoreactivity in the assay of CNP-22. In this context, studies in the authors' laboratory show that the p.Arg117Gly mutation in the ring retains normal immunoreactivity when the Phoenix assay is used to measure CNP-22.

Since profoundly reduced postnatal growth in mice with homozygous mutations in *NPPC* (long bone abnormal, lbab) is rescued by CNP agonists [66], early detection of this putative genetic disorder in humans will be important, as the condition is likely to be uniquely responsive to exogenous CNP. Dominant activating mutations of *FGFR3*, such as those causing achondroplasia, result in profoundly disproportional short stature and are associated with inappropriately raised plasma concentrations of both CNP and NTproCNP [50] in both children and adults. Presumably, the increase in circulating levels in achondroplasia (Fig. 4) is due to inhibited intracellular CNP signaling by overactive FGFR3 signaling [67–69], although the precise molecular events underlying this feedback regulation are poorly understood.

Whether plasma CNP products are perturbed in other skeletal dysplasias is being studied and could reveal other conditions associated with CNP resistance or changes in CNP clearance. In the light of known associations of plasma CNP products with genetic disorders already identified, some prediction on the likely changes in plasma levels can be made concerning those that are yet to be identified in humans. By analogy, an increase in plasma CNP products is anticipated in LOF mutations affecting components further downstream in the signaling pathway. For example, LOF mutations affecting cGMP protein kinase type 2 (*PRKG2*) are a cause of diminished growth in mice [70] and cattle [71]. *PRKG2* is a recognized target of cGMP in chondrocytes. Gain-of-function mutations in *NPR3* (the clearance receptor) would be expected to diminish bone growth, reduce plasma CNP, and increase NTproCNP, raising the NTproCNP-to-CNP ratio. The same directional change in ratio for ANP and BNP would also be expected. On the other hand, mutations causing a gain-of-function mutation in osteocrin (an endogenous ligand with strong affinity for *NPR3*) would be expected to have the opposite impact, and a phenotype similar to that of the LOF mutation in *NPR3* [72]. In view of the limited knowledge of mechanisms underlying many growth disorders, it is likely that measurements of CNP products will be revealing in some areas and possibly lead to novel treatments.

Future Research and Conclusions

Interpreting plasma CNP products in clinical settings is a rapidly evolving field highly dependent on context and defining an appropriate reference range. In children with a disorder of growth, age, gender, and concurrent height velocity are important factors to consider when interpreting results. Comparatively little is known of the day-to-day variations in plasma levels, the possible influence of diurnal fluctuations in growing children, or the influence of changing contributions from other tissues to plasma concentrations. All deserve closer study.

Further, with respect to circulating levels, presumably all growth plate tissues involved in endochondral growth contribute to varying degrees, and at different times, whereas only selected bones contribute to height. Differential rates of epiphyseal closure (e.g., vertebrae vs. femoral-tibial) may therefore distort relationships of plasma NTproCNP with height velocity. Despite these uncertainties, evidence that levels of plasma NTproCNP change promptly in response to interventions, and precede detectable changes in height velocity, suggest potentially important applications in pediatric practice. For example, in children with congenital adrenal hyperplasia, avoiding excessive glucocorticoid doses (which suppress height velocity and will reduce NTproCNP) – yet pre-

venting increased endogenous androgen secretion (which accelerates height velocity and likely increases NTproCNP) – is frequently challenging and could be facilitated by judicious NTproCNP monitoring, allowing adjustment in doses well before changes in height velocity can be detected. Similarly, plasma CNP products may prove to be useful in predicting an abrupt increase in height velocity in precocious puberty and may assist in clinical decisions related to the timing of growth plate closure and surgical treatment in subjects with scoliosis [73].

It is anticipated that formal studies on large groups of children receiving rhGH for short stature will test the predictive value of CNP assays for height response, their use in monitoring compliance with treatment, and monitoring and dose titration of drugs potentially harmful to growth. In this context, changes in concentrations in an individual during short time intervals are more likely to be informative than a single measurement. As shown already for those rare mutations affecting CNP-dependent disorders of bone growth, measurements of plasma CNP products may provide a much-needed window into the peptide's role at the level of the growth plate itself in a range of pathophysiological states. Should long-term use of CNP agonists become available for the treatment of specific skeletal disorders, conceivably measurements of plasma NTproCNP could be useful in dose monitoring. Here, the rationale is based on (1) likely interference by the administered CNP agonist in the measurement of endogenous bioactive CNP and (2) current data showing that enhanced (CNP-induced) intracellular activity has the capacity to reduce endogenous CNP production, reducing the plasma concentration of bioinactive propeptides such as NTproCNP. However, the extent to which results may guide an individual's management will depend on the assay's reproducibility and signal-to-noise ratio in the context of changing growth plate activity during relatively small time periods – all of which remain to be clarified for assays used commercially.

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Statement of Ethics

The authors have no ethical conflicts to disclose.

Disclosure Statement

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Author Contributions

All three authors contributed equally to this work.

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