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Original Article

Is Plasma C-Type Natriuretic Peptide Level Suitable for Diagnosing and Typing Skeletal Dysplasia?

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ABSTRACT

Objective: Skeletal dysplasia is a heterogeneous group of diseases that lead to abnormal enchondral ossification and typing of the disease is quite complex. C-type natriuretic peptide (CNP), one of the members of the natriuretic peptide family, has been implicated in bone development, and CNP levels are high in some types of skeletal dysplasia. The aim of this study was to evaluate the use of CNP as a marker for skeletal dysplasia types and to investigate its role in typing.

Methods: Thirty-seven patients aged six months to 18 years [26 (70.3%) girls] were included in this cross-sectional study from among 75 skeletal dysplasia patients. All subjects were physically examined; anthropometric measurements were obtained, and bone surveys were evaluated. ELISA was used to assess CNP plasma levels. Forty-nine healthy children aged six months to 18 years [24 girls (49%)] comprised the control group.

Results: The CNP concentration of the patient group (n=37) was 1.31±1.40 ng/mL which was similar to the control group (n=49) at 1.04±1.40 ng/mL (p=0.207). However, the CNP concentration of patients with achondroplasia (n=17) was significantly higher (1.79±1.64 ng/mL) than the control group (p=0.032).

Conclusion: Our study contributes evidence concerning CNP values of both healthy children and children with skeletal dysplasia. Compared with healthy children, those with achondroplasia have elevated plasma levels of CNP. Further larger studies are necessary to assess the use CNP as a marker for the diagnosis and typing of skeletal dysplasia.

Keywords: Achondroplasia, c-type natriuretic peptide, short stature, skeletal dysplasia

INTRODUCTION

C-type natriuretic peptide (CNP) is a member of the natriuretic peptide family and plays a key role in regulating endochondral bone development. Although CNP can be expressed in many tissues, such as cartilage, bone, brain, endothelium, smooth muscle, and heart, it is mostly synthesized in the hypertrophic zone of the growth plate.[1,2]

The CNP is a powerful positive regulator of linear growth.[3,4] CNP released from the endothelium initiates cartilage matrix synthesis and stimulates chondrocyte proliferation and differentiation.[1,2] Furthermore, CNP plays a role in bone development. Experimental

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studies have shown that changes in the CNP gene lead to short stature in knockout mice.[3] Plasma CNP levels were found to be high in adult patients with achondroplasia, thanatophoric dysplasia, and hypochondroplasia.[4]

Skeletal dysplasia is a heterogeneous group of syndromes accompanied by malformations of the skeleton with enchondral ossification. Skeletal dysplasia comprises 461 different diseases that are classified into 42 groups.[5] A detailed history, physical examination, advanced laboratory studies, and a multidisciplinary approach are crucial for the differential diagnosis of skeletal dysplasia. The aims of this study were to evaluate the possibility of using CNP as a marker for skeletal dysplasia types.

MATERIALS AND METHODS

This study was a case-control study. It was approved by the Ege University Faculty of Medicine Ethics Committee (decision dated: 18.01.2017, with approval number 16-8/9). The study was supported by Scientific Research Projects, with grant no. 2018-TIP- 031. Written consent was obtained from all the children and their parents who agreed to participate in the study.

Study Subjects

Subjects were recruited from among 75 patients with skeletal dysplasia followed up in our pediatric endocrinology outpatient unit. The subjects included were between the ages of 6 months and 18 years. The 38 subjects that were excluded either declined to participate, had other systemic diseases such as congenital cardiac defects, were being treated with growth hormones, were on other medical therapy, or were out of the age range of 6 months and 18 years. Thirty-seven of the 75 patients agreed to participate and were included in the study. Fifty healthy children and their parents who attended our general pediatrics outpatient unit were informed about the study, and 49 healthy children were randomly included in the study as a control group. All followed up patients and controls who met the inclusion criteria were included in the study. Post-hoc power was calculated from the mean of the concentration in healthy subjects and patients with skeletal dysplasia. Accordingly, the power of the study was calculated as 0.612.

Study Procedure

A physical examination was performed, and anthropometric measurements were obtained for all subjects. Weight measurements were evaluated by a Turkish Standards Institution (TSI)-approved Baster weighing instrument, with 0.1 kg intervals, and height measurements were made using a Baster-brand measuring instrument with TSI-approved 0.1 cm intervals. Head circumference and chest circumference were measured with an elastic measuring instrument with TSI-approved 1 mm intervals on a precision tape measure. Sitting height was measured using a sit-down Harpenden sitting height table. Standard deviation values of weight, height, head circumference, and body mass index (BMI) were calculated according to the norms of Turkish children developed by Neyzi et al.[6]. The standard deviation of the sitting height and the standard deviation of the sitting height/aspect ratio were evaluated using the Tanner-Whitehouse method.[7]

The puberty stage was classified according to the Tanner scale. Bone surveys were evaluated in the Department of Pediatric Radiology. Bone age was evaluated using the Greulich and Pyle atlas.[8] All subjects who were clinically suspected of skeletal dysplasia underwent radiographic evaluation after whole-body assessment of anterior/posterior and lateral radiographs. The diagnosis of skeletal dysplasia was confirmed by the pediatric radiologist.

Venous blood samples (1 mL) were drawn into EDTA tubes for the measurement of CNP in both the skeletal dysplasia group and the control group. Human CNP (2',3'-cyclic-nucleotide 3'-phosphodiesterase) enzyme-linked immunosorbent assay for quantitative detection of CNP in serum or plasma (Wuhan Fine Biotech Co., Ltd., Wuhan, Hubei, China) was used according to the manufacturer's protocol. The CNP kit we used was sensitive to values of 0.188 ng/mL and could detect values in the range 0.313-20 ng/mL. Plasma samples were isolated by using the method of centrifugation of the blood at 1000xg for 10 minutes and then stored at -80 °C until assayed. The supernatant was collected, and each sample was tested at least twice. We excluded laboratory errors, as shown in the figures below. The healthy group and the patient group perfectly fitted polynomial curves (Figure 1).

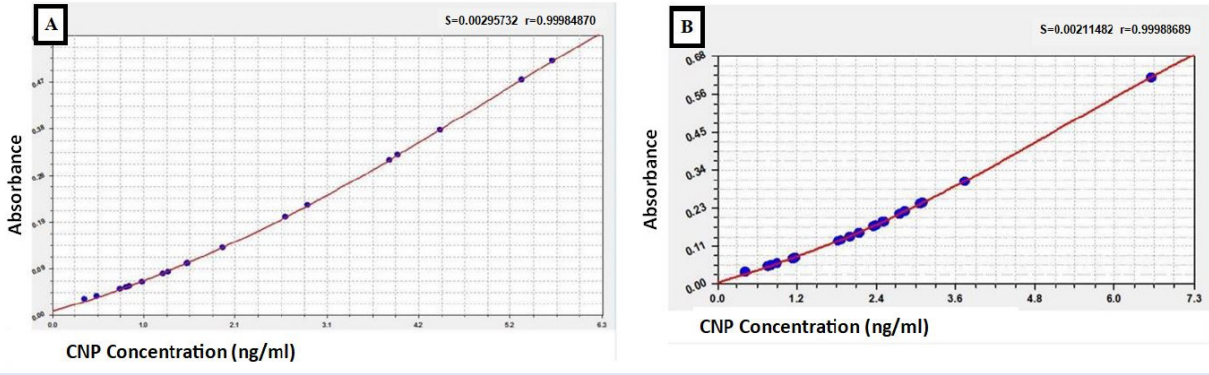

Figure 1. A) Healthy group polynomial curve fit (<0.313 ng/mL excluded), B) Patient group polynomial curve fit (<0.313 ng/mL excluded)

Statistical Analysis

The SPSS, version 21.0 (IBM Corp., Armonk, NY, USA) was used to obtain descriptive statistics of continuous data with mean, standard deviation, median, and lowest and highest values, and of categorical data using frequency and percentage values. The distribution of the data sets was assessed using the Shapiro-Wilk test.

All patients with skeletal dysplasia were compared with the control group, and the subgroup of patients with achondroplasia was also compared with the control group. Age groups were classified as: 1) 0-1 year; 2) 1-5 years; 3) 5-9 years; and 4) >9 years, based on age-dependent changes in height velocity.

The Mann-Whitney U test was used for independent group comparisons, and the Kruskal-Wallis test was used for comparison between independent multiplex groups with continuous variables. Spearman's correlation coefficient was used to determine the direction and degree of linear relationships between CNP concentration and other continuous variables, which included body weight, height, sitting height, BMI, and bone age.

Since CNP concentration did not show a normal distribution according to gender and groups, "sex effect," "group effect," and "gender x group interaction" were analyzed by using non-parametric methods using factorial design * SAS software (Version 9.3; procedure: mixed; SAS Institute, Cary, NC, USA). This approach was also used when the age effect was grouped and analyzed. The significance level was determined as 0.05 in all analyses, and the Bonferroni correction was used in binary comparisons.[9]

RESULTS

The study consisted of 86 participants, including 37 (43%) patients with skeletal dysplasia and 49 (57%) healthy subjects. Of these participants, 59% (n=51) were male, 41% (n=35) were female, and 36% (n=31) were younger than 4 years , 35% (n=30) were between 5 and 9 years , and 29% (n=25) were older than 9 years.

The skeletal dysplasia group consisted of achondroplasia (45.9%, n=17), spondyloepiphyseal dysplasia (16.2%, n=6), metaphyseal dysplasia (13.5%, n=5), epiphysial dysplasia (5.4%, n=2), hypochondroplasia (5.4%, n=2), acromesomelic dysplasia (2.7%, n=1), and unclassified skeletal dysplasia (10.8%, n=4).

In all participants consisted of controls and skeletal dysplasia groups, no significant difference was found between the median CNP and the genders (p=0.082). The median CNP in the skeletal dysplasia group (n=37) was 0.423 (0.31-6.59) ng/mL. The median CNP in the control group (n=49) was 0.313 (0.31-5.725) ng/mL (p=0.207) (shown in Table 1).

The median CNP was significantly higher in the skeletal dysplasia group (p=0.039). When the CNP was compared by gender, the median CNP in boys was significantly lower (p=0.015). Group *sex interaction was not statistically significant (p=0.103) (shown in Table 2).

The median CNP between the skeletal dysplasia group and the healthy group (p=0.187) and between age groups was not different (p=0.456), and the age interaction between the groups *was not statistically significant (p=0.256) (shown in Table 3).

The median CNP was significantly different between the patients with achondroplasia and the control group (p=0.038), whereas no difference was found in terms of CNP between age groups (p=0.814). Group * age interaction was not statistically significant (p=0.319) (shown in Table 3).

In the achondroplasia group (n=17), the median CNP was 1.87 (0.31- 6.59) ng/mL and this was significantly higher than the healthy control group (p= 0.032) (shown in Table 4).

Anthropometric measurements were significantly lower in the skeletal dysplasia group and in the achondroplasia group than in the healthy group (p<0.001) (shown in Table 5).

There was no correlation between CNP and groups' anthropometric measurements, except for sitting height SDS (shown in Table 5).

DISCUSSION

The C-type natriuretic peptide is an essential regulator of skeletal development. Mutations that cause loss of function of CNP receptor, natriuretic peptide receptor-B (*NPR-B*, gene *NPR2*), cause short stature, autosomal recessive skeletal dysplasia, acromesomelic dysplasia Maroteaux type (AMDM).[1,10,11] Considering that 1 in 700 people carries the NPR2 mutation, the reason for the idiopathic short stature phenotype seen in 1 in 30 people might be carrying the *NPR2* mutation.[10,12] Similar phenotypic features were observed in some individuals that overexpressed CNP due to balanced translocations.[1]

Achondroplasia is the most common form of skeletal dysplasia ranging from 1 in 10,000 to 40,000 newborns worldwide.[13] Fibroblast Growth Factor Receptor 3 (*FGFR3*) mutations cause achondroplasia.[13,14] FGFR-3 activates many cascades in the growth plate, such as the signal transducers and activators of the transcription (STAT1) pathway and the MAPK kinase (MEK)/ERK MAPK pathway. These two pathways inhibit CNP intracellular signaling, blocking chondrocyte proliferation and differentiation.

Table 1. Comparison of gender related CNP concentration (ng/mL) values in all population and comparison of CNP Concentrations (ng/mL) in patient, achondroplasia and control groups

Variables		$\tilde{X}$ [Min; Max]
CNP (ng/mL)	Female (n=35)	0.42 [0.31; 6.59][a]
	Male (n=51)	0.31 [0.31; 3.76][a]
	Patient (n=37)	0.42 [0.31; 6.59][b]
	Achondroplasia (n=17)	1.79 [0.31; 6.59][c]
	Control (n=49)	0.31 [0.31; 5.72]

$\tilde{X}$: Median, CNP: C-type natriuretic peptide, Min: Minimum, Max: Maximum

*p<0.05: Level of significance *Mann-Whitney U Test

[a]p= 0.082[mw] : compared with gender groups

[b]p= 0.207[mw] : compared with control group

[c]p= 0.032[mw] : compared with control group

Table 2. Comparison of gender related CNP concentration (ng/mL) values

	Patient	Control	p-value*		
Gender	X̃ (n) [Min; Max]	X̃ (n) [Min; Max]	Gender Effect	Group Effect	Interaction
Female	1.87 (11) [0.31; 6.59]	0.31 (24) [0.31; 5.72]	0.015	0.039	0.103
Male	0.31 (26) [0.31; 3.76]	0.31 (25) [0.31; 2.93]			

X̃: Median, CNP: C-type natriuretic peptide, Min: Minimum, Max: Maximum

*p<0.05: Level of significance *Non-parametric method in factorial design

Table 3. Comparison of distribution of CNP plasma levels in different age groups

	Patient	Achondroplasia	Control	Patient-Control p-value*			Achondroplasia-Control p-value*		
Age	X̃ (n) [Min; Max]	X̃ (n) [Min; Max]	X̃ (n) [Min; Max]	Gender Effect	Group Effect	Interaction	Gender Effect	Group Effect	Interaction
0-1 y.	- (0)	- (0)	3.28 (2) [1.54; 4.44]	0.456	0.187	0.256	0.814	0.038	0.319
1-5 y.	0.42 (10) [0.31; 2.85]	1.17 (6) [0.31; 2.85]	0.31 (19) [0.31; 2.93]						
5-9 y.	1.51 (14) [0.31; 6.59]	2.14 (6) [0.31; 6.59]	0.78 (16) [0.31; 5.38]						
>9 y.	0.31 (13) [0.31; 3.1]	0.81 (5) [0.31; 3.11]	0.31 (12) [0.31; 5.7]						

X̃: Median, CNP: C-type natriuretic peptide, Min: Minimum, Max: Maximum

*p<0.05: Level of Significance *Non-parametric method in factorial design

Overactivity of FGFR-3 inhibits NPR-B. Without an effective receptor, CNP can not initiate intracellular CNP/cyclic guanosine monophosphate (cGMP) signalization. As a result, the plasma CNP levels will be elevated.[12,15] We thought that this well-known mechanism could help typing and diagnosing skeletal dysplasia according to plasma CNP level degrees. Our study found that the level of CNP was higher in patients with achondroplasia than in the reference population (p=0.032).

In the literature, few studies have evaluatedthe CNP plasma level in skeletal dysplasias. Most of them are experimental animal studies.[12,16-19] To our knowledge, CNP level in children with skeletal dysplasia is not known. In the literature, a few studies investigated CNP levels of humans with skeletal dysplasia, which was consisted of patients in adulthood and childhood.[4]

Olney et al.[4] investigated CNP resistance in a group consisting of 63 participants, which consisted of 20 adults with achondroplasia, six children with hypochondroplasia, two children with tanatophoric dysplasia, and four children and one adult with acromesomelic dysplasia, Maroteaux type (AMDM). CNP and NTproCNP were higher in patients with achondroplasia. Similarly, CNP and NTproCNP levels were increased in adult patients with achondroplasia. The NTproCNP/CNP ratio was a measure of CNP degradation, and no difference was found from the reference population. They also showed elevated CNP levels in two children with thanatophoric dysplasia, but their sample size was too small for statistical evaluation. That study showed a positive correlation between NTproCNP level and growth rate in children with achondroplasia and in the reference population. NTproCNP and CNP levels were higher in patients with hypochondroplasia and achondroplasia than in the reference population. They showed increased proCNP products in skeletal dysplasias and CNP resistance in tissues.[4] The results in the achondroplasia group were similar to our study. We observed an apparent elevation in CNP levels in the achondroplasia group (p=0.032). However, since the number of patients with other skeletal dysplasias was insufficient to make reliable statistical analysis, no comparison between CNP level in these small sub-groups and the reference population was attempted (shown in Table 4). The other types of skeletal dysplasia were infrequent. The other studies could not obtain statistical confirmation in the literature. When we compared the patient group with the reference population, we found no difference in CNP levels (p=0.42). Indirectly, we can say that CNP levels are not elevated in all types of skeletal dysplasia. Elevated CNP level is significant for achondroplasia.

Although experimental animal studies showed that CNP levels were potential biomarkers of long bone growth,[12,16-19] it was hard

to use CNP level in daily clinical practice. There are few suitable assays, and there are no reference ranges in CNP measurements. We believe that our study contributed to the literature the measurement of CNP level in skeletal dysplasia.

To understand the other factors that affected CNP levels, we discussed the studies about CNP levels in healthy subjects. CNP levels vary with age, and there is no significant difference between the genders.[20] Del Ry et al.[20] recommended that at least five different reference ranges should be used when evaluating CNP. Therefore, we classified CNP levels according to age groups. We did not include newborns because the CNP level in the newborn period is variable. In a study by Olney et al.[21], both CNP and NTproCNP levels showed an evident variation based on age. CNP and NTproCNP showed very high levels in newborns in the first week of life, followed by a downward trend after six weeks. CNP levels did not change with pubertal status, and there was no difference between boys and girls, as we also found in our study.[21] Our findings supported a study that found no significant difference between male and female populations.[20] However, CNP levels were higher in female patients. We found no interaction with gender and skeletal dysplasia effect (p=0.103). While the gender distribution was similar in the whole group and in the healthy control group, the skeletal dysplasia group had male dominance. Although the age distribution was homogeneous in both groups, we found no correlation between CNP plasma level and age. Del Ry et al.[20] had a lot of newborn subjects in their study. Bone development and height velocity were the fastest in the newborn period. We had no newborns so that we might not find difference. There was a need for studies with a more significant number of healthy participants to evaluate the reference range.

In 2014 Topçu et al.[22] published a study that established a reference value for Turkish children in Ankara and Denizli and compared the relationship between NT-proCNP level and height velocity. In this study, plasma NTproCNP concentration negatively correlated with age, body weight, and height in children. Gender was not a factor affecting age-related plasma NTproCNP concentration until puberty. Contrary to other studies, the plasma NTproCNP concentration in overweight/obese children in the Turkish population was significantly lower than in normal-weight children. In our study, we excluded obese individuals as they could affect the results in the reference group, and there was no clear evidence concerning CNP concentrations in obese children compared to normal-weight peers in the literature. We did not find any significant relationship between body weight, height, age, gender, and CNP levels in either the skeletal dysplasia group or the healthy group.

Our study was more comprehensive than other studies because it included skeletal dysplasia patients and healthy subjects. Thus, we compared many auxological parameters, such as body weight, height, head circumference, sitting height, BMI, and bone age, gender, and age. While there was no clear consensus on the CNP level reference range and the factors affecting its outcome, our study contributed to the literature by providing the CNP values of healthy children and children with skeletal dysplasia.

Table 4. Types of skeletal dysplasia and CNP (ng/mL)

Types of skeletal dysplasia	N (%)	X̃ [Min; Max]
Achondroplasia	17(45.9%)	1.87 [0.31; 6.59]
Spondyloepiphyseal dysplasia	6 (16.2%)	0.61 [0.31; 2.78]
Metaphyseal dysplasia	5(13.5%)	0.31 [0.31; 3.76]
Epiphysial dysplasia	2(5.4%)	0.31 [0.31; 0.31]
Hypochondroplasia	2(5.4%)	0.54 [0.31; 0.76]
Chromosomal dysplasia	1(2.7%)	0.31 [0.31; 0.31]
Other	4(10.8%)	0.73 [0.31; 0.76]

X̃: Median, CNP: C-type natriuretic peptide, Min: Minimum, Max: Maximum

Table 5. Correlation of CNP concentration and anthropometric measurements of designated groups

Variables	Group (n)	X̃ [Min; Max]	p	ρ*
Weight SDS	Patient (37)	-1.45 [-14.5; 2.29]	0.95	0.11
	Ach. (17)	-1.57 [-14.5; 1.60]	0.96	-0.01
	Control (49)	0.15 [-1.67; 1.27]	0.55	0.09
Height SDS	Patient (37)	-4.57 [-14.73; 1.93]	0.72	-0.06
	Ach. (17)	-5.05 [-14.73; 1.93]	0.37	0.23
	Control (49)	0.04 [-1.21; 1.44]	0.17	0.2
Sitting Height SDS	Patient (37)	-2.115 [-9.72; 0.47]	0.99	0.001
	Ach. (17)	-1.40 [-6.76; 0.39]	0.66	-0.12
	Control (49)	-0.21 [-1.75; 1.21]	0.05	0.29
BMI SDS	Patient (37)	1.08 [-5.06; 4.23]	0.47	0.12
	Ach. (17)	1.58 [-5.06; 4.23]	0.49	-0.18
	Control (49)	0.12 [-1.95; 1.06]	0.32	0.15

X̃: Median, CNP: C-type natriuretic peptide Ach.: Patients with achondroplasia, SDS: Standard deviation score, BMI: Body mass index, Min: Minimum, Max: Maximum
p<0.05: level of significance; ρ correlation coefficient; *Bonferroni correction

Study Limitations

This study had some limitations. Since the different skeletal dysplasia groups, with the exception of the achondroplasia group, did not have sufficient numbers the utility of CNP as a biomarker for other skeletal dysplasia types could not be evaluated. Our study was designed as a pioneer study. We hope that this study will act as the spur and a pilot for a nationwide study. We showed that the small group of achondroplasia patients was discriminated from the other types of skeletal dysplasias by considering CNP concentration alone.

CONCLUSION

In our study, we investigated the effects of body weight, height, sitting height, BMI, bone age, gender and age on CNP levels in osteochondroplasia. There was no clear consensus on the CNP level reference range or the factors affecting its outcome. In this context, our study only contributed to the formation of reference values in achondroplasia patients. To establish a definite relationship, studies involving large numbers of different types of osteochondroplasia are required.

Our study showed that plasma CNP levels were higher in patients with achondroplasia. We believe that our study has produced some promising findings and may provide the basis for large samples derived from multicenter or national studies. To conclude that CNP can be used in the typing of all skeletal dysplasias, more clinical studies with molecular genetic analyses are needed.

Acknowledgments

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Ethics

Ethics Committee Approval: It was approved by the Ege University Faculty of Medicine Ethics Committee (decision dated: 18.01.2017, with approval number 16-8/9).

Informed Consent: Published written consent was obtained from parents and their children.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: S.K.Ç., D.G., S.Ö., H.A., Ş.D., Concept: D.G., S.Ö., Ş.D., Design: D.G., Ş.D., Data Collection or Processing: S.K.Ç., D.G., S.Ö., H.A., E.I., Ş.D., Su.Ö., Analysis or Interpretation: S.K.Ç., Ş.D., Su.Ö., Literature Search: S.K.Ç., E.I., Ş.D., Writing: S.K.Ç., Ş.D., Su.Ö.

Conflict of Interest: No conflict of interest was declared by the authors.

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