Bi-allelic Loss-of-Function Mutations in the NPR-C Receptor Result in Enhanced Growth and Connective Tissue Abnormalities

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The natriuretic peptide signaling pathway has been implicated in many cellular processes, including endochondral ossification and bone growth. More precisely, different mutations in the NPR-B receptor and the CNP ligand have been identified in individuals with either short or tall stature. In this study we show that the NPR-C receptor (encoded by *NPR3*) is also important for the regulation of linear bone growth. We report four individuals, originating from three different families, with a phenotype characterized by tall stature, long digits, and extra epiphyses in the hands and feet. In addition, aortic dilatation was observed in two of these families. In each affected individual, we identified a bi-allelic loss-of-function mutation in *NPR3*. The missense mutations (c.442T>C [p.Ser148Pro] and c.1088A>T [p.Asp363Val]) resulted in intracellular retention of the NPR-C receptor and absent localization on the plasma membrane, whereas the nonsense mutation (c.1524delC [p.Tyr508*]) resulted in nonsense-mediated mRNA decay. Biochemical analysis of plasma from two affected and unrelated individuals revealed a reduced NTproNP/NP ratio for all ligands and also high cGMP levels. These data strongly suggest a reduced clearance of natriuretic peptides by the defective NPR-C receptor and consequently increased activity of the NPR-A/B receptors. In conclusion, this study demonstrates that loss-of-function mutations in *NPR3* result in increased NPR-A/B signaling activity and cause a phenotype marked by enhanced bone growth and cardiovascular abnormalities.

The importance of the natriuretic peptide signaling pathway in the regulation of cardiovascular and renal homeostasis was first identified more than 30 years ago.[1] More recently, studies in both humans and mice have shown that the natriuretic peptides are also important for regulating endochondral ossification and linear bone growth.[2] Genetic alterations in *NPPC* (MIM: 600296) and *NPR2* (MIM: 108961) (the genes encoding the CNP ligand and the NPR-B receptor, respectively) can result in either short or tall stature, depending on the nature of the mutation.[3–14] Increased activity of the NPR-B/CNP signaling pathway causes tall stature (MIM: 615923) while decreased NPR-B/CNP signaling activity results in short stature phenotypes, such as acromesomelic dysplasia Maroteaux type (AMDM [MIM: 602875]) or "idiopathic" short stature (MIM: 616255).[2,3,5–7,9]

The natriuretic signaling pathway can be activated by three main natriuretic peptides (NPs): atrial natriuretic peptide (ANP [MIM: 108780]) and B-type natriuretic peptide (BNP [MIM: 600295]), which are both expressed and stored in granules of the atrial myocytes in mammalian hearts,[15,16] and C-type natriuretic peptide, which is expressed by a broad range of cell types.[17–19] The NPs are produced as prohormones that are subsequently cleaved to yield the N-terminal bio-inactive products (NTproNP) and the mature active peptides, namely $ANP_{1\text{-}28}$, $BNP_{1\text{-}32}$, $CNP_{1\text{-}53}$, and/or $CNP_{1\text{-}22}$.[1,20,21] NPs activate the pathway by binding to one or more of three distinct natriuretic peptide receptors, namely NPR-A, NPR-B, and NPR-C, which are encoded, respectively, by *NPR1* (MIM: 108960), *NPR2*, and *NPR3* (MIM: 108962). NPR-A and NPR-B are highly homologous guanylyl cyclase receptors which, upon ligand binding, catalyze the conversion of intracellular guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP).[22,23] NPR-A preferentially binds ANP and BNP, while NPR-B binds CNP with the highest affinity.[24] NPR-C differs from the other receptors in its activity, binding capacity, and expression.[1,25] Of all NP receptors, NPR-C is the most abundantly and widely expressed[1] and in contrast to the other receptors, NPR-C binds all three NPs with similar affinity.[26] The receptor lacks guanylyl cyclase activity but can inhibit adenylyl cyclase activity via Gi-dependent signaling in certain tissues.[27] The NPR-C receptor mainly acts as a clearance receptor. Clearance of NPs from the extracellular environment via NPR-C is one of several mechanisms regulating concentrations of active

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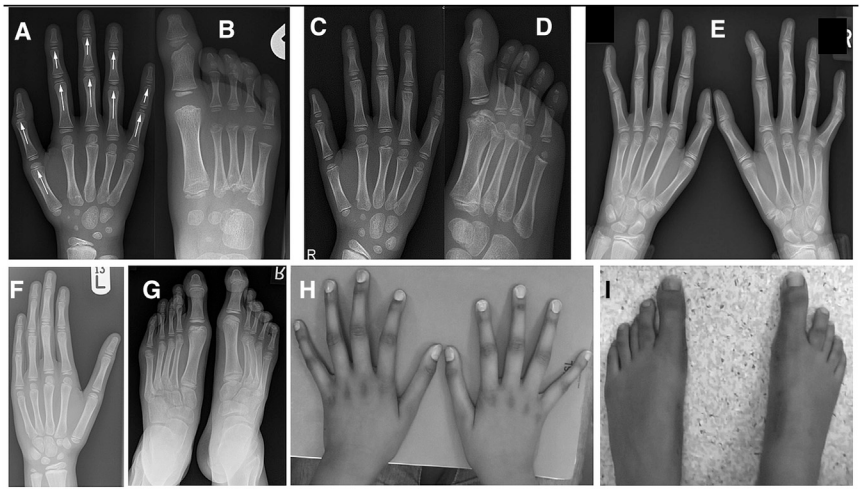

Figure 1. Radiographic and Clinical Images of the Hands and Feet

(A and B) Radiographs from individual 1 (reproduced with permission from de Jong et al.[56]). (A) Right hand image taken at the age of 6 years shows an extra epiphysis (indicated by white arrow) at multiple sites. There is also a pseudoepiphysis at the base of the second metacarpal. (B) Right foot image taken at the age of 5 years shows the presence of an extra epiphysis at the head (distal end) of the first metatarsal and the distal ends of the second to fifth proximal phalanges.

(C and D) Radiographs from individual 2 (reproduced with permission from de Jong et al.[56]). Images were taken at the age of 5 years and show the relatively long halluces (D) and extra epiphyses in hands (C) and feet (D).

(E) Radiograph of both hands from individual 3 taken at the age of 11 years 8 months. The image shows long and slender tubular bones, especially in both thumbs. The presence of an extra epiphysis is best seen at the distal end of both metacarpals I.

(F–I) Radiographic and clinical images of the hands and feet from individual 4. The radiographs were taken at the age of 7 years and 10 months. The tubular bones in the left hand are long and slender, especially the proximal and middle phalanges and metacarpal I, that in addition show an extra epiphysis at their distal end (F). The same pattern is observed in both feet with large metatarsals I and proximal phalanges (G). Clinical photos of the hands (H) and feet (I) show the long fingers and toes, most pronounced for the halluces, which is typical and therefore representative for all affected individuals.

NPs.[28] The relative contributions of clearance and other degradative mechanisms are likely to be tissue specific[28] but remain to be clarified in humans. In mice with homozygous loss-of-function (LoF) mutations in NPR-C, a variety of phenotypes have been reported.[29] All NPR-C mutant mice show an increased body length, longer tubular bones, and kyphosis. Other variable features include delayed endochondral ossification, increased bone turnover, arachnodactyly, enlarged vertebrae, reduced body weight and fat mass, and reduced blood pressure,[29–32] indicating that NPR-C plays a role in many tissues and processes.[29]

In this study, we investigated four individuals from three different families presenting with tall stature and long digits (arachnodactyly). In addition, all individuals were slender and their feet showed disproportionately long halluces (Figure 1). Extra epiphyses were noted in the hands and feet and this radiographic sign served as an important diagnostic hallmark (Figure 1). Furthermore, in two of the affected individuals, dilatation of the aorta was observed, and in three, hypermobility of the joints was present (Table 1) (see Supplemental Note). Mutations in Fibrillin 1 (*FBN1* [MIM: 134797]), known to cause Marfan syndrome (MIM: 154700), were excluded in all individuals prior to this study. Genomic DNA of all affected individuals and family members was isolated from blood using standard protocols. All individuals gave informed consent to the study. The study was approved by the ethical committee of the Antwerp University Hospital (registration number: B300201730930). Whole-exome sequencing in families 1 and 3 and targeted screening of all coding regions of *NPR3* using Sanger sequencing in family 2 resulted in the identification of bi-allelic mutations in *NPR3* (GenBank: NM_001204375.1) in all four affected individuals (Table 1). All mutations are submitted to the corresponding Leiden Open Variant Database (LOVD: npr3.lovd.nl).[33] The two sibs from family 1 were compound heterozygous for the c.442T>C (p.Ser148Pro) and c.1524delC (p.Tyr508*) mutations. Individual 3 (family 2) was homozygous for the c.1088A>T (p.Asp363Val) mutation whereas individual 4 (family 3) was homozygous for the c.248delT (p.Val83Glyfs*105) mutation. Segregation analysis in all three families revealed co-segregation of the variants with the abnormal phenotype. These findings are in line with genome-wide association studies (GWASs) showing that genetic variations in and nearby the *NPR3* locus affect human adult height,[34–39] and are also in line with the phenotype of the *Npr3* mutant mice described above. Taken together, these reports provide additional evidence that mutations in *NPR3* are the genetic cause of the phenotype in the studied individuals. *In silico* analysis using different prediction programs supported a disease-causing effect for all identified variants (Table S1). In addition, the variants were either not listed in the ExAC,[40] gnomAD,[40] and dbSNP databases or reported only in very low frequencies but never in a homozygous state. According to the prediction program HOPE,[41] the p.Ser148Pro substitution results in the loss of a hydrogen bond in the extracellular region of the protein and the p.Asp363Val substitution changes the hydrophobicity of the protein, which can also result in loss of hydrogen bonds and/or disrupt the correct folding of the protein. Loss of a hydrogen bond or disturbed folding can ultimately impair trafficking of the receptor to the membrane.

To test this hypothesis, HEK293T cells were transfected with plasmids encoding either wild-type, p.Ser148Pro, or p.Asp363Val mutant C-terminal GFP-tagged NPR-C. The

Table 1. Overview of the Phenotype and Genotype in the Four Affected Individuals

	Individual 1	Individual 2	Individual 3	Individual 4
Family	family 1	family 1	family 2	family 3
Ethnicity	Dutch	Dutch	Pakistani	NA
Age (years)	11	10	14	8.5
Gender	male	male	male	female
Height	+3.03 SDS	+3.43 SDS	+4.41 SDS	+4.76 SDS
Weight/height ratio	−4.1 SDS	−2.07 SDS	−1.2 SDS	−1.04 SDS
Blood pressure (mm Hg)	99/62	92/62	95/54	NA
Extra epiphyses in fingers and toes	+	+	+	+
Long digits (especially halluces)	+	+	+	+
Aorta dilatation	+3.78 SDS	−	+3 SDS	−
Joint hypermobility	+	+	+	−
Additional clinical features	mild pectus excavatum	NA	clinodactyly, camptodactyly, mild motor coordination problems, mild learning disability, myopia	NA
NPR3 (NM_001204375.1) mutation(s)	compound heterozygous c.442T>C (p.Ser148Pro) c.1524delC (p.Tyr508*)	compound heterozygous c.442T>C (p.Ser148Pro) c.1524delC (p.Tyr508*)	homozygous c.1088A>T (p.Asp363Val)	homozygous c.248delT (p.Val83Glyfs*105)

NA: no data available

wild-type construct was purchased from GeneCopoeia and the mutations were introduced using the QuickChange site directed mutagenesis II kit from Agilent. The presence of the mutation and the absence of "off target" errors were confirmed by Sanger sequencing of the complete coding region. The results in Figure 2 clearly demonstrated that both mutant NPR-C receptors were not present on the cell membrane and therefore not able to fulfill their normal function. The presence of a signal peptide in NPR-C and the cellular localization of the mutant NPR-C observed during this experiment (Figure 2) suggested that the mutant receptor was most likely retained in the endoplasmic reticulum-Golgi network. A similar effect has been reported for human loss-of-function (LoF) mutations in the NPR-B receptor[9] and murine LoF mutations in NPR-C.[29]

In addition to the two missense variants, we also identified a heterozygous nonsense variant (p.Tyr508*) in the last exon and a homozygous frameshift variant (p.Val83Glyfs*105) in the first exon, both resulting in a premature stop codon. It is known that nonsense mutations at the end of the protein have a higher likelihood of escaping nonsense-mediated mRNA decay and therefore produce a C-terminally truncated protein.[42,43] To investigate this, RNA was isolated from blood of individuals 1 and 2 and their parents using the PAXgene Blood RNA system (QIAGEN). cDNA synthesis was performed with the SuperScript III First-strand Synthesis System (Thermo Fisher Scientific). Sanger sequencing of cDNA revealed that for both affected sibs in family 1, the only cDNA recovered contained the p.Ser148Pro variant (Figure S4), indicating that the p.Tyr508* variant does cause nonsense-mediated mRNA decay. Unfortunately, no mRNA was available from family 3 in which the p.Val83Glyfs*105 variant was identified, but, based on the location of the mutation in the first exon of the gene, it is most likely that it also results in nonsense-mediated mRNA decay.

As we have demonstrated that the identified mutations result in the absence of functional NPR-C receptors on the cell membrane, we next investigated the effect of these mutations on the clearance of NPs from the extracellular environment. A previous study in *Npr3*$^{-/-}$ mice showed that [^{125}I]ANP clearance from plasma was reduced but plasma concentrations of ANP and BNP were not raised and were in fact slightly lower than normal.[32] Plasma CNP was not measured. In the absence of functional NPR-C on the cell membrane, one would predict that the clearance of the bioactive natriuretic peptides (but not the N-terminal bio-inactive products [NTproNPs], since these are not cleared by the NPR-C receptor) would be reduced, resulting in relatively increased plasma levels of these bioactive products. To investigate this hypothesis, levels of CNP, BNP, ANP, and their corresponding N-terminal bio-inactive products were measured in plasma collected from individual 1 at the age of 11 years and in individual 3 at the age of 13 years. Unfortunately, individuals 2 and 4 did not consent for these experiments. NPs and NTproNPs were measured by radioimmunoassay after extraction over C18 SepPac cartridges (Waters Corp) as

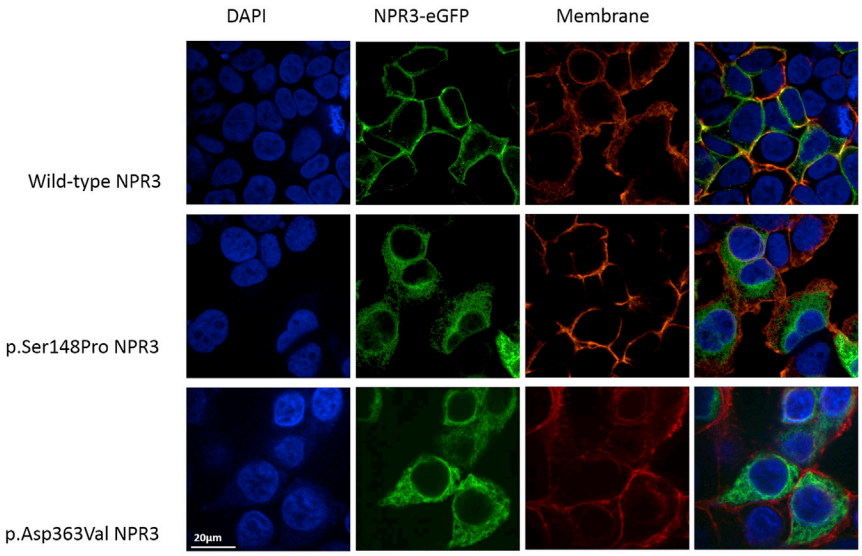

Figure 2. Cellular Localization of Wild-Type and Mutant (p.Ser148Pro and p.Asp363Val) NPR3
The nuclei are stained blue using DAPI and the cell membrane is marked red using Alexa Fluor wheat germ agglutinin (WGA) 555 (Thermo Fisher Scientific). Images are taken with a Nikon Eclipse Ti-E inverted microscope attached to a microlens-enhanced dual spinning disk confocal system (UltraVIEW VoX; PerkinElmer) equipped with 405, 488, and 561 nm diode lasers for excitation of blue, green, and red and far-red fluorophores, respectively. Magnification 60×.

previously described.[44–46] At the time of sampling, the annualized height velocity of individual 1 was 8.9 cm/year (height velocity standard deviation score: + 6.17);[47] concurrent height velocity was unavailable for individual 3. In both affected individuals, a decreased NTproNP/NP ratio for all three NPs and increased plasma cGMP levels were found (Table 2). The highly discrepant lower levels of NTproCNP (despite the accelerating skeletal growth as documented in individual 1) are probably due to local negative feedback mechanisms.[5,48] Previously, a study in healthy children showed that the age-dependent changes in both CNPs and NTproCNPs are highly correlated (r = 0.57)[44] and are closely associated with concurrent height velocity. This implies that in normal settings, including situations with enhanced growth, plasma concentrations of both active and bio-inactive CNP forms should be similarly raised. This is clearly not the case in the individuals we investigated in this study where the positive CNP SDS (+1.61; +6.4, respectively) is in line with a positive height velocity SDS (+6.17; not available, respectively) but not with the negative NTproCNP SDS (−0.74; −0.05, respectively). Together, these findings indicate a reduced CNP clearance on the one hand and a reduced CNP production on the other hand. The latter is reflected by the lower levels of NTproCNPs and is presumably caused by a local (paracrine) negative feedback loop.[5,48,49] Although plasma volume and renal function were not measured here, presumably similar negative feedback mechanisms[50,51] underpin the abnormally low ratios of the ANP and BNP peptides. Furthermore, the plasma level of cGMP, the downstream product of NPR-A and NPR-B receptor activation, was relatively high in both individuals. These data indicate that loss of NPR-C results in increased or persistent NPR-A and NPR-B signaling due to the increased availability of the bioactive natriuretic peptides. By measuring both bioactive and bio-inactive products, we have now provided evidence in strong support for both the increase in circulating levels of bioactive forms and also reduced production of NPs in the absence of functional NPR-C. Collectively, our findings suggest that low NTproCNP/CNP ratios may be a distinctive biochemical signature of bi-allelic *NPR3* mutations, contrasting with other states of increased CNP pathway activity causing enhanced skeletal growth where concordant directional changes in both CNPs and NTproCNPs are found.[5,44,52] Extending these observations by studying additional affected subjects is now clearly indicated.

Interestingly, the phenotype caused by bi-allelic LoF mutations in *NPR3* is very similar to the one caused by mono-allelic gain-of-function mutations in *NPR2*,[5–7] which is consistent with the different roles both receptors play in the natriuretic signaling pathway. Both phenotypes share tall stature and long digits, especially the halluces, as common features. We were intrigued by the presence of multiple extra epiphyses in the tubular bones (especially middle and proximal phalanges) of the hands and feet in the affected individuals (Figure 1), suggesting that this may be unique to NPR-C LoF. We therefore carefully re-evaluated the published radiographs of the individuals reported with heterozygous activating *NPR2* mutations. Close examination of the radiographs of the individual with the p.Ala488Pro variant in *NPR2*, published by Miura and colleagues (Figure 2),[6] also shows the presence of multiple extra epiphyses in the hands. Extra epiphyses in the distal parts of the appendicular skeleton have not been reported in the mouse models.[29–32] In the *Npr3*-null mice and in the homozygous Kylb (*Npr3* mutant) mice, a delayed appearance of the secondary ossification centers has been observed[31,32] as well as an increased bone turnover.[29,32] Thus, although not unique to NPR-C LoF, extra epiphyses may be (directly or indirectly) responsible for the enhanced longitudinal growth of the involved digits, especially as extra epiphyses are also observed in disproportionately long fingers of individuals affected with spondylomegaepiphyseal-metaphyseal dysplasia (MIM: 613330).

Chronic extra-skeletal effects of sustained increases in bioactive NPs in humans are unknown but important to

Table 2. Overview of the Levels of ANP, BNP, and CNP, the N-Terminal Bio-inactive Products, and cGMP in Plasma of Individuals 1 and 3

	Individual 1	Individual 3	Reference Range
NTproCNP (pmol/L)	28.5 (−0.7 SDS)	37.4 (−0.05 SDS)	22–52[a]
CNP (pmol/L)	2.5 (1.6 SDS)	6.8 (6.4 SDS)	0.9–2.9[a]
Ratio NTproCNP/CNP	11.4	5.5	21–27[a]
NTproBNP (pmol/L)	8.6	5.3	2–50[b]
BNP (pmol/L)	5.3	2.9	3–12[b]
Ratio NTproBNP/BNP	1.6	1.8	2.6–4.3[b]
NTproANP (pmol/L)	307	268	200–1,260[b]
ANP (pmol/L)	33.7	24.4	4–27[b]
Ratio NTproANP/ANP	9.1	11	20–140[b,c]
cGMP (nmol/L)	7.3	6.1	0.2–2.8[d]

SDS: age adjusted SD.
[a]Childhood reference values[44] (interquartile range – males aged 1–19 yr)
[b]Normal adult reference range[45] (95% confidence interval – adults aged 40–80 yr)
[c]Unpublished data
[d]Childhood reference values[57]

consider in view of the well-recognized lipolytic, metabolic, antifibrotic, and vasodilating effects of NPs.[1,21,53] All affected individuals described in this paper exhibit reduced body weight (Table 1), similar to reports in *Npr3*-null mice and mice with spontaneous mutations in *Npr3*.[29–32] This finding is consistent with the important role of NP signaling in the regulation of body weight and fat mass.[54] The finding of aortic root dilatation is also important. NPR-C is expressed in vascular smooth muscle cells, endometrium, and fibroblasts. In light of the well-documented antifibrotic and vasodilator actions mediated by both NPRA/B receptors,[1] the presence of aortic dilatation and joint laxity is notable. In mice, the cardiovascular phenotype of NPR-C LoF has not been investigated, although chronic and sustained blockade of NPR-C inhibits cardiac fibrosis and remodeling.[55] In two *Npr3* mutant mouse models, sudden death has been reported.[31,32] In our study, progressive aortic dilatation was observed in the older two subjects (Supplemental Note). Therefore, it will be important to closely follow up all affected individuals since it is possible that this cardiovascular complication is age dependent. To further assess the relevance and role of *NPR3* in the development of aortic root dilatation, we screened a cohort of 480 individuals with a referring diagnosis of Marfan syndrome or Loeys-Dietz syndrome but without a pathogenic variant in any of the analyzed aneurysm-associated genes. No *NPR3* mutation, however, was found in any of these individuals, indicating that variants in *NPR3* are probably not a common cause of aortic root dilatation (data not shown). Finally, it is interesting to note that mild tricuspid valve insufficiency and mild mitral valve prolapse have been reported in an individual with skeletal overgrowth due to a balanced translocation causing *NPPC* overexpression.[4]

In summary, our study demonstrates that bi-allelic inactivating mutations in *NPR3* can cause a phenotype characterized by tall stature, long digits (especially the halluces), and variable connective tissue abnormalities, including aortic dilatation and joint hypermobility. A reduced clearance of natriuretic peptides by the defective NPR-C receptor and a consequentially increased activity of the NPR-A/B receptors are the most likely underlying pathophysiologic mechanisms. The presence of extra epiphyses in the hands and feet is intriguing and may reflect the effect of aberrant natriuretic peptide signaling on endochondral ossification. It is also an important diagnostic sign that allows the clinician to distinguish this disorder from other heritable connective tissue diseases characterized by tall stature and aortic dilatation, such as Marfan syndrome and Loeys-Dietz syndrome.

Supplemental Data

Supplemental Data include four figures, two tables, and Supplemental Note (case reports) and can be found with this article online at https://doi.org/10.1016/j.ajhg.2018.06.007.

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Declaration of Interests

T.C.R.P. and E.A.E. have a patent filed entitled "Assessment of skeletal growth using measurements of NT-CNP peptides."

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Web Resources

GenBank, https://www.ncbi.nlm.nih.gov/genbank/
LOVD, http://www.lovd.nl/3.0/home
OMIM, http://www.omim.org/

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