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Hypercalciuria in children with haemophilia suggests primary skeletal pathology

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Summary

Several factors can compromise optimal bone mass accrual during childhood and predispose to osteoporosis later in life. Patients with haemophilia are at risk of low bone mass already during childhood, partly due to reduced physical activity related to joint bleeds and haemarthrosis. The introduction of primary prophylaxis with regular infusions of the deficient coagulation factor has enabled reduction or prevention of haemophilic arthropathy. Finnish children with severe haemophilia start prophylaxis early and are encouraged to participate in physical activities. We hypothesized that prophylactic therapy would ensure normal childhood bone mass development. We carried out a case-control study in 29 children with haemophilia (**2 mild, 6 moderate, 21 severe**) and 58 age-matched controls. Their bone health was determined by fracture history, blood and urine biochemistry, bone densitometry and spinal imaging. Bone mineral density was lower in children with haemophilia but there was no evidence for significantly increased fracture rate. The patients had significantly higher urinary calcium excretion and higher serum calcium concentration, and reduced bone resorption as compared with the controls. Our findings suggest primary skeletal pathology, resulting in increased urinary calcium loss and altered bone metabolism, which may over time contribute to the development of osteoporosis in patients with haemophilia.

Keywords: hypercalciuria, bone mineral density, osteoporosis, children, haemophilia

Introduction

Osteoporosis, decreased bone strength with increased fracture risk, affects mainly the elderly. However, the basis of osteoporosis can be established already during childhood if peak bone mass (PBM) attainment is compromised.

Genetic factors account for most of the variation in PBM. Other factors include inadequate dietary intake of calcium and vitamin D, reduced physical activity, chronic illnesses, hormonal deficiencies and some medications, especially glucocorticoids. In clinical practice, the diagnosis of osteoporosis is mostly based on dual-energy X-ray absorptiometry (DXA). Reduced bone mineral density (BMD) is associated with susceptibility to fractures in all age groups (Boot *et al*, 2010).

Haemophilia is an X-chromosomal inherited bleeding disorder characterized by bleeding tendency due to missing or deficient clotting factor VIII (FVIII) (haemophilia A) or IX (FIX) (haemophilia B). The clinical presentation and severity vary according to factor levels (in severe form, the FVIII or FIX is $< 1\%$ of normal activity, moderate $\geq 1 - 4\%$ and mild $\geq 5 - 25\%$). In severe haemophilia, the often spontaneous bleeds in joints and muscles lead to haemarthrosis and chronic arthropathy. Severe haemophilia is most often diagnosed within the first year of life. In moderate haemophilia bleeding episodes occur usually after small traumas or injuries. In the patient with mild disease, the spectrum of bleeding manifestations includes easy bruising and postsurgical bleeds. With modern prophylaxis (i.e. regular infusion of the missing coagulation factors) severe haemophilia can be rendered clinically to moderate disorder by preventing spontaneous joint bleeds. The children with prophylactic therapy are encouraged to participate in normal physical activities.

Several chronic illnesses predispose to osteoporosis. Also patients with severe haemophilia are at risk of osteoporosis (Gallacher *et al*, 1994; Wallny *et al*, 2007; Iorio *et al*, 2010). Increasing evidence suggests that even children with haemophilia are at risk for reduced BMD (Barnes *et al*, 2004; Tlacuilo-Parra *et al*, 2008). Several risk factors can contribute to these findings. First, children with haemophilia are likely to be less active in sports than their healthy peers. This may correlate to the severity of haemophilia, the number of previous joint bleeds and the availability of prophylactic treatment. Children who develop inhibitory antibodies against the coagulation factor needed are especially prone to spontaneous bleeds before successful immunotolerance induction (ITI). Secondly, sedentary lifestyle is associated with reduced sunlight exposure and vitamin D deficiency. Thirdly, chronic systemic inflammation due to hepatitis C or other blood-borne viruses may predispose to reduced BMD (Masaki *et al*, 1998; Katsarou *et al*, 2010). Furthermore, it has been speculated that chronic blood loss induces osteoporosis through functional interaction between haematopoietic and bone tissues (Gurevitch & Slavin, 2006).

Since the introduction and better availability of plasma-derived products of higher purity as well as recombinant concentrates in haemophilia treatment during the past 20 – 30 years, the prognosis and life expectancy of patients with haemophilia have improved remarkably. As life expectancy approaches that of the general male population, we face new challenges with elderly haemophilia patients and their age-related illnesses. Osteoporosis in the advanced age can significantly complicate the treatment of haemophilic arthropathy. Achieving the optimal PBM is therefore especially important in patients with haemophilia or other severe bleeding disorder. Nilsson and co-workers (1992)

1
2 showed that it is possible to preserve joint function by preventing bleedings with early prophylaxis. Recent studies
3 (Khawaji *et al*, 2009; 2010) have indicated that early-onset prophylactic treatment can help to maintain mobility and
4 preserve normal BMD. At our center the principal treatment regimen for children with severe haemophilia has been
5 from the late 1990s regular continuous treatment as primary prophylaxis (PP), started before the age of 2 years
6 without previous joint bleed or after the first joint bleed. Considering these protective circumstances their skeletal
7 characteristics were expected to be similar to healthy population. This case-control study was undertaken to assess
8 bone health and relevant life-style factors, including dietary intakes of calcium and vitamin D, and physical activity in
9 children with haemophilia.
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22 Patients and Methods

23 *Patients and study protocol*

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25 This one-center case-control study included children with haemophilia **who were aged > 4 years. Twenty two of**
26 **them were** on regular continuous treatment, defined as PP when started before age 2 years without previous joint
27 bleed or after the first joint bleed (Donadel-Claeyssens *et al*, 2006) or as secondary prophylaxis (SP) when started
28 after 2 years or after more than 2 joint bleeds. The subjects with SP include one boy with severe haemophilia and
29 inhibitor, one with delayed diagnosis of haemophilia and one with moderate to severe haemophilia. The
30 prophylactic clotting factor therapy in haemophilia A is typically 20 – 40 IU/kg three times a week or every other day
31 and in haemophilia B, 30 – 50 IU/ kg twice weekly or every third day. The patients are encouraged to participate in
32 sports activities. Contact sports such as ice hockey are allowed before 12 – 14 years of age but not encouraged in the
33 advanced series without tailoring of the treatment regimen. None of the children have hepatitis B, C or HIV infection.
34 **Clinically the joint bleeds had not led to long term functional disability in any of the patients. None of the patients**
35 **had a history of high blood pressure or haematuria.**
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47 An information letter and a consent form were sent to the parents of 33 patients with haemophilia followed at
48 Hospital for Children and Adolescents, University of Helsinki, Finland. One patient with severe haemophilia A was
49 from Vietnam and one with moderate haemophilia A from India, all others were of Caucasian origin. The Research
50 Ethics Committee of Helsinki University Hospital approved the study and a written informed consent was obtained
51 from all study participants and/or their guardian. With the exception of two patients with mild haemophilia, all
52 consented to participate in the study (31/33, 94%).
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57 Clinical data were collected from the patients' medical records. The patients were interviewed and clinically
58 examined by SR or AM on their hospital follow-up visit. Anthropometry, bone mineral and body composition
59 measurements and laboratory samples were obtained during the same visit.
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2 Two age and height-matched controls for each patient were selected from a representative cohort of apparently
3 healthy Finnish school children from the same district (Viljakainen *et al*, 2010). The original cohort comprised 202
4 children and adolescents from 7 to 19 years of age; 62% were girls. Background characteristics, lifestyle factors and
5 medical history were collected with questionnaires and interview. Anthropometry, bone mineral and body
6 composition measurements and laboratory samples were obtained during the study visit using the same methods as
7 for the patients. With the exception of controls for two 4-year-old boys with haemophilia all the selected controls
8 were male.

17 *Bone densitometry and radiographic evaluation*

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19 Whole body (WB), lumbar spine (LS) (L1-L4) and total hip BMD (g/cm²), bone area and bone mineral content (BMC)
20 were measured with DXA (Hologic Discovery A, Waltham, USA). All the values were transformed into Z-scores (the
21 difference of the measured value from the mean sex- and age-specific reference value in SD units) using the
22 equipment-specific reference database for United States Caucasian Children. Body composition was analyzed with
23 the manufacturer's software to separate lean body mass (LBM) and fat mass (FM). Percent fat was calculated as fat
24 mass divided by total mass. For the detection of possible vertebral compression deformities, lateral images of the
25 thoracic and lumbar spine were acquired using the DXA scanner (IVA, instant vertebral assessment). The vertebrae
26 were compared with adjacent vertebrae and graded depending on percent reduction in anterior, mid- or posterior
27 vertebral height. A 20% reduction in vertebral height was used as cut off to define abnormal morphology (Mäkitie *et*
28 *al*, 2005).

29
30 Bone age of the patients was determined from a plain radiograph of the left hand according to Greulich and Pyle
31 (1959). Both the bone age and IVA radiographs were evaluated by an experienced paediatric endocrinologist (OM)

42 *Growth assessment*

43
44 Height was measured without shoes using a standard wall-mounted Harpenden stadiometer. Weight was measured
45 using a standard electronic scale. The SD score for height and height-adjusted weight (a percentage of the mean
46 ratio of normal population of the same gender and height) were obtained using the Finnish standards (Sorva *et al*,
47 1990, Pere *et al*, 2000). Body mass index (BMI) was calculated as weight in kilograms divided by square of height in
48 meters (kg/m²). Pubertal maturation of the patients was assessed clinically according to Tanner (1962), by sex
49 steroid concentrations and the bone age. Assessment of pubertal maturation of the controls was based on serum
50 gonadotrophin and sex steroid concentrations only. For data analysis the subjects were divided into three subgroups
51 based on pubertal maturation (prepubertal, pubertal and postpubertal).

59 *Lifestyle factors*

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2 Together with their parents the subjects filled in a questionnaire on background characteristics, lifestyle factors and
3 medical history; information was reviewed and completed together with a paediatrician during the follow-up visit.
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5 For all the fractures, also the localization and trauma mechanism were recorded.
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8 The physical activity included regular every-day activities (e.g. walking to school), activity at school, and both guided
9 and unguided leisure-time activities. Duration, frequency and intensity of activity sessions were recorded. A total
10 physical activity score was obtained as a sum for a whole week. Physical activity scores of 13.5, 17 and 20
11 correspond to 1, 1.5 and 2 hours daily activity, respectively, which meet the recommendation for this age group
12 (Nordic Council of Ministers, 2004). In addition, duration of each activity was multiplied with an intensity factor
13 according to Neville *et al* (2002) to obtain scores for intensity of physical activity and strenuous exercise, expressed
14 as a total for a whole week. Physical activity score was considered low when less than 13, corresponding to less than
15 one hour of daily activity. Intensity of physical activity score included in addition the intensity of the sports activity
16 (range 3.0-34.0).
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24 Dietary intakes of vitamin D and calcium were calculated using a food frequency questionnaire (FFQ), which is a
25 validated semi quantitative questionnaire covering over 70 foods (Outila *et al*, 2001). The nutrient contents of the
26 foods were calculated using the Finnish National Food Composition Database (Fineli®, version 2001), which is
27 maintained by the Nutrition Unit, National Public Health Institute of Finland. The calculated total intake of vitamin D
28 included also intake from supplements. If FFQ was not returned (8 patients and 13 controls subjects), dietary intakes
29 of vitamin D and calcium were calculated by a nutritionist either from 4-day food records or from a structured
30 interview during the out-patient visit.
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38 *Laboratory measurements*

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40 Morning blood samples after overnight fast were assayed for testosterone, 25-hydroxy vitamin D (25-OHD), intact
41 parathyroid hormone (PTH), calcium (Ca), phosphate (**Pi**), alkaline phosphatase (ALP), carboxyterminal telopeptide of
42 type I collagen (ICTP), and procollagen type I aminoterminal propeptide (PINP). Concentration of calcium (**u-Ca**),
43 phosphate (**u-Pi**) and creatinine (**u-Crea**) and type I collagen cross-linked N-telopeptide (INTP or NTx) was measured
44 from second void urine samples. All laboratory samples for patients and controls were obtained **between October**
45 **and April when the amount of sunlight is minimal** except in three patients with severe B haemophilia whose
46 samples were obtained in late summer and therefore excluded from the analyses. **The measurements were in all**
47 **study subjects based on a single sample, obtained similarly in the patients and the controls.** All blood and urine
48 measurements were analyzed in the Central Laboratory of Helsinki University Hospital.
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58 *Statistical analyses*

59 Statistical analyses were performed with SPSS for Windows, Version 17.0 for Windows (SPSS Inc. Chicago, IL).
60 Comparison of two groups was performed with Independent samples t-test and in case of categorical variables with
Chi Square. Pearson correlation was utilized to identify associations between variables. Multivariate analyses were

1
2 performed to investigate differences in BMD after adjusting for confounder e.g. intensity of physical activity. If
3 variables were not normally distributed corresponding non-parametric tests were applied. Results are expressed as
4 means with 95% confidence limits or SDs. P values <0.05 were considered significant.
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10 **Results**

11 *Demographic and anthropometric data*

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16 The final study group included 31 haemophilic boys aged between 4 and 16 years. The clinical characteristics of the
17 patients are presented in Table I. The median age at the start of the prophylactic treatment was 0.97 years (mean
18 1.47). Seven of the total cohort (24%) and 6 (29%) of the patients with severe haemophilia had a history of no joint
19 bleeds. For the remaining 22 patients, the number of joint bleeds (either self-reported or documented) varied
20 between 1 and 11 (mean 3.1, median 2). The anthropometric characteristics of the patients and controls were
21 similar (Table II). The number of overweight or obese patients was slightly higher in patient group. Also, the mean
22 fat% Z-scores were somewhat higher in cases compared to controls. Neither of these figures was statistically
23 significant and there were no differences in BMI or LBM between the groups.
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31 *Lifestyle factors*

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33 Data for vitamin D intake and calcium were available for 29 patients and 52 controls. Fourteen of the patients (48 %)
34 and 32 of the controls (55%) received vitamin D supplements. The daily total intakes of vitamin D ranged from 4.3 to
35 34.3 µg/day for the patients and from 4.3 to 15 µg /d for the controls, and were lower than recommended in 8
36 patients (28%) and 13 controls (28%). The daily intakes of calcium were below the recommendation for 2 patients
37 and 1 control. There were no significant differences in vitamin D or calcium intakes between the groups (Table III).
38 Altogether 3/28 of the patients with bleeding disorder and 4/43 of the controls were considered physically inactive
39 (score <13). The intensity for physical activity score was significantly lower in the patient group (p=0.002). (Table III).
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46 *Bone density and fractures*

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49 The z-scores for BMD are shown in Table IV. The BMDs for LS, femur and WB were lower in the patients but this
50 reached statistical significance only in the WB z scores (p=0.007). As the intensity of physical activity was lower in the
51 patient group we hypothesized that this could be the factor behind the difference. However, the difference persisted
52 even after adjusting the WB z-scores for physical activity (p= 0.044). When evaluated separately in subgroups
53 according to pubertal maturation (pre-, mid- and postpubertal) the difference between patients and controls
54 remained in the older groups but not in prepubertal children. There were no significant differences in lean mass and
55 fat mass between the two groups indicating that the trend for lower physical activity had not resulted in altered
56 body composition in the patients (Table II). The fat% Z-scores correlated inversely with BMD Z-score in femur and
57 WB (r=-0.207, p<0.05 and r=-0.438, p=0.001, respectively).
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1
2 Asymptomatic vertebral fractures assessed by IVA were present in 4 (15 %) of the patients and 3 (4.8 %) controls.
3
4 (Chi-Square; $p=0.135$). Three patients had one vertebral wedge fracture (anterior vertebral height loss $>20\%$) and
5
6 one had one crush fracture (vertebral height loss of 20%) with normal bone densities, whereas all the three control
7
8 patients had one vertebral wedge deformity.

9
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11 Three (10%) patients had had one or more traumatic fractures and one had clavicular fracture at birth. Of the
12
13 controls, 12/52 (23%) had a history of at least one traumatic fracture (Chi-Square; $p=0.26$).
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15 16 *Biochemical findings*

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18 Urine calcium secretion (U-Ca/Crea) was significantly higher in the patient group ($p=0.001$) (Figure 1; Table V) and 3
19
20 patients (12.5 %) with haemophilia had hypercalciuria (U-Ca/Crea >0.70 mmol/mmol) while hypercalciuria was
21
22 observed in only 2 (3.5%) of the controls. Calcium excretion was not associated with age, pubertal stage, severity of
23
24 the haemophilia, number of joint bleeds, prophylaxis, history of inhibitors, or BMD **in our relatively small cohort**.

25
26 Urinary calcium excretion correlated inversely with PTH ($r = -0.314$, $p = 0.004$) and the bone resorption marker ICTP
27
28 ($r = -0.276$, $p=0.012$) and positively with 25-OHD ($r = 0.289$, $p = 0.008$) and urinary Pi ($r = 0.290$, $p = 0.008$). There were
29
30 no differences in mean serum 25-OH-D concentrations between the study group and the controls (Table V), the
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32 median concentrations being 47 (range 18-90) and 45 (range 17-77) nmol/L for the patients and the controls,
33
34 respectively. The concentration was suggestive of vitamin D insufficiency (<50 nmol/L) in 13 patients (50%) and 37
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36 (66%) controls and of severe D vitamin deficiency (below 25 nmol/L) in 2 (8%) patients and 1 (2%) control. No
37
38 significant differences were detected in the serum bone turnover markers PINP, ICTP, or ALP between the two
39
40 groups but the bone resorption marker U-INTP was significantly lower in the patient group ($p=0.002$). Consequently,
41
42 the ratio S-PINP/U-INTP was significantly higher in the patients as compared with the controls ($p=0.009$).

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44 Interestingly, the patients also had higher serum Ca ($p=0.006$) concentration.
45

46 **Discussion**

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48 Several previous studies have suggested a connection between haemophilia and osteoporosis. A recent meta-
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50 analysis, which summarized the results of seven case-control studies, comprising 111 paediatric and 101 adult
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52 haemophilia patients, confirmed an association between haemophilia and low BMD (Iorio *et al*, 2010). Most of the
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54 studies have involved adults and since the number of paediatric studies is limited it has remained unknown whether
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56 compromised bone health is secondary to joint bleeds and physical inactivity. The findings of the present study,
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58 involving children with early-onset prophylactic coagulation factor treatment, suggest that in addition to life-style
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60 factors also primary factors, especially hypercalciuria, contribute to impairment of bone health and development of
osteoporosis over time even in prophylactically treated patients.

1
2 In this study we examined 29 Finnish children with haemophilia and 58 matched controls. The study group did not
3 differ significantly from the control group regarding calcium or vitamin D intake or other lifestyle factors, although
4 there was a trend towards lower physical activity. **Approximately half of the study subjects received vitamin D**
5 **supplements, which are presently recommended to all Finnish children.** Although the BMD in the haemophilia
6 patients was within normal range, their whole body BMD was significantly lower than in the healthy controls. Our
7 results indicate that in spite of prophylaxis, haemophilia patients are at risk of lower BMD compared to their peers.
8 This finding becomes more apparent after puberty. While lower physical activity seems a possible explanation, it
9 does not alone account for the difference. Low BMI or lean body mass, but also overweight and obesity may
10 compromise bone mass accrual (Dimitri *et al*, 2010; Viljakainen *et al*, 2010). No significant differences in lean mass
11 and fat mass were observed between our patients and control subjects.
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20 Previous paediatric studies have involved variable patient populations and assessment of confounding factors has
21 often been inadequate. One of the largest paediatric studies consisting of 62 haemophilic children (35% severe) was
22 reported by Tlacuilo-Parra in 2008. None of the patients were on prophylactic therapy and all had a history of one or
23 more joint bleeds. There were no differences in dietary calcium intake whereas, as expected with on-demand
24 therapy, there was a significant difference in physical activity between the patients and controls. The lumbar spine
25 BMD in patients was significantly lower than in controls; the authors proposed that this was due to the lower
26 physical activity. Recently the same group compared serum bone turnover markers in 69 children with haemophilia
27 and normal vs. low BMD. They found that children with haemophilia and low LS BMD had lower osteocalcin levels
28 indicating diminished osteoblastic activity but found no differences in bone resorption markers (Tlacuilo-Parra 2010).
29 An Australian study reported reduced areal BMD z-scores in 19 children with severe haemophilia A compared with
30 healthy controls (Barnes *et al*, 2004). The mean age of the patients was 12.2 years; all had severe haemophilia. Most
31 (79%) of the participants were on prophylactic treatment. The reduction in BMD correlated with the severity of
32 haemophilic arthropathy. However, diet, other lifestyle factors or laboratory tests were not taken into account.
33 Furthermore, neither the age of prophylaxis onset nor the number of joint bleeds was reported. Another recent
34 study (Christoforidis A *et al*, 2010) used both quantitative ultrasound sonography (QUS) and DXA to assess bone
35 status in 27 children or young adults (mean age 12,3 years) with moderate or severe haemophilia A, most of whom
36 were on either primary or secondary prophylaxis. Their bone properties were within normal limits. This study also
37 lacked a normal control group and lifestyle factors or laboratory tests were not evaluated. In line with these previous
38 studies, our findings suggest that early-onset prophylactic treatment is likely to have a favorable effect on BMD and
39 allow nearly normal physical activity. However, despite successful prevention of joint bleeds and inactivity, our study
40 subjects tended to have lower BMD than age- and height-matched controls suggesting that the compromised bone
41 health in haemophilia is not caused by lifestyle factors only.
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Importantly we observed significantly increased urine calcium secretion in our study cohort as compared with the controls, and 3 patients with haemophilia had pathological hypercalciuria (U-Ca/Crea >0.70 mmol/mmol).

Hypercalciuria is a known risk factor for osteoporosis and fractures; the pathophysiology is multifactorial (Srivastava

1
2 & Alon, 2007; Zerwekh 2010). Garcia-Nieto *et al.* (1997) showed that one third of 73 children with idiopathic
3 hypercalciuria had osteopenia. Penido *et al.* (2003) reported reduction in BMD among 88 children with idiopathic
4 hypercalciuria. They suggested altered bone metabolism as a mechanisms for lower BMD in these patients. Our
5 hypercalciuria. They suggested altered bone metabolism as a mechanisms for lower BMD in these patients. Our
6 finding of significantly higher urinary calcium in haemophilia patients than in their healthy peers suggests primary
7 pathology in bone metabolism, which in turn may result in impaired bone mass accrual and low BMD in haemophilia
8 patients. The cumulative effects of hypercalciuria over years may become fully evident only in an older patient
9 group.
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16 The mechanisms behind the high urine calcium excretion in children with haemophilia remain unclear. None of the
17 patients were immobilized, their calcium and vitamin D intakes were comparable to the controls and none had S-25-
18 OHD values exceeding the normal range. Considering the higher p-Ca and consequently lower PTH, one can presume
19 that the hypercalciuria is a result of increased renal filtration load of calcium possibly secondary to reduced bone
20 metabolism and mineralization and not due to increased bone resorption, as high urinary calcium was associated
21 with lower PTH and lower bone resorption markers ICTP and INTP. This finding is in contrast with a recent report on
22 adult Greek haemophiliacs who had increased levels of bone resorption markers as compared to their controls
23 (Katsarou *et al.*, 2010). However, their study group differed from ours on several other aspects as well, including high
24 number of HIV and HCV seropositivity and arthropathy. **As our study group was relatively small and the
25 observations were based on single samples only the biochemical findings need to be confirmed in future studies
26 involving larger numbers of patients.**
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36 Although hypercalciuria has not been evaluated or reported in previous studies, other findings have suggested
37 primary skeletal pathology in haemophilia. Manco-Johnson *et al.* (2007) studied the effect of prophylactic treatment
38 compared with enhanced on demand therapy on the joints of 65 boys with severe haemophilia A. Joint preservation
39 was evaluated by magnetic resonance imaging (MRI) and plain-film radiography. A subset of patients with
40 abnormal MRI had no history joint bleeds. The authors proposed that deterioration of joints evidenced by MRI is
41 caused by microhaemorrhage into the joints or subchondral bone. The same mechanism may also underlie the
42 altered bone metabolism and hypercalciuria seen in our patients. Gurevitch and Slavin hypothesized in 2006 that
43 chronic blood loss resulting in overexertion of haematopoietic system plays an important role in the etiology of
44 osteoporosis. They speculated that blood loss creates pressure on the haematopoietic system leading to both
45 osteogenic activity through stimulation of haematopoietic system including osteoclasts and enhancement of bone
46 development though increased production and maturation of osteoblasts.
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56 **Chronic microhaemorrhage could also induce chronic inflammation, which has been associated with low BMD. As
57 inflammatory cytokines were not evaluated we can not rule out this possibility. However, assuming that the
58 number of joint bleeds correlates with the level of inflammatory cytokines one would expect reduced BMD to
59 become more evident with higher number of joint bleeds. This was not observed in our study.**
60

1
2 Oral vitamin K antagonists are used as anticoagulants by patients with thrombosis or risk of thrombosis. A possible
3 known side effect is reduction in **BMD**. A Greece group assessed the BMD and bone metabolism markers of 23
4 children with long-term oral anticoagulant therapy and the bone metabolism markers of 25 age matched controls
5 (Avgeri *et al*, 2008). As expected, the patient group had considerably lower vitamin K dependant coagulation factors
6 (F II, VII, IX, X, protein S, protein C) than the controls due to anticoagulation. There were several lines of evidence
7 supporting the association between osteopenia and long-term oral anticoagulation: signs of osteopenia in majority
8 of the patients in DXA scanning, higher osteoclast activity markers, lower osteoblast activity markers and higher PTH.
9 Nevertheless, hypercalciuria was not connected to oral anticoagulation and suggests that impaired coagulation
10 cascade does not *per se* lead to high urinary calcium excretion.
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19 The age-specific rates of chronic renal disease in persons with haemophilia were higher in every age group than
20 among US males (Kulkarni *et al*, 2003). The reasons underlying chronic renal disease in haemophilia patients are
21 multifactorial. However, in the light of our findings, the role of hypercalciuria needs to be investigated in future
22 studies.
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27 In conclusion, the findings of our study indicate that even though BMD in boys with haemophilia is within normal
28 range, the BMD values tend to be lower than in healthy peers. Importantly, our study identified hypercalciuria as a
29 novel risk factor for osteoporosis. This finding suggests that in addition to life-style factors, such as reduced physical
30 activity, primary skeletal pathology contributes to the development of osteoporosis in patients with haemophilia.
31 The clinical implications of high urinary calcium excretion **and its causal relationship with low** bone mass and renal
32 function **impairment** remains unclear **in patients with haemophilia**. Further studies are needed to elucidate the
33 mechanisms behind hypercalciuria and its long-term skeletal consequences.
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Table I. Clinical characteristics of the patients with haemophilia.

	Age	Diagnosis	Age at diagnosis	Treatment	Age at start of prophylaxis	History of inhibitor	Joint bleeds		
							0	1-3	>4
13	16.4	mild haemophilia A	0	D		no		x	
14	16.7	mild haemophilia A	0.5	D		no		x	
15	8.3	moderate haemophilia A	1.8	D		no			x
16	11.3	moderate haemophilia A	1.1	P	2.8	no	x		
17	16.1	moderate haemophilia A	1.1	D		no		x	
18	8.0	moderate haemophilia B	0	D		no		x	
19	10.7	moderate haemophilia B	0.5	D		no			x
20	10.8	moderate haemophilia B	0	P	8.7	no			x
21	4.2	severe haemophilia A	0.1	P	1	no	x		
22	4.8	severe haemophilia A	0.6	P	0.9	no	x		
23	8.5	severe haemophilia A	0	P	0.9	no	x		
24	8.7	severe haemophilia A	0.3	P	0.6	no		x	
25	8.8	severe haemophilia A	1	P	1.3	yes	x		
26	9.2	severe haemophilia A	0	P	0.7	no	x		
27	10.1	severe haemophilia A	0	P	0.6	yes		x	
28	10.5	severe haemophilia A	0	P	0.9	yes		x	
29	11.2	severe haemophilia A	0	P	0.6	no		x	
30	12.2	severe haemophilia A	1	P	1.2	no			x
31	12.6	severe haemophilia A	0	P	2.3	no		x	
32	14.6	severe haemophilia A	0.2	P	0.9	no			x
33	14.6	severe haemophilia A	1.1	P	3.6	yes			x
34	14.9	severe haemophilia A	0.5	ITI	3.9	yes*			x
35	15.1	severe haemophilia A	0.2	P	1.1	no			x
36	16.0	severe haemophilia A	5.5	P	5.5	no			x
37	6.4	severe haemophilia B	0	P	1	no	x		
38	6.4	severe haemophilia B	0	P	1	yes*, LT			x
39	8.6	severe haemophilia B	0.5	P	0.6	no		x	
40	9.3	severe haemophilia B	0.6	P	0.7	no		x	
41	13.7	severe haemophilia B	0.6	P	1	no		x	

P=prophylaxis, D= on demand, ITI= immunotolerancy induction. Prophylaxis for haemophilia A was given every other day and for haemophilia B every 3rd – 5th day, *inhibitor at the study time, LT=low titer

Table II. Age and anthropometrical data of the 29 patients with haemophilia and the 58 control subjects.

	mean \pm SD (n)		p value
	patient (29)	control (58)	
Age (years)	11.0 \pm 3.6	11.6 \pm 2.8	0.32
Weight (kg)	44.2 \pm 21.6	45.3 \pm 18.1	0.80
Height (cm)	147 \pm 21	150 \pm 18	0.39
Height z-score	0.3 \pm 1.1	0.5 \pm 1.0	0.46
Relative weight (%)*	13.9 \pm 21.6	8.0 \pm 18.8	0.21
Lean mass (kg)	31.4 \pm 12.6	33.1 \pm 12.1	0.54
Fat mass (kg)	13.2 \pm 11.3	11.5 \pm 7.4	0.44
Overweight/obese (%)	35.5	24.6	0.28**
BMI (kg/m ²)	19.3 \pm 4.5	19.2 \pm 4.0	0.92
Fat % z score	-0.039 \pm 1.41	-0.266 \pm 1.17	0.44
Puberty			
prepubertal	19	38	
midpubertal	2	6	0.84**
postpubertal	8	14	

* height adjusted weight

** Chi-Square test

Review

Table III. Dietary intakes of vitamin D and calcium and physical activity among patients and controls (mean \pm SD)

Variable	(mean \pm SD)		p value
	patients (29)	controls (45)	
Calcium intake (mg)	1570 \pm 540	1550 \pm 490	0.86
Dietary Vitamin D intake (mg)	8.2 \pm 3.1	8.4 \pm 3.3	0.79
Total vitamin D intake(mg)	12.8 \pm 7.3	11.9 \pm 5.8	0.58
Physical activity score	17.6 \pm 5.1	19.6 \pm 3.2	0.064*
Intensity of physical activity	17.3 \pm 7.8	23.0 \pm 7.0	0.002

* Mann-Whitney test

Table IV. Bone densitometry findings for patients and controls according to pubertal maturation

Variable	patients (27)	controls (58)	p value
lumbar spine z-score			
all	0.137 ± 1.093	0.381 ± 0.866	0.270
prepubertal	0.188 ± 0.959 (n=17)	0.292 ± 0.772 (n=38)	0.671
mid/postpubertal	0.050 ± 1.344 (n=10)	0.550 ± 1.022 (n=20)	0.265
femur z-score			
all	-0.052 ± 1.116	0.362 ± 0.851	0.063
prepubertal	0.041 ± 0.864	0.479 ± 0.848	0.084
mid/postpubertal	-0.210 ± 1.493	0.140 ± 0.832	0.414
whole body z-score			
all	-0.067 ± 1.167	0.512 ± 0.737	0.007
prepubertal	0.235 ± 0.700	0.550 ± 0.722	0.138
mid/postpubertal	-0.580 ± 1.612	0.440 ± 0.780	0.017*

* Mann-Whitney test

Peer Review

Table V. Biochemical findings in the patients with haemophilia and the controls. The values are given as mean \pm SD.

Variable	Patients (26)	Controls (57)	p value
S-25-OH-D	47 \pm 17	44 \pm 12	0.351
S-PTH	32 \pm 22	42 \pm 23	0.073
S-ALP	230 \pm 58	228 \pm 65	0.857
S-Ca	2.40 \pm 0.08	2.34 \pm 0.09	0.006*
S-Pi	1.37 \pm 0.19	1.46 \pm 0.18	0.042*
S-PINP	547 \pm 172	564 \pm 197	0.700
S-ICTP	14.6 \pm 4.0	15.6 \pm 4.5	0.348
U-Ca/U-Crea	0.361 \pm 0.29	0.181 \pm 0.18	0.001*
U-Pi/U-Crea	2.62 \pm 1.31	2.35 \pm 2.53	0.616
U-INTP	448 \pm 231	604 \pm 300	0.021*
S-PINP/U-INTP	1.144 \pm 0.72	1.05 \pm 0.40	0.009*

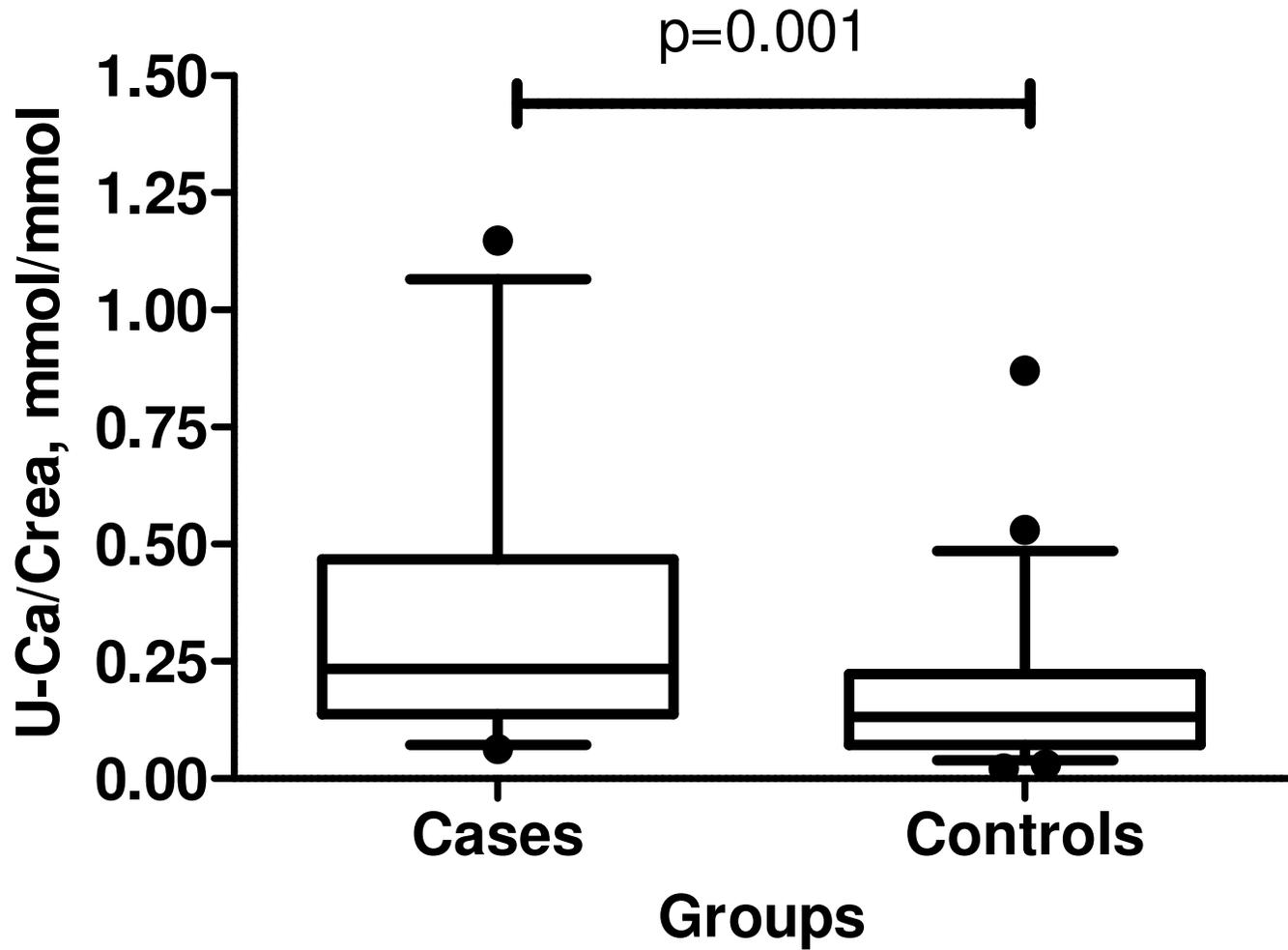
S, Serum; U, Urinary; 25-OHD, 25-hydroxy vitamin D; PTH, intact parathyroid hormone; ALP, alkaline phosphatase; Ca, calcium; Pi, phosphate; PINP, procollagen type I aminoterminal propeptide; ICTP, carboxyterminal telopeptide of type I collagen; Crea, Creatinine; INTP, type I collagen cross-linked N-telopeptide

*The differences remained even after adjusting for age.

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2 **Figure legends**
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7 Figure 1. Urinary calcium excretion in patients and healthy controls.
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For Peer Review



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