Collagen X Marker Levels are Decreased in Individuals with Achondroplasia

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Abstract
Collagen X marker (CXM) is a degradation fragment of collagen type X. It is a real-time biomarker of height velocity with established norms. Plasma C-type natriuretic peptide (CNP) and NTproCNP levels have also been found to correlate with growth velocity in the general population and are elevated in individuals with achondroplasia compared with age- and sex-matched controls. Collagen X marker levels in people with fibroblast growth factor receptor 3 (FGFR3)-opathies have never been systematically measured. The objective of this study was to measure CXM in a population of dwarfism caused by FGFR3-opathies. Using the same cohort in which CNP and NTproCNP levels were previously measured, archived serum aliquots from 63 children with achondroplasia, six with hypochondroplasia, and two with thanatophoric dysplasia had CXM concentrations measured. Results were plotted against age- and sex-specific norms, and standard deviation scores were plotted for comparison between clinical diagnoses. CXM levels were significantly decreased ($p < 0.0001$) in children with achondroplasia compared with age- and sex-matched controls. Temporal patterns of change in CXM levels were sex-dependent. As the FGFR3 pathway was more constitutively active, CXM levels decreased. New tools are emerging to study impact of skeletal dysplasia on growth plate regulation and function.

Keywords CNP · CXM · Achondroplasia · Biomarker · Growth plate · FGFR3

Introduction
Fibroblast growth factor receptor 3 (FGFR3) regulates bone growth in chondrocytes. Activating mutations in FGFR3 lead to the skeletal dysplasias hypochondroplasia, achondroplasia, and thanatophoric dysplasia. During postnatal skeletal growth, increased signaling of FGFR3 leads to a potent suppression of chondrocyte proliferation and differentiation, resulting in decreased linear bone growth [1].

C-type natriuretic peptide (CNP) is a small, single-chain peptide produced in the hypertrophic zone chondrocytes within the growth plate. It acts on its receptor (NPR-B) to stimulate chondrocyte differentiation and proliferation. C-type natriuretic peptide is a potent positive regulator of linear growth and correlates with growth velocity [2]. Its pro-peptide is cleaved into an inactive amino-terminal fragment (NTproCNP) and the active peptide CNP. Both of these peptides are measurable in the blood. Bio-inactive NTproCNP is not subject to the same clearance pathways as CNP, and, hence, levels in serum reflect CNP production more accurately. In children, plasma levels of CNP and NTproCNP are high in infancy and decrease with age until puberty when they rise again, only to decline soon after to low stable adult levels [2, 3].

The FGFR3 and CNP signaling pathways interact both directly and indirectly in chondrocytes, especially through the Erk MAP kinase pathway [4]. Notably, plasma
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The CXM concentration of each dysplasia sample was determined using the 4 parameter logistic (4PL) nonlinear regression model of the calibration curve using Gen5 software provided by BioTek (Winooski, VT) (R² > 0.95 was deemed acceptable per assay). NTproCNP was assayed as described [5].

Data analysis

Data for CXM are summarized as medians with interquartile ranges (25th–75th percentiles). Reference comparison data for CXM levels were acquired from previously published norms of CXM in healthy individuals [7]. Standard deviation scores (SDS) for FGFR3-OPathy cohort CXM levels were calculated by measuring residuals of FGFR3-OPathy cohort data against a nonparametric Nadaraya-Watson kernel regression estimate on reference data (age vs. CXM, bandwidth of kernel regression was 2.5 years). Statistical analysis with SDSs was conducted on residuals from a nonparameterized regression model in order to maximize statistical power, consequently limiting sources of type II error. Error bars for SD values represent a 95% confidence interval in the age-adjusted difference between reference and cohort CXM values. Significance testing between reference/cohorts was performed via ANOVA and post-hoc via Tukey’s honestly significant difference means comparison test. Two tiers of significance were assumed for p values: < 0.05 and < 0.0001.

Parametrized fourth-order polynomial quantile regression lines (5th / 50th / 95th percentile) for CXM levels in reference data were drawn in R with use of the packages ‘quantreg,’ ‘tidyverse,’ and ‘ggplot2.’ The algorithmic method employed for polynomial quantile regression is a modified version of the Barrodale and Roberts algorithm described in Koenker and d’Orey [9]. This method was selected because of its optimal efficiency in computing the full quantile regression process in programs with fewer than a thousand observations [9]. Quantile regressions accounted for the full reference dataset in male/female/combined without exception [7]. Figures were drawn to age 18 for pediatric focus in FGFR3-OPathy cohorts. The CXM and NTproCNP levels in all 63 individuals younger than 18 years in the achondroplasia cohort were compared, with fourth-order polynomial least-squares regressions drawn for trends. Squares of the Pearson product-moment correlation coefficients (R²) for each regression were included. Statistics were calculated using RStudio (Version 1.4.1103, ©2009–2021 RStudio, PBC).
Results

Subject characteristics are shown in Table 1. There were 63 children with achondroplasia, 6 with hypochondroplasia, and 2 with thanatophoric dysplasia. In children with achondroplasia, CXM was significantly lower ($p < 0.0001$) than in the general population. There was not a statistically significant difference between children with hypochondroplasia and the general population, although the trend seemed to imply a mild decrease. The two children with thanatophoric dysplasia had very low CXM levels compared with their average-stature peers, which was statistically significant although the $n$ was quite small.

The CXM levels of each subject were plotted against age and separated by sex, as seen in Fig. 1. Superimposed on the data are regression lines for CXM levels by sex in the general population [7]. The majority of data points for females with achondroplasia are at or below the mean for the general population; this was true for many of the males with achondroplasia as well.

This is further illustrated in Fig. 2, where regression lines for both the general population as well as children with achondroplasia are all plotted on one graph in 2A and separated by sex in 2B. Figure 2A illustrates the difference between CXM levels of the two populations ($p < 0.0001$). The CXM levels are significantly decreased in children with achondroplasia, especially early in childhood when they are typically quite elevated. Additionally, there are some notable differences between the sexes. Females with achondroplasia deviate more from the general population than males. In males, CXM levels appear to overlap the general population between the ages of 5 and 10 years old. However, while that period is the absolute peak for children with achondroplasia, it is part of a gradual incline for the reference population. This differs greatly from the female data, which

**Table 1** Characteristics of Individuals with fibroblast growth factor receptor 3 (FGFR3)-opathies

|                          | Hypochondroplasia | Achondroplasia | Thanatophoric Dysplasia |
|--------------------------|-------------------|----------------|-------------------------|
| No                       | 6                 | 63             | 2                       |
| Sex (M/F)                | 3/3               | 32/31          | 2/0                     |
| Age (range in years)     | 8.6 (6.3 to 11.4) | 4.7 (0.2 to 17.3) | 2.5 (2.3 to 2.7) |
| CXM Total (ng/ml)$^a$     | 19.6 (19.3 to 21.2) | 21.8 (15.6 to 25.3) | 4.9 (4.6 to 5.2) |
| CXM Total $^b$ SDS       | −0.61 (−1.63 to 0.41) | −0.82 (−1.16 to −0.49)** | −2.68 (−4.44 to −0.92)* |
| CXM Male $^b$ SDS        | −0.39 (−1.85 to 1.07) | −0.59 (−1.06 to −0.11)** | −2.73 (−4.52 to −0.94)* |
| CXM Female $^b$ SDS      | −0.82 (−1.60 to 0.50) | −1.05 (−1.48 to −0.61)** | N/A |

$^a$Data are median (interquartile range)

$^b$Standard deviation score (SDS): average residual vs. Reference (Total, Male [M], or Female [F])/reference SD (95% confidence interval)

* $p < 0.05$ vs. Reference

** $p < 0.0001$ vs. Reference

CXM collagen X marker

Fig. 1 Collagen X marker (CXM) levels by age and sex. Achon achondroplasia, Hypo hypochondroplasia, TD thanatophoric dysplasia
show a significant decrease in CXM levels for females with achondroplasia at all ages until close to skeletal maturity \((p < 0.0001)\).

As a biomarker of phenotype, CXM is further substantiated when levels are separated by diagnosis. The CXM standard deviation scores (SDS) for children with hypochondroplasia, achondroplasia, and thanatophoric dysplasia are plotted in Fig. 3 in comparison with the reference data. There is a statistically significant decrease in the CXM level in children with achondroplasia compared with the general population \((p < 0.0001)\). Additionally, as the FGFR3 pathway is more constitutively active, CXM levels appear to decrease.

It is evident CXM levels are low in children with achondroplasia \((p < 0.0001)\). However, the correlation between CXM and height velocity is insignificant \((R^2 = 0.14)\). This is quite different from the strong correlation observed in the general population \((R^2 = 0.64)\) [6, 7].

Levels of NTproCNP and CXM in the samples collected of the 63 pediatric subjects with achondroplasia are plotted against age on the same graph in Fig. 4. These graphs are overlaid to highlight the similar appearance of the regression line on each. In the first 2 years of life, values of both analytes are higher—CXM more so than NTproCNP. Whereas after 4 years of age NTproCNP is relatively stable, CXM exhibits marked perturbation in years 4 to 8. In contrast to NTproCNP, CXM levels clearly decline in later childhood through adolescence. Although NTproCNP is significantly associated with growth velocity \((R^2 = 0.42)\), and CXM is not, their associations with age are similar \((R^2 = 0.40\) and 0.43, respectively).
Discussion

C-type natriuretic peptide and NTproCNP have been established as growth plate biomarkers that correlate with height velocity and bone growth. Recently, CXM has been identified as a new downstream biomarker of linear growth [6, 7]. For each of these biomarkers, reference data have been established based on levels measured in the general population. Each of these biomarkers can serve as new tools to study the impact of skeletal dysplasias on growth plate regulation and function. Evidence is emerging that these biomarkers are different in individuals with skeletal dysplasia when compared with typically developing individuals [5]. This study adds to the growing literature by illustrating how CXM is decreased in children with FGFR3-opathies when compared with their peers.

Importantly, we observed a significant impact of sex on the profile of CXM changes in early life. Although the impact of sex on long bone growth in achondroplasia has not been extensively studied, differences do appear to be present in mice models. Wagner et al. demonstrated that bone length in male mice at 2 weeks of age is reduced compared with females, suggesting that the inhibitory effects of the FGFR3 gain-of-function mutation occur earlier in males [10]. A trend for lower levels of CXM in male infants in our study aligns with this finding. They also found that CNP overexpression in achondroplasia mice differentially increases female long bone growth. This is supported in Yasoda et al. [11], who also demonstrated a sex-differential in findings with a CNP-overexpressing achondroplasia mouse model, with females more responsive to the growth-promoting effects than males. Taken together, animal models suggest that female growth plates are more sensitive to CNP signaling, while male growth plates are more sensitive to FGF signaling at very young ages. Our study describes a distinct difference in CXM levels between boys and girls with achondroplasia. Despite similar growth patterns in both sexes [12, 13], girls showed consistently decreased CXM levels throughout childhood. The precise reason for this is unknown but might relate in part to the sex differences seen in the mouse models. Closer study of serial changes in CXM and growth velocity in a larger number of carefully matched boys and girls with achondroplasia can be expected to clarify this important issue.

As a biomarker of phenotype, CXM is further substantiated in this study. As shown in Fig. 3, there is a statistically significant decrease in the CXM level in children with achondroplasia compared with the general population. Additionally, CXM levels decrease further with increasing severity of constitutive activation of the FGFR3 pathway. This is in contrast to CNP levels, which increase in a step-wise fashion with these diagnoses. In achondroplasia, for example, despite CNP levels being elevated and positively correlated with growth velocity [5], CXM is low. Likewise, in thanatophoric dysplasia, where the FGFR3 pathway is even more overactive, CNP levels are even higher and CXM levels are even lower, although this was with a small sample size.

Furthermore, it has been previously shown that there is crosstalk between the FGFR3-activated MAPK-pathway and the CNP signaling pathway, both being mutually inhibitory [14]. Overactivation of the MAPK pathway by
activation mutations of FGFR3 reduce natriuretic peptide receptor 2 (NPR2, the CNP receptor) signaling, causing CNP resistance. This study further supports this hypothesis, as evidenced by CXM being low in individuals with FGFR3 overactivity despite high levels of CNP. Collectively, these findings provide biochemical support for increase in the physiological driver of endochondral growth (CNP) proximal to the lesion and concurrent decrease in products of the growth plate hypertrophic chondrocytes (CXM) distal to the lesion. The disturbed architecture of the growth plate in achondroplasia may account for lack of any significant association of CXM with long bone growth. Increased variation in CXM may reflect significant shifts in growth channels, as observed in Del Pino et al. [15], contributing to the lack of association with linear growth.

It is interesting to note CXM levels in children with FGFR3-opathies appear to be most dramatically decreased in early childhood when these levels are usually quite elevated in the general population. Figure 4 highlights the similarities of the biomarker regression lines in children with achondroplasia as well as the trend toward a lack of pubertal peak with instead what appears to be a pubertal plateau in both biomarkers. This may further support the idea that children with achondroplasia do not experience the puberty-related increased growth velocity seen in the general population. A longitudinal study with multiple CXM measurements over many years would be helpful in further characterizing the lack of pubertal peak seen in these children.

This study has limitations. Previous studies have noted that there is a CXM diurnal variation of 26% [6]. However, reanalysis of the reference data using the hours of this study’s sample collection yielded a lower variance of 18%. Additionally, the ELISA assay is not commercially available. However, the laboratory that developed the ELISA assay ran both the reference samples and the samples from this study contemporaneously with the same controls and calibrator sets and under the same conditions, in hopes of mitigating confounding factors. Finally, as skeletal dysplasias are rare conditions, the numbers in this study are small and, therefore, power and statistical significance are limited. With small numbers, it can be challenging to interpret findings. Also, with cross-sectional data, it can be difficult to determine whether trends are true or from sampling errors. This study found differences between the sexes with a greater variation between females with and without achondroplasia compared with the males. Additional data collection is needed to further analyze if this is a true finding.

Future studies should focus on analyzing CXM levels in children, not only with FGFR3-opathies but also other skeletal dysplasias, to further assess how CXM is affected by these changes in growth plate physiology and the potential role of CXM as a biomarker of growth in these populations.

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Authors’ Contribution RSC guarantor, data acquisition, interpretation, writing, RCO study design, data analysis, interpretation, writing, ALD data acquisition, interpretation, writing, RFC data acquisition, WGM study design, data acquisition, CPD data acquisition, CJB data acquisition, DAC data analysis, interpretation, WAH data acquisition, data analysis, BJ data acquisition, data analysis, EAE study design, interpretation, writing, TCRP study design, interpretation, writing, MBB study design, data acquisition, data analysis, interpretation, writing.

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Data Availability Some or all datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

Declarations

Conflicts of interest CJB, ALD, BJ, DAO’C, RCO have no disclosure information relevant to this work. CPD received lecture fees from BioMarin. RFC & WAH are inventors on a patent entitled, “Type X collagen assay and methods of use thereof,” filed by Shriners Hospital for Children. EAE & TCRP have filed a patent entitled, “Assessment of skeletal growth using measurements of NT-CNP peptides.” MBB, RFC, WAH, have consulted for TherAchon/Pfizer and QED. MBB, RFC, EAE, WAH, WGM have consulted for BioMarin. WAH has consulted for Relay Therapeutics.

MBB, WAH have consulted for Ascendis. MBB and RSC receive research funding from BioMarin, TherAchon/Pfizer, Ascendis, and QED.

Ethical Approval The original study was approved by the Nemours Florida Institutional Review Board. The study was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Informed Consent All children had written parental permission obtained. Parental permission forms included authorization to archive and use blood samples for future research studies.

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