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Bone Resorption in Syndromes of the Ras/MAPK Pathway

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Abstract

Background—Disorders of the Ras/MAPK pathway have an overlapping skeletal phenotype (eg. scoliosis, osteopenia). The Ras proteins regulate cell proliferation and differentiation and NF1 individuals have osteoclast hyperactivity and increased bone resorption as measured by urine pyridinium crosslinks [pyridinoline (Pyd) and deoxypyridinoline (Dpd)]. Pyd and Dpd are hydroxylysine derived cross-links of collagen found in bone and cartilage and excreted in the urine. Dpd is most abundant in bone. The aim of this study was to evaluate if other syndromes of the Ras/MAPK pathway have increased bone resorption, which may impact the skeletal phenotype.

Methods and Results—Participants: [Noonan syndrome (n=14), Costello syndrome (n=21), and cardiofaciocutaneous (CFC) syndrome (n=14)]. Pyridinium cross-links from two consecutive first morning urines were extracted after acid hydrolysis and analyzed by High Performance Liquid Chromatography. Three separate analyses of covariance (ANCOVA) were performed to compare Pyd, Dpd, and Dpd/Pyd ratio of each group to controls after controlling for age. Data were compared to 99 healthy controls.

Conclusions—The Dpd and the Dpd/Pyd ratio were elevated ($p < 0.0001$) in all 3 conditions compared to controls suggesting that collagen degradation was predominantly from bone. The data suggest that the Ras/MAPK signal transduction pathway is important in bone homeostasis.

Keywords

bone; cardiofaciocutaneous syndrome; Costello syndrome; Noonan syndrome; pyridinium

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Conflicts of Interest: None

INTRODUCTION

Mutations in a variety of Ras/mitogen-activated protein kinase (MAPK) pathway associated genes are causative for several distinct but clinically overlapping disorders. Neurofibromatosis type 1 (NF1) is due to loss of function mutations in *NF1*. Noonan syndrome is a heterogeneous condition and most have mutations in either *PTPN11*, *SOS1*, *KRAS*, *RAF1*, *NRAS*, or *SHOC2* (1–6). Cardiofaciocutaneous (CFC) syndrome is due to mutations in *BRAF*, *MAP2K1*, *MAP2K2*, and *KRAS* (6–8). Costello syndrome is exclusively due to mutations in *HRAS* (9). These genes play a role in the Ras/MAPK signal transduction pathway, and Ras proteins are expressed in osteoprogenitor cells (10–13). The musculoskeletal system is frequently observed in several of the Ras/MAPK pathway syndromes suggesting that activation of the Ras/MAPK pathway impacts cells regulating bone development and homeostasis.

Skeletal abnormalities including osteoporosis are seen in NF1 (14–29). The skeletal findings of Noonan and CFC syndrome are not as well delineated but include short stature, scoliosis, and chest wall deformities (30–34). The skeletal findings of Costello syndrome have been better characterized in small cohorts and include scoliosis, hip dysplasia, chest wall deformities, short stature, and osteopenia (35–36). White et al. reported on bone mineral density (BMD) of 8 individuals with Costello syndrome; all had osteopenia and 3 of the 8 individuals were symptomatic suggesting that decreased BMD in Costello syndrome is common and clinically significant (35).

Based on the musculoskeletal phenotypes, one infers that activation of the Ras pathway could impact bone cellular processes. Evaluation of markers of bone resorption provides a good correlation with bone mass and insight into bone homeostasis (37–40). Collagen molecules are bound together by “crosslinks” which provide strength to bone. As bone is resorbed by osteoclastic activity, the degraded crosslinks of the mature collagen, termed pyridinium crosslinks, are excreted in the urine. The urinary pyridinium crosslinks, pyridinoline (Pyd) and deoxypyridinoline (Dpd), provides an excellent inference of bone resorption (38–46). Pyd and Dpd are found in bone and cartilage, but bone has the highest content of Dpd (42, 43, 46). We previously reported that individuals with NF1 have increased markers of bone resorption (47), and osteoclasts have been shown to be hyperactive in NF1 (10, 48). Therefore, we hypothesized that other syndromes of the Ras/MAPK pathway have increased bone resorption.

MATERIALS AND METHODS

Subjects

Physical examinations and medical histories were obtained on individuals with Noonan, Costello, and CFC syndromes. Individuals were examined by a medical geneticist (DS) and the diagnosis confirmed clinically by phenotype with supportive genotypic information when available. Individuals with recent radiographically confirmed fractures were not included. Families completed questionnaires on calcium intake estimates (49), and metabolic cost (MET) per week based on past-year physical activity levels (50). Pyridinium crosslink data from a local regional cohort of healthy children (N=99) were used for comparison (ages 1–17 years). Given the age range of the controls, and to minimize potential effects related to age, only individuals ≤31 years were included in the syndromic groups.

Written informed consent was obtained and the study was approved by the Institutional Review Board at the University of Utah.

Measurements

Urine from two first morning voids, when possible, was obtained for the extraction and analysis of total, free and the peptide-bound pyridinium crosslinks, Pyd and Dpd, by High Performance Liquid Chromatography (HPLC) according to established procedures (44, 47). In brief, urine was hydrolyzed and pyridinium crosslinks were isolated using a cellulose column and analyzed by reverse-phase HPLC. Pyridinium crosslink concentration was calculated using a 4-levels calibration curve obtained with an external standard. Urinary pyridinium crosslink concentration was normalized to urinary creatinine, measured by an Olympus AU 400 and calculated as $\mu\text{mol/mol}$ creatinine. The average of the two first-morning urine samples was used to minimize the effect of the day-to-day variation in the excretion of the crosslinks.

Mutation Analysis

DNA was extracted either from peripheral blood or buccal cheek cells through standard protocols at the University of Utah Center for Clinical and Translational Science Core Facility using a commercial kit manufactured by QIAGEN (Valencia, CA).

PTPN11, SOS1, HRAS, BRAF, MEK1, MEK2 Sequencing—Sanger sequencing of PCR products amplified from genomic DNA was performed using standard techniques on exons 3, 8, and 13 of *PTPN11*, all exons of *SOS1*, exon 2 of *HRAS*, exons 6, 11, 12, 14 and 15 of *BRAF*, and exons 2 and 3 of *MEK1* (*MAP2K1*) and *MEK2* (*MAP2K2*). Conditions and primer sequences are available upon request.

Data Analysis

Standard univariate descriptive statistics were used to summarize the distributions of the pyridinium crosslink measurements for each syndrome, and scatter plots with regression curves were used to examine the joint relationships of the crosslink measurements with age and syndrome.

Three separate analyses of covariance (ANCOVA) models were performed to compare mean levels of Pyd, Dpd, and the Dpd/Pyd ratio between each of the Costello, CFC, and Noonan syndrome groups to the control group after adjusting for age. In sensitivity analyses, expanded models with quadratic terms for age and interactions between age and syndrome group were examined to confirm the robustness of the age-adjusted comparisons to potential violations of the basic ANCOVA model. Adjusted means of pyridinium crosslink measurements were calculated to provide estimates of the mean crosslink measurements given the average age of 10.4 years for the full cohort. All hypothesis tests were performed using a 2-sided level of 0.05, without adjustment for multiple comparisons. All statistical analyses were performed using SAS 9.2 (SAS Institute Inc., NC).

RESULTS

The cohort consisted of 148 participants: Noonan syndrome (n=14), CFC syndrome (n=14), Costello syndrome (n=21), controls (n=99) (Table I).

Mean ages differed significantly among the four groups ($p < 0.001$) (Table I). Two separate consecutive first morning urine samples were available in the majority of individuals.

The mean past-year physical activity estimation of those who completed questionnaires for the syndromic groups was 29.2 MET/week (range 2.6 – 121; median 16.5; n=27) [Costello syndrome: mean 38.4, range 6.1–121, median 17.5, n=11; CFC syndrome: mean 19.3, range 4.1–82.2, median 14.6, n=9; Noonan syndrome: mean 27.4, range 2.6–59.7, median 26.3,

n=7]. Two individuals with Costello syndrome utilized a wheelchair, and two individuals with CFC syndrome used “walkers”. Activity estimation levels were not available in controls, however, mean levels for a cohort of 241 healthy children have been reported previously to be 38.1 MET/week (range 0.6–316.8; median 25.6) (23). Mean calcium intake estimations for the syndromic groups were 1379 mg/day (range 157–3161, median 1193, n=35) [Costello syndrome: mean 1449, range 423–3161, median 1293, n=14; CFC syndrome: mean 1114, range 157–2875, median 1019, n=13; Noonan syndrome: mean 1686, range 493–2692, median 1558, n=8]. Calcium intake estimations were not available in controls, but average ranges based on age and sex for healthy individuals <30 years in one report were 918–1296 mg/day (52).

Dual energy x-ray absorptiometry (DXA) measurements performed previously on a clinical or research basis were available on 9 individuals (Table II).

Mutations in *HRAS* were identified in all individuals with Costello syndrome in which adequate DNA was available: p.G12S (n=17), p.G13C (n=2), p.G13D (n=1). In one individual in which adequate DNA was not available, an *HRAS* mutations leading to the following predicted amino acid change (p.A146V) was verbally reported by parents.

Upon sequencing of *PTPN11* exons 3, 8, and 13, mutations were identified in 6 individuals with Noonan syndrome: p.N308D (n=2), p.D106A (n=1), p.N58D (n=1), p.N58H (n=1), p.Y63C (n=1). A mutation in *SOS1* (p.T266K) was identified in one individual with Noonan syndrome. In 2 individuals, mutations were identified in a clinical laboratory but not confirmed in our laboratory: *RAF1* (p.P261A), *PTPN11* (p.F285S). No causative mutations were identified in 5 individuals after sequencing of *SOS1* and *PTPN11* (exons 3, 8, 13), reviewing available medical records, and assessing parental report of possible mutations.

Upon sequencing of *BRAF* exons 6, 11, 12, 14 and 15, mutations were identified in 5 individuals with CFC syndrome: p.S467A (n=1), p.F468S (n=1), p.G469E (n=1), p.T470del (n=1), p.L597V (n=1). A mutation in *MEK1* was identified in one individual (p.K59del), and a mutation in *MEK2* was identified in another (p.Y134C). In the remaining individuals with CFC syndrome in which adequate quality DNA was not available or genes or exons not selected for sequencing, mutations in the following genes were reported by parents: *BRAF* (n=2), *MEK2* (n=2), *KRAS* (n=1), unknown (n=2).

After adjustment for age, Pyd was elevated in each syndromic group compared to controls, with statistical significance reached for the Noonan and Costello syndrome groups [adjusted mean differences \pm SE vs. controls were 85.1 ± 23.8 ($p=0.0005$) for Noonan syndrome, 45.3 ± 20.8 ($p=0.0311$) for Costello syndrome, 34.8 ± 23.4 ($p=0.1397$) for CFC syndrome (Fig. 1, Table III)].

Age-adjusted Dpd levels were significantly elevated in all 3 syndromic groups compared to controls [adjusted mean differences \pm SE vs. controls were 35.0 ± 5.9 ($p<0.0001$) for Noonan syndrome, 28.2 ± 5.8 ($p<0.0001$) for Costello syndrome, 28.2 ± 5.8 ($p<0.0001$) for CFC syndrome (Fig. 2, Table III)]. The age adjusted differences among the four groups were larger relative to the variability in the data for Dpd (increase in R^2 attributable to group differences = 24%) than for Pyd (increase in R^2 = 21%), and age-adjusted Dpd/Pyd ratios were also significantly elevated in all three syndromic groups vs. controls (adjusted mean differences vs. controls were 0.06 ± 0.013 ($p<0.0001$) for Noonan syndrome, 0.07 ± 0.011 ($p<0.0001$) for Costello syndrome, 0.08 ± 0.013 ($p<0.0001$) for CFC syndrome (Fig. 3, Table III). While formal adjustments for multiple comparisons were not included in the original analysis plan, the age adjusted comparisons of Dpd and of Dpd/Pyd between each syndrome group and controls remained statistically significant at the 0.001 level after post-

hoc Bonferroni adjustment for 9 separate comparisons (3 syndromic groups vs. control comparisons for each of three outcomes).

Age-adjusted elevations in Dpd and the Dpd/Pyd ratio vs. controls were similar between the Noonan, CFC, and Costello syndrome groups, and no statistically significant differences ($p < 0.05$) were observed between the syndrome groups for any of the pyridinium crosslink measurements.

The Dpd/Pyd ratio was not significantly correlated with physical activity level estimation (MET/wk) in the 27 participants with physical activity level data (Pearson partial $R = 0.22$, $p = 0.29$).

One individual with Costello syndrome was receiving pamidronate. Two individuals with Noonan syndrome, 2 individuals with Costello syndrome and 1 individual with CFC syndrome were receiving growth hormone. Given the potential effect of these therapeutic agents on bone resorption the data were re-analyzed excluding these individuals and the comparisons of each syndrome group with controls and between syndrome groups were similar.

DISCUSSION

Individuals with selected syndromes of the Ras/MAPK pathway (i.e. Noonan syndrome, CFC syndrome, and Costello syndrome) have increases in urinary pyridinium crosslinks similar to what we have previously reported in NF1 (least squares mean for individuals with NF1 with a skeletal dysplasia: Pyd = 233 $\mu\text{mol/mol}$ creatinine, Dpd = 71 $\mu\text{mol/mol}$ creatinine, Dpd/Pyd ratio = 0.31) (47). Dpd and the Dpd/Pyd ratio were significantly elevated in all syndromes suggesting that the increase in pyridinium crosslink excretion is primarily from bone resorption.

The exact etiology of the increase in the excretion of bone resorption markers is not known and likely multifactorial. Our data from individuals with Noonan, CFC, and Costello syndromes support the hypothesis that bone resorption is increased in syndromes associated with increased Ras signaling. It is likely that additional factors contribute to the increased bone resorption markers such as inactivity, hypotonia, and poor motor function. However, based on results from the *Nf1*^{+/-} mouse model, it is known that the myeloid and mesenchymal progenitor cells function abnormally (10–13), and *in vitro* studies on NF1 human osteoclasts have increased lytic activity suggesting that increased signaling through the Ras/MAPK pathway impacts bone remodeling (10, 48). Functional assays of osteoclasts from individuals with Noonan, CFC and Costello syndromes will help determine the direct relationship of increased Ras signaling on osteoclast cellular functions.

Decreased BMD has been reported in Costello syndrome and NF1 (19–29, 35), and was also observed clinically from DXA scans in small numbers of individuals with Costello, CFC, and Noonan syndromes. It is possible that the decreased BMD is appropriate for size given the association of short stature with these syndromes, yet several of the previously reported individuals with Costello syndrome had significant fractures associated with osteoporosis (35). Even if generalized skeletal abnormalities are common, there are discrepancies in the focal skeletal phenotype of the Ras/MAPK pathway syndromes (eg. sphenoid wing dysplasia and tibial dysplasia only observed in NF1). This is likely secondary to somatic events (53, 54), modifier genes, expression patterns of the various causative genes, and activation of other effector pathways.

The clinical consequences of the increased bone resorption are still not well known and there may be compensatory effects by the mesenchymal lineages. Although bone resorption

is increased, many of the musculoskeletal findings such as pectus abnormalities and hand anomalies are likely not related to osteoclast functions suggesting that the Ras/MAPK signal transduction pathway impacts other cell lineages important in musculoskeletal development.

A number of limitations make interpretation of our results difficult. The rarity of the syndromes, particularly CFC syndrome and Costello syndrome, results in a small cohort of individuals. In addition, co-morbidities may contribute to bone resorption. For example, one individual with Costello syndrome was receiving pamidronate infusions, which interferes with osteoclast function likely decreasing the bone resorption markers. Also, 5 individuals were receiving growth hormone injections which potentially could increase bone resorption markers (55). Any factors decreasing bone loading could also increase bone resorption markers. The mean MET/week for the combined syndromic groups was lower compared to previous reports of healthy children (23) and decreased physical activity from a number of co-morbidities such as hypotonia, scoliosis, seizures, etc. may contribute to increased bone resorption. However, there was no statistically significant correlation between the Dpd/Pyd ratio and physical activity level estimation (MET/wk). Interestingly the mean MET/week for the Costello syndrome group was similar to previous reports of healthy children, but the effectiveness of performing activities that put forces upon bone may be impaired.

Although bone resorption was increased in the syndromic groups compared to controls, there were no significant differences between the phenotypically categorized Noonan, CFC, and Costello syndrome groups. Assessment of bone resorption based on the various genotypes was difficult to assess give small number of individual genotypes. Future studies with larger numbers of participants will be needed to examine correlations of genotypes with bone resorption.

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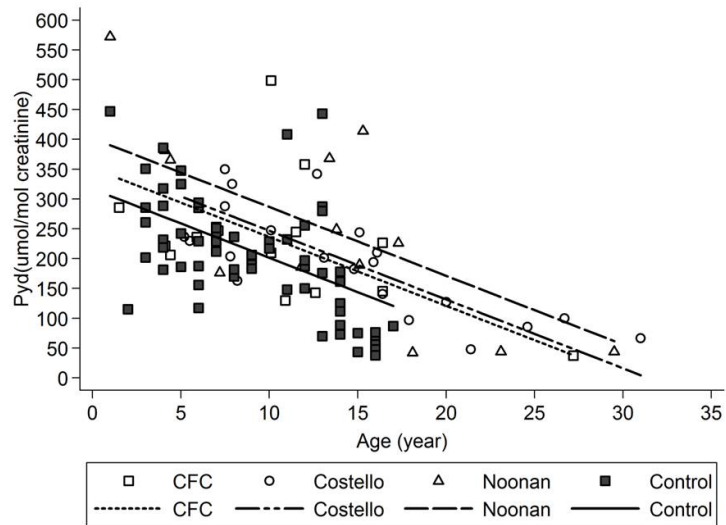


Figure 1. Comparisons of Pyridinoline (Pyd) Among Syndromic Groups
 Pyd (umol/mol creatinine) in each syndromic group (Noonan syndrome, Costello syndrome and cardiofaciocutaneous (CFC) syndrome) was compared to controls using age-adjusted analyses of covariance (ANCOVA) adjusted for age shown graphically.

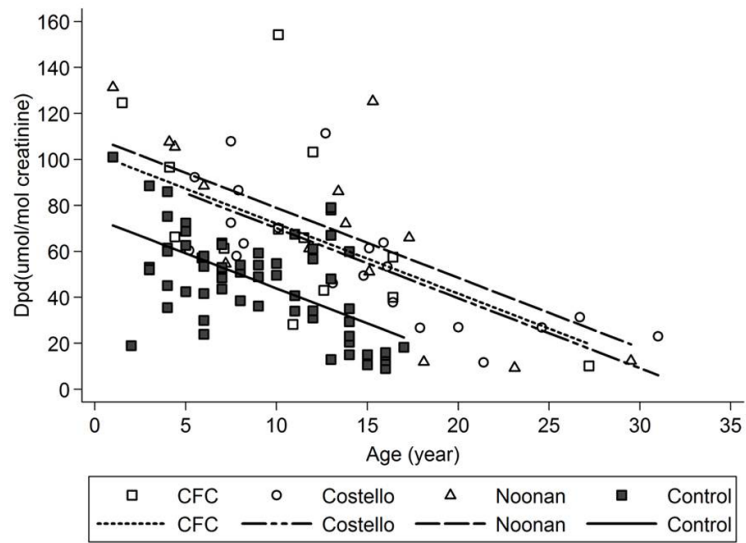


Figure 2. Comparisons of Deoxypyridinoline (Dpd) Among Syndromic Groups
 Dpd (umol/mol creatinine) in each syndromic group (Noonan syndrome, Costello syndrome and cardiofaciocutaneous (CFC) syndrome) was compared to controls using age-adjusted analyses of covariance (ANCOVA) and shown graphically.

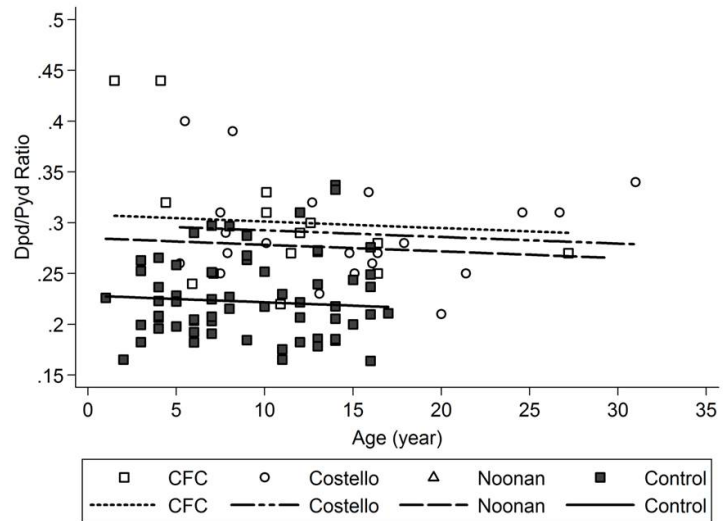


Figure 3. Comparisons of Deoxypyridinoline/Pyridinoline (Dpd/Pyd) Ratio Among Syndromic Groups

Dpd/Pyd ratio in each syndromic group (Noonan syndrome, Costello syndrome and cardiofaciocutaneous (CFC) syndrome) was compared to controls using age-adjusted analyses of covariance (ANCOVA) and shown graphically.

Table 1
 Characteristics of Patients(age)and the Unadjusted Average of Pyridinium Crosslink Measurements for Syndromic Groups

	Controls (N=99)	Noonan Syndrome(N=14)	CFC Syndrome (N=14)	Costello Syndrome (N=21)	P value*
Age (yrs) (mean ±SD)	9.06 ± 4.35	12.86 ± 7.89	10.73 ± 6.49	14.57 ± 7.21	0.0002
Pyd(umol/molcrt)(mean ±SD)	212.24 ± 95.37	253.50 ± 156.52	227.81 ± 109.20	194.34 ± 88.61	0.38
Dpd(umol/molcrt)(mean ±SD)	46.78 ± 20.85	70.23 ± 40.33	69.87 ± 38.50	56.25 ± 27.68	0.0008
Dpd/Pyd ratio (mean ±SD)	0.22 ± 0.04	0.28 ± 0.03	0.30 ± 0.07	0.29 ± 0.05	<0.0001

* Overall comparison of means among syndromic groups using Analysis of Variance (ANOVA) F-Test.

Pyd=pyridinoline; Dpd=deoxypyridinoline; crt=creatinine

Table II

Dual Energy X-ray Absorptiometry Bone Mineral Density (BMD) Scores

<u>Study Participant</u>	<u>Body region imaged</u>	<u>Derived scores for BMD</u>
Costello #8	whole body	z-score (-4.6)
	lumbar spine	z-score (-4)
	distal femur	z-score (-6.8)
Costello #10	whole body	z-score (-1.4)
	lumbar spine	z-score (-2.4)
	lumbar spine	t-score (-4.1)
Costello #15	femoral neck	t-score (-2.3)
	radius	t-score (-5.6)
	total hip	z-score (-2.8)
Costello #17	lumbar spine	t-score (-3.7)
	femoral neck	t-score (-2.0)
	radius	t-score (-5.2)
Costello #24	lumbar spine	z-score (-2.4)
	femoral neck	z-score (-1.9)
	total hip	z-score (-2.7)
Costello #25	forearm	z-score (-3.6)
	whole body	z-score (-1.3)
	lumbar spine	z-score (-1.3)
CFC #16	femoral neck	z-score (-1.7)
	lumbar spine	t-score (-1.6)
	femoral neck	t-score (-1.2)

Table III

The Comparisons of Adjusted Means in Syndromic Groups with Controls

	Control		Noonan Syndrome		CFC Syndrome		Costello Syndrome	
	LS Mean ³ ± SE ³	p-value ¹	LS Mean ³ ± SE ³	Diff. ³ ± SE	LS Mean ³ ± SE ³	Diff. ³ ± SE	LS Mean ³ ± SE ³	Diff. ³ ± SE
Pyridinium Crosslink Pyd ² (umol/molert)	197.30 ± 8.38	0.0005	282.42 ± 22.08	85.12 ± 23.83	232.12 ± 21.86	34.82 ± 23.44	242.62 ± 18.60	45.32 ± 20.82
Dpd ² (umol/molert)	42.84 ± 2.8	<0.0001	77.86 ± 5.48	35.03 ± 5.92	71.01 ± 5.43	28.17 ± 5.82	68.99 ± 4.62	26.16 ± 5.17
Dpd/Pyd ratio	0.22 ± 0.004	<0.0001	0.28 ± 0.01	0.057 ± 0.013	0.30 ± 0.01	0.080 ± 0.013	0.29 ± 0.01	0.070 ± 0.011

Note:

¹ Comparison with control² Pyd=pyridinoline; Dpd=deoxypyridinoline; crt=creatinine³ LS=least square, SE=standard error of LS mean, Diff. =difference